Volume 297, number 3, 229–232 FEBS 10674 February 1992

Receptor-mediated endocytosis of the intrinsic factor–cobalamin complex in HT 29, a human colon carcinoma cell line

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Received 4 November 1991; revised version received 12 December 1991

A HT 29 cell line derived from human colonic carcinoma was shown to express the intrinsic factor receptor, with about 5000 binding sites per cell and an association constant of 20×10^9 l/mol at pH 7.4 and 4°C. The number of binding sites increased dramatically between 7 and 10 days of culture time. Endocytosis of the intrinsic factor–cobalamin–receptor complex was inhibited by two ways: at 4°C and at 37°C by incubating the cells with vinblastine, monensin and chloroquine. The plasma membrane receptor was cross-linked to [^{57}Co]cobalamin–intrinsic factor and solubilized with Triton X-100. The cross-linked complex had a relative molecular mass of 330 kDa in native PAGE.

Intrinsic factor receptor; Endocytosis, HT 29 cell-line; Colonic carcinoma

1. INTRODUCTION

Assimilation of cobalamin (vitamin B12) requires its intraluminal binding with intrinsic factor (IF), a protein secreted by gastric parietal cells, and the subsequent binding of the intrinsic factor–cobalamin complex (IF-Cbl) to a specific receptor [1]. The receptor is located only in the distal ileum of adult humans, but it is also detected in the jejunum and in the colon of fetus, before 25 weeks of gestational age [2,3]. Recently, we have reported the synthesis of transcobalamin II and of a receptor for IF-Cbl in HT 29 cells [4,5]. HT 29 is a cell line, derived from a human colon carcinoma, which shares the same brush border enzymes than fetal cells and grows as polarized monolayers in culture [6]. Here, we have studied the physicochemical properties and the function of the IF-Cbl receptor in this cell line.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Cells were a subpopulation of HT 29 cells [7] adapted to a glucose-free medium [8] and reversed for several passages in a glucose containing medium. These cells maintained their differentiation characteristics. The cells were grown in minimum essential medium (Gibco, Paisley, Renfrewshire, Scotland, UK) supplemented with 10% (v/v) fetal calf serum and 1% antibiotics. Stock cultures were grown in 25-cm^2 flasks and placed in a 37°C incubator in a humidified CO_2/air (1:9) atmosphere.

2.2. *Binding study of [^{57}Co]Cbl-IF to cells*

The cells (6×10^4) were seeded in 25-cm^2 flasks and allowed to grow for 15 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 incubated for 15 min in the same buffer solution containing either 20 mmol/l EDTA or 0.05% bovin trypsin, washed again twice in the initial buffer solution and rested for 1 h before use. Aliquots of 10^6 cells were incubated for 1 h at 4°C with an increased amount of [^{57}Co]Cbl-IF (15–150 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 centrifugated at 700×g for 5 min and washed twice in the incubation buffer before counting 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 a Scatchard plot [9]. The ^{57}Co-labeled Cbl-IF stock solution (0.74 pmol IF-Cbl/ml) was prepared by incubating 20 µl human gastric juice (IF concentration of 37 pmol/ml) with 74 pmol cobinamide and 0.74 pmol cyano[^{57}Co]Cbl (Radiochemical Centre, Amersham, UK) in 10 mmol/l Tris-HCl buffer (pH 7.4) containing 50 mmol/l mannitol, for 15 min.

2.3. *Binding of [^{57}Co]Cbl-IF to plasma membranes*

The enriched fraction of plasma membranes was obtained according to the Ca^{2+}-aggregation method of Kessler et al. [10]. Alkaline phosphatase was determined using paranitrophenylphosphate as a substrate [11]. The protein content was assayed by the method of Bradford et al. [12]. The protein concentration and the specific activity of the enriched fraction were respectively estimated 325 µg/ml and to 22 U/mg protein. The binding of [^{57}Co]Cbl-IF to plasma membranes was studied as described above for intact cells using 200 µl of membrane fraction per test. The membranes were centrifuged at 15,000×g for 10 min and washed twice in the incubation buffer before counting the γ-radioactivity of the pellet.

