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Effect of Disulfide Bonds of Transcobalamin II Receptor on Its Activity and Basolateral Targeting in Human Intestinal Epithelial Caco-2 Cells*

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Transcobalamin II-receptor (TC II-R) contains 10 half-cysteines, of which 8 are involved in intramolecular disulfide bonding. Reduction followed by alkylation with *N*-ethylmaleimide (NEM) of the 62-kDa TC II-R monomer *in vitro* or treatment of human intestinal epithelial Caco-2 cells with low concentrations (10^{-6} M) of NEM resulted in TC II-R exhibiting a loss of ligand binding and an increase in its apparent molecular mass by 10 kDa to 72 kDa. Domain-specific biotinylation studies using NEM-treated filter-grown cells revealed loss of TC II-R but not cation-independent mannose 6-phosphate receptor protein at the basolateral cell surface. Pulse-chase labeling of NEM-treated cells with [^{35}S]methionine revealed that the modified 72-kDa TC II-R, like the native 62-kDa TC II-R in untreated cells, turned over rapidly with a $t_{1/2}$ of 7.5 h and was sensitive to treatment with peptide *N*-glycosidase F, sialidase alone, or sialidase and *O*-glycanase but not to treatment with endoglycosidase H. Labeled 72-kDa TC II-R, which was retained intracellularly following treatment of Caco-2 cells with methyl methanethiosulfonate, returned to the basolateral cell surface following withdrawal of cells from methyl methanethiosulfonate treatment and exposure to dithiothreitol. Based on these results, we suggest that formation and maintenance of intramolecular disulfide bonds of TC II-R is important for its acquisition of ligand binding and post-trans-Golgi trafficking to basolateral surface membranes but not for its turnover and exit from the endoplasmic reticulum or trafficking through the Golgi.

Transcobalamin II-receptor (TC II-R)[1] is a single chain glycoprotein with a molecular mass of 62 kDa (1) containing about 27% carbohydrate. It promotes plasma transport of cobalamin (Cbl; vitamin B_{12}) bound to transcobalamin II (TC II) (2). TC II-R functions as a noncovalent homodimer with a molecular mass of 124 kDa in tissue membranes (1), and its dimerization occurs not in the ER, where it is a monomer, but rather in the plasma membrane (3). In addition, the dimerization of TC II-R is a membrane fluidity-driven event requiring a highly ordered rigid bilayer (4). In polarized epithelial Caco-2 cells, TC II-R is predominantly present in basolateral membranes (5), where it functions in the delivery of Cbl to be utilized as coenzymes (6). Treatment of the basolateral surface of Caco-2 cells with TC II-R antiserum *in vitro* or circulating TC II-R antibody *in vivo* results in a failure of TC II-Cbl uptake, causing intracellular Cbl deficiency (6). These studies have suggested that plasma membrane delivery of TC II-R is extremely important for cells to obtain their Cbl from the circulation to maintain their normal metabolic, differentiation, and proliferation status. Despite the importance of plasma membrane expression of TC II-R in the tissue transport of Cbl, no information is available regarding its intracellular trafficking and plasma membrane expression.

Recent studies have shown that formation of intracellular inter- or intrachain disulfide bonds and their final rearrangement by thiol-disulfide exchange reaction is one of the co/post-translational modifications of some proteins (7, 8). The correct formation of intrachain and, in the case of oligomeric proteins, interchain disulfide bonds is crucial for the proper assembly of secretory and plasma membrane proteins, which in turn determines their stability, intracellular transport, maturation, and function (9–11). Inhibition of disulfide bond formation by DTT treatment has been shown to affect secretion of albumin and H1 subunit of asialoglycoprotein receptor from fibroblasts (12) and of gp80 (clusterin, apolipoprotein J) in polarized Madin-Darby canine kidney cells (13). However, the secretion of non-disulfide bond-containing proteins such as α_1-antitrypsin (14) in HepG2 cells or of a 20-kDa peptide derived from osteoponin in Madin-Darby canine kidney cells (13) is not affected. These studies have proposed that the retention of proteins in the ER following treatment with DTT is due to a failure of these proteins to form disulfide bonds, which then fold improperly. As a consequence, these misfolded proteins fail to achieve a conformation essential for their function as well as their exit from the ER for further processing and targeting (15).

Human TC II-R contains 10 half-cysteines (16), and recent immunoblot studies of placental plasma membrane proteins under nonreducing and reducing conditions (1) have shown that TC II-R dimers contain intramolecular disulfide bonds that, upon reduction, increase the apparent molecular mass of the dimer by 20 kDa. Taken together, these results have suggested that some if not all of the SH groups of TC II-R monomer may be involved in the formation of intramolecular disulfide

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[1] The abbreviations used are: TC II-R, transcobalamin II-receptor; TC II, transcobalamin II; Cbl, cobalamin (vitamin B_{12}); DMEM, Dulbecco's modified Eagle's medium; IAM, iodoacetamide; NEM, *N*-ethylmaleimide; MMTS, methyl methanethiosulfonate; CI-MPR, cation-independent mannose 6-phosphate receptor; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; TGN, trans-Golgi network; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; 2-ME, 2-mercaptoethanol; Endo H, Endo-β-*N*-acetylglucosaminidase; PNGase, peptide:*N*-glycosidase; PBS, phosphate-buffered saline; NSF, *N*-ethylmaleimide-sensitive factor.

