Synthesis and secretion of a cobalamin-binding protein by HT 29 cell line

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An HT 29 cell line derived from human colonic carcinoma was shown to synthesize and release a cobalamin-binding protein. The cobalamin-binding protein was classified as transcobalamin (TC). By gel filtration on Sephacryl S200 HR, we observed that the secreted protein bound to cobalamin had the same size as plasma transcobalamin. Like transcobalamin, the cobalamin-binding protein bound cobalamin but not cobinamide. Purification of the cobalamin-binding protein was performed by heparin–Sephacryl affinity chromatography and by Sephacryl S200 gel filtration. The molecular mass of the purified protein was estimated at 44 kDa by SDS/PAGE. The isoelectric point was determined to be 6.4. The purified cobalamin-binding protein reacted with an antiserum produced against human transcobalamin. A 44 kDa band was also identified by SDS/PAGE of an immunoprecipitated homogenate from HT 29 cells labelled with [³⁵S]methionine and in a Western blot of cell homogenates. The secretion of the cobalamin-binding protein was maximal between 10 and 12 days of cell culture and was inhibited by cycloheximide.

INTRODUCTION

Cobalamin (Cbl; cyanocobalamin is vitamin B-12) is assimilated in the distal ileum by a receptor-mediated endocytosis of the vitamin bound to a specific protein named intrinsic factor (IF) (Seetharam & Alpers, 1985; Guéant & Gräsbeck, 1990). In the blood, Cbl is bound either to transcobalamin (TC) or to another Cbl-binding protein, haptocorrin (HC) [previously named transcobalamin I or R binder (Nexø & Olesen, 1982)]. TC is the plasma binding protein for Cbl that facilitates its receptor-mediated uptake by perfused tissues (Youngdahl-Turner & Rosenberg, 1978). Its origin remains an open question. Several locations for secretion and synthesis of TC have been demonstrated in cultured cells such as fibroblasts (Berliner & Rosenberg, 1981), bone-marrow cells (Frater-Schröder *et al.*, 1985), human and rat hepatocyte (Hall *et al.*, 1985; Fiedler-Nagy *et al.*, 1975), and endothelial cells from human umbilical vein (Quadros *et al.*, 1989; Carmel *et al.*, 1990).

In the present paper we demonstrate the secretion and the synthesis of a TC protein by a cell line, HT 29, derived from a colonic carcinoma (Fogh & Trempe, 1975). The cells resemble foetal enterocytes and are present in culture as polarized monolayers (Rousset, 1986).

EXPERIMENTAL

Cell culture

Cells were a subpopulation of HT 29 cells (Fogh & Trempe, 1975) adapted to a glucose-free medium (Zweibaum *et al.*, 1985) and reversed for several passages in a glucose-containing medium. These cells maintained their differentiation characteristics (A. Zweibaum, personal communication). The cells were grown in minimum essential medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 10% (v/v) fetal-calf serum (FCS) and 1% antibiotics. Stock cultures were grown in 25 cm² flasks and placed in 37 °C incubator in a humidified CO₂/air (1:9) atmosphere.

Gel filtration

Gel filtration of unsaturated TC was performed on a Sephacryl S200 HR column (100 cm × 1.5 cm) (Pharmacia–LKB, Uppsala, Sweden). Media from 8–14 days of cell culture were collected, concentrated 50-fold and dialysed against 0.02 M-Tris/HCl buffer, pH 8, containing 1 M-NaCl. A 100 µl sample was consecutively incubated with 10 µl (90 pmol) of cobinamide (Cbi) (in order to saturate HC present in FCS) for 10 min and with 20 µl of cyano[⁵⁷Co]cobalamin (CN[⁵⁷Co]Cbl) (0.74 pmol) (Amersham France SA, Les Ulis, France) for 20 min before gel filtration of the sample. It was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions (1 ml each) were collected for detection of the CN[⁵⁷Co]Cbl by γ-radiation counting.

