THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 14, Issue of April 7, pp. 8152–8157, 1995
Printed in U.S.A.

Membrane Expression and Interactions of Human Transcobalamin II Receptor*

(Received for publication, July 11, 1994, and in revised form, January 27, 1995)

Santanu Bose‡, Shakuntla Seetharam§, and Bellur Seetharam‡§¶

From the Departments of ‡Biochemistry and §Medicine, Medical College of Wisconsin and Veterans Administration Medical Center, Milwaukee, Wisconsin 53226

Antiserum raised to purified 62-kDa human placental transcobalamin II receptor (TC II-R) has been used to study its synthesis and membrane expression. The antiserum immunoprecipitated a 45-kDa protein from the cell-free translation using human kidney mRNA and recognized a single 124-kDa band on immunoblotting of placental and other human tissue membranes, and quantitation of the blots revealed high levels of TC II-R expression in the human kidney followed by placenta, intestine, and liver. Triton X-100 extraction of placental membranes resulted in the complete (100%) solubilization of the receptor, and immunoblotting of the Triton X-100-soluble fraction revealed a single band of 62 kDa. Lipid extraction of placental membranes with a mixture of chloroform:methanol (2:1) followed by immunoblotting revealed a single band of molecular mass 62 kDa. The molecular mass of the pure Triton X-100-bound receptor increased on SDS-polyacrylamide gel electrophoresis from 62 to 124 kDa upon its insertion in liposomes prepared using egg phosphatidylcholine and cholesterol. Chemical cross-linking of native membrane- or lipid vesicle-bound TC II-R or detergent-soluble extracts of the membrane with ^{125}I-TC II-cobalamin revealed that both the 124- and 62-kDa forms of the receptor were active in ligand binding. Based on these results we suggest that TC II-R is synthesized as a single polypeptide of 45 kDa, and following its maturation (involving *N*- and *O*-glycosylation) the 62-kDa mature receptor is expressed in plasma membranes as a noncovalent dimer of 124 kDa. The dimerization of TC II-R in the plasma membranes is due to its interactions with annular lipids.

The cellular delivery of cobalamin (Cbl[1]: vitamin B_{12}) is mediated by transcobalamin II (TC II), a 45 kDa non-glycoprotein plasma transporter of Cbl (1). Cobalamin complexed to TC II is endocytosed by a specific plasma membrane receptor (TC II-R) (2). The high affinity saturable binding of TC II·Cbl complex to plasma membranes of liver (3), kidney (4, 5), and placenta (6, 7) has been demonstrated, suggesting that the TC II-R is expressed in many tissues. A highly pure TC II-R from human placenta has been obtained and reported to be a 50–55-kDa glycoprotein containing about 33% carbohydrate (8) based on sucrose density centrifugation and amino sugar analysis. There are reports (9–11) based on animal studies that kidney avidly takes up more TC II·Cbl than other tissues, including liver. These studies have suggested the possibility that the renal uptake of Cbl bound to TC II occurs either directly from the circulation or indirectly via passage through liver and thus may be the most important organ to take up Cbl bound to TC II. It has been suggested that the kidney in many species acts as the storage organ of Cbl (5, 12). Cobalamin stored in the kidney is thought to be delivered back into the circulation in times oftissue need and excreted in urine once the renal threshold of storage is reached (13, 14). The bi-directional movement of Cbl in the kidney is thought to be mediated by TC II and in support of this hypothesis are our recent findings demonstrating the secretion of TC II in both apical and basolateral directions in renal epithelial cells (15). Despite the importance of TC II-R in maintaining Cbl flux across plasma membranes by mediating the uptake and tissue exchange of Cbl, the details of its synthesis and membrane expression TC II-R in human is not known. The current work was undertaken to address these issues.

The results of the current study show that human TC II-R is synthesized as a single polypeptide of molecular mass 45 kDa, glycosylated to contain both *N*- and *O*-linked sugars and the resulting mature receptor of molecular mass 62 kDa is expressed in the plasma membranes as a functional noncovalent dimer. Furthermore our results also show that the dimerization of TC II-R is due to its interactions in the membrane with annular lipids.

MATERIALS AND METHODS

Human placenta was obtained from unidentified donors who had normal vaginal delivery at the Doyne Hospital, Milwaukee, WI. The placenta was washed with normal saline, cut into small pieces, and stored at −70 °C until use. Pieces of human liver, kidney, and intestine were obtained from the autopsy specimens of unidentified patients. The tissue pieces were stored at −70 °C until use. For the preparation of total RNA and mRNA, a surgical piece of human kidney frozen at −70 °C was obtained as a gift from the Nephrology Unit of Froedtert Memorial Lutheran Hospital, Milwaukee, WI. The following reagents and chemicals were purchased commercially as indicated: [^{57}Co]cyanocobalamin (specific activity, 15 μCi/μg) and carrier free Na^{125}I (Amersham Corp.); rabbit serum (Life Technologies, Inc.); Trans^{35}S-label (>1000 Ci/mmol, containing 70% methionine, 15% cysteine, 7% methionine sulfone, 3% cysteic acid, and the rest other sulfur compounds) (DuPont NEN); protein A, lactoperoxidase, neuraminidase from *Clostridium perifringens*, and cholesterol (Sigma); endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus*, peptide:*N*-glycosidase from *Flavobacterium meningosepticum*, and *O*-glycosidase from *Diplococcus pneumoniae* (Boehringer Mannheim); disuccinimidyl suberate and iodogen (Pierce); and egg phosphatidylcholine (Avanti Polar Lipids). Pure human TC II was a gift from the late Dr. Charles A. Hall (Nutrition Assessment Laboratories, VA Hospital, Albany, NY). Antiserum to rat renal intrinsic factor receptor (16) and human TC II (17) was prepared as described earlier.

