Function and stability of human transcobalamin II: role of intramolecular disulfide bonds C98-C291 and C147-C187

Seema Kalra,1 Ning Li,1 Shakuntla Seetharam,1 David H. Alpers,2 and Bellur Seetharam1,3
Division of Gastroenterology and Hepatology, Departments of 1Medicine and 3Biochemistry, Medical College of Wisconsin and Veterans Administration Medical Center, Milwaukee, Wisconsin 53295; and 2Washington University School of Medicine, St. Louis, Missouri 63110

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Kalra, Seema, Ning Li, Shakuntla Seetharam, David H. Alpers, and Bellur Seetharam. Function and stability of human transcobalamin II: role of intramolecular disulfide bonds C98-C291 and C147-C187. Am J Physiol Cell Physiol 285: C150–C160, 2003. First published March 26, 2003; 10.1152/ajpcell.00496.2002.—The current studies have investigated the role of three disulfide bonds of human transcobalamin II (TC II), a plasma transporter of cobalamin (Cbl; vitamin B12), in its function and stability. When translated in vitro in the presence or absence of microsomal vesicles, TC II constructs with a single substitution, C3S or C249S, demonstrated synthesis of a stable functional protein. However, TC II synthesized in the presence of microsomal vesicles using constructs with a single (C98S, C147S, C187S, C291S), double (C3/147S, C98/147S) or triple (C3/98/147S) substitution was unstable. In the absence of microvesicular vesicles, the percentage of binding to Cbl-Sepharose matrix by TC II expressed by constructs C3S, C147S, C291S, C98/147S, or C3/98/147S was 100, 49, 52, and 35%, respectively. Upon their reductive alkylation, the binding of TC II expressed by these constructs was reduced to ~25–30%. TC II constructs C3S or C249S, when expressed in TC II-deficient fibroblasts, produced a stable functional protein, but those expressed by constructs C147S, C187S, C291S, C3/147S, C98/147S, or C3/98/147S were rapidly degraded. The intracellular degradation of TC II expressed by these constructs was inhibited by lactacystin or MG-132 but not by the lysosomal degradation inhibitors ammonium chloride or chloroquine. These studies suggest that optimal binding of Cbl by human TC II has been shown (8) to release Cbl bound to it, suggesting that one or more of its three disulfide bonds may play a direct or indirect role in Cbl binding and, hence, are essential for the functional integrity of the Cbl binding pocket (31), no direct evidence exists for such a proposal. However, bovine TC II has been shown by chemical analysis to contain three disulfide bonds that are formed utilizing the six conserved residues (8) whose locations vary very insignificantly in mammalian Cbl binding proteins, including human and rat TC II. Furthermore, reductive alkylation of bovine holo-TC II has been shown (8) to release Cbl bound to it, suggesting that one or more of its three disulfide bonds could play a direct or indirect role in Cbl binding by apo-TC II.

Despite the identification of six conserved residues in identical positions in all human Cbl binders (33), and in human TC II, there are two additional cysteine residues that are not present in either human IF (16) or HC (17). Although the conserved cysteine residues of these proteins have been proposed to form disulfide bonds that are important for their folding and, hence, are essential for the functional integrity of the Cbl binding pocket (31), no direct evidence exists for such a proposal. However, bovine TC II has been shown by chemical analysis to contain three disulfide bonds that are formed utilizing the six conserved residues (8) whose locations vary very insignificantly in mammalian Cbl binding proteins, including human and rat TC II. Furthermore, reductive alkylation of bovine holo-TC II has been shown (8) to release Cbl bound to it, suggesting that one or more of its three disulfide bonds could play a direct or indirect role in Cbl binding by apo-TC II.

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their role in intracellular folding, stability, secretion, and function. Such studies have been hampered so far because many human cells in culture synthesize and secrete endogenous TC II (33), making them unsuitable as an expression system. In this study, these mutant cells have been used for transient expression of wild-type and cysteine mutants of human TC II.

The results of this study using both cell free and intracellular synthesis of apo-TC II show that TC II expressed after disruption of the disulfide bond formed between C3 or C249 was able to escape the quality control mechanism of the endoplasmic reticulum (ER), retaining all the properties of wild-type TC II, Cbl, and TC II-R binding and the ability to mediate Cbl transport into cells. However, disruption of the other two disulfide bonds of TC II, C98-C291 and C147-C187, resulted in decreased Cbl and its receptor binding as well as its rapid degradation by the proteasome system.

### MATERIALS AND METHODS

**Materials.** The following were commercially purchased as indicated: [57Co]Cbl (1.3 μCi/μg; ICN Radiochemicals, Irvine, CA); [35S]methionine (1,175 Ci/mmol) and N-[3H]ethylmaleimide (60 Ci/nmol; NEN Life Science Products), 5,5’-dithiobis-2-nitrobenzoic acid (DTNB; Pierce); Sepharose, iodoacate, CNBr-Sepharose, and chloroquine (Sigma-Aldrich, St. Louis, MO); lactacytin and MG-132 (Calbiochem, La Jolla, CA); Fluoro-Hance, used for autoradiography (Research Products International, Mount Prospect, IL); canine pancreatic microsomal membranes and the TNT quick coupled transcription/translation system (Promega, Madison, WI); QUSO-G32 (Philadelphia Quartz, Valley Forge, PA); and vector pcDNA3 and Lipofectamine Plus (Invitrogen, Carlsbad, CA). Monocarboxylic acid derivative of Cbl linked to Sepharose was prepared as described earlier (1). Antiserum to human TC II was raised in New Zealand White rabbits as described earlier (27). The affinity-purified antiserum (anti-sel) was used for immunoprecipitation of labeled TC II from cell culture media.

