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Receptor binding of transcobalamin II-cobalamin in human colon adenocarcinoma HT 29 cell line

Rhizlane Bougrine,[‡] Catherine Masson, Renée Hatier,* Ebba Nexø,[†] Jean-Pierre Nicolas, and Jean-Louis Gueant

*Laboratory of Molecular and Cellular Nutrition and INSERM U 308, Faculté de Médecine, B.P. 184, 54505 Vandoeuvre-les-Nancy Cedex, France; *Laboratoire d'Histologie-Embryologie, Faculté de Médecine, B.P. 184, 54505 Vandoeuvre-les-Nancy Cedex, France; [†]Department of Clinical Biochemistry, University Hospital of Aarhus, Denmark; and [‡]Laboratoire de Biochimie et pharmacognosie, Université Moulay Ismail, Faculté des Sciences, Meknès, Maroc*

Transcobalamin II is the blood cobalamin binding protein that delivers cobalamin to target cells via a receptor-mediated endocytosis. We have studied the receptor binding of human transcobalamin II in differentiated HT 29 cells. The on-rate constant and the off-rate constant were estimated at 5.8 nM^{-1} min^{-1} and 0.015 min^{-1}, respectively. Scatchard analysis of the transcobalamin II-[^{57}Co] cobalamin binding to HT 29 cells showed a Ka at 0.14 pM^{-1} and 31,000 receptor sites per 15 days-aged cell. The binding was inhibited by EDTA and nearly abolished at pH 5.0. The transcobalamin II-[^{57}Co] cobalamin bound to plasma membrane was eluted as a high molecular cross-linked complex in Superose 6 gel filtration. Electron microscope radioautography showed the endocytosis of iodinated rabbit transcobalamin II in HT 29 cells. In conclusion, transcobalamin II receptor is present in HT 29 cells and may be involved in a receptor-mediated endocytosis of transcobalamin-cobalamin complex. (J. Nutr. Biochem. 7:397–402, 1996.)

Keywords: transcobalamin; vitamin B12; cobalamin; colon adenocarcinoma; HT 29 cell line; receptor mediated endocytosis

Introduction

Assimilation of cobalamin (Cbl; cobalamin is vitamin B12) requires its intraluminal binding to a gastric transport protein:intrinsic factor (IF), and the subsequent receptor-mediated endocytosis of the Cbl-IF complex in the ileum mucosa.[1] Transcytosis of cobalamin in the ileum involves a transfer of cobalamin to another binding protein named Transcobalamin II (TCII).[2,3] TCII is synthesized by two colon carcinoma cell lines that resemble fetal enterocyte, CaCo2, and HT 29.[4,5,6] The brush border membrane of these cell lines shares also the IF receptor.[5,7]

TCII is the blood cobalamin binding protein that delivers cobalamin to target cells via a receptor-mediated endocytosis.[8,9,10,11] This receptor-mediated endocytosis of Cbl-TCII has been described for various cells such as fibroblasts, placental, and leukemia cells[9,10,11,12,13] and more recently germ cells.[14] Endocytosis of TCII leads to the lysosomial degradation of TCII, subsequent intracellular conversion of Cbl to coenzymes, and recycling of the TCII-receptor.[15]

In polarized cells such as ileal cells and colon carcinoma cell lines, dietary cobalamin can be delivered to the cell from the apical side. No information has been provided until now among the possibility for the cells to internalize cobalamin via a receptor-mediated endocytosis of TCII-Cbl. The aim of the present study was, therefore, to investigate the presence of a TCII-receptor in HT 29 cells.