Purification of Placental TC II-R—TC II-R was purified essentially as described by Seligman and Allen (8) with the following modifications.

* This work was supported by NIDDK Grant 26638 and Veterans Administration Grant 7816-01P. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom all correspondence should be addressed: MACC Fund Center, Rm. 6061, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Tel.: 414-456-4655; Fax: 414-259-1533.

[1] The abbreviations used are: Cbl, cobalamin; TC II, transcobalamin II; TC II-R, transcobalamin II receptor; PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum.

Human placenta was cut into small pieces and homogenized in a Waring blender at top speed in 50 mM acetate buffer, pH 5.5, containing 140 mM Nacl and 5 mM EDTA. The homogenate was incubated for 1 h at 22 °C with stirring and centrifuged at 25,000 × *g* for 30 min. The total placental membrane was resuspended and homogenized in the same buffer, incubated, and centrifuged as before. Detergent extraction, ammonium sulfate precipitation of the solubilized fraction, and the affinity chromatography of TC II-R were carried out exactly according to Seligman and Allen (8).

Preparation of TC II-R Antiserum—TC II-R (12 μg/lane) was subjected to SDS-PAGE (5%) under non reducing conditions. The protein band corresponding to 62 kDa was cut out from four to five lanes and was homogenized in 1 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 140 mM NaCl, 0.1 mM PMSF. The gel slurry was mixed with 1 ml of complete Freund's adjuvent. Three ml of such a mixture corresponding to approximately 30 μg of TC II-R was subcutaneously injected into multiple sites on the back of New Zeland White rabbits. The rabbit was boosted with 15 μg of TC II-R mixed in incomplete Freund's adjuvent after 10 days, and 5–10 ml of blood was drawn every week following the booster dose.

Immunoblotting of Membrane- and Lipid Vesicle-bound TC II-R—Total membranes (5–50 μg of protein) prepared from the human tissues and egg PC/cholesterol vesicles containing TC II-R (10 μg) were subjected to SDS-PAGE (7.5%) under nonreducing conditions according to Laemmli (18). The proteins separated were transferred to nitrocellulose membranes and probed with diluted (1:1000) antiserum to TC II-R and ^{125}I-protein A according to Burnette (19). The transfer time varied from 45 to 90 min depending on the physical nature of the TC II-R. Usually the monomer form of the receptor was transferred within 45 min and the dimer in 90 min. The blots were visualized following autoradiography. Pre-absorbed antiserum was obtained by incubating 100 μl of undiluted antiserum with 5 mg of human kidney membranes (90 pmol of TC II·Cbl binding ability) for 6 h at 22 °C. The mixture was centrifuged for 15 min at 17,000 × *g*. The supernatant (350 μl) was collected, diluted, and used for immunoblotting. The absorption of TC II-R antibody to the renal membranes was confirmed by the absence of inhibition of TC II · [^{57}Co]Cbl binding to TC II-R even when 200 μl of absorbed antiserum was used in the binding assay. In some experiments immunoblotting was carried out using Triton X-100 (1%) solubilized supernatant fraction (10 μg of protein) or the insoluble pellet fraction (75–300 μg of protein) from placental membranes. Total membranes (5 mg of protein) in 10 mM Tris-HCl buffer, pH 7.5, containing 140 mM NaCl and 0.1 mM PMSF were treated with Triton X-100 for 12 h at 5 °C and centrifuged for 30 min at 25,000 × *g*. The supernatant was carefully removed, and the pellet fraction was suspended and homogenized in same buffer and made up to the same volume as the supernatant. In addition the placental membranes (10–15 mg of protein) that were subjected to lipid extraction using a 2:1 mixture of chloroform and methanol were also used for immune blotting. After careful removal of the organic phase, the protein felt was suspended and homogenized in 5 ml of Tris-buffered saline and used (10 μg of protein) for immunoblotting studies.

Preparation of Egg PC/Cholesterol Liposomes—The liposomes were prepared essentially according to Low and Zilversmit (20). Briefly, egg PC and cholesterol (molar ratio 1:0.25) were dried under a stream of nitrogen. The dried lipids were rehydrated with 1 ml of 10 mM Tris-HCl, pH 7.4, containing NaCl (140 mM), cholate (40 mM), PMSF (0.1 mM), TC II-R (15 μg), sonicated, and dialyzed for 48 h with exchange of 2 liters every 12 h of Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 0.1 mM PMSF. After dialysis, the lipid vesicle bound TC II-R was collected by centrifugation at 100,000 × *g* for 2 h. The pellet was resuspended in the dialysis buffer and was used for SDS-PAGE.