**Human and rat TC II cDNA clones.** Human TC II cDNA clone was isolated from a human endothelial cDNA library as described previously (20). Rat TC II cDNA clone was isolated by screening a visceral yolk sac library from a 19-day-old pregnant rat cDNA expression library constructed in the vector λ-gt11 by using human TC II cDNA as a probe. The sequence of rat TC II has been submitted to the GenBank (accession no. AF054810).

**Titration of pure human TC II for thiol groups.** Pure recombinant TC II (25 μg) was reduced with 2-mercaptoethanol (40 mM) for 1 h in the dark in Tris-glucose buffer, pH 8.0, containing 0.2 mM EDTA. Both reduced and unreduced TC II were incubated with DTNB (10 μg) for 1 h at 22°C. The absorbency of the samples was measured at 412 nm, and the spectrometric titration of the thiol groups was calculated on the basis of the known extinction coefficient of DTNB (13,600 mol⁻¹cm⁻¹) according to Habeeb (14). Titration of pure TC II (5 μg) was carried out by using N-[3H]ethylmaleimide (750,000 dpm/100 fmol) under reducing and nonreducing conditions as described above for DTNB titration. The samples were dialyzed and the bound radioactivity was counted.

In vitro mutagenesis of cysteine residues of human TC II. In vitro mutagenesis of the two nonconserved and the six conserved cysteine residues to serine residue was carried out using the PCR-based Quick-Change site-directed mutagenesis kit from Stratagene (La Jolla, CA). For generation of double and triple cysteine mutants, mRNAs containing the required single or double mutation were used as templates. All mutants were sequenced to confirm mutation of cysteine at the desired position(s).

**Cell culture and transient transfection of wild-type and mutant TC II constructs in human TC II deficient fibroblasts.** Stock cultures of normal and TC II-deficient fibroblasts were routinely cultured in 5% CO₂ in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 0.25 mM nonessential amino acids, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.75 mg/ml amphotericin). Fibroblasts cultured in the absence of antibiotics and grown to 60–70% confluence were used for transient transfection. The wild-type or cysteine mutant TC II constructs (5 μg) were transfected by using the Lipofectamine method. After 20 h of transfection, the medium was harvested, and the binding of [57Co]Cbl by the culture medium (1 ml) was determined with 1 pmol of [57Co]Cbl using the albumin-coated charcoal absorption method (13). At 20 h posttransfection, the cells were metabolically labeled with [35S]methionine (25 μCi/ml) for 8 h, and the medium was collected for further processing. In other experiments that tested the effect of ammonium chloride (100 μg/ml), chloroquine (100 μg/ml), lactacytin (30 μM), or MG-132 (15 μM) on the degradation of TC II, cells were labeled for 1 h with [35S]methionine at 20 h posttransfection. Cell lysates were prepared from washed, labeled cells that were suspended in 1 ml of Tris-buffered saline, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride and 2 mM benzamidine. The cell suspension was sonicated for 30 s and then treated with 0.5% Triton X-100 and 0.1% sodium deoxycholate. The cell lysate was obtained by centrifugation.

**Immunoprecipitation of labeled TC II from cell culture media.** Cell culture media (5 ml) from transfected cells were treated with 15 μl of undiluted antiserum to human TC II. After a 12-h incubation at 5°C, a 1:1 suspension of protein A-Sepharose (50–150 μl) was added and incubated for 2 h at 22°C. The radioactivity was collected by centrifugation, washed, released with SDS-sample buffer, and subjected to nonreducing SDS-PAGE.

Precipitation of the labeled media with QUSO-G32. In some experiments the labeled media (1–2 ml) were treated with QUSO-G32 (5–10 mg), and the resins collected by centrifugation were then processed for SDS-PAGE as described previously (28).
**SDS-PAGE analysis and fluorography.** $[^{35}S]$methionine-labeled TC II ($[^{35}S]$-TC II) obtained by immunoprecipitation of cell culture medium (10,000–60,000 dpm) or the cell lysate was subjected to nonreducing SDS-PAGE (7.5–10%). The gels were fixed, treated with Fluor-Hance for 30 min, dried, and subjected to fluorography. In some experiments, purified recombinant TC II (1 μg) was subjected to SDS-PAGE under both nonreducing and reducing conditions. Recombinant TC II protein or $[^{35}S]$-TC II translated from the constructs C3S, C3/147/S, C98/147/S, or C9/98/147/S were reduced by incubation in the dark for 30 min with 2-mercaptoethanol (10 mM) and then alkylated with excess iodoacetate (200 mM). The reduced and nonreduced samples were subjected to SDS-PAGE. The TC II protein bands were visualized by silver nitrate staining, and $[^{35}S]$-TC II bands were visualized by fluorography. All SDS-PAGE experiments were carried out at least three to four times, and the data from a typical representative electrophoresis gel are shown.