Methods and materials

Cell culture

Cells were a subpopulation of HT 29 cells[16] adapted to a glucose free medium[17] and reversed for several passages in a glucose

Address reprint requests to Professor J.L. Guéant, Medical Faculty, University of Nancy, BP 184, 54505, Vandoeuvre-les-Nancy Cedex, France.
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655 Avenue of the Americas, New York, NY 10010

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containing medium. Those cells maintained their differentiation (A. Zweibaum, M. Rousset, personal communication). The cells were grown in minimum essential medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 10% fetal calf serum (FCS) and 1% antibiotics as described recently.[6,7] The cells were grown in 25 cm^2 flasks and placed in a 37°C incubator in a humidified atmosphere with 10% CO_2 and 90% air.

Preparation of semi-purified TCII saturated with [^{57}Co] Cbl and of ^{125}I-iodide-rabbit TCII

The [^{57}Co] Cbl-TCII complex was obtained by Sephacryl S300 gel filtration of human serum incubated with [^{57}Co] cobalamin. The serum was from a patient having a cobalamin deficiency, with a blood cobalamin level lower than 50 fmoles/ml and a TCII binding capacity estimated at 1,010 fmoles/mL. Briefly, 1 ml of serum was incubated with 3.7 pmoles (1 μCi) of [^{57}Co] cobalamin (Amersham, France SA, Les Ulis, France) for 20 min at room temperature. The sample was run on Sephacryl S300 HR (Pharmacia, LKB, Uppsala, Sweden). The column (2.5 × 100 cm) was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 0.7 M NaCl and 0.02% NaN_3 (w/v). The eluted fractions corresponding to the [^{57}Co] Cbl-TCII complex were pooled and used for binding experiments with the cells. Under these conditions, labeled cobalamin represented more than 95% of cobalamin bound to TCII and specific activity was in order of 0.27 Ci/μmole. An aliquot of the [^{57}Co] Cbl-TCII pool was run in an analytical Superose 12 column (1.0 × 30 cm) to verify that the tracer was bound to TCII. The tracer-protein solution (0.12 pmoles) was incubated successively with 0.37 pmoles of [^{57}Co] Cbl (to saturate the Cbl binding protein) and 10 μl of a 100-fold diluted anti-TCII rabbit serum, as described previously. The experiment was repeated without incubation with anti-TCII rabbit serum. Both samples were filtered in the Superose 12 column, using the buffer solution described above at a flow rate of 0.3 mL/min.

Rabbit TCII was purified by E. Nexø[18] and iodinated by the method of Markwell[19] adapted as described.[7] The specific activity of the iodinated protein was at 0.2 Ci/mmole.

Binding study of [^{57}Co] Cbl-TCII to HT 29 cells

The cells (6×10^4) were seeded in 25 cm^2 flasks and allowed to grow for 7 to 21 days. The cells were washed twice with 5 ml of 0.01 mol/l Tris/HCl buffer (pH 7.4) containing 50 mM mannitol and were incubated for 15 min in the same buffer solution containing 20 mmol/L EDTA. They were washed again twice in the initial buffer solution and rested for 1 hr before use. Aliquots of 0.5 × 10^6 cells were incubated for 1 hr at 4°C with increasing amount of [^{57}Co]Cbl-TCII (3–112 fmol), either in the presence of 1 mmol/l $CaCl_2$ (for total binding determination) or of 5 mmol/L EDTA (for non-specific binding determination). The cells were centrifuged at 700 × g for 5 min and washed twice in the incubation buffer before counting the γ-radioactivity of the pellet. Specific binding was estimated from the difference between total binding and non-specific binding. The association constant and number of binding sites were calculated from Scatchard plot.[20] Binding experiments were performed in duplicate and the data from the experiment that were statistically the most significant were taken into account.

Binding experiments of iodinated rabbit TCII to HT29 cells was performed by the same protocol except that aliquots of 10^6 cells were incubated with 20 to 1,150 fmoles of labeled TCII.