2.4. *Cross-linking of [^{57}Co]Cbl-IF to plasma membranes*

150 pmol of [^{57}Co]Cbl-IF were incubated with 1 mg enriched membrane fraction in 60 mmol/l HEPES (pH 7.5) containing either 1 mmol/l $CaCl_2$ or 5 mmol/l EDTA, for 1 h at room temperature. The pellet was washed twice in the incubation buffer and incubated with 2 µmol dithiobissuccinimidyl propionate (DTSP) in one ml incubation buffer for 15 min, as described [13]. The reaction was stopped by adding 0.5 ml of 60 mmol/l ammonium acetate. The pellet was washed twice in 20 mmol/Tris-HCl buffer (pH 8.0) containing 0.7 mol/l NaCl and 5 mmol/l EDTA and resuspended in the same buffer containing

Abbreviations: IF, intrinsic factor; Cbl, cobalamin.

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1% Triton X-100. After 24 h incubation, the supernatant was run on Superose-6B (Pharmacia LKB, Uppsala, Sweden) gel filtration. The column (0.5 × 15 cm) was eluted with the Tris-HCl buffer solution described above at a flow rate of 0.1 ml/min. The eluted fractions, corresponding respectively to IF-Cbl receptor and IF-Cbl peaks, were pooled and the radioactive material was precipitated in chloroform and methanol as described [14]. After evaporation and dissolution in 50 μl distilled water, the samples were subjected to native PAGE in 4–16% gradient gels. The gels were dried and subjected to radioautography for 1 month, using X-ray OMAT plates. ^{14}C-labeled proteins were used as markers.

3. RESULTS AND DISCUSSION

A typical hyperbolic saturation curve of IF-Cbl specific binding to HT 29 cells was obtained, corresponding to about 5000 binding sites per cell and to an association constant of 20×10^9 (Fig. 1). On the other hand, a 2-fold lower number of binding sites and a same affinity were obtained with trypsin treated cells. This showed that the binding sites were accessible to extracellular proteases. The number of binding sites increased dramatically between 7 and 10 days and remained stable thereafter (Fig. 2). We have also observed such an increase, after the same culture time, for the synthesis of transcobalamin II by HT 29 cells [4]. This delay corresponded to the time needed by the cells to reach confluency [6].

Our results presented some evidences of receptor mediated endocytosis of IF-Cbl, although this mechanism was not directly demonstrated.

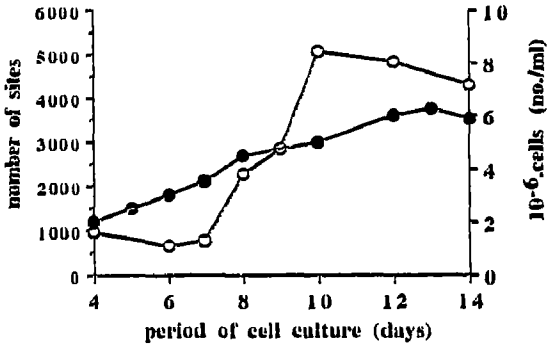

Fig. 2. IF-Cbl receptor sites (○) and HT 29 cell growth (●) as a function of culture time. Each open circle corresponds to a determination by Scatchard plot as described in Fig. 1.

The cell uptake of IF-Cbl was higher at 37°C than at 4°C (Figs. 3 and 4). Internalization of the tracer was observed only when the cells were incubated at 37°C with the tracer; it increased slowly and reached 40% of the cellular radioactivity after 60 min incubation (Fig. 3). Endocytosis of IF-Cbl has been reported in vivo in guinea pig ileum by electron microscope radioautography [15] and in vitro, in ileal isolated cells [16] although an immunocytochemical electron microscopic study failed to demonstrate it [17]. Endocytosis of IF-Cbl was inhibited by pretreating the cells for 1 h with either EDTA, vinblastine, or monensin. Monensin was