In vitro translation and processing of wild-type and cysteine mutants of TC II. The wild-type and cysteine mutant constructs of human TC II were transcribed and translated by the TNT quick coupled system as described by the manufacturer. In some experiments, canine pancreatic microsomal membranes (1 μl) were added during transcription and translation. Total translation mixture (1 μl) was boiled with SDS-sample buffer and subjected to nonreducing SDS-PAGE (10%), and the bands were visualized by fluorography.

Functional determination of translated products. To determine Cbl or TC II-R binding ability of the translated products, the total translation mixture (2 μl) was diluted to 300 μl with Tris-buffered saline, pH 7.4, and allowed to react with 100 μl of a 1:1 suspension of Cbl-Sepharose or TC II-R-Sepharose for 2 h at 5°C. The reaction mixture was then microfuged, and the beads were washed exhaustively by using 1.5 ml of Tris-buffered saline for each wash. After 15–20 washes, the radioactivity bound to the beads was determined and expressed as a percentage of trichloroacetic acid (5%)-precipitable radioactivity obtained from 2 μl of the translation mixture using wild-type TC II cDNA. To determine the ability of various constructs bearing a single substitution to promote Cbl uptake by fibroblasts, the unlabeled media from cells transfected with these constructs for 20 h were harvested and then incubated with $[^{57}Co]$Cbl (0.5 pmol) for 30 min. The media were added to nontransfected postconfluent TC II-deficient fibroblasts. After 2 h of incubation at 37°C, the cells were harvested and washed with 10 mM K-phosphate buffer, pH 5.0, containing 5 mM EDTA to remove surface-bound radioactivity, and the cells were then counted for internalized $[^{57}Co]$Cbl in a gamma counter.

**RESULTS**

Location of cysteine residues in human TC II. Human TC II contains eight cysteine residues (Fig. 1). Sequence alignment of human TC II with other mammalian Cbl binders, rat IF, and porcine HC shows that six of its eight cysteine residues are conserved (Fig. 1, filled stars) and are located in identical positions (20). In addition, human TC II, which is 95–98% identical to bovine and rat TC II, also contains two nonconserved cysteine residues at positions 65 and 78 (Fig. 1, open star) that are absent in both bovine TC II (8) and human IF or HC (33). The presence of six conserved cysteine residues in all Cbl binding proteins has led to the speculation that they are utilized to form three disulfide bonds that could be important in the regulation of many of the properties of Cbl binding proteins. In contrast, the two nonconserved cysteine residues present in human and rat TC II are replaced by serine residues in bovine TC II, rat IF, or human HC, and this replacement is thought to represent neutral polymorphic changes and thus may not participate in disulfide bond formation. To address these issues, both the conserved and nonconserved cysteine residues of human TC II were selected for in vitro mutagenesis. However, before these studies were initiated, it was first important to confirm that, like bovine TC II (8), the human analog also contains three disulfide bonds. For this purpose, we used purified human recombinant TC II obtained by expressing human TC II cDNA in the baculovirus expression system.

**Purified human TC II contains three disulfide bonds.** Before titration for total and free sulphydryl (SH) groups, the purity of recombinant TC II was confirmed by SDS-PAGE (Fig. 2). The purified recombinant human TC II demonstrated a single band of 43 kDa under nonreducing conditions (Fig. 2, lane 1) and 47 kDa under reduced conditions (lane 2). The difference in the...
mobilizes resulting in an apparent increase of molecular mass of 4 kDa suggested the presence of disulfide bonds in human TC II. The phenomenon of reduced mobility on SDS-PAGE following reductive alkylation of single polypeptide chain proteins containing intramolecular disulfide bonds, such as TC II, is well known (5, 23, 24, 29).

Purified human recombinant TC II, when titrated under nonreducing and reducing conditions, revealed the presence of two and eight free SH groups, respectively (Table 1), indicating that human TC II in its native form contains two free cysteine residues and six cysteine residues that are utilized to form three disulfide bonds. To determine the effect of disruption of these disulfide bonds and the role, if any, of the free cysteine residues in the various properties of TC II, TC II mutants were initially generated in which each of the eight cysteine residues were changed one at a time to a serine residue. To express these mutant constructs in a human cell, we chose human foreskin fibroblasts isolated from Cbl-deficient children because they do not synthesize TC II. However, it was first important to determine that these mutant human cell lines are able to synthesize and secrete TC II when transfected with wild-type TC II cDNA.