Binding study of [^{57}Co] Cbl-TCII to plasma membranes

The enriched fraction of plasma membranes was prepared as described.[21] The protein concentration and the alcaline phosphatase specific activity of the enriched fraction were at 250 μg/mL and at 16.4 ± 5.1 units/mg protein (n = 4), respectively. Time-course of the binding of [^{57}Co] Cbl-TCII to plasma membrane was determined by incubating 140 fmoles of the tracer with 2 ml of plasma membrane suspension in a total volume of 10 ml in presence of incubation buffer (see previous discussion). Aliquots of 1 mL suspension were taken off after 2 to 32 min incubation time. Membranes were washed and counted as described above. Inhibition of the binding by non-labeled TCII was studied by the same protocol, excepting that 900 fmoles of non-labelled Cbl-TCII were added 15 min. after the addition of the tracer to the plasma membrane suspension.

Aliquots of 200 μL membrane fraction were incubated for 1 hr at 4°C with an increasing amount of [^{57}Co] Cbl-TCII (2 to 35 fmoles) either in the presence of 1mM $CaCl_2$ (for total binding determination) or of 5 mM EDTA (for non-specific binding determination). Incubation was performed in a total volume of 1 ml with 20 mM Tris/HCl, pH 7.4, containing 120 mM NaCl, 1% BSA (w/v). The membranes were centrifuged at 15,000 × g for 15 min and washed twice in the incubation buffer before counting the γ-radioactivity of the pellet. Association constant and number of binding sites were calculated from Scatchard plot performed in duplicate.[20]

Binding of [^{57}Co] Cbl-TCII to plasma membrane as a function of pH

Aliquots of 200 μL of plasma membrane fraction were incubated with 17.5 fmoles of the tracer in 1 mL buffer for 1 hr at room temperature at a pH ranging from 5 to 9. Incubation buffer solutions were either 50 mM sodium acetate, pH 5 or 6, or 25 mM Tris/HCl, pH ranging from 7 to 9. Each buffer solution contained 1 mM $CaCl_2$ for specific binding or 5 mM EDTA for non-specific binding.

Cross-linking of [^{57}Co] Cbl-TCII to plasma membrane

Seven hundred fmoles of cyano [^{57}Co] Cbl-TCII were incubated with 1 mg plasma membrane suspension in 60 mM HEPES (pH 7.5) containing either 1 mM $CaCl_2$ or 5 mM EDTA (for control experiment of non-specific binding), for 1 hr at room temperature. After centrifugation at 15,000 × g for 15 min, the pellet was washed twice with HEPES buffer and incubated with 2 μmol dithio-bissuccinimidyl propionate (DTSP) for 15 min as described.[22] The reaction was stopped by adding 0.5 ml of 60 mM ammonium acetate. The pellet was washed twice in 20 mM Tris/HCl buffer, pH 8.0, containing 0.7 M NaCl and 5 mM EDTA. It was resuspended in the same buffer in presence of 2% Triton X-100. After 24 hr incubation, the supernatant was run on Superose 6 B mini-gel filtration. The column (0.5 × 20 cm) was eluted with Tris/HCl buffer, pH 8.0, containing 0.7 M NaCl and 5 mM EDTA at a flow rate of 0.1 mL/min. The receptor [^{57}Co] Cbl-TCII and the [^{57}Co] Cbl-TCII complexes were detected by γ-counting of the eluted 0.1 mL fractions.

Endocytosis of iodinated TCII

Radioautography was performed on HT 29 cells (10^6/assay) incubated with [^{125}I]-TCII as described, except that incubation was at room temperature for 10 min. Radioautography was performed on cells (10^6/assay) incubated with [^{125}I]-TC as described, at room temperature for 10 min. The cells were washed and fixed for electron microscope radioautography. Radioautography processing was performed on ultra-thin sections using an Ilford emulsion

and Microdol-X (Kodak) developer, with 2 months of exposure at 4°C as described.[23]