Cell-free Translation and Immunoprecipitation of TC II-R—Total RNA from human kidney was isolated by the guanidine thiocyanate method of Chomczynski and Sacchi (21). Poly(A)$^+$ RNA was isolated using the PolyATract® mRNA isolation system (Promega), and 2 μg was used for translation in reticulocyte lysate system as described before (22). The Triton X-100 extract of the ^{35}S-labeled product was immunoprecipitated with TC II-R-specific antiserum (2 μl). In some experiments, the labeled extract was preincubated with TC II-R (2 μg) for 30 min prior to the addition of TC II-R antiserum.

Iodination of Human TC II—Human TC II (5 μg, 40 kDa as assessed by SDS-PAGE) was iodinated with 0.5 mCi of Na^{125}I and IODO-GEN, as recommended by the manufacturer. The iodinated TC II was separated on a Sephadex column in 10 mM Tris-HCl buffer, pH 7.5, containing 140 mM NaCl and 1 mg/ml bovine serum albumin. The recovery was estimated to be about 80%, which gave a specific activity of approximately 75–80 μCi/μg.

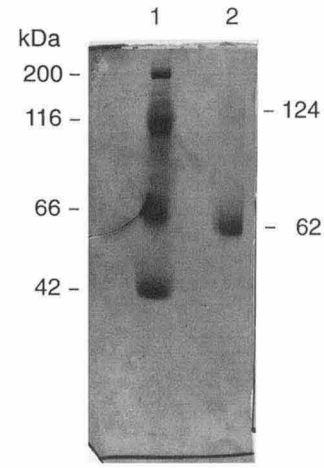

FIG. 1. **SDS-PAGE of purified human placental transcobalamin II receptor.** Purified receptor (5 μg) was subjected to SDS-PAGE (7.5%) and visualized with silver nitrate stain.

Cross-linking of Native Membrane and Liposomal Bound TC II-R—Placental membranes (25–30 μg of protein), Triton X-100 extract (25 μg of protein), liposomal bound TC II-R (10 μg of protein), and pure TC II-R (2 μg) were incubated in a volume of 750 μl with ^{125}I-TC II·Cbl (5 ng of protein and 240,000 dpm) containing 0.1 mM HEPES buffer, pH 8.0, 0.1 mM NaCl in the presence and absence cold TC II·Cbl (500 ng) for 2 h at 25 °C. The samples were then incubated with 4 mM disuccinimidyl suberate for 30 min at 25 °C. The cross-linking reaction was stopped by the addition of glycine (0.1 mM). The reaction tubes containing the placental membranes, and the liposomes were centrifuged and the pellet fraction was washed with the incubation buffer. The samples were then analyzed on nonreducing SDS-PAGE (5%). The bands were visualized after autoradiography.

Other Methods—Total membranes from the indicated tissues were prepared by centrifuging a 10% (w/v) homogenate prepared in 10 mM Tris-HCl buffer, pH 7.5, containing NaCl (140 mM), PMSF (0.1 mM) at 100,000 × *g* for 1 h. The resulting pellet was collected and resuspended in the same buffer, homogenized, and used for immunoblotting studies. Preparation of placental ER membranes were obtained as follows. A 10% homogenate was prepared in 10 mM potassium phosphate buffer, pH 6.8, containing 0.25 M sucrose and 5 mM EDTA in Potter-Elvehjem homogenizer. The homogenate was sequentially centrifuged at 666 × *g*, 10,000 × *g*, and 27,000 × *g* for 20 min. The supernatant obtained following the 27,000 × *g* spin was centrifuged for 2 h at 100,000 × *g*, and the pellet obtained was taken to represent the ER fraction. Protein concentration was determined using the Bradford assay with bovine serum albumin as the standard (23). TC II-R assays were performed using partially purified TC II from freshly drawn rabbit blood. The rabbit serum was subjected to partial purification according to Lindemans *et al.* (5). The preparation of TC II·[^{57}Co]Cbl complex for receptor assays was according to Gottlieb *et al.* (24). Immunoprecipitation of the labeled ligand with TC II antiserum confirmed that all the labeled Cbl was bound to TC II. TC II-R activity was determined using the pure receptor or Triton X-100 extracts from tissue homogenates essentially according to Seligman and Allen (8). Immunoblocking of the binding of TC II·[^{57}Co]Cbl to TC II-R was carried out by preincubating the pure TC II-R (0.5 μg) with antiserum (1–20 μl) at 22 °C for 30 min, followed by the addition of the ligand (1.5 pmol) and an additional incubation for 60 min at 22 °C. The bound and the free ligand were separated on DEAE-Sephadex columns as described previously (8).

RESULTS AND DISCUSSION

Synthesis and Membrane Expression of TC II-R—In order to study the synthesis and membrane expression of TC II-R, antiserum was raised to human placental TC II-R purified essentially according to Seligman and Allen (8). The purified receptor demonstrated a single major band of 62 kDa and a minor band of 124 kDa on nonreducing SDS-PAGE (Fig. 1) and the eluate of both of these bands were active in ligand, TC II·[^{57}Co]Cbl binding (data not shown). The size of the major pure TC II-R noted is about 10 kDa higher than the value of 50 kDa (8) or 58 kDa (25) reported by earlier studies, and this

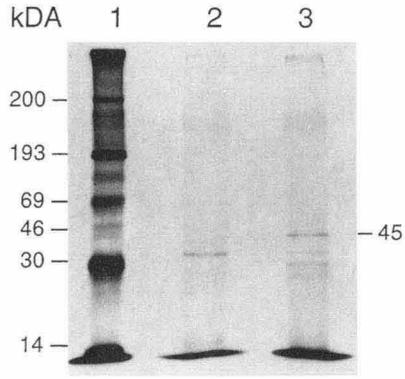

FIG. 2. **Cell-free translation and immunoprecipitation of TC II-R.** Human mRNA from kidney was translated in the reticulocyte translation system, and the translated proteins were extracted with Triton X-100 and immunoprecipitated in the presence (*lane 2*) and absence (*lane 3*) of excess cold endogenous TC II-R. Other details of translation, extraction, and immunoprecipitation are provided under "Materials and Methods."