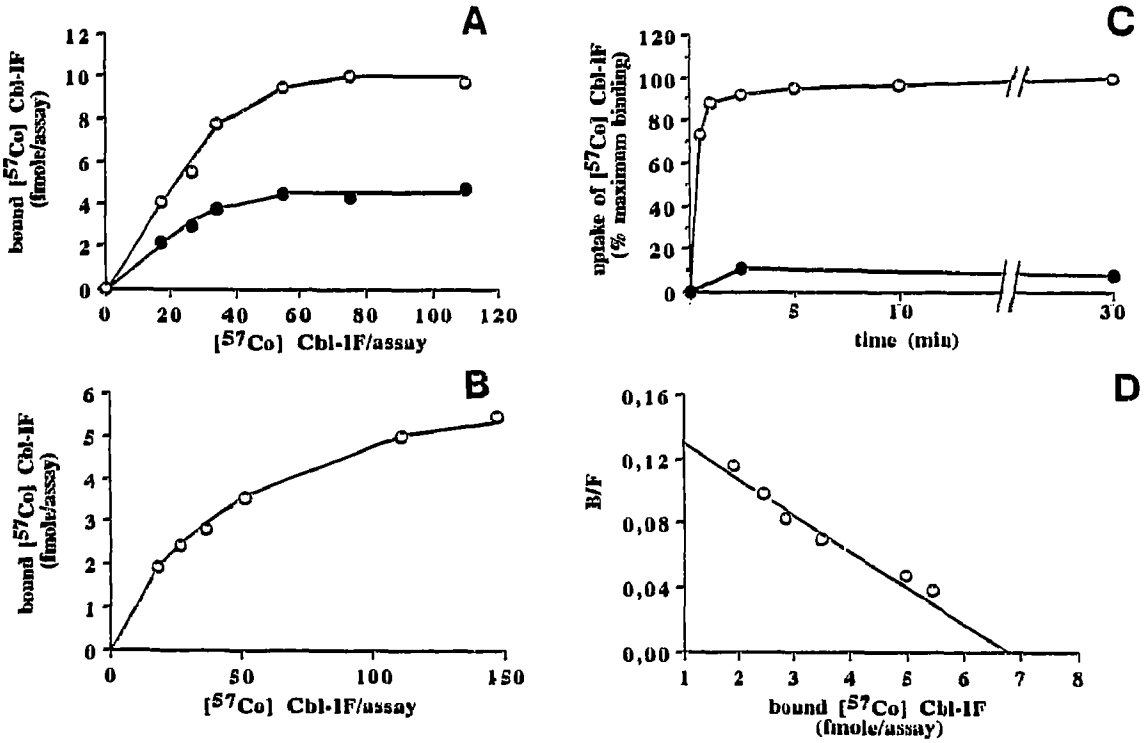

Fig. 1. Saturation curve of the binding of [^{57}Co]Cbl-IF (15–150 fmol/assay) to a suspension of HT 29 cells (A) and to an enriched preparation of HT 29 plasma membranes (65 μg protein/assay) (B) at pH 7.4, in the presence of 1 mmol/l $CaCl_2$. Two protocols for preparing the cell suspension were tested, with either 0.05% trypsin (●) or 5 mmol/l EDTA (○). C: time course of the binding of [^{57}Co]Cbl-IF to HT 29 plasma membranes in the presence of 1 mmol/l $CaCl_2$ (○) or 5 mmol/l EDTA (●). A scatchard plot of the experiment B is presented in D.