Results

Semi-purified [^{57}Co] Cbl-TCII was collected from Sephacryl S300 gel filtration of human serum incubated with labelled cobalamin. A typical elution profile is shown in *Figure 1*. The tracer-protein complex was eluted at a retention time of 60 min in Superose 12 analytical gel filtration (*Figure 1*). It shifted into the "void volume" elution position when the sample was preincubated with anti-TCII rabbit serum, showing that the tracer was bound to TCII, only (*Figure 1*). The binding of [^{57}Co] Cbl-TCII to HT 29 plasma membrane fraction increased as a function of incubation time (*Figure 2*). Maximum binding was observed after about 15 min of incubation. The on-rate constant was estimated at about 5.8 $nM^{-1}min^{-1}$ (*Figure 2*). Dissociation

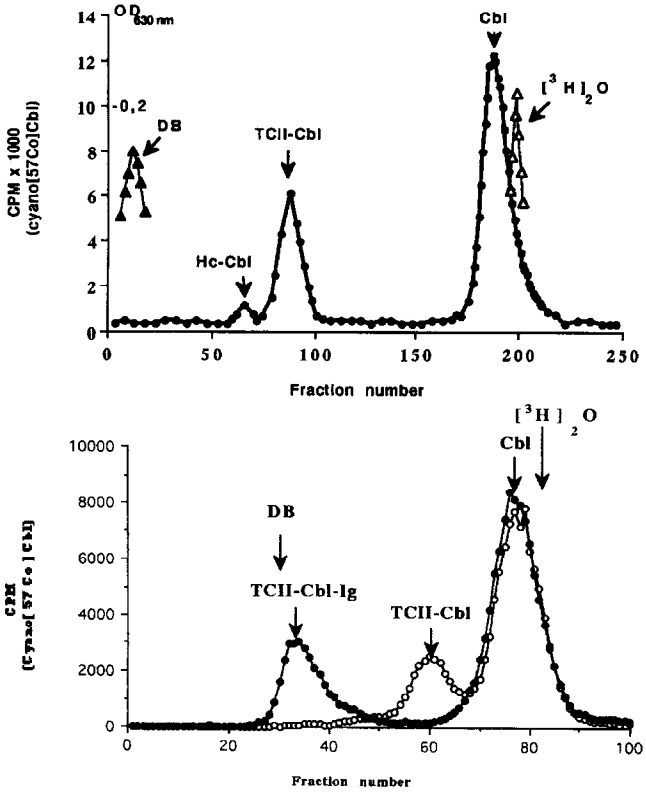

Figure 1 *Top:* Sephacryl S300 gel filtration of [^{57}Co] cobalamin-transcobalamin II (Cbl-TCII) complex. Human serum was incubated with [^{57}Co] cobalamin as described under the "Methods and materials" and run in a 2.5 × 100 cm column eluted at a flow rate of 0.5 ml/min. The tracer was eluted as a haptocorrin-cobalamin complex (Hc-Cbl) and a transcobalamin II-cobalamin complex. *Bottom*: An aliquot of the labeled Cbl-TCII peak eluted from Sephacryl S300 chromatography was incubated (●) or not (○) with anti-TCII rabbit serum and run in an analytical Sephacryl S200 HR gel filtration column (0.5 × 30 cm) eluted at a flow rate of 0.3 ml/min. In absence of incubation with anti-TCII serum, the labeled Cbl-TCII complex was eluted with a retention time at 60 min. When the tracer-protein complex was incubated with the anti-TCII serum, the peak shifted in the void volume, corresponding to the formation of a specific immune complex. Void volume and total volume were determined by using dextran blue 2000 (DB) and tritiated water ([3H]$_2$O), respectively.