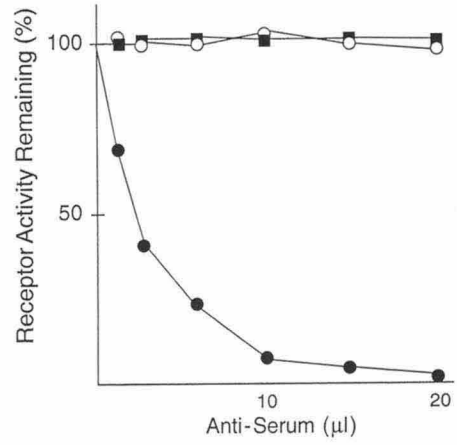

FIG. 3. **Immunoblocking of the binding of TC II · [^{57}Co]Cbl to purified TC II-R.** Pure TC II-R (0.5 μg) was preincubated with the indicated amounts of antiserum to human TC II-R (●), TC II (■), and rat intrinsic factor-Cbl receptor (○) for 1 h at 22 °C. TC II·[^{57}Co]Cbl (1.5 pmol) was then added and assayed for ligand binding. The activity is expressed as the percentage of TC II-R activity in a sample incubated without serum.

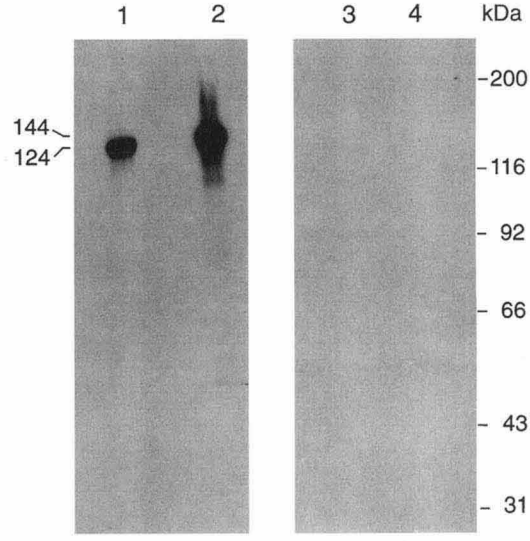

FIG. 4. **Western blotting of human placental membranes.** Placental membranes (10 μg of protein) were incubated with (*lanes 2* and *4*) and without (*lanes 1* and *3*) 2-mercaptoethanol (2.5%) for 1 h at 22 °C and then subjected to SDS-PAGE (7.5%). The separated proteins were transferred to nylon membrane and probed with unabsorbed (*lanes 1* and *2*) or absorbed (*lanes 3* and *4*) anti-serum. The diluted antiserum was absorbed with total human kidney membranes.

difference could be due to the differences in the methods employed to determine the size of the receptor. Antiserum prepared for the 62-kDa TC II-R immunoprecipitated a major protein band of 45 kDa from the detergent extract of the translated product obtained using *in vitro* translation of human kidney mRNA in the reticulocyte lysate translation system (Fig. 2, *lane 3*). Confirmation that the immunoprecipitated 45-kDa product as TC II-R was obtained when competition with excess cold TC II-R (*lane 2*) did not reveal the 45-kDa product. However, a 30-kDa band was immonuprecipitated even in the presence of cold TC II-R. The origin of this band is not known, but it may represent an immunocross-reactive protein, unrelated to TC II-R. The origin of two or three bands around 30 kDa and lower seen in *lane 3* is not known but the bands of molecular mass < 30 kDa may represent proteolytic fragments of TC II-R as these bands also disappeared during immunoprecipitation of labeled product with cold TC II-R. It is interesting to note the primary translation in a cell free system of TC II-R and its ligand TC II (26) is around 45 kDa. This result confirms the earlier observation (8) based on quantitative amino acid comparison that TC II-R and TC II have a similar polypeptide length. To the best of our knowledge TC II-R and TC II may represent a unique receptor-ligand system in which both molecules have similar sized polypeptides. The difference of 17 kDa in the molecular mass of the mature (62 kDa) (Fig. 1) and the TC II-R obtained by cell-free translation (45 kDa) (Fig. 2) is due to the addition of both *N*- and *O*-linked oligosaccharides following its synthesis and maturation. Based on the mobility shifts of the pure TC II-R by peptide:*N*-glycosidase F and by sialidase and *O*-glycanase the total carbohydrate on TC II-R was estimated to account for about 17 kDa or 27.5% of its total molecular mass of 62 kDa (data not shown). Earlier studies based on the amino sugar and gas chromatography analysis of purified TC II-R (8) or on *N*- and *O*-glycosidase digestion of placental detergent extract cross-linked to ^{125}I-TC II (25) have reported total carbohydrate of TC II-R to be 33 and 29%, respectively.