 transient transfecion of wild-type TC II in TC II-deficient human skin fibroblasts. When the mutant cell line (WG 1346) was transfected with the vector alone and metabolically labeled with [35S]methionine, SDS-PAGE of the TC II antiserum-immunoprecipitated material obtained using the medium did not reveal synthesis of TC II (Fig. 3, lane 1). A similar lack of TC II synthesis by mutant cell line WG 1276 was reported earlier (21). However, low levels of endogenous TC II synthesis could be detected in nontransfected normal skin fibroblasts (lanes 2 and 3). When transfected with wild-type TC II cDNA, the TC II-deficient fibroblasts from patient WG 1346 (lane 4) and WG 1276 (lane 5) revealed a single band of molecular mass of 43 kDa representing nonaggregated TC II. In addition, because of overexpression and the highly hydrophobic nature of TC II, its aggregates that were also immunoprecipitated with TC II antiserum could be identified at the top of the gel (lanes 4 and 5). Because of lower levels of expression, the aggregated form of TC II was absent (lanes 2 and 3) when the labeled medium from normal fibroblasts was immunoprecipitated and subjected to SDS-PAGE. Compared with the levels of the

Table 1. Titration of total and free SH groups of human TC II

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<tr>
<th>Conditions</th>
<th>DTNB</th>
<th>[3H]NEM</th>
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<tbody>
<tr>
<td>No additions</td>
<td>2 ± 0.10</td>
<td>2 ± 0.14</td>
</tr>
<tr>
<td>With 2-ME</td>
<td>8 ± 0.19</td>
<td>8 ± 0.20</td>
</tr>
</tbody>
</table>

Results shown under both conditions are means ± SD of 4 different titration experiments. SH, sulphydryl; TC II, transcobalamin II; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); [3H]NEM, N-[3H]ethylmaleimide; 2-ME, 2-mercaptoethanol.

Fig. 3. SDS-PAGE analysis of TC II synthesized in normal and TC II-deficient fibroblasts after transfection with wild-type TC II cDNA. Human skin fibroblasts were labeled with [35S]methionine after transfection with vector alone into patient cell line WG 1346 (lane 1), without transfection in normal human skin fibroblasts MCH 24 (lane 2) or MCH 55 (lane 3), or after transfection with wild-type TC II cDNA into patient cell lines WG 1346 (lane 4) or WG 1276 (lane 5). The labeled media were then treated with antiserum to human TC II, and the immunoprecipitated radioactivity was subjected to nonreducing SDS-PAGE. The bands were visualized by fluorography. Other details are provided in MATERIALS AND METHODS.

43-kDa form of TC II secreted by normal fibroblasts, the mutant cells secreted nearly 30- to 40-fold higher levels of labeled TC II when transfected with wild-type TC II cDNA. These studies clearly indicated that TC II-deficient fibroblasts are an excellent choice to study the synthesis and secretion of endogenously expressed TC II.

[57Co]Cbl binding by culture media of transfected cells. The culture media of cells transfected with wild-type TC II cDNA revealed nearly a 40- to 45-fold stimulation of Cbl binding activity compared with cells transfected with the vector alone (Table 2). Similar high levels of Cbl binding were also noted when the cells were transfected with mutant TC II constructs with a single (C65S, C78S, C3S, C249S) or double (C65/78/78/S) substitution. In contrast, Cbl binding activity could not be detected in the medium of cells transfected with mutant TC II constructs containing a single (C98S, C147S, C187S, C291S), double (C3/147/S, C98/147/S), or triple (C98/147/S, C65/78/78/S) substitution. These results indicate that two of six conserved cysteine residues, C3 and C249, and the two nonconserved cysteine residues, C65 and C78, are not important for Cbl binding, whereas the four conserved cysteine residues, C98, C147, C187, or C291, may play an important role in the folding of TC II, which in turn may be essential for Cbl binding, secretion, interaction with TC II antiserum, or intracellular stability. To
To test this possibility, the labeled culture media obtained from cells transfected with the indicated TC mutant constructs were treated with QUSO-G32, a resin that specifically binds to TC II (4, 28). When the radioactivity bound to QUSO-G32 was washed and subjected to SDS-PAGE (Fig. 4C), labeled TC II could be detected from the media of cells transfected with the wild-type and C3S or C249S mutant constructs but not in the media of cells transfected with constructs with a single (C98S, C147S, C187S, C291S), double (C3/147/S, C98/147/S), or triple (C3/98/147/S) substitution. These data clearly indicated that failure to detect labeled TC II in the culture media of cells transfected with these constructs was not due to the inability of TC II synthesized to react with TC II antisera but, rather, to either lack of their synthesis or rapid degradation. Although TC II synthesized and secreted from the mutant constructs C3S and C249S was able to bind Cbl, we wanted to examine whether they were also able to bind to their cell surface receptor and mediate Cbl transport into cells.

Binding to TC II-R and Cbl transport. When the labeled media from cells transfected with the wild-type, C3S, and C249S constructs were allowed to react with Sepharose-TC II-R affinity matrix, the radioactivity bound to the beads revealed on SDS-PAGE (Fig. 5A) a single band of 43–47 kDa. This band was absent when the labeled culture media from cells transfected with constructs C98S, C147S, C187S, or C291S were treated with Sepharose-TC II-R. These results clearly established that TC II expressed by C3S and C249S, like the wild-type TC II, was also able to bind to its

Fig. 4. SDS-PAGE analysis of [35S]-labeled TC II ([35S]-TC II) synthesized after transfection of the wild-type and indicated mutant constructs of human TC II. The media (A) or the cell lysates (B) from cells labeled for 8 and 1 h, respectively, were treated with antiserum to human TC II and protein A-Sepharose. In some experiments (C), the labeled media (1 ml) were treated with 5 mg of the resin QUSO-G32 for 2 h. The bound resin and the immunoprecipitated radioactivity were washed and subjected to nonreducing SDS-PAGE. The bands were visualized by fluorography. Other details are provided in MATERIALS AND METHODS. V, vector alone; Wt, wild type.