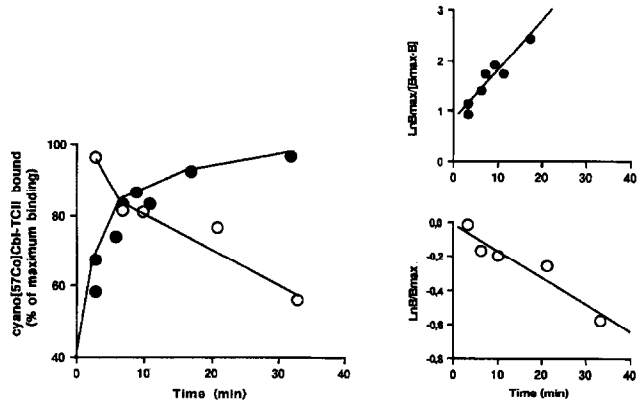

Figure 2 Time-course of [^{57}Co]-cobalamin-transcobalamin II binding to plasma membrane fraction from HT 29 cells. Association (●) was performed at 4°C using 140 fmol of [^{57}Co] cobalamin-transcobalamin II incubated with 2 ml of plasma membrane fraction in a final volume of 10 mL. One ml aliquot of the mixture was collected at incubation time ranging from 2 to 32 min. Competitive dissociation (○) was obtained by incubating the membranes with the tracer in presence of a 6.4 fold excess of non-labeled saturated transcobalamin II, 15 min. after the addition of the tracer. Plots were used for determining the on-rate constant (up right) and off-rate constant (bottom right). Non-specific binding of the tracer was determined by incubating the membranes with the tracer in presence of EDTA. It was estimated at 2.2%.

of the tracer was determined in presence of 6.4 fold excess of non-labeled cobalamin-TCII, with an off-rate constant at about 0.015 min^{-1} (*Figure 2*). From these experiments, the association constant was calculated at 0.39 pM^{-1}, which was about 2 fold lower than the value estimated from Scatchard analysis of binding experiments with cell membrane fraction. Binding of [^{57}Co] Cbl-TCII complex to plasma membrane was saturable, with a typical hyperbolic curve. Scatchard analysis showed a single class of binding sites with a Kass at 0.8 pM^{-1} and a specific binding activity at 80 fmoles/mg protein. A 20 fold excess of non-labeled TCII saturated with Cbl was able to displace 93% of [^{57}Co] TCII from the membrane fraction. The binding was inhibited by incubating the membrane fraction in presence of 5 mM EDTA instead of $CaCl_2$ (*Figure 2*). EDTA was used further to estimate non-specific binding.

Incubation of HT29 cell suspension with increasing amount of the tracer produced also a typical hyperbolic curve. The Scatchard analysis of the binding of [^{57}Co] Cbl-TCII to cell suspension showed a single class of binding sites with a Kass at 0.14 pM^{-1} for 15 days-aged cells. The Kass was at 3 nM^{-1} when iodinated rabbit TCII was used as a tracer (*Figure 3*). The number of binding sites was at 5,100, 31,000, and 21,600 for cells aged 7, 15, and 24 days, respectively.

Binding of [^{57}Co] Cbl-TCII to plasma membrane from HT 29 cells was pH-dependent. Maximum binding was obtained at neutral pH whereas abolition of the binding was observed at pH 5.0. We did not examine pH below 5 as binding of [^{57}Co] Cbl to TCII decreased below this pH value (data not shown). The pH-dependent binding of labeled TCII to plasma membranes from HT 29 cells were similar to that observed with the plasma membrane fraction from placental cells and hog intestine enterocytes (*Figure*

