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Pamela Youngdahl-Turner, Leon E. Rosenberg

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Research Article

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Binding and Uptake of Transcobalamin II by Human Fibroblasts

PAMELA YOUNGDAHL-TURNER and LEON E. ROSENBERG, *Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510*
ROBERT H. ALLEN, *Division of Hematology-Oncology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110*

ABSTRACT We have used purified, ^{125}I-labeled human transcobalamin II (TC II), saturated with cobalamin (Cbl), to study the uptake process for the TC II-Cbl complex by intact normal cultured human skin fibroblasts. We have also investigated the possibility that a defect in one step of this process underlies that inborn error of Cbl metabolism—designated cbl C—in which mutant cells are unable to retain Cbl intracellularly or convert it to its coenzyme forms. TC II-Cbl binding at 4°C reached a plateau after 3–4 hr; 95% of the bound ^{125}I was releasable with trypsin. Binding of TC II-Cbl at 4°C could be inhibited by human and rabbit TC II-Cbl and human TC II devoid of Cbl but not by other Cbl-binding proteins, albumin, or free Cbl. Specific binding reached saturation at ≅5 ng TC II/ml (0.13 nM) and could be inhibited by ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid. At 37°C, the TC II-Cbl complex was internalized as shown by a progressive decrease in the trypsin-releasable fraction of bound ^{125}I. After 2 h at 37°C, increasing amounts of acid-soluble ^{125}I were found in the incubation medium indicating that the labeled TC II was being degraded. Chloroquine, an inhibitor of lysosomal proteolysis, prevented this degradation. The binding, internalization, and degradation of TC II-Cbl by cbl C cells was indistinguishable from that by control cells. Our studies provide additional support for the concepts: (*a*) that the TC II-Cbl complex binds to a specific cell surface receptor through a site on the TC II; (*b*) that the interaction between the receptor and TC II is calcium dependent; (*c*) that the TC II-Cbl is internalized via endocytosis; (*d*) that the degradation of TC II and release of Cbl from the complex occurs in lysosomes. We also conclude that the defect in cbl C must reside at some step beyond this receptor-mediated uptake process.

INTRODUCTION

Transcobalamin II (TC II)[1] is the primary cobalamin (Cbl; vitamin B_{12}) transport protein found in the plasma of man and other mammals. It has been shown to facilitate Cbl uptake by a variety of tissues in vivo and by various cell types in vitro (1–3). Several different lines of experimentation using radioactive Cbl bound to unlabeled TC II have been employed to study the Cbl uptake process. Cellular uptake studies have suggested that the TC II-Cbl binds to the cell surface before the internalization of the Cbl (4, 5). A specific receptor for the TC II-Cbl complex in rat liver and human placental membranes has been identified and is assumed to mediate this TC II-Cbl binding (6, 7). Based on sub-cellular fractionation studies after intravenous injection of Cbl in rats, Newmark et al. (8) and Pletsch and Coffey (9) proposed that the TC II-Cbl complex then enters the cell via endocytosis and is transiently localized in secondary lysosomes where the TC II is degraded and the Cbl is released into the cytoplasm. Subsequently, Cbl is converted to its coenzyme forms, adenosylcobalamin and methylcobalamin. Because of a lack of a label on the protein moiety of the TC II-Cbl complex, the evidence for internalization and degradation of the TC II has been indirect, although recent in vivo studies using [^{57}Co]Cbl bound to ^{125}I-labeled TC II (10) have shown that there is

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Dr. Allen's current address is Division of Hematology, University of Colorado Medical School, Denver, Colo.

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[1] *Abbreviations used in this paper*: Cbl, cobalamin; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; PBS, phosphate-buffered saline; TC II, transcobalamin II.

initially a coordinate clearance of both labels from rabbit plasma by a variety of tissues and that the TC II is rapidly degraded in this process.

Fibroblasts from patients expressing the cbl C mutation are deficient in the synthesis of both Cbl coenzymes, adenosylcobalamin and methycobalamin (11, 12). Previous studies have shown that whereas the initial uptake of labeled Cbl bound to TC II appeared to be comparable in cbl C and control cells, the cbl C cells failed to retain Cbl and bind it to an intracellular protein or proteins (13) which probably represent the two Cbl-dependent apoenzymes: methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carbonyl-mutase, EC 5.4.99.2) and N^5-methyltetrahydrofolate:homocysteine methyltransferase (5 - methyltetrahydropteroyl - L - glutamate:L-homocysteine S-methyltransferase, EC 2.1.1.13) (14, 15). Because these studies did not distinguish between cell surface binding and internalization, they did not exclude the possibility that the underlying defect in the cbl C cells resides in some process mediating the uptake of TC II-Cbl or the sub-cellular distribution of free Cbl.

This paper presents additional studies of the TC II-Cbl uptake process in normal cultured human skin fibroblasts. We have used purified ^{125}I-labeled human TC II saturated with Cbl (^{125}I-TC II) to investigate directly the binding, uptake, and site of degradation of the complex. We have also studied cbl C cells to test the hypothesis that a defect in one step of the uptake process could lead to the failure of the mutant cells to retain Cbl and convert it to its coenzyme forms.