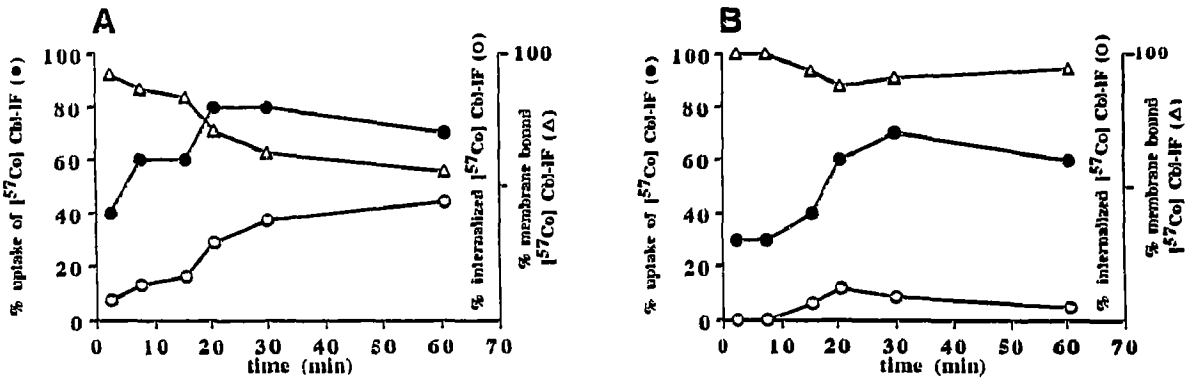

Fig. 3. Time course of the endocytosis of 295 fmol of [^{57}Co]Cbl-IF by HT 29 cells (10^7 per assay). The experiment was performed either at 37°C (A) or at 4°C (B) in 10 ml of 10 mmol/l Tris-HCl buffer (pH 7.4) containing 120 mmol/l NaCl and 1 mmol/l $CaCl_2$. Uptake of the tracer (●) corresponded to the percentage of radioactivity detected in the cell pellet, membrane binding (△) to the percentage of pellet radioactivity removed by washing the cells with 50 mol/l sodium acetate (pH 4) containing 120 mol/l sodium chloride and 5 mmol/l EDTA and internalized IF-Cbl (○) to the remaining radioactivity of washed cells. Each point is the mean of duplicated experiments.

less effective than vinblastine and vinblastine less effective than EDTA (Fig. 4). Vinblastine had the same disruptive effect than colchicine on microtubules. The effect of colchicine has already been reported elsewhere for rat kidney receptor [18]. In the present case, vinblastine could inhibit both receptor-mediated endocytosis of IF-Cbl and externalization of the insaturated receptor. The pretreatment of cells by an increasing amount of chloroquine provoked a drug-dependent inhibition of the IF-Cbl uptake. The maximum effect was observed with 10 μmol of chloroquine. It can be explained by an decrease of the cell uptake of IF-Cbl due to an inhibition of the pH-dependent dissociation of the internalized receptor from IF-Cbl since this drug increases the pH of the acidic compartment of the cell [19]. The fact that IF-Cbl binding to HT 29 membranes was inhibited at pH 5 made this explanation likely (data not shown). The pH increase can also inhibit the lysosomal enzyme activity since this activity is optimum at acidic pH [19]. Previous data have shown that chloroquine impaired assimilation of Cbl in vivo, in the mouse [20,21].

The binding of [^{57}Co]Cbl-IF to the enriched fraction of plasma membranes was maximal in about 5 min (Fig. 1). It was inhibited significantly by EDTA (Fig. 1). The affinity was similar to that obtained with intact cells and the specific activity of the IF receptor was estimated to 105 fmol/mg protein (Fig. 1). The cross-linking of [^{57}Co]Cbl-IF to the membrane receptor was inhibited by 95.6% in the presence of a 100-fold excess of non-labeled IF-Cbl. It was also inhibited by 90% when the membrane fraction and the tracer were incubated in the presence of EDTA. The cross-linked solubilized Cbl-IF– receptor complex was eluted as a high molecular mass complex, in the same elution position as Dextran blue, in superose 6 mini gel filtration (Fig. 5). Covalent binding of IF-Cbl to the receptor was attested by the absence of dissociation of the complex in the presence of EDTA. The complex was dissociated in presence of β-mercaptoethanol (Fig. 5). The IF-Cbl receptor peak

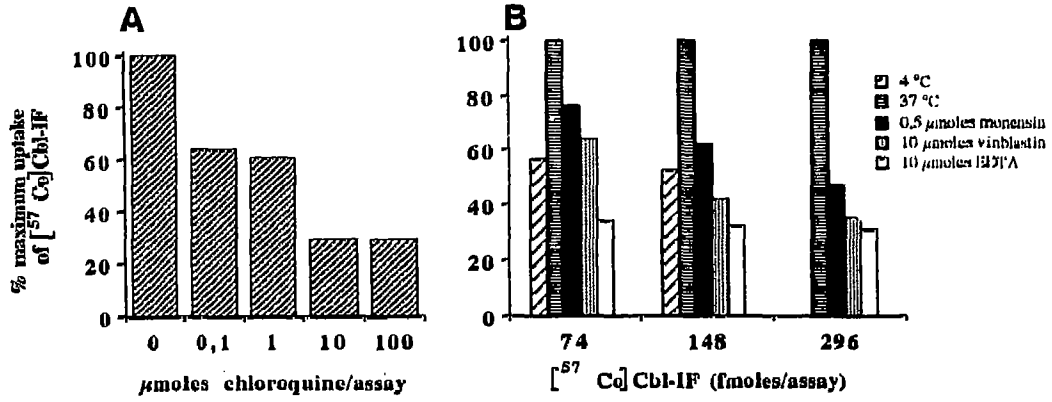

Fig. 4. The effect of increasing concentrations of chloroquine on the uptake (at 37°C for 1 h) of 295 fmol [^{57}Co]Cbl-IF by HT 29 cells (A). The uptake was also studied by incubating 10^6 cell with increasing concentrations of the tracer for 1 h either at 4°C or at 37°C after 1 h preincubation with EDTA (10 μmol/ml), monensin (0.5 μmol/ml) and vinblastine (10 μmol/ml) (B). The incubation volume was 1 ml per assay.