**Table 2. 57Co/Cbl binding activity in culture media of TC II-deficient fibroblasts transfected with wild-type and cysteine-serine substitutions of human TC II**

<table>
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<th>Construct</th>
<th>Conserved</th>
<th>[57Co/Cbl Bound] fmol/ml medium</th>
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<tr>
<td>Vector</td>
<td></td>
<td>5 ± 2</td>
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<tr>
<td>Wild type</td>
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</tr>
<tr>
<td>C3S</td>
<td>Yes</td>
<td>225 ± 15</td>
</tr>
<tr>
<td>C65S</td>
<td>No</td>
<td>200 ± 11</td>
</tr>
<tr>
<td>C78S</td>
<td>No</td>
<td>198 ± 6</td>
</tr>
<tr>
<td>C98S</td>
<td>Yes</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>C147S</td>
<td>Yes</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>C187S</td>
<td>Yes</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>C249S</td>
<td>Yes</td>
<td>225 ± 13</td>
</tr>
<tr>
<td>C291S</td>
<td>Yes</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>C65/78S</td>
<td></td>
<td>205 ± 10</td>
</tr>
<tr>
<td>C3/147S</td>
<td></td>
<td>10 ± 5</td>
</tr>
<tr>
<td>C98/147S</td>
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<td>9 ± 5</td>
</tr>
<tr>
<td>C65/78/147S</td>
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<td>11 ± 7</td>
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<tr>
<td>C3/98/147S</td>
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 Constructs with indicated single, double, or triple mutation were transfected for 24 h. The medium was used for [57Co]Cbl binding assay. The binding values are reported as means ± SD of duplicate assays from 4 separate transfection experiments. Bcl, cobalamin.

To address these issues, we treated the labeled media with human TC II antiserum and tested for the synthesis and secretion of TC II.

**SDS-PAGE analysis of [35S]-TC II.** The culture media of cells transfected with the wild-type or C3S, C65S, C78S, or C249S mutant constructs revealed a single band on nonreducing SDS-PAGE (Fig. 4A). However, TC II obtained from constructs with substitutions at C3 or C249 had less mobility on SDS-PAGE, which resulted in an apparent increase in their molecular mass by 4 kDa. No labeled TC II could be detected when the cells were transfected with the vector alone or with TC II constructs with a single (C98S, C147S, C187S, C291S), double (C98/147/S, C3/147/S), or triple (C3/98/147/S) substitution.

To determine whether the lack of Cbl binding (Table 2) and a failure to detect labeled TC II in the culture media of cells transfected with some of the mutant constructs (Fig. 4A) was due to a secretory defect, SDS-PAGE analysis of the cell lysate (Fig. 4B) was carried out using cells that were labeled for only 60 min. Immunoprecipitation of cell lysates with TC II antiserum revealed labeled TC II in the cell lysates obtained from cells transfected with the wild-type or C3S, C65S, C78S, or C249S mutant constructs. No labeled TC II could be detected in lysates of cells transfected with constructs bearing single (C98S, C147S, C187S, C291S), double (C3/147/S, C98/147/S), or triple (C3/98/147/S) substitution. These data indicate that lack of Cbl binding or a failure to detect labeled TC II noted in the cell culture media of cells transfected with these TC II constructs was not due to their lack of secretion or cellular retention. However, the possibility existed that the inability to detect labeled TC II in the medium or cell lysate after transfection of some of these constructs may be due to their inability to react with TC II antisera, thus not allowing their detection on SDS-PAGE.

**C DISULFIDE BONDS OF HUMAN TRANSCOBALAMIN II**

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<tr>
<th>DISULFIDE BONDS</th>
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<td>C65/78S</td>
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receptor. In support of this finding, TC II secreted by cells transfected with C3S and C249S constructs was also able to promote Cbl transport into fibroblasts (Fig. 5B) with about 80 and 60% efficiency compared with the wild-type TC II, respectively. In contrast, the cell culture media obtained from cells transfected with constructs C98S, C147S, C187S, or C291S were unable to promote Cbl transport.