METHODS

Materials. Tissue culture medium was purchased from Grand Island Biological Co., Grand Island, N. Y., except the fetal calf serum, which was obtained from Flow Laboratories, Inc., Rockville, Md. Hepes was obtained from Sigma Chemical Co., St. Louis, Mo. and Calbiochem, San Diego, Calif. Chloroquine, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), and bovine serum albumin were purchased from Sigma Chemical Co.

Human TC II was purified from normal human plasma and labeled with ^{125}I as described (10, 16). The labeled protein contained 0.2–0.4 mol of iodine/mol of TC II. It was stored at −25°C in 1.0 M NaCl containing 0.01 M Tris/HCl, pH 8.0, and 0.05 mg bovine serum albumin/ml. To remove free iodine which slowly eluted from the protein during prolonged storage, aliquots were thawed and dialyzed overnight against two changes of 100 vol of 1.0 M NaCl plus 0.01 M Tris/HCl, pH 8.0. The dialysis tubing and all glassware which came into contact with the TC II were soaked in the same buffer containing bovine serum albumin before use. The ^{125}I-TC II was used within 1 mo after labeling or dialysis. All experiments were done with ^{125}I-TC II saturated with cyanocobalamin. For some experiments, the ^{125}I-TC II was also labeled with cyano[^{57}Co]cobalamin([^{57}Co]Cbl)(sp act 22 μCi/nmol).

The unlabeled, purified human and rabbit TC II (10), and granulocyte R-binder (17) were stored at −25°C in 0.75 M NaCl containing 0.05 M potassium-phosphate, pH 7.5.

Cell culture. Six human diploid skin fibroblast lines were used: three from controls (lines 82, 83, 87) and three from cbl C mutants (78, 178, 287). Cells were studied between their 9th and 27th passage in culture, except line 178 which was between passage 28 and 39. Growth medium was Eagle's minimal essential medium supplemented with 1% (vol/vol) nonessential amino acids, 10% (vol/vol) fetal calf serum, and 100 μg/ml of kanamycin. Stock cultures were maintained at 37°C in 32-oz glass bottles which were tightly capped after being gassed with 5% CO_2/95% air. For binding and uptake experiments, cells were harvested from confluent monolayers with trypsin and plated onto 100-mm plastic tissue culture dishes at 7.8–8.8 × 10^5 cells/dish. The dishes were incubated at 37°C in a humidified, 5% CO_2/95% air atmosphere. Experiments were done on the 4th day after plating when the cells had been confluent for 1 day.

Binding and uptake of TC II. Studies at 4°C were carried out with the dishes on ice in a cold room. The cells were incubated for 15 min in growth medium to bring the temperature to 4°C. The medium was aspirated, the cells were washed twice with 5 ml of cold Dulbecco's phosphate-buffered saline (PBS), and 6 ml of incubation medium was added. The incubation medium was identical to the growth medium except that it contained 10 mM Hepes, pH 7.4, and the ^{125}I-TC II. Fetal calf serum has no detectable TC II as measured by saturation of the serum with [^{57}Co]Cbl and chromatography on Sephadex G-150.[2] After incubation, aliquots of medium were assayed for ^{125}I, and the remainder was aspirated. In most experiments, the cells were washed four times with 5 ml PBS at 4°C and then were brought to room temperature. 1 ml of 0.2 N NaOH was added to the dish, and the monolayer was dissolved. The solubilized cells were transferred to a plastic counting tube, and the dish was washed twice using 1 ml of 0.2 N NaOH. After counting ^{125}I in a Packard automatic gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.), an aliquot was taken for determination of cell protein by the method of Lowry et al. (18). In those experiments designed to distinguish cell surface radioactivity from internalized radioactivity, the cells were harvested by adding 1 ml of 0.125% trypsin in Dulbecco's calcium- and magnesium-free PBS after washing. The dishes were incubated at 37°C until the cells were floating. The cell suspension was transferred to a 15-ml plastic conical centrifuge tube, and the dish was washed twice with cold growth medium. The cell suspension was centrifuged at 1,100 *g* for 5 min at 4°C. The trypsin supernate was removed and counted; the cell pellet was dissolved in 0.2 N NaOH, counted, and then the cell protein determined.

Studies at 37°C were carried out in an identical manner except that the 15-min incubation for temperature equilibration was omitted. A humidified, CO_2-incubator was used for the incubation. Binding and uptake of TC II are defined as the amount of ^{125}I associated with the cells at 4° and 37°C, respectively.

Preparation of cell extracts by sonication and Sephadex G-150 chromatography of cell extracts were carried out as described (13).

Degradation of TC II. After incubation of the ^{125}I-TC II with cells, 1 ml of the medium was transferred to a conical centrifuge tube and 0.25 ml of 50% trichloroacetic acid was

[2] Mellman, I. S., P. Youngdahl-Turner, and L. E. Rosenberg. Unpublished observations.

added. After incubation for at least 30 min at 4°C, the precipitate was removed by centrifugation at 600 *g* for 15 min. The supernate was removed and counted; a blank value was determined by incubating medium in dishes without cells under the same conditions. Degradation of TC II is defined as the total amount of acid-soluble ^{125}I formed in the presence of cells less the amount formed in the absence of cells. The amount of free iodine in the acid-soluble ^{125}I was determined by chloroform extraction according to Goldstein and Brown (19).