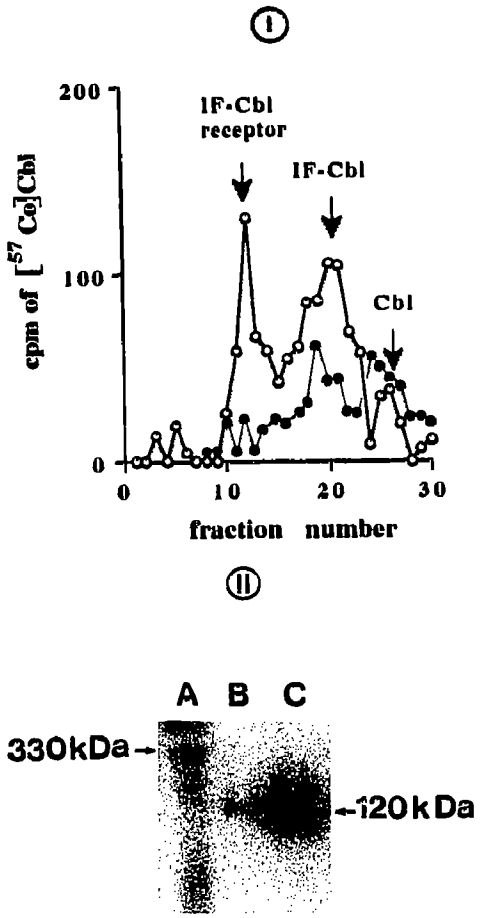

Fig. 5. Superose 6 gel filtration of the Triton X-100-solubilized holoreceptor after chemical cross-linking of [^{57}Co]Cbl-IF to the HT 29 plasma membranes. Elution was performed in the presence of 5 mmol/l EDTA with (●) or without preincubation (○) in 2.5% (vol/vol) β-mercaptoethanol. The receptor-Cbl-IF peak was eluted in the same position as Dextran blue 2000; it was dissociated by β-mercaptoethanol treatment. The [^{57}Co]Cbl-IF was eluted with a retention time of 21 min. The corresponding peaks had respective M_rs of 330 kDa and of 120 kDa in the radio-autogram of native PAGE (lanes A and B). [^{57}Co]Cbl-IF had an M_r of 120 kDa (lane C) when it was treated with cross-linking agent in absence of membrane receptor.

and the IF- Cbl peak eluted in gel filtration had respective M_rs of 330 kDa and 120 kDa in native PAGE (Fig. 5). This indicated that the IF-Cbl complex may act as a dimer, as suggested before by Gräsbeck et al. [22].

Recently, the transcytosis of Cbl bound to IF and the presence of IF receptor have been reported in $CaCo_2$ cell, another cell line which derives from colon carcinoma [23]. The ^{35}S-labeled receptor was immunoreactive to anti-dog receptor antibodies and the immunoprecipitated material had a molecular mass estimated to 230 kDa in SDS-PAGE [23]. Both HT 29 and $CaCo_2$ cells, although of colon origin, express the IF-Cbl receptor but also brush border enzymes that make them look like fetal cells [6]. The expression of IF-Cbl could therefore be due to fetal reversion since the presence of IF-Cbl receptor has been recently reported by us in the fetal colon, before 25 weeks of gestational age [2,3].

In conclusion, we have described the presence of a receptor in HT 29 cells which is functionally efficient for endocytosis of IF-Cbl. This receptor may be considered as a marker of the functional fetal properties of this cell. Our data made HT 29 a valuable cell model to prepare cDNA probes of the human IF receptor and to study the trafficking of Cbl through the intestinal epithelium.

Acknowledgements: We are grateful to Dr. A. Zweibaum (INSERM U178, Villejuif, France) who prepared and gave us the differentiated HT 29 cells adapted to a glucose-containing medium.

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