TC saturated with CN[⁵⁷Co]Cbl was assayed with 25 µl of human placenta membrane (11 mg of protein/ml) prepared as described (Nexø & Hollenberg, 1980) in 1 ml of 0.02 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and either 2 mM-CaCl₂ or 25 mM-EDTA. The same protocol was performed with rabbit TC saturated with Cbl (3 nmol/ml) in the same buffer containing 25 mM-EDTA. The samples were incubated for 2 h at room temperature. TC-bound and free Cbl were separated by filtration through 0.2 µm-pore-size cellulose acetate filters (Sartorius, Göttingen, Germany).

Isoelectrofocusing

The TC sample used was collected after filtration through Sephacryl S200 HR. Isoelectrofocusing was performed on a 110 ml LKB column (8101) using a 0–50% sucrose gradient in 5 M-urea. The pH ranged from 2.5 to 8.

Purification of TC

Purification of TC was performed with 500 ml of cell medium as starting material. The Cbl-binding proteins were saturated by consecutive incubation with 72.5 nmol of Cbi and with a mixture of 0.725 nmol of CNCbl and 0.725 pmol of CN[⁵⁷Co]Cbl (30 min

Abbreviations used: TC, transcobalamin; Cbl, cobalamin (vitamin B-12); IF, intrinsic factor; HC, haptocorrin; FCS, foetal-calf serum; Cbi, cobinamide; CN[⁵⁷Co]Cbl, cyano[⁵⁷Co]cobalamin; DPBS, Dulbecco modified phosphate-buffered saline without Ca²⁺ or Mg²⁺.

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for each incubation). The preparation was adsorbed to a heparin-Sepharose 4CL column (10 cm $\times$ 0.5 cm) (Pharmacia-LKB). The affinity-chromatography column was washed with 200 ml of 0.02 M-Tris/HCl buffer, pH 8, containing 1 mM-EDTA. The TC fraction was eluted with the same buffer containing 0.5 M-NaCl and subsequently filtered through the Sephacryl S200 HR column as described above. The TC fraction was detected by γ -radiation counting of labelled Cbl. The final preparation was submitted to SDS/PAGE (Technical File no. 110; Pharmacia-LKB) in an automatic system (Phast System; Pharmacia-LKB).

TC secretion

Cells (6×10^4) were seeded on 24-well plates under the same conditions as for the stock culture. Medium from four wells was collected. The concentration of unsaturated TC was determined by isotope-dilution assay. We used the heparin-Sepharose assay of TC described by van Kapel *et al.* (1988) to study the TC secretion by HT 29 cells as a function of time. Controls for non-specific binding to heparin were done using purified pig HC and human gastric juice preincubated with Cbl (in order to saturate HC). Less than 1% of either hog HC or human IF saturated with labelled Cbl was adsorbed to heparin.

Effect of cycloheximide

Cells (6×10^4 /ml) were seeded on six-well plates and allowed to grow until confluent. Cells were cultivated in the absence of FCS for 24 h before cycloheximide was added. The effect of cycloheximide was tested in triplicate at two concentrations (1 and 10 μ g/ml). The medium was removed after either 24 or 48 h in order to measure the TC concentration.

Radioisotope assay of the Cbl-binding capacity of TC

We used the method described by van Kapel *et al.* (1989). A 500 μ l portion of culture supernatant was incubated with 10 μ l of Cbl (7.4 μ mol/ml) for 5 min. A 50 μ l portion of a Cbl mixture (2.95 pmol of CNCbl/0.74 pmol of CN 57 Co]Cbl in 1 ml of 0.02 M-Tris/HCl buffer, pH 8, containing 1 mM-EDTA) was then added for 20 min with gentle agitation. The reactant mixture was completed with 440 μ l of 0.04 M-Tris/HCl buffer, pH 8, containing 2 mM-EDTA. After incubation, 300 μ l of heparin-Sepharose suspension (1 g swollen in 12 ml of distilled water containing 1 mM-EDTA) was added for 2 h with agitation. The mixture was centrifuged at 1000 g and washed three times with 0.02 M-Tris/HCl buffer, pH 8, containing 1 mM-EDTA. The concentration of TC was estimated from the radioactivity in the pellet and the specific radioactivity of the CN 57 Co]Cbl. Results were expressed as fmol of Cbl bound to TC/ml.