RESULTS

Effect of iodination on TC II function. To determine if the iodination procedure alters or interferes with the function of TC II in our experimental system, we compared the uptake of unlabeled TC II saturated with [^{57}Co]Cbl (TC II-[^{57}Co]Cbl) to that of ^{125}I-labeled TC II also saturated with [^{57}Co]Cbl (^{125}I-TC II-[^{57}Co]Cbl). Uptake of the ^{57}Co label by control fibroblasts at 37°C was followed for 6 h. As shown in Fig. 1, the uptake of [^{57}Co]Cbl bound to iodinated TC II was ≅70% of that bound to unlabeled TC II. The uptake of free [^{57}Co]Cbl was ≅4% of that bound to unlabeled TC II (data not shown). After a 6-h incubation with either the single- or double-labeled TC II, cell extracts were prepared and chromatographed on Sephadex G-150 (Fig. 2). The elution pattern of the ^{57}Co label was almost identical in both cases. Each showed a major peak of radioactivity which contains the two Cbl-dependent apoenzymes,[3] and minor peaks at the void volume and regions corresponding to TC II and free Cbl. Thus, the iodinated TC II retained its functional role in the delivery of Cbl to the cell for subsequent metabolism and association with the Cbl-dependent apoenzymes even though its uptake

[3] Mellman, I. S., H. F. Willard, and L. E. Rosenberg. Manuscript in preparation.

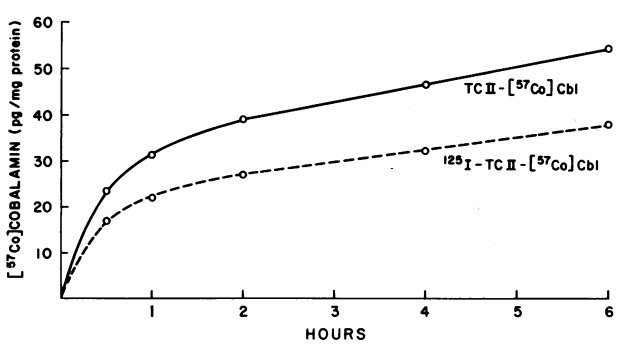

FIGURE 1 Uptake of [^{57}Co]Cbl bound to unlabeled or ^{125}I-labeled TC II by human fibroblast monolayers. Control cells were incubated at 37°C with TC II-[^{57}Co]Cbl or ^{125}I-TC II-[^{57}Co]Cbl at 2.8 ng TC II/ml. At the times indicated, the cells were washed, solubilized in NaOH, and then counted. A representative experiment is shown; points are the mean of duplicate determinations.

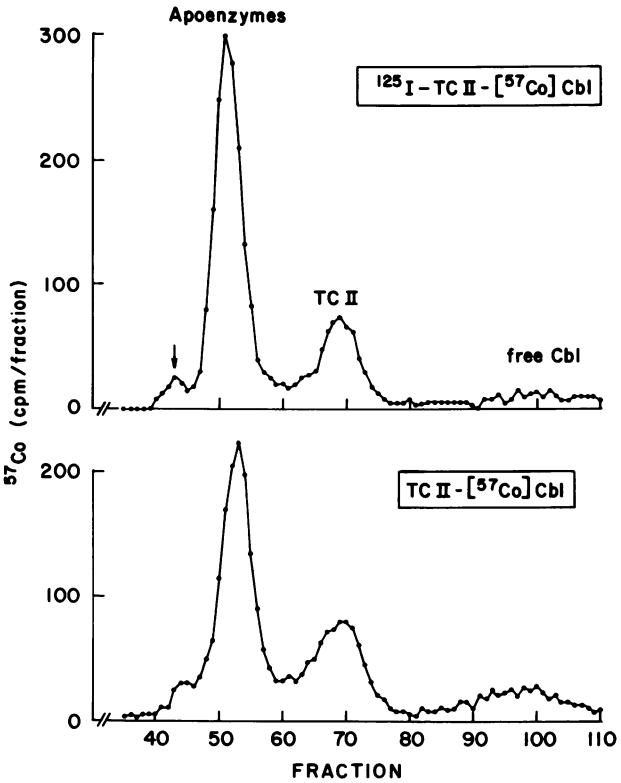

FIGURE 2 Sephadex G-150 elution pattern of ^{57}Co in fibroblast extracts. Control cells were incubated at 37°C with TC II-[^{57}Co]Cbl or ^{125}I-TC II-[^{57}Co]Cbl at 2.8 ng TC II/ml for 6 h. Cell extracts were then prepared by scraping the cells into 0.15 M NaCl containing 50 mM potassium phosphate, pH 7.4. After sonication, the extract was centrifuged for 15 min at 40,000 *g*, loaded onto a Sephadex G-150 column (1.6 × 100 cm) and eluted with the same buffer. 1.85-ml fractions were collected and counted for ^{57}Co radioactivity. The arrow indicates the void volume. See Rosenberg et al. (13) for details.

was less efficient than that of noniodinated TC II. Schneider et al. (10) earlier presented evidence that the iodinated TC II retains its function in their in vivo studies.