Confirmation that the antiserum raised in rabbits to the 62-kDa band is indeed specific for TC II-R was obtained when incubation of the receptor with graded amounts of antiserum was able to inhibit the binding of TC II·[^{57}Co]Cbl to the pure receptor (Fig. 3). About 100% inhibition was observed (Fig. 3) with 20 μl of undiluted antiserum. Using the same volume, antiserum to the ligand, human TC II, or rat intrinsic factor-cobalamin receptor did not demonstrate any inhibition. These results show that TC II-R antibody, like the antibody raised against intrinsic factor-cobalamin receptor (27), is a blocking antibody and is specific for TC II-R. In addition, lack of inhibition by antiserum to the ligand, TC II, and intrinsic factor receptor further suggest that TC II-R antiserum is specific.

In order to further test the specificity of the antiserum, immunoblotting studies were carried out with human placental membranes (Fig. 4). Although a single band was revealed in these membranes, surprisingly the molecular mass of this band was 124 kDa under nonreducing (Fig. 4, *lane 1*) and 144 kDa (Fig. 4, *lane 2*) under reducing conditions, respectively, and not 62 kDa. The specificity of this reaction was confirmed when the diluted antiserum treated with human kidney membranes (see later) failed to recognize the same bands (*lanes 3* and *4*). These results suggested that the antiserum to TC II-R is specific for TC II-R and that the receptor exists possibly as a noncovalent dimer in the placental membranes. In addition, the apparent increase in molecular mass of membrane bound mature TC

II-R by 20 kDa upon reduction suggested that the receptor does not contain disulfide-linked polypeptides but contains intramolecular disulfide bonds which upon reduction may alter the compactness of the membrane receptor thus altering it's mobility. Similarly the apparent molecular mass of the pure TC II-R increased from 62 to 72 kDa upon reductive alkylation (data not shown). This result is consistent with the idea that pure TC II-R does not contain disulfide linked subunits and that the intramolecular disulfide bonds of the receptor remain intact during it's transport to and following insertion in the plasma membrane. Similar observations of decreased mobility on SDS-PAGE following reduction of the cation-independent mannose 6-phosphate receptor (28), and other proteins (29) have been noted and has been attributed to the presence of intramolecular disulfide bond clusters.

Since the immunoblotting of placental membranes revealed a single band of 124 kDa, it is likely that TC II-R exists as a homodimer in these membranes. In addition, the dimerization of TC II-R appears to be noncovalent and is a property of the membrane bound receptor. In order to verify whether the TC II-R exists as a homodimer in other tissue membranes and to evaluate the relative distribution of TC II-R in other human tissue membranes, immunoblotting studies using total membranes from human placenta, intestine, kidney, and liver (Fig. 5) were carried out. A single band of 124 kDa was revealed in all the tissue membranes. Although the immune cross-reactive band was easily visible using 10 μg of membrane protein from placenta, intestine, and kidney, 50 μg of liver membrane protein was needed to visualize the band. Quantitation of these bands revealed that the relative distribution of TC II-R was highest in the kidney (100%) followed by placenta (28%), intestine (18%), and liver (2%). Although on a protein basis, TC II-R levels in the liver were lower relative to kidney or placenta or intestine, significant amounts of TC II-R will also be expressed in the liver, considering its larger mass. The high levels of TC II-R in these human tissues is consistent with the flux of Cbl bound to TC II that cross plasma membranes of these tissues in order to maintain the special functions or characteristics of these organs such as Cbl storage (liver), plasma Cbl uptake, storage, and either excretion or tissue exchange (kidney), maternal-fetal transfer (placenta), and rapid proliferation and differentiation of absorptive enterocytes (intestine).

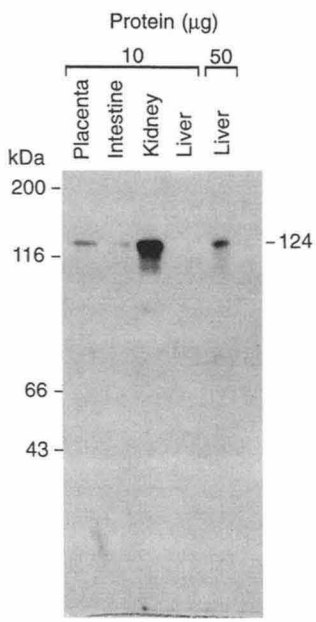

FIG. 5. **Immunoblotting of human tissue total membranes.** Total membranes prepared from the indicated tissues were obtained by centrifuging a 10% homogenate (prepared in 10 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 0.1 mM PMSF) at 100,000 × *g* for 1 h. Indicated amounts of membrane protein were subjected to SDS-PAGE (7.5%), and the separated proteins were transferred to a nylon membrane (transfer time 90 min), probed with TC II-R antiserum and ^{125}I-protein A, and subjected to autoradiography.