Western blotting of the cell homogenate

Anti-(human TC) was produced by immunizing a rabbit with intradermal injection of approx. 0.5 nmol of purified human TC mixed with complete Freund's adjuvant (Behringwerke), followed by booster injections with 0.5 nmol of human TC mixed with incomplete Freund's adjuvant 2 weeks, 1 month and 2 months after the first injection. Human TC was purified from pleural and ascitic fluid obtained from therapeutic punctures by the same method as described for rabbit TC (Nexø *et al.*, 1977).

Cells (6×10^4) were seeded in 25 cm² flasks and allowed to grow for 6, 10 and 15 days. The cell culture was thereafter grown in absence of FCS at confluence of the cells. Before use, the cells were washed twice with 5 ml of DPBS (Dulbecco modified phosphate-buffered saline without Ca²⁺ or Mg²⁺; Gibco), scraped off, then resuspended in 0.01 M-Tris/HCl buffer, pH 7.1, containing 50 mM-mannitol, aprotinin (1000 units/ml) and 0.4 mM-phenylmethanesulphonyl fluoride. The cells were homo-

genized in a Weathon-Potter homogenizer and sonicated for 20–30 s. Homogenate (50 μ l; 200 μ g of protein) was mixed with 50 μ l of the same buffer containing 2% (w/v) Triton X-100 and shaken at 4 °C for 1 h. After centrifugation at 27000 g for 15 min, the supernatant was used for SDS/PAGE studies. A 50 μ l portion of 0.02 M-Tris/HCl buffer, pH 6.8, containing 20% (v/v) glycerol, 4% (w/v) SDS and 0.1% (w/v) Bromophenol Blue, was added to the supernatant. The samples were boiled for 3 min at 100 °C and resolved by 10%-PAGE as described by Laemmli (1970). After electrophoresis the gel was equilibrated in 2.5 mM-Tris containing 0.192 M-glycine and 20% (v/v) methanol. The proteins were transferred to a nitrocellulose membrane using a semi-dry Transblot apparatus (Bio-Rad, Richmond, CA, U.S.A.). The transfer lasted 1 h at 25 V. The immunoblotting was performed as described (Johnson *et al.*, 1984). Anti-human TC rabbit serum was used at a dilution of 1:5000 in 0.02 M Tris/HCl buffer, pH 8.0, containing 2% (w/v) glycine, 3% (w/v) milk proteins, 0.15 M-NaCl and 0.1% (v/v) Tween 20. After washing the membrane with the same buffer, immunodetection was carried out using the biotin-alkaline phosphatase reaction (Vectastain kit; Vector Laboratories, Burlingame, CA, U.S.A.). TC was also detected from cell homogenates by using dot-blot instead of Western blot. In that case, 1 μ g of protein/spot was put on the membrane. The detection was performed as described for the Western-blot procedure.

[³⁵S]Methionine labelling

HT 29 cells (6×10^4 /ml) were cultured in a 25 cm² bottle for 10 days. The monolayer was then washed twice for 30 min with DPBS and for 30 min with methionine-free medium in the absence of FCS. [³⁵S]Methionine (0.025 μ mol/ml; 1000 Ci/mmol) (Amersham France) was added in methionine-free medium for 24 h. After incubation, the cell medium was discarded and the cells were prepared for immunoprecipitation as described by Le Bivic *et al.* (1989). Anti-TC antiserum was used at a dilution of 1:500.