Kinetics of ^{125}I-TC II binding. Binding studies were carried out at 4°C to minimize endocytosis and, therefore, internalization of the protein (20). A concentration of 2.5 ng of TC II protein/ml of medium (0.07 nM) was used to approximate plasma TC II-Cbl concentration. When three different control fibroblast lines were incubated with ^{125}I-TC II for up to 6 h, the results shown in Fig. 3 were obtained. Total binding of TC II to the cells increased for 3–4 h before reaching a plateau. At the longest interval examined, 2% of the medium TC II was bound to cells. Little difference between control lines was observed.

Specificity of ^{125}I-TC II binding. The specificity of TC II binding was tested by measuring the binding of ^{125}I-TC II at 4°C for 4 h in the presence of various

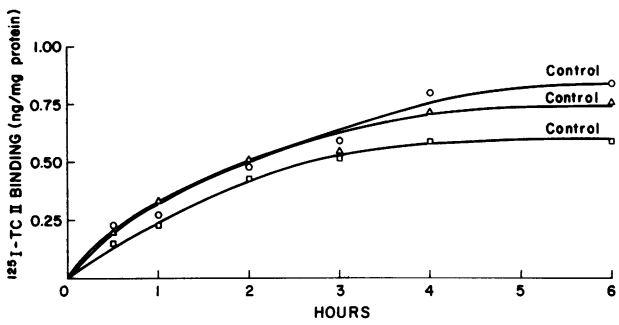

FIGURE 3 Binding of ^{125}I-TC II to control fibroblast monolayers as a function of time. Cells were incubated at 4°C for the times indicated with ^{125}I-TC II at 2.5 ng/ml. Representative experiments for three different control cell lines are shown; points are the mean of duplicate determinations.

unlabeled potential competitors (Fig. 4). The concentration of ^{125}I-TC II saturated with Cbl, i.e. holo-TC II, was constant, and a 5- to 100-fold excess of the competitor was added. Unlabeled human holo-TC II inhibited ^{125}I-TC II binding by 80% maximally, implying that only 20% of the observed binding is nonspecific. Rabbit holo-TC II also inhibited binding markedly (Fig. 4A). At low concentration ratios, it was a less effective inhibitor than holo-TC II. In fact, three times as much rabbit holo-TC II was required to yield half-maximum inhibition as observed with human holo-TC II. This suggests that rabbit TC II has a lower affinity for the human TC II receptor than does its human counterpart. Another human Cbl-binding protein, granulocyte R-binder, as well as ovalbumin and bovine albumin were tested as inhibitors of TC II binding. No inhibition of binding was observed with any of these proteins, even when they were added at a 500-fold excess. These data indicate that the cell surface receptor is specific for TC II.

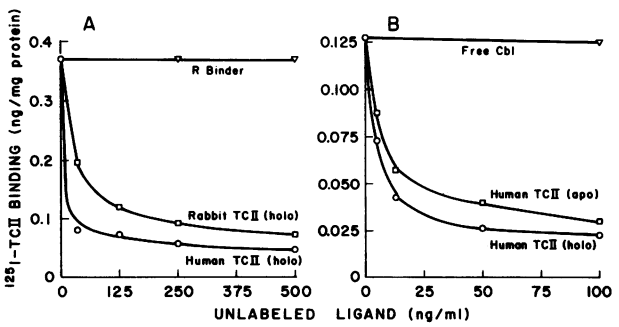

FIGURE 4 Inhibition of ^{125}I-TC II binding by unlabeled ligands. The ^{125}I-TC II and unlabeled ligands were added to control cells at the same time, and incubated for 4 h at 4°C. The data are the mean of duplicate observations in representative experiments. (A) Effect of human and rabbit holo-TC II and granulocyte R-binder on ^{125}I-TC II (2.5 ng/ml) binding. (B) Effect of human holo- and apo-TC II and free Cbl on ^{125}I-TC II (1.0 ng/ml) binding.

We used human apo-TC II, that is, TC II devoid of cobalamin, to investigate the nature of the recognition site on the TC II-Cbl complex. As shown in Fig. 4B, apo-TC II inhibited binding maximally by 77%. Its apparent affinity for the TC II receptor was approximately half that of holo-TC II. This might be due to conformational differences between apo- and holo-TC II, but it could also reflect a reduced stability of apo-TC II to freezing and thawing (21). Free Cbl did not inhibit the binding at molar concentration ratios up to 30,000. These results indicate that the cell surface receptor recognizes a site on the TC II moiety of the complex.