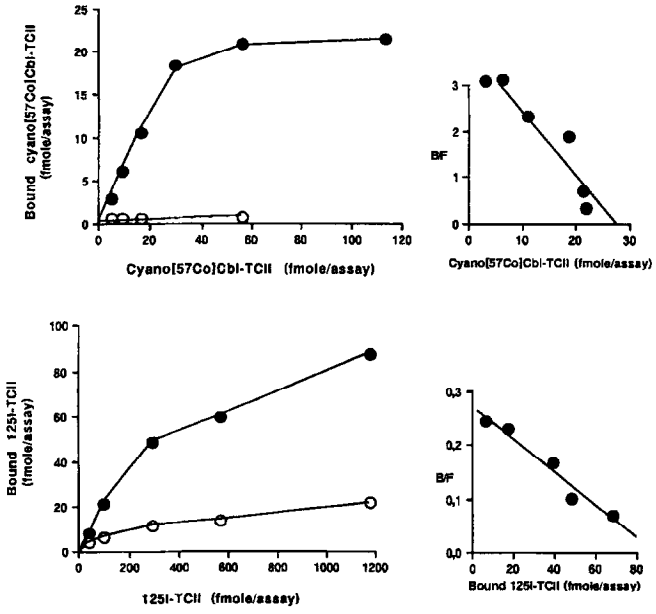

Figure 3 Saturation curve (up left) and Scatchard plot (up right) of the binding of [^{57}Co] cobalamin to a HT 29 cell suspension (0.5 10^6 cells per test tube) at 4°C for 1 h. Saturation curve (bottom left) and Scatchard plot (bottom right) of the interaction between HT 29 cells (10^6 per test tube) and iodinated rabbit TC II, at 4°C for 1 hr. The amount of the tracer bound to cells is estimated by subtracting non-specific binding (determined in presence of 5 mM EDTA, O) from total binding (determined in presence of 1 mM-$CaCl_2$, ●).

4). Non-specific binding of the tracer to plasma membrane from HT 29 cells was studied at pH 5, 6, and 7.4, in presence of EDTA; it was respectively estimated at 1.8%, 1.4%, and 2.2%.

The [^{57}Co] Cbl-TCII complex was cross-linked to the plasma membrane binding site before its solubilization in 2% Triton X-100. It was eluted as a high molecular mass aggregate in Superose 6B gel filtration as it was eluted in the same position than dextran blue (*Figure 5*). This receptor peak was not observed when the cross-linking experiment was performed in presence of EDTA instead of $CaCl_2$ showing that it did not correspond to a non-specific binding of the tracer.

Electron microscopy radioautography of HT 29 cell suspension incubated with rabbit iodinated TCII showed the association of silver grain to endosomes (*Figure 6*).

Discussion

The HT 29 cells used in this study were adapted in a glucose-free medium. HT 29 can share a brush border membrane and the same enzymes than fetal enterocytes.[24] These cells can help to elucidate the Cbl metabolism in intestinal mucosa because it expresses the IF-receptor in the brush border membrane and this receptor is involved in a receptor-mediated endocytosis of the IF cobalamin complex.[25] HT 29 cells are also able to synthesize TCII.[26] Similar observations have been made with CaCo2, another colon carcinoma cell line.[27]

The present study is the first one describing the presence of TCII-specific binding sites on plasma membrane of intestinal cells. Cross-linking experiments with plasma membrane showed that these binding sites correspond to a triton X-100 soluble receptor (*Figure 5*). This receptor is also present in mature intestinal cells as we observed a TCII binding capacity in a membrane preparation from hog ileal mucosa (*Figure 4*). The functional characteristics of this receptor are close to those previously described for TCII receptor of placental cells, fibroblasts, germ cells and leukemia cells.[11,12,14,29–31] The TCII binding to the receptor is pH-dependent, it requires calcium, and it is involved in the endocytosis of TCII.

The affinity of the receptor for TCII-Cbl is about 10 fold higher in HT 29 cells than in other mammalian cells, including malignant cells such as K 562 and HL 60 leukemia cells.[31] However, the affinity of the solubilized TCII-Cbl receptor purified from human placenta is only 2 fold lower than that of the present study.[13] In our study, the affinity is much lower with iodinated purified rabbit TCII. In fact, it has been previously shown that rabbit TCII has a lower affinity than human TCII for leukemia cell receptor,[31] human placenta receptor, and rabbit liver receptor.[12] In addition, one may assume that iodination of the molecule has an affect on its binding activity for both the cobalamin and the receptor. We could not check that rabbit TCII remained completely saturated after iodination. The TCII receptor has a higher affinity for the Cbl-TCII complex than for apo-TCII[32] and this may also explain its low affinity for iodinated rabbit TCII. It is however worth noting that the number of receptor detected by employing [^{57}Co] Cbl-TCII and [^{125}I]-TCII is approximately the same.