Membrane Interactions and Dimer Formation of TC II-R—The expression of a single species of TC II-R of molecular mass 124 kDa in the tissue membranes suggested that during its purification involving initial extraction with Triton X-100, the dimer is converted to a monomer or alternatively the monomeric form of the receptor is free in the membranes and is extracted by the detergent Triton X-100, whereas the dimeric form bound tightly to cytoskeletal elements is not. In order to test this hypothesis immunoblotting analysis was carried out using the native placental membranes or the Triton X-100-solubilized supernatant and the Triton X-100-insoluble pellet (Fig. 6) prepared from the native membranes. The 124-kDa dimer was present in the native membranes and could be detected following the transfer of the separated proteins for 90 min (Fig. 6A, *lane 1*) but not for 45 min (Fig. 6*B*, *lane 1*). The 62-kDa monomeric form on the other hand could be detected in the Triton X-100-soluble fraction following a transfer time of 45 min (Fig. 6*B*, *lane 8*) but not 90 min (Fig. 6*A*, *lane 2*). The 62-kDa monomer (Fig. 6*B*, *lanes 10, 11,* and *12*) or the 124-kDa dimer forms (Fig. 6*A*, *lanes 4–6*) of TC II-R could not be detected in the Triton X-100-insoluble pellet using 75 (*lanes 4* and *10*), 150 (*lanes 5* and *11*), and 300 (*lanes 6* and *12*) μg of protein, whereas the dimeric form or the monomeric forms in the native

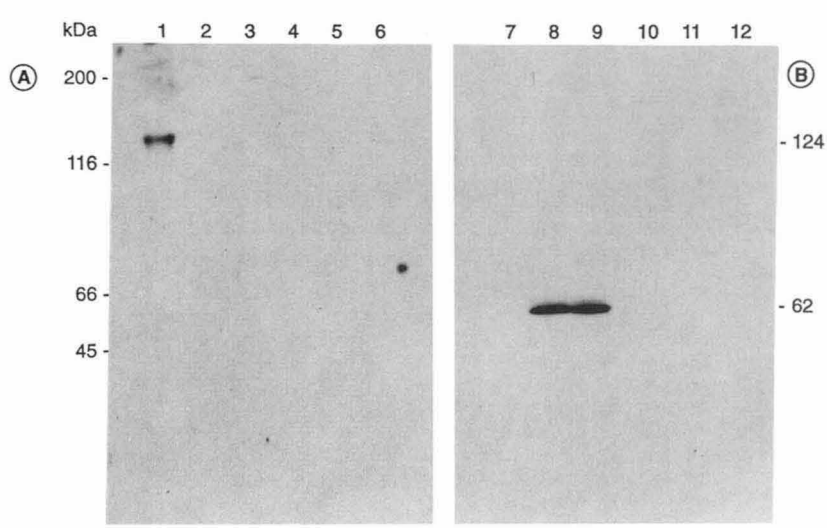

FIG. 6. **Immunoblotting of placental membranes treated with Triton X-100 or chloroform:methanol mixture.** Placental membrane protein (10 μg, *lanes 1* and *7*), Triton X-100-soluble fraction (10 μg of protein, *lanes 2* and *8*), Triton X-100-insoluble pellet fraction (protein: 75, 150, and 300 μg, respectively, in *lanes 4 and 10, 5* and *11*, and *6* and *12*) and chloroform:methanol delipidated membranes (10 μg of protein, *lanes 3* and *9*) were subjected SDS-PAGE (7.5%). The separated proteins were blotted to nitrocellulose membranes for 90 (*A*) or 45 (*B*) min and probed with TC II-R antiserum and ^{125}I-protein A. Other details of membrane treatment are provided under "Materials and Methods."

membranes or Triton X-100-soluble fraction could be detected with as little as 10 μg of protein or less. This result suggested that the dimeric form of the receptor is the only form of the receptor present in the membranes and that solubilization and the ensuing delipidation might be responsible for the total conversion of the dimer to the monomer. In order to test this possibility the membranes were delipidated with a chloroform: methanol mixture. The membrane fraction thus delipidated demonstrated the presence of the 62-kDa monomer (Fig. 6*B, lane 9*) but not the 124-kDa dimer (Fig. 6*A, lane 3*). Furthermore metabolic labeling studies using human colon adenocarcinoma (Caco-2) cells, known to express TC II-R[2] with [^{3}H]myristate or [^{3}H]palmitate or [^{3}H]mevanalactone did not reveal any type of co- or post-translational modifications involving covalent fatty acid acylation or isoprenylation of TC II-R (data not shown). These observations indicated that the dimerization of the TC II-R probably occurs following its membrane assembly and is due to strong lipid protein interactions involving the hydrophobic TC II-R and annular membrane lipids. Furthermore these observations also indicated the absence of any role of cytoskeletal proteins or of covalently bound lipids in either the anchoring of TC II-R to the membrane or in the conversion of the monomer to the dimer form of TC II-R.