Saturability of ^{125}I-TC II binding. Fig. 5 shows the binding of TC II to control cells as a function of TC II concentration. Because we had shown that there is significant nonspecific binding of the ^{125}I-TC II, parallel sets of cells were used to correct for this. In one set, total binding of ^{125}I-TC II to the cells was measured; in the second set, binding of ^{125}I-TC II in the presence of a large excess of unlabeled TC II was determined. This binding in the presence of unlabeled TC II is defined as nonspecific binding; it is a nonsaturable process and is a linear function of TC II concentration. Specific binding is derived by subtracting the nonspecific binding from the total binding and, as shown in Fig. 5, is saturable. The amount of nonspecific and total binding varied between experiments; the specific binding curve was reproducible between experiments and among different cell lines. From the plateau of the specific binding curve, we estimate that there are 3,000–4,000 specific binding sites/cell. From

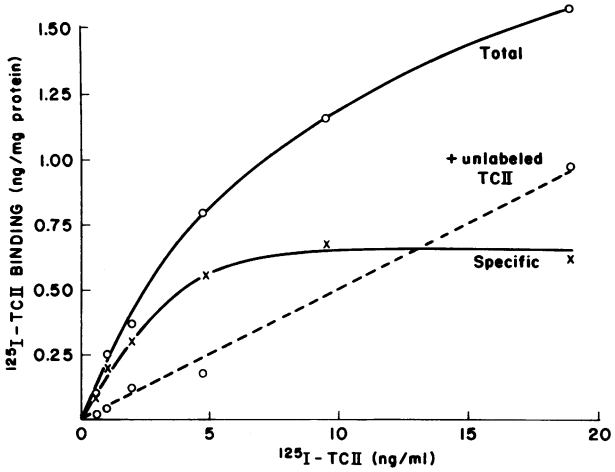

FIGURE 5 Binding of ^{125}I-TC II as a function of TC II concentration. Control cells were incubated at 4°C for 4 h with ^{125}I-TC II at the indicated concentrations. Unlabeled human holo-TC II at 475 ng/ml was added at the same time as the labeled TC II. Data from a representative experiment is shown; points are the mean of duplicate determinations. See text for details.

TABLE I
Effect of Calcium on ^{125}I-TC II Binding by Fibroblast Monolayers

Conditions	Inhibition of total binding
	%
Calcium-free medium	0
+ EGTA (2 mM)	65
+ Unlabeled TC II (250 ng/ml)	69
+ EGTA (2 mM) and unlabeled TC II (250 ng/ml)	72

Control cells were preincubated at 4°C for 15 min and washed with Dulbecco's calcium- and magnesium-free PBS. Calcium-free medium (modified Eagle's medium with Earle's spinner salts—Grand Island Biological Co.) containing 10% dialyzed fetal calf serum, 10 mM Hepes and indicated inhibitors was added. 15 min later, ^{125}I-TC II was added to a final concentration of 2 ng/ml. After incubation for 1 h, the cells were washed with calcium- and magnesium-free PBS, solubilized, and then counted. The data are the average of duplicate determinations in two experiments.

the concentration of TC II giving half-maximal specific binding, we derive a dissociation constant of ≅50 pM.

Role of calcium in ^{125}I-TC II binding. It has been shown in other cell studies that calcium plays a role in the interaction between the TC II-Cbl complex and the cell surface (4, 7). Table I shows the results of experiments testing the role of calcium in this system. Simply assaying TC II binding in calcium-free medium did not inhibit total binding. Adding EGTA, a specific calcium chelator, inhibited binding by 65%, a value very similar to that observed with an excess of unlabeled TC II. Addition of both EGTA and unlabeled TC II inhibited binding no more than either agent alone indicating that EGTA inhibits only specific binding and implying that calcium plays a role in the specific binding of TC II to the cell surface receptor.

Uptake of ^{125}I-TC II. At 37°C the uptake and metabolism of labeled TC II differed considerably from that observed at 4°C (Fig. 6). At 4°C, binding of ^{125}I-TC II to the cells was relatively slow, and no acid-soluble label was found in the medium over the 6-h incubation (Fig. 6B). At least 95% of the label was releasable by trypsin (Fig. 6A), suggesting that the association of TC II with the cells at 4°C reflects only cell surface binding. At 37°C, on the other hand, the association of labeled TC II with the cells was more rapid, reaching a plateau by 2 h (Fig. 6D). This association represents both binding to the cell surface and internalization of the TC II, as shown by a rapid and