RESULTS AND DISCUSSION

When the concentrated cell medium was incubated with labelled Cbl, two Cbl binders were detected by gel filtration at the same elution position as gastric HC and human plasma TC respectively (Fig. 1). The 'HC' peak disappeared when the concentrate was preincubated with Cbl before addition of labelled Cbl, indicating that the protein eluted as TC bound Cbl but not Cbl. The same holds true for plasma TC. In comparison, a 'void-volume' peak, referred to as 'TC₀', an 'HC' peak and a very small peak corresponding to TC were observed when either concentrated culture medium or FCS was submitted to gel filtration. The 'TC₀' peak may consist of aggregates of TC and HC (Carmel, 1979; Hall, 1988). A low level of TC in foetal serum has been reported previously in the serum of 20-week human foetus (Sonnenborn & Iwanik, 1974; Hansen *et al.*, 1989). A main isoprotein at pH 6.4 was detected by isoelectrofocusing of the TC peak (Fig. 2). This isoprotein corresponded to one of the isoproteins of TC which are detected in human plasma (Hansen & Frater-Schröder, 1987). A minor peak was also observed at pH 4.9. The binding of labelled TC (prepared from cell-culture medium) on human placenta membranes was estimated to be 32.0 ± 5.4 fmol/mg of protein ($n = 4$) in the presence of CaCl₂. When the experiment was performed in the presence of EDTA instead of CaCl₂, a $56 \pm 4.8\%$ ($n = 4$) inhibition of this binding was observed. Assuming that the binding observed in presence of EDTA represented non-specific binding, we estimated that 15 fmol of TC-labelled Cbl complex were bound/mg of human placenta membrane protein. When the experiment was per-

Fig. 1. Sephacryl S200HR gel filtration of FCS (a) and concentrated culture medium (b) saturated with labelled Cbl

In both cases, two peaks were identified; the first peak was eluted in the void volume. It was identified as a TC and HC aggregate named TC₀ usually observed in gel filtration of Cbl-binding protein from human plasma. The second and the third peak corresponded to the elution position of plasma HC and to free Cbl respectively. (c) The same experiment was performed with concentrated cell medium obtained after 12 days of cell growth. Two peaks were eluted, which corresponded to HC and TC. Arrows indicate elution positions of standard molecules [in order of elution: Dextran Blue (DB); plasma HC, IF from human gastric juice, plasma TC and ³H₂O].

Fig. 2. Isoelectric focusing of secreted TC

Isoelectric focusing was performed as described in the Experimental section. The pI of TC was estimated at 6.4. A minor peak was also observed (with a pI of 4.9).

formed in the presence of saturated rabbit TC, an inhibition of 84.1 % ($n = 2$) of the specific binding of labelled human TC was observed. These results were very convincing, even though the non-specific binding was relatively high.

The Cbl-binding protein of the cell medium was purified by

Fig. 3. PAGE of TC from HT29 cells

Lane A, Molecular-mass markers; lane B, SDS/PAGE of purified TC; lane C, Western blotting of cells homogenate from 10-day cells; TC was detected on nitrocellulose membrane after electrotransfer by using an anti-(human TC) serum; lane D, radioautography of [³⁵S]-methionine-labelled proteins (after 24 h incubation of cells with [³⁵S]methionine) immunoprecipitated with anti-(human TC) serum.

Fig. 4. Dot-blot of TC from HT 29 cells

A 1 µg portion of protein from the samples was laid on a nitrocellulose sheet. TC was detected with anti-(human TC) serum (samples: 1, 2 and 3, cell homogenates at 6, 10 and 15 days respectively; 4, BSA; 5, TC purified from medium removed from HT29 cells).

affinity chromatography and gel filtration. It had a molecular mass of 44 kDa on SDS/PAGE (Fig. 3) which was the same molecular size as human plasma TC (Quadros *et al.*, 1989). In our purification procedure, TC was saturated with CNCbl/labelled CNCbl. The recovery, estimated from γ -radiation counting, was only 10.2% and 6.4% after heparin-Sepharose chromatography and Sephacryl chromatography respectively; 2 µg of purified TC could be obtained from 500 ml of cell medium. The purified protein reacted with rabbit anti-(human TC), as shown by dot-blot (Fig. 4). By the same procedure, TC was also detected in cell homogenates at different ages of the culture, but not in the corresponding cell medium (Fig. 4).