The specific binding activity of our plasma membrane preparation was in the same order of magnitude as that reported by Nexø et al. for placenta plasma membrane.[12] However, the number of binding sites was about 10 fold and

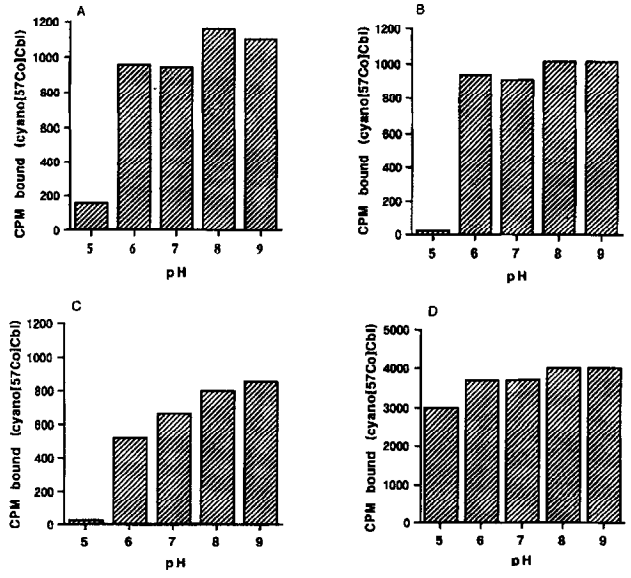

Figure 4 pH-dependent-binding of [^{57}Co] transcobalamin II to brush border plasma membranes from HT 29 cells (A), placenta (B), hog intestine (C). The membrane fractions were prepared as described in reference 21. In the three cases, the binding was decreased at pH 5.0. The pH dependent binding of cyanocobalamin was also determined in the same pH conditions (D).

50 fold higher than those reported respectively for K 562 and HL 60 leukemia cells and for rabbit germ cells.[14,31] HT 29 cell line is known to overexpress several intestinal functional proteins such as sucrase isomaltase, intrinsic factor receptor and TCII, by comparison with colon epithelial cells or ileal enterocytes.[24,25,27,33,34] This could be also the case for the TCII receptor. Additional studies should be performed with ileal intestinal mucosa to confirm this hypothesis. The number of receptor sites increased up to 31,000 per cell at 15 days of culture and decreased later. The same changes have been previously observed with IF-receptor and TCII synthesis in HT 29 cells.[25,26] It could be the consequence of the establishment of polarity of the cells, as HT 29 cells reach confluency between 7 and 10 days.[24]

The receptor is responsible for the specific endocytosis of TCII-Cbl. This receptor-mediated endocytosis has been demonstrated in various cells such as leukemia cells, fibroblasts, and germ cells.[10,11,14] The pH-dependent binding of TCII to the receptor provokes a release of TCII-Cbl in the acidic compartment of the cell. The degradation of TCII in lysosomes leads to the release and intracellular conversion of cobalamin into coenzymes.[10] Our findings are in agreement with this description of TCII cellular metabolism as we observed a pH-dependent binding of TCII to the receptor