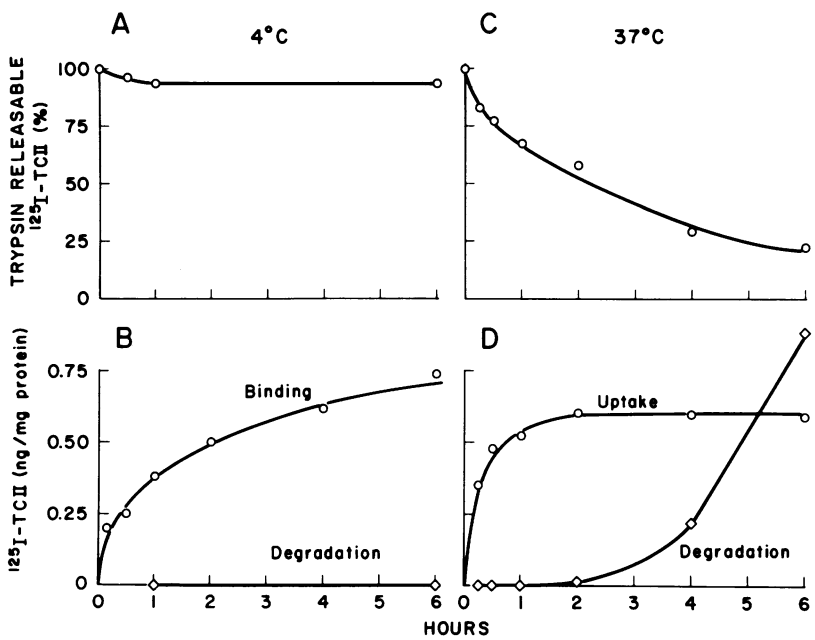

FIGURE 6 Interaction of ^{125}I-TC II with control fibroblast monolayers at 4° and 37°C. Cells were incubated with 2.5 ng ^{125}I-TC II/ml. At the times indicated, cells were harvested with trypsin. Binding and uptake are the amount of label in the trypsin supernate plus the cell pellet at 4° and 37°C, respectively. Degradation is the cell-dependent increase of acid-soluble label found in the medium. The trypsin-releasable TC II is the percent of ^{125}I-TC II associated with the cells (including that degraded by the cells) found in the trypsin supernate. A representative experiment is shown; points are the mean of duplicate determinations. (A) Trypsin-releasable ^{125}I-TC II bound to the cells at 4°C. (B) Binding and degradation of ^{125}I-TC II at 4°C. (C) Trypsin-releasable ^{125}I-TC II bound to the cells at 37°C. (D) Uptake and degradation of ^{125}I-TC II at 37°C.

significant decrease in the percentage of label which can be released from the cells by trypsin (Fig. 6C).

Degradation of ^{125}I-TC II. A second process that occurs at 37°C is cell-mediated degradation of the ^{125}I-TC II. After a lag of ≅2 h, an increasing amount of acid-soluble material was found in the medium (Fig. 6D). This acid-soluble ^{125}I was not extracted into chloroform and was, therefore, probably still bound to amino acids. We interpret the presence of this cell-dependent acid-soluble material as evidence that the TC II has been taken up by cells and then degraded to amino acids or small peptides which subsequently appear in the medium.

The lysosomotropic agent chloroquine was used to investigate the nature of the cellular degradation of TC II. Chloroquine has been shown to accumulate in lysosomes of cultured cells and to inhibit lysosomal degradative process in vivo (22). Chloroquine had no effect on the binding of ^{125}I-TC II to cells at 4°C (data not shown). The effect of 50 μM chloroquine on the kinetics of ^{125}I-TC II uptake and degradation is shown in Fig. 7. Under these conditions virtually no degradation of TC II occurred. Chloroquine had no effect on the initial uptake of TC II by cells. The apparent increase in uptake at later times can be accounted for by the absence of release of ^{125}I-TC II degradation product from chloroquin-treated cells, which appeared heavily vacuolated due to accumulation of lysosomes. These data suggest that TC II is localized within lysosomes after internalization and that its degradation is dependent on lysosomal proteases.

cbl C Mutants. We have compared the binding, uptake, and degradation of ^{125}I-TC II in cbl C lines with that in control cells. As shown in Fig. 8, we observed no consistent difference in the kinetics of total binding of labeled TC II to mutant cells. Furthermore, no significant difference was observed in the shape or plateau of the specific binding curves determined in

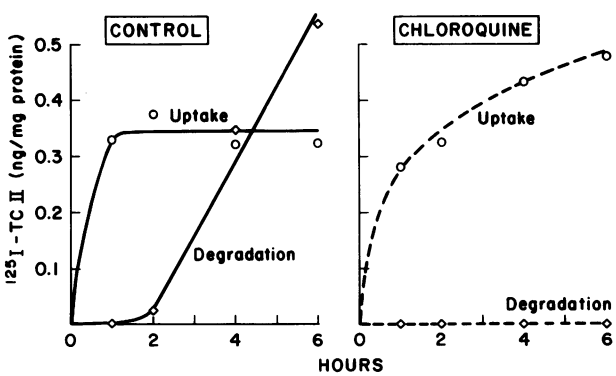

FIGURE 7 Effect of chloroquine on the kinetics of ^{125}I-TC II uptake and degradation by fibroblast monolayers. (Left): Control cells were incubated with 2.5 ng ^{125}I-TC II/ml at 37°C. (Right): Control cells were preincubated with 50 μM chloroquine for 1 h at 37°C. After washing several times with Dulbecco's PBS, medium containing 2.5 ng ^{125}I-TC II/ml and 50 μM chloroquine was added. At the times indicated, the uptake and degradation of TC II was determined. A representative experiment is shown; points are the mean of duplicate determinations.