Western blotting of the homogenates showed a main band with the same molecular mass as the protein purified from cell medium (Fig. 3). Minor bands, which may correspond to non-specific binding, were also observed. The immunoprecipitation of [³⁵S]methionine-labelled proteins showed a main band with an estimated molecular mass of 44 kDa (Fig. 3). Several minor bands were also observed that may correspond to non-specific precipitation.

The TC secretion was maximal between 10 and 12 days of culture and decreased later (Fig. 5). Incubation of the cells during 24 or 48 h with cycloheximide reduced TC secretion (Fig. 6). The TC secretion was restored in 24 h when cycloheximide was removed from the cell-culture medium after a previous incubation with 1 µg of cycloheximide/ml for 24 or 48 h. The inhibiting effect of cycloheximide on HT 29 secretion of TC was about 20% greater than that obtained with endothelial cells (Quadros *et al.*, 1989).

The secretion of TC has been demonstrated in different cells. Most of them were non-polarized cells whose own uptake of Cbl is facilitated by TC. In cultured endothelial cells from human

Fig. 5. Concentration of TC in culture medium as a function of culture time

Each point corresponds to the mean of four measurements.

Fig. 6. Effect of cycloheximide on TC secretion

The experiment was performed in triplicate. The cells were incubated in the presence of either 1 µg or 10 µg of cycloheximide/ml for 24 or 48 h. The TC concentration in the cell medium was determined by the radioisotope-dilution assay as described in the Experimental section. 'Cyc' is the amount of cycloheximide added (µg/ml).

umbilical vein (Quadros *et al.*, 1989) the nascent TC binds the Cbl in the medium and facilitates its uptake by a specific membrane receptor. Identical results have been obtained with cultured Hep G2 hepatoma cells (Hall *et al.*, 1985; Hall & Colligan, 1989), human skin fibroblasts and bone-marrow cells (Frater-Schröder *et al.*, 1985). In these studies, TC release was correlated with the Cbl level of the culture medium.

In contrast, HT 29 cells and enterocytes are polarized cells (Rousset, 1986). HT 29 cells derive from pathological cells and present foetal characteristics (Rousset, 1986). In the ileal enterocyte, TC seems to be involved in the absorption of Cbl from the intestinal lumen. In inherited TC deficiency, the Schilling test (Hall, 1988) is abnormal, suggesting that TC is needed for the enterocyte transport of Cbl. One piece of evidence for the role of TC in Cbl assimilation was provided by Chanarin *et al.* (1978). They saturated the plasma Cbl binders of volunteers with CNCbl and then gave them an oral dose of CN[⁵⁷Co]Cbl. After 4 h, CN[⁵⁷Co]Cbl was detected in the plasma as a TC-Cbl complex, indicating that the passage of CN[⁵⁷Co]Cbl through the enterocyte was responsible for its binding to TC. In another study TC was detected in guinea-pig enterocyte by immunofluorescence (Rothenberg *et al.*, 1978). The presence of TC in the enterocyte can be explained either by an uptake of TC from plasma or by synthesis *in situ*. Our study is consistent with the synthesis of TC by enterocytes if one considers that HT 29 cells are functionally close to enterocytes [they share the same brush-border enzymes and a specific receptor for IF-Cbl complex (Guéant *et al.*, 1990)].

Recently, a Cbl-binding protein having the same molecular

size as TC was observed in the culture medium of another carcinoma cell line, CaCo2, suggesting synthesis of this protein (Dix *et al.*, 1990). The meaning of this synthesis remains unclear; it should be studied in regard to its role in Cbl assimilation. Another hypothesis is that the TC and HC may be candidates for 'tumour protein' in hepatoma and lung cancer respectively (Sheppard *et al.*, 1984).

In conclusion, HT 29 cells synthesize and release a Cbl-binding protein that we have classified as TC. The HT 29 cells could be a model for studying the vectorial passage of Cbl through the intestine and to obtain cDNA probes in order to demonstrate TC synthesis in mature enterocytes.

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