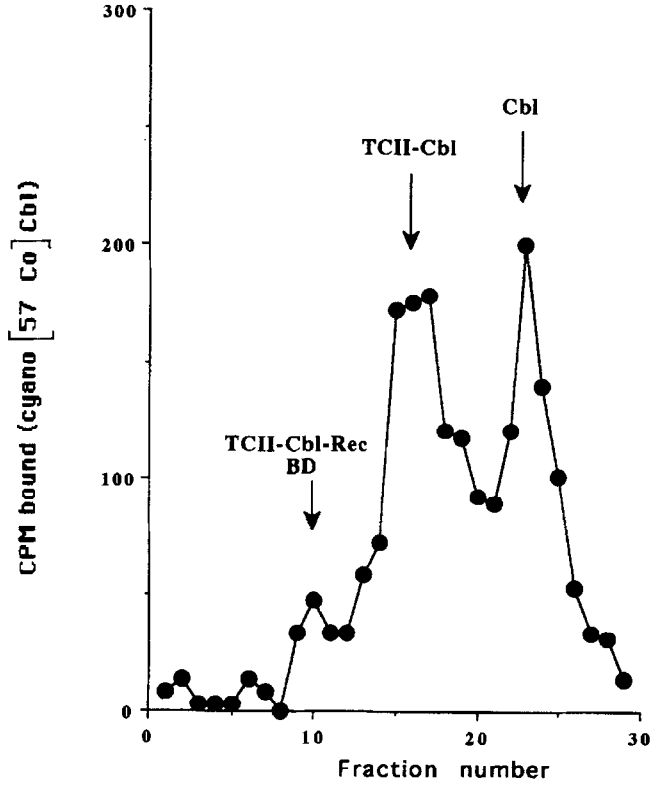

Figure 5 Superose 6 mini-gel filtration of the Triton X-100 solubilized holoreceptor after chemical cross linking of [^{57}Co]-cobalamin-transcobalamin II (TCII-Cbl) to the HT 29 plasma membranes in presence of either 1 mM-$CaCl_2$ (●) or 5 mM EDTA (○). Elution was performed in the presence of 5 mM-EDTA. The [^{57}Co] cobalamin-transcobalamin II-receptor peak (Cbl-TCII-Rec) was eluted as a high molecular mass complex, with the same retention time as Dextran blue 2000 (DB). This peak was not observed when the experiment was performed in presence of EDTA instead of $CaCl_2$.

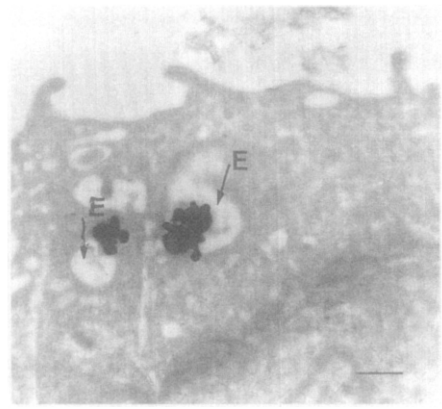

Figure 6 Electron microscope radioautography of HT 29 cell suspension after incubation with iodinated rabbit transcobalamin II. Incubation conditions are described under method section. Silver grain were predominantly associated with the membrane of endosomes (E), bar = 0.6 μm.

and an endocytosis of iodinated TCII. However, this study is preliminary and needs additional experiments to describe precisely by radioautography the intracellular trafficking of iodinated TCII in HT 29 cells. In addition, the basolateral and apical localization of the receptor needs to be determined.

The TCII receptor could be involved in the transcytosis of Cbl in intestinal cells. It has been shown in guinea pig ileal cells that Cbl was transferred from IF to TCII within the cell.[2] The cellular compartment that is involved in this transfer is unknown, but indirect evidence such as the inhibitory effect of chloroquine on transcytosis of Cbl[25,35] suggests that the acidic prelysomal and lysosomial compartments are potential candidates. If this were the case, TCII-receptor could not be implicated in the sorting of TCII-Cbl, as it has no binding activity at pH 5.0 (*Figure 4*). In fact, it is more likely that the TCII-receptor is involved in the uptake of Cbl from blood by intestinal cells. This means that Cbl could be internalized in intestinal cells in two ways: one is the IF-receptor mediated endocytosis of intraluminal food Cbl and the other is the TCII-receptor mediated endocytosis of cobalamin from blood.[27]

In conclusion, a TCII-receptor is present in HT 29 cell line. This receptor may be responsible for a specific endocytosis of the TCII-Cbl complex, as observed previously for other cells.

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