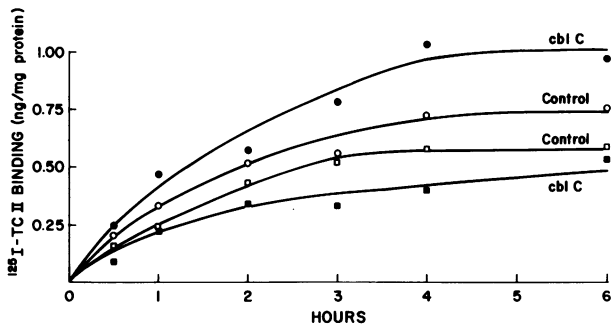

FIGURE 8 Kinetics of ^{125}I-TC II binding to cbl C and control cells at 4°C. Cells were incubated with 2.5 ng ^{125}I-TC II/ml at 4°C. Representative experiments for two control and two cbl C lines are shown; points are the mean of duplicate determinations.

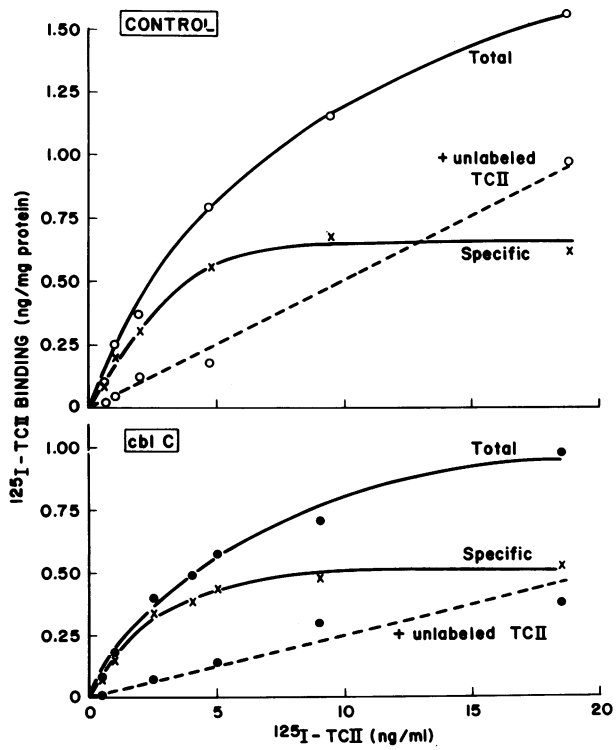

FIGURE 9 ^{125}I-TC II binding to cbl C and control cells at 4°C as a function of TC II concentration. Cells were incubated at 4°C for 4 h with ^{125}I-TC II at the indicated concentrations. Unlabeled holo-human TC II at 474 ng/ml was added at the same time as the labeled TC II. A representative experiment for a cbl C and control line are shown; points are the mean of duplicate determinations.

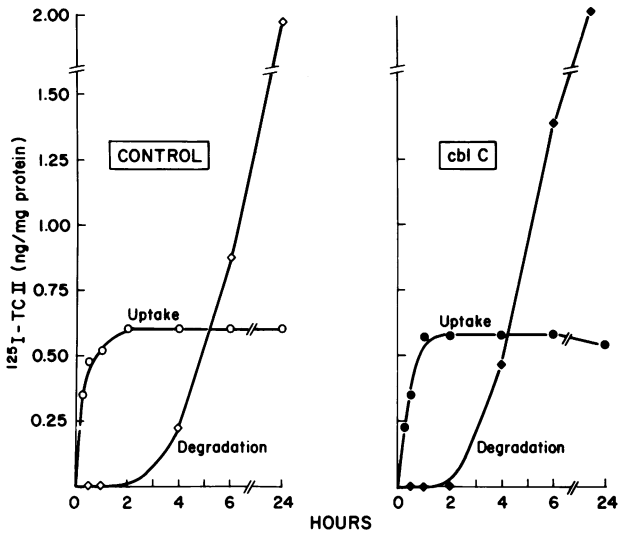

FIGURE 10 Kinetics of ^{125}I-TC II uptake and degradation in cbl C and control cells at 37°C. Cells were incubated with 2.5 ng ^{125}I-TC II/ml at 37°C. Representative experiments are shown; points are the mean of duplicate determinations.

control and cbl C lines (Fig. 9). Finally, no difference in ^{125}I-TC II uptake and degradation at 37°C was found when mutant and control cells were compared (Fig. 10).

DISCUSSION

The results presented in this paper are consistent with the model shown in Fig. 11. We have demonstrated that there is a specific receptor for TC II-Cbl on the surface of cultured human skin fibroblasts. This receptor resembles that found in human placental membrane preparations (7) in that it exhibits high affinity for the TC II-Cbl complex, somewhat reduced affinity for apo-TC II, and no affinity for free Cbl. The data presented by Fiedler-Nagy et al. (6) also suggest that rat liver membrane preparations have a high affinity binding site for the TC II-Cbl complex which also recognizes apo-TC II. Thus, evidence from all three systems indicates that the TC II-Cbl complex binds to a high affinity receptor through a site on TC II. Moreover, we have found that the interaction between TC II and the human fibroblast receptor is dependent on calcium, an observation in accord with those made using the placental membrane system and other intact cells (4, 5, 23).