Together, these cell transfection experiments suggested the possibility that the TC II mutant constructs bearing single (C98, C147, C187, C291), double (C3/C147, C98/C147), or triple substitution at all the six conserved positions (Fig. 6A), double substitution at C3/C147 or C98/C147, or triple substitution at C3/C98/C147 (Fig. 6B) translated well with demonstration of a single band on SDS-PAGE. However, when translated in the presence of pancreatic membranes (Fig. 6A and B, lanes 2), the translated products from the wild-type, C3S, and C249S constructs demonstrated a single band with slightly increased mobility consistent with signal pep-
DISULFIDE BONDS OF HUMAN TRANSCOBALAMIN II

raphy. However, the relative intensities of the bands obtained after translation in the absence and presence of microsomal membranes were similar. In contrast, the intensities of TC II bands were dramatically reduced when the translation of TC II constructs C98S, C147S, C187S, or C291S (Fig. 6A, lanes 2) or C3/147/S, C98/147/S, or C3/98/147/S (Fig. 6B, lanes 2) were carried out in the presence of microsomal membranes. This finding indicated increased instability of TC II when translated and allowed to undergo cotranslational translocation with one or none of the disulfide bonds intact and appears to depend on the presence of both C147-C187 and C98-C291 disulfide bonds.

To test whether the in vitro translated TC II was able to bind Cbl, we allowed equal amounts of translation reaction containing similar amounts of radioactivity to react with Sepharose-Cbl affinity matrix (Fig. 6, C and D). The Cbl binding by [35S]-TC II translated in the absence of microsomal vesicles (Fig. 6A and B, lanes 1) by the wild-type and mutant constructs containing a single substitution of the six conserved cysteine residues was very similar (Fig. 6C, open bars), indicating that Cbl binding by unprocessed (lack of signal peptide cleavage) TC II is not directly affected by mutation of any one of the six conserved residues. However, when the translation was carried out in the presence of microsomal vesicles (Fig. 6, A and B, lanes 2), only the constructs C3S or C249S demonstrated Cbl binding similar to the wild type. Mutant constructs C98S, C147S, C187S, or C291S demonstrated nearly 80–90% decreased Cbl binding consistent with the percent loss of intensities of TC II band (Fig. 6C, filled bars). In contrast to Cbl binding, the loss of TC II-R binding by TC II translated in the presence of pancreatic microsomes was less (25–45%) (data not shown) for all the mutant constructs bearing a single substitution, except for construct C291S, where the receptor binding declined to about 80%. The persistence of Cbl and receptor binding by unprocessed TC II expressed by constructs bearing a single substitution was a surprising result and could arise as a result of their ability to form the two other disulfide bonds because single substitution disrupts formation of only one of the three disulfide bonds of TC II. To test this possibility, we tested the ability to bind Cbl and receptor of TC II synthesized by in vitro translation using mutant constructs bearing double and triple substitutions in the presence and absence of microsomal vesicles.

In contrast to wild-type TC II, TC II synthesized in the presence of pancreatic microsomes from constructs with double (C3/147/S, C98/147/S) or triple (C3/98/147/S) substitution were highly unstable (Fig. 6D), and Cbl binding (Fig. 6D) declined by more than 90%, consistent with a total loss of TC II protein. Interestingly, TC II synthesized in the absence of microsomal vesicles by the same constructs was stable but demonstrated a 50–70% loss of Cbl binding (Fig. 6D) and a 25–50% loss in receptor binding (data not shown). These latter results indicated that loss of Cbl binding by native TC II may be due to disruption of all its three disulfide bonds. To examine this possibility, we determined Cbl binding by unprocessed TC II expressed by constructs bearing a single, double, or triple substitution with and without their reduction.

Reductive alkylation decreases Cbl binding of TC II expressed in vitro. SDS-PAGE analysis (Fig. 7A) of unprocessed TC II expressed by mutant constructs C3S, C98/147/S, C98/147/S, or C3/98/147/S revealed synthesis of a stable form of TC II whether they were reduced (Fig. 7A, lane 2) or not (lane 1). However, after reduction, Cbl binding by TC II expressed by all the mutants constructs and even the wild-type TC II declined by 70–90% (Fig. 7B). These results clearly indicated that for optimal Cbl binding by apo TC II, at least two disulfide bonds formed by C98, C147, C187, and C291 are essential.

In addition to their role in Cbl binding, the two disulfide bonds formed by C98, C147, C187, and C291 also appear to be essential for conferring both extracellular and intracellular stability of TC II. Because TC II expressed by these mutant constructs became unsta-

Fig. 7. Effect of reduction of the wild-type and cysteine mutants of TC II on Cbl binding. A: [35S]-TC II (10–12,000 dpm) unreduced (lanes 1) or reduced and alkylated (lanes 2) expressed in vitro in the absence of microsomal vesicles from the indicated constructs were subjected to SDS-PAGE, and the bands were visualized by fluorography. B: binding of unreduced (open bars) and reduced (filled bars) [35S]-TC II to Sepharose-Cbl. Values are expressed as a percentage of the TCA-precipitable radioactivity present in 2 μl of reaction obtained after translation and represent means ± SD of 4 different binding and translation experiments.
ble during their in vitro cotranslational translocation, it is likely that they are targets for early detection by the quality control mechanism of ER and are most probably degraded by the proteasome system. To test this possibility, and to verify whether lysosomal degradation is involved as well, we carried out transfection studies using inhibitors of both lysosomal and proteasomal degradation machinery.