Direct evidence of lipid involvement in the dimerization of TC II-R was obtained when the receptor was inserted in lipid vesicles. The receptor associated with the lipid vesicles revealed on SDS-PAGE a higher molecular mass of around 124 kDa, twice the size of the Triton X-100 micellar bound receptor (Fig. 7*A*). Upon immunoblotting, TC II-R associated with the lipid vesicles (Fig. 7*B, lane 3*) had a molecular mass of 124 kDa, similar to TC II-R present in the native renal (*lane 1*) and placental (*lane 2*) membranes. These results have confirmed the observation that delipidation and relipidation result in the formation of monomer or dimer, respectively, and that the dimerization of TC II-R in native membranes is due to association with plasma membrane lipids. The TC II-R expressed in the plasma membranes of several rat tissues and in cultured cells is also a dimer with a molecular mass of 124 kDa, suggesting that dimerization of TC II-R occurs in all the tissue/cellular plasma membranes of other species.[2]

The functional significance of expression of the dimeric form of TC II-R in plasma membranes is not known. Cross-linking of ^{125}I-TC II · Cbl (Fig. 8) to the native membrane (Fig. 8*A, lane 1*) or its detergent extract (Fig. 8*A, lane 4*) or lipid vesicle-bound TC II-R (Fig. 8*B, lane 5*) or the pure TC II-R (Fig. 8*B, lane 8*) show that both the dimeric and monomeric form of the receptor are functional in ligand binding. The specificity of ligand binding is borne out by the observation that prior incubation with 100-fold molar excess of cold TC II·Cbl abolished the binding of ^{125}I-TC II·Cbl to TC II-R fractions (*lanes 2, 3, 6,* and *7*). Based on the size of the cross-linked products, the membrane-bound dimer binds 2 mol of the ligand, and it is thus possible that the expression of the dimeric form of TC II-R may help in facilitating rapid clearance of circulatory TC II·Cbl known to occur *in vivo* (30).

Earlier studies have shown that TC II-R is a very hydrophobic protein capable of binding 1.25 times its own weight of Triton X-100 (8). Our studies suggest further that the dimerization is the result of strong lipid-protein interaction within the lipid microenvironment of the plasma membrane. It is interesting to note that the molecular mass of TC II-R from placental microsomes is 62 kDa (data not shown). This observation suggests that the receptor is either a monomer in the ER, or the interaction between the two monomers in the ER membrane is not of sufficient strength that it can be dissociated by treatment with SDS. If the receptor is indeed a true monomer in the ER membrane, the ensuing folding alterations that occur during the processing (following its exit from ER) might expose the hydrophobic surfaces of the receptor to favor a much stronger monomer-monomer interaction in the plasma membrane. On the other hand, the qualitative and quantitative differences in the lipids of the ER and plasma membrane may

[2] S. Bose and B. Seetharam, unpublished observations.

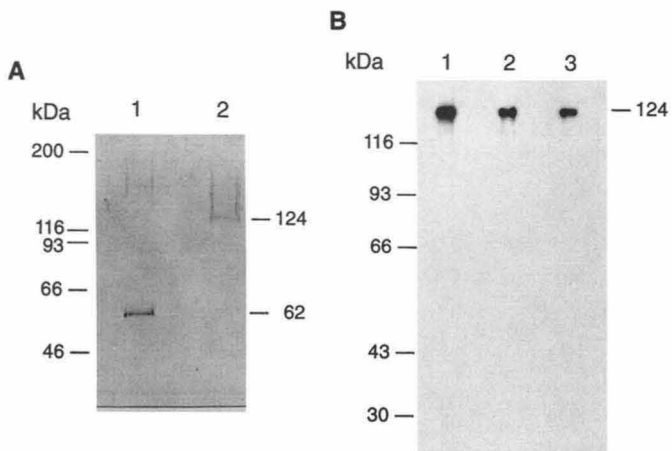

FIG. 7. **SDS-PAGE and immunoblot analysis of purified TC II-R associated with egg PC and cholesterol vesicles.** Egg PC/cholesterol liposomes (molar ratio 1:0.25) were prepared by the cholate dialysis method. During dialysis, TC II-R (10 μg) was also present. After dialysis the receptor-lipid fraction was centrifuged for 2 h at 100,000 × *g*. The lipid pellet was reconstituted in Tris-buffered saline and subjected for SDS-PAGE and either stained for protein with silver nitrate (*A*) or used for immunoblotting (*B*). *A*: *lane 1*, pure receptor (5 μg) and *lane 2*, liposomal bound TC II-R (3 μg). *B*, total membrane protein from human kidney (5 μg), human placenta (10 μg) and liposomal bound TC II-R (5 μg) were used for SDS-PAGE and immunoblotting.

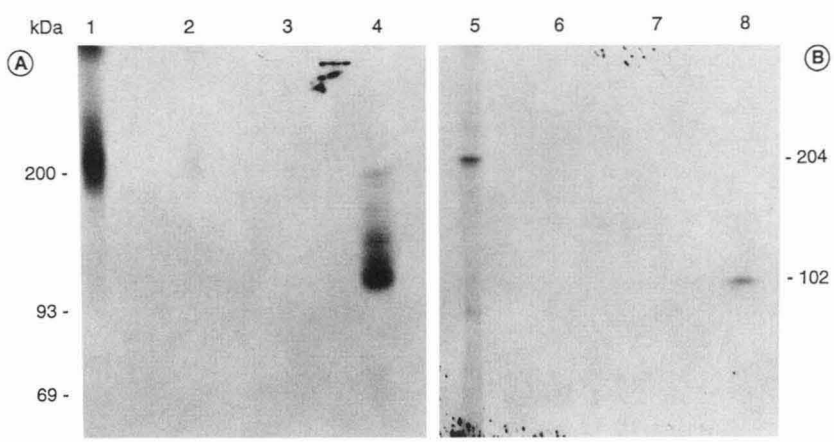

FIG. 8. **Cross-linking of membrane-bound and membrane free TC II-R.** *A*, placental membranes (*lanes 1* and *2*) or Triton X-100 extract (*lanes 3* and *4*) were cross-linked with ^{125}I-TC II in the absence (*lanes 1* and *4*) and presence (*lanes 2* and *3*) of 100-fold molar excess of TC II·Cbl. *B*, TC II-R bound to egg PC-cholesterol liposomes (*lanes 5* and *6*) or pure TC II-R (*lanes 7* and *8*) was cross-linked with ^{125}I-TC II·Cbl in the absence (*lanes 5* and *8*) and presence (*lanes 6* and *7*) of 100-fold molar excess of TC II·Cbl. The cross-linked products were analyzed of SDS-PAGE (5%) and visualized by autoradiography. Other details are provided under "Materials and Methods."