Once bound to a cell surface receptor, the TC II-Cbl complex could theoretically be metabolized in two different ways. The complex could be dissociated at the cell surface and its individual components (TC II and Cbl) metabolized independently thereafter. Alternatively the intact complex could enter the cell and be dissociated subsequently. Past and present data strongly favor the latter possibility. Pletsch and Coffey (24) identified the TC II-Cbl complex in lysosomal fractions of rat hepatic parenchymal cells. Schneider et al. (10) noted coordinate clearance of doubly labeled TC II-Cbl from rabbit plasma into several tissues. Our present data, also, indicate that a process conforming to adsorptive endocytosis (20) is responsible for internalization of the TC II-Cbl complex, and its subsequent localization in lysosomes.

From our data we conclude that it is also within lysosomes that TC II is degraded. We measured TC II degradation by following the appearance of acid-soluble ^{125}I in the medium. Such acid-soluble radioactivity was not found at 4°C or in the absence of viable cells, and it did not reflect trivial elution of iodide from the labeled protein. Rather, acid-soluble ^{125}I appeared only after a distinct time lag at 37°C, as would be expected if uptake of the TC II-Cbl complex by the cell was a necessary precedent for its degradation. Furthermore, chloroquine, a known inhibitor of lysosomal proteolysis (22), inhibited TC II degradation markedly. Finally, preliminary experiments from our laboratory show that chloroquine also inhibits the release of [^{57}Co]Cbl from the TC II-Cbl

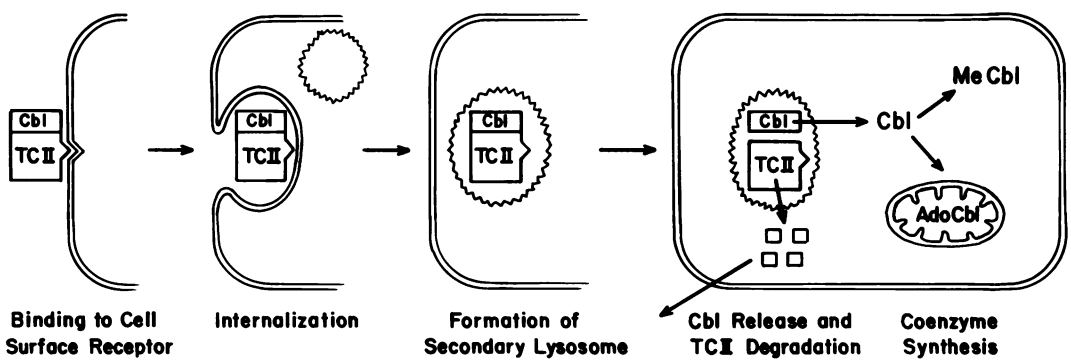

FIGURE 11 Schematic representation of TC II binding, uptake, and degradation by intact human fibroblasts. See discussion for details. AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin.

complex.[4] All of these results support the contention that TC II is degraded to free amino acids within lysosomes, that these amino acids then leave the lysosome (and appear as ^{125}I-labeled amino acids in experiments like ours) and that such degradation is a necessary precondition for release of Cbl from the lysosome.

The scheme we and others have presented for the binding, uptake, and lysosomal degradation of TC II is similar to the mechanism proposed for the uptake of some other proteins. Ashwell and Morell (25) have demonstrated the existence of an asialoglycoprotein receptor which mediates the internalization and lysosomal catabolism of asialoglycoproteins and the granulocyte Cbl-binding protein (17) by hepatic parenchymal cells. Goldstein and Brown (26) have defined an analogous pathway in their extensive studies of the binding, uptake and degradation of low density lipoprotein by human fibroblasts. Low density lipoprotein uptake is, in another respect, functionally analogous to TC II uptake in that both proteins serve as carriers mediating the delivery of cell nutrients, cholesterol, and Cbl, respectively. Although the low density lipoprotein receptor appears to be under feedback regulation dependent on intracellular cholesterol content, preliminary results from our laboratory indicate that TC II receptor availability is not modulated by prior exposure to TC II or free Cbl and thus may not be involved in a similar regulatory mechanism.[2]

The data with respect to the cbl C mutants show that these cells are comparable to control cells in their binding, uptake, and degradation of TC II. This evidence leads us to conclude that the underlying defect in the cbl C cells must reside at some step beyond the receptor-mediated uptake of Cbl. Although the formal possibility remains that the Cbl is not released from lysosomes to the cytoplasm in these cells, this seems unlikely because the lysosomal degradation of TC II is completely normal and because the total Cbl content of cbl C cells is reduced, not increased. We have postulated that cbl C cells lack an intracellular cobalamin-binding protein (13). Although we now feel that this protein is not a unique binder but rather represents the Cbl-dependent apoenzymes, N^5-methyltetrahydrofolate:homocysteine methyltransferase and methylmalonyl-CoA mutase (14, 15), the fact remains that these cells lack an intracellular Cbl-binding activity. We are currently investigating the possibility that the cbl C cells have a primary defect in one of the Cbl-dependent apoenzymes whose role in intracellular Cbl metabolism involves binding and cellular retention as well as enzymatic catalysis. Alternatively, some as yet undefined step in Cbl activation which is necessary for Cbl binding to the above names apoenzymes may be defective in the mutant cells.

[4] Youngdahl-Turner, P., R. H. Allen, and L. E. Rosenberg. Unpublished observations.

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