**Substitutions of conserved C98, C147, C187, or C291 result in degradation of TC II by the proteasome system.** When the transfected cells were labeled for 1 h in the presence of ammonium chloride or chloroquine and the labeled medium was treated with QUSO-G32, labeled TC II could be detected on SDS-PAGE (Fig. 8) only with the wild-type TC II, not with mutant TC II constructs at C98S, C147S, C187S, or C291S. However, when similar studies were carried out using proteasome degradation inhibitors lactacystin or MG-132, labeled TC II could be detected with all the mutant constructs, once again using QUSO-G32. In the medium of cells treated with lactacystin, antiserum to human TC II was able to precipitate only the wild-type TC II, not mutant TC II constructs C98S, C147S, C187S, or C291S. Similar results were obtained with labeled medium from cells treated with MG-132 (data not shown). Interestingly, the labeled proteins obtained from cells incubated with either lactacystin or MG-132 were able to bind to Sepharose-Cbl (Fig. 8B) or Sepharose-TC II-R (Fig. 8C) matrices. The amount of radioactivity bound to either of the matrices was very similar and was about 75–90% of that bound when the wild-type TC II cDNA was transfected. However, when similar experiments were carried out using the constructs with the double and triple mutations, the binding of labeled TC II to either of the affinity matrices declined between 70 and 85% (data not shown). Together, these in vitro and in vivo studies showed that substitutions at conserved C98, C147, C187, or C291 disrupting two of the three disulfide bonds of TC II resulted in 1) loss of function, 2) inability to interact with TC II antiserum, and 3) targeting for rapid degradation by the proteasomal system. The various effects on the properties of TC II expressed in cells due to single substitutions of cysteine residues are summarized in Table 3.

**DISCUSSION**

Bovine TC II contains three intramolecular disulfide bonds that are formed utilizing its six conserved cysteine residues, and their assignment for disulfide bond formation are C3-C252, C98-C293, and C147-C190 (8). When bovine holo-TC II is reduced in vitro, it undergoes spectral changes and Cbl bound to it is released, suggesting that disulfide bonds of TC II may play a role in Cbl binding. Despite these studies, it is not known whether 1) all three disulfide bonds equally contribute for Cbl binding by apo TC II, 2) their disruption affects its ability to bind to its receptor, or 3) the three disulfide bonds have any role in its intracellular trafficking, stability, and secretion. The current studies have addressed these issues with the use of human TC II, which, like bovine TC II (8), also contains three disulfide bonds (Table 1), has 95–98% identity with rat and...
bovine TC II, and contains six conserved cysteine residues whose locations vary very insignificantly (Fig. 9) from that of bovine TC II (8).

In this study we have expressed various cysteine mutants of TC II in an in vitro expression system as well as by transient transfection in human TC II-deficient fibroblasts. TC II is not a glycoprotein (25) and is not known to undergo any other type of post-translational modification other than the formation of intramolecular disulfide bonds. Thus, when expressed in human cells, folding alterations that would affect its function and stability would largely be dependent on the posttranslational formation of intramolecular disulfide bonds. Moreover, the availability of human TC II-deficient skin fibroblasts that are unable to synthesize TC II due to lack of its mRNA (21) have made our studies easier, because absence of any endogenous TC II expression will completely eliminate background noise. When the wild-type TC II cDNA was expressed in these mutant cells, a 30- to 40-fold increased synthesis (Fig. 3) and secretion of functional TC II was observed. This observation has confirmed our earlier finding that a failure to synthesize TC II in these mutant cells is due to lack of TC II transcript (19, 21).

The six conserved cysteine residues of human TC II, by analogy to bovine TC II, will be utilized to form the three disulfide bonds C3-C249, C98-C291, and C147-C187. Mutational analyses of these six conserved cysteine residues have allowed us to make important conclusions regarding the role of these three disulfide bonds on the many properties of human TC II. The major conclusions from these studies are that the disulfide bond formed between C3 and C249 has no role in either function or stability of human TC II, whereas the two disulfide bonds formed between C98 and C291 and between C147 and C187 are important for both.

The lack of any role for the C3-C249 disulfide bond is borne out by the observations that TC II synthesized in vitro (Fig. 6) and in transfected cells (Table 2, Fig. 4) by constructs C3S and C249S were able to bind Cbl and its receptor, undergo secretion, and promote Cbl transport into cells (Fig. 5). It is important to note that even though we have been able to demonstrate gross Cbl binding by TC II expressed by constructs C3S and C249S, our studies do not allow us to make any conclusions regarding their relative affinities for Cbl binding. Because the wild-type TC II binds to Cbl with very high affinity with an association constant of $10^{11} \text{M}^{-1}$ (2), these mutant proteins with even a 100- to 1,000-

**Table 3. Summary of effect on various properties of human TC II expressed in fibroblasts following single substitution of its eight cysteine residues**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Human TC II Construct</th>
</tr>
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<tbody>
<tr>
<td>Location of cysteine</td>
<td>Wt C65, C78, C3 or C249 → S</td>
</tr>
<tr>
<td>Additions</td>
<td>None Chloroquine/NH4Cl Lactacystin/MG-132</td>
</tr>
<tr>
<td>Cbl binding</td>
<td>+ + − − + +</td>
</tr>
<tr>
<td>Receptor binding</td>
<td>+ + − − − +</td>
</tr>
<tr>
<td>Secretion</td>
<td>+ + − − − +</td>
</tr>
<tr>
<td>Intracellular stability</td>
<td>+ + − − − +</td>
</tr>
<tr>
<td>Ab precipitation</td>
<td>+ + + + − −</td>
</tr>
<tr>
<td>QUSO G-32</td>
<td>+ + + + − −</td>
</tr>
</tbody>
</table>

Wt, wild type; Ab, antibody.