also be responsible for the presence of monomer or dimer in these membranes, respectively. Further studies are needed to elucidate the structural elements of TC II-R important in the formation of noncovalent dimers in native plasma membranes and the specific lipids that help mediate this event.

In conclusion, the current study has examined some of the molecular properties of TC II-R and its expression in human tissue membranes. These studies have provided insights into the role of membrane lipids in the formation of noncovalent homodimers of TC II-R in human tissue plasma membranes. With the availability of nanomole amounts of the receptor and mono-specific antiserum, further studies on its structure and regulation of expression are now possible.

Acknowledgment—We thank Dr. Nancy Dahms for critical evaluation of the manuscript.

REFERENCES

1. Quadros, E. V., Rothenberg, S. P., Pan, Y.-C. E., and Stein, S. (1986) *J. Biol. Chem.* **261,** 15455–15460
2. Youngdhal-Turner, P., Rosenberg, L. E., and Allen, R. H. (1978) *J. Clin. Invest.* **61,** 133–141
3. Nexo, E., and Hollenberg, M. D. (1980) *Biochim. Biophys. Acta* **628,** 190–200
4. Scott, J. S. D., Bowman, E. P. W., and Cooksley, W. G. E. (1985) *Clin. Sci.* **68,** 357–364
5. Lindemans, J., Van Kappel, J., and Abels, J. (1986) *Scand. J. Clin. Lab. Invest.* **46,** 223–232
6. Fidler-Nagy, C., Rowley, G. R., Coffey, J. W., and Miller, O. N. (1975) *Br. J. Haematol.* **31,** 311–321
7. Friedman, P. A., Shia, M. A., and Wallace, J. K. (1977) *J. Clin. Invest.* **59,** 51–58
8. Seligman, P. A., and Allen, R. H. (1978) *J. Biol. Chem.* **253,** 1766–1772
9. Scott, J. S. D., Treston, A. M., Bowman, E. P. W., Owens, J. A., and Cooksley, W. G. F. (1984) *Clin. Sci.* **67,** 299–306
10. Okuda, K. (1962) *J. Nutr.* **77,** 131–136
11. Okuda, K. (1962) *Nature* **195,** 292–293
12. Dryden, L. P., and Hartman, A. M. (1966) *J. Nutr* **90,** 382–386
13. Grossowicz, N., Jablonska, M., Izak, G., and Rachmilewitz, M. (1970) *Am. J. Clin. Nutr.* **23,** 127–131
14. Yamada, S., Rittinen, L., Majuri, R., Fakuda, M., and Grasbeck, R. (1991) *Kidney Int.* **19,** 289–294
15. Ramanujam, K. S., Seetharam, S., and Seetharam, B. (1991) *Biochem. Biophys. Res. Commun.* **179,** 543–550
16. Ramanujam, K. S., Seetharam, S., Ramasamy, M., and Seetharam, B. (1991) *Am. J. Physiol.* **260,** G416–G422
17. Seetharam, B., Levine, J. S., Ramasamy, M., and Alpers, D. H. (1988) *J. Biol. Chem.* **263,** 4443–4449
18. Laemmli, U. K. (1970) *Nature* **227,** 680–685
19. Burnette, W. N. (1981) *Anal. Biochem.* **7,** 195–203
20. Low, M. C., and Zilversmit, D. B. (1980) *Biochemistry* **19,** 3913–3918
21. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
22. Seetharam, S., Dahms, N., Li, N., Ramanujam, K. S., and Seetharam, B. (1991) *Biochem. Biophys. Res. Commun.* **177,** 751–756
23. Bradford, M. M. (1976) *Anal. Biochem.* **112,** 195–203
24. Gottlieb, C., Lau, K. S., Wasserman, L. R., and Herbert, V. (1965) *Blood* **25,** 875–884
25. Quadros, E. V., Sai, P., and Rothenberg, S. P. (1994) *Arch. Biochem. Biophys.* **308,** 192–199
26. Li, N., Seetharam, S., Lindemans, J., Alpers, D. H., Arwert, F., and Seetharam, B. (1993) *Biochim. Biophys. Acta.* **1172,** 21–30
27. Seetharam, B., Alpers, D. H., and Allen, R. H. (1981) *J. Biol. Chem.* **256,** 3785–3790
28. Sahagian, G. G., and Neufeld, E. F. (1983) *J. Biol. Chem.* **258,** 7121–7128
29. Li, W., and Schlessinger, J. (1991) *Mol. & Cell Biol.* **11,** 3756–3761
30. Allen, R. H. (1975) *Prog. Hematol.* **9,** 57–84