To the best of our knowledge, this finding is the first to demonstrate that TC II deficiency can be corrected at a cellular level.

The two nonconserved residues of human TC II, C65 and C78, are not involved in a disulfide bond between themselves or any of the other six conserved cysteine residues. This conclusion is based on the following observations: 1) titration of native human TC II shows the presence of two free SH groups (Table 1); 2) substitution of one or both of these two cysteine residues with serine had no effect on Cbl binding (Table 2); 3) synthesis and secretion of TC II was not affected when cells were transfected with TC II constructs C65S or C78S (Fig. 4A); and 4) functional bovine TC II (8) contains serine at this position. Thus replacement of cysteine by serine at these two positions may represent neutral polymorphism. It is well known that TC II is a tolerant protein and exhibits neutral polymorphism (22) without loss of function (15).

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fold decrease in their affinity for Cbl would still bind to it avidly. Further kinetic analysis of Cbl binding by these mutant proteins would require preparation of sufficient amounts of these mutant proteins.

The two disulfide bonds formed by the conserved cysteine residues C98, C147, C187, and C291 play an important role in the stability and function of human TC II. The following lines of evidence support such a conclusion. First, TC II expressed by mutant constructs C98S, C147S, C187S, and C291S was unstable when expressed in cells (Fig. 4, A and B) or in vitro in the presence of pancreatic microsomes (Fig. 6A). Second, the double and triple mutants C3/147/S, C98/147/S, and C3/98/147/S, when expressed in vitro and allowed to undergo cotranslational translocation, were almost completely degraded (Fig. 6B) with nearly 90–95% loss of both Cbl (Fig. 6D) and TC II-R binding (data not shown). Third, TC II expressed in vitro by the construct C3S, which is still able to form two disulfide bonds, C147-C187 and C98-C291, lost 75% of its Cbl binding ability when it was reduced and alkylated (Fig. 7). Fourth, TC II expressed by constructs C3/147/S, C98/147/S, or C3/98/147/S, which are still able to form either single disulfide bond C98-C291 or C3-C249 or no disulfide bond, lost 40, 60, or 75% of its Cbl binding ability, respectively, without reduction. Upon reductive alkylation, Cbl binding was further reduced to 75–80%. Finally, loss of 60% Cbl binding activity by TC II construct C98/147/S suggests that all or some of the Cbl binding by TC II expressed by constructs C3S or C249S is due to the presence of disulfide bonds C98-C291 and C147–187. Together, these studies have underscored the importance of disulfide bonds C98-C291 and C147–187 in Cbl binding and stability and support earlier studies that have shown the release of Cbl from holo-TC II after disruption of its disulfide bonds by reduction (8).

In contrast to decreases in Cbl binding, decreases in TC II-R binding by TC II expressed in vitro in the presence of pancreatic microsomes by constructs C98S, C147S, or C187S were less marked. It is likely that a partially degraded piece(s) of TC II may be able to bind to TC II-R. In support of this suggestion are recent observations from our laboratory (Li N, Bose S, Kalra S, Seetharam S, and Seetharam B, unpublished observations) that have shown TC II-R binding activity of different cysteine mutants of bovine pancreatic trypsin inhibitor (18) expressed in the yeast system. These observations suggest strongly that not all three disulfide bonds of human TC II may be topologically equivalent in mediating its trafficking out of ER, protecting the Cbl binding site, stability, and secretion.

It is interesting to note that the highly identical region (Fig. 9) that is common to all mammalian TC II and other Cbl binding proteins important in Cbl binding (Li N, Seetharam S, and Seetharam B, unpublished observations) is encompassed by all three disulfide bonds. However, disulfide bonds C147-C187 and C98-C291 appear to maintain their integrity. In addition, none of the three disulfide bonds of TC II appears to have any direct role in the secretion of TC II. This observation is not too surprising, because earlier studies (3, 12, 30) have suggested that disulfide bonds in secretory proteins have no role in constitutively secreted proteins (26, 32) such as TC II, whereas they can influence secretion of proteins that are transported by the regulated pathway.

In summary, TC II expressed in cells after disruption of the C3-C249 disulfide bond, though misfolded as ascertained by changes in mobility on SDS-PAGE, was stable, secreted, and functional. However, disruption of disulfide bonds C98-C291 or C147-C187 resulted in expression of TC II that was also misfolded, as ascertained by its inability to react with TC II antiserum, and was rapidly degraded by the proteasomal system. Thus, in the context of their cellular expression and intracellular folding, not all six conserved cysteine residues of human TC II are equivalent.

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Present address of N. Li: Department of Dermatology, University of North Carolina, Chapel Hill, NC 27599.

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