bonds, which remain intact during its transport to and dimerization in the plasma membranes. Furthermore, the increase in apparent molecular mass of the reduced TC II-R dimer may be related to its attainment of an extended or a more linear structure. However, it is not known whether the TC II-R monomers contain intrachain disulfide bonds that, when reduced, form extended structures and whether such a modification has any effect on their ligand binding, turnover, and trafficking to the basolateral membranes. The current studies have addressed these issues *in vitro* using pure TC II-R and *in vivo* using human intestinal epithelial Caco-2 cells as a model system. The results of this study show that TC II-R monomers contain four intramolecular disulfide bonds that are required for activity and two free SH groups that have no role in ligand binding. Sulfhydryl group modification of either isolated TC II-R monomer or that expressed in Caco-2 cells by SH-modifying agents demonstrated a more linear extended form of an inactive receptor with a molecular mass of 72 kDa. The extended 72-kDa form of TC II-R, like the native 62-kDa form, turned over rapidly in cells with a $t_{1/2}$ of 7.5 h. In addition, it was able to exit ER and transit through the Golgi as revealed by the sialylation of both its *N*- and *O*-linked sugars. However, the 72-kDa form of TC II-R was unable to undergo transport from the trans-Golgi to its destination in the basolateral membranes.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The following chemicals were purchased as indicated: [^{57}Co]CN-Cbl (15 μCi/μg) and carrier-free Na^{125}I (17.4 Ci/mg) (Amersham Corp.); [^{35}S]methionine (1175 Ci/mmol) and [^{3}H]NEM (60 Ci/mmol) (NEN Life Science Products); ^{125}I-protein A (>30 Ci/μg) (ICN Radiochemicals, Irvine, CA); *N*-ethylmaleimide, iodoacetamide, methyl methanethiosulfonate, dithiothreitol, protein A-Sepharose, and sialidase from *Clostridium perifringens* (from Sigma); 5,5′-dithiobis(2-nitrobenzoic acid), sulfosuccin imidobiotin and disuccinimidyl suberate (Pierce); Millicell-HA culture plate inserts (Millipore); cellulose nitrate membranes (Schleicher and Schuell). Endo-β-*N*-acetylglucosaminidase from *Streptomyces plicatus*, Peptide-*N*-glycosidase from *Flavobacterium meningosepticum*, and *O*-glycanase from *Diplococcus pneumonia* (Boehringer Mannheim). All chemicals purchased were used as such except that iodoacetamide was recrystallized in water. Pure TC II-R (specific activity, 14–15 nmol of ligand binding/mg of protein) was purified from human placenta, and its antiserum was prepared as described earlier (1). Antiserum to bovine CI-MPR was a gift from Dr. Nancy M. Dahms (Department of Biochemistry, Medical College of Wisconsin, Milwaukee). Partially purified human TC II used for ligand binding was prepared from human plasma according to Lindemans *et al.* (17). Human TC II was a gift from Charles A. Hall (Nutrition Assessment laboratory, Albany, NY). Pure TC II (5 μg) or streptavidin (50 μg) was iodinated with 0.5 mCi of Na^{125}I and IODO-GEN as recommended by the manufacturer. The iodinated proteins were separated from free iodine on a Sephadex G-25 column using 10 mM Tris-HCl, pH 7.5, buffer containing 140 mM NaCl and 1 mg/ml bovine serum albumin. The specific activity of ^{125}I-TC II and ^{125}I-streptavidin was between 70 and 80 μCi/μg.

Reductive Modification of SH Groups of TC II-R and Activity Measurements—Pure TC II-R (5 μg) was treated with 130 mM of iodoacetamide (IAM) or NEM or methyl methanethiosulfonate (MMTS) for 1 h at room temperature in the presence or absence of 4 M urea or 2-mercaptoethanol (40 mM). The receptor was first incubated with 4 M urea (15 min) or 2-mercaptoethanol (1 h in the dark) prior to the addition of the SH-modifying agent. In some experiments, the receptor was first treated with urea for 15 min followed by 2-mercaptoethanol treatment for 1 h and finally treated with the SH reagent. All samples (1.5 ml) were dialyzed for 12 h against 5 liters of 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 0.1 mM PMSF. The dialyzed samples were assayed for TC II-[^{57}Co]Cbl (1.5 pmol) binding by the DEAE-Sephadex method of Seligman and Allen (16). TC II-R that was reduced and treated with each of the SH-modifying agents was treated with DTT (200 mM), dialyzed, and assayed for the ligand binding as stated above. In some experiments, TC II-R reduced in the presence of urea and treated with IAM, NEM, or MMTS was cross-linked with ^{125}I-TC II-Cbl (200,000 dpm/2 pmol Cbl binding capacity) in the presence and absence of excess (20-fold) unlabeled TC II-Cbl. Reduced and SH group-modified TC II-R (2 μg) was incubated with and without cold TC II-Cbl for 30 min at room temperature. The samples were then incubated with ^{125}I-TC II-Cbl for 2 h at room temperature, the cross-linking agent disuccinimidyl suberate (4 mM) was added, and they were incubated for an additional 30 min. The reaction was stopped by the addition of glycine (0.1 mM). The reaction mixture was subjected to nonreducing SDS-PAGE, and the bands were visualized by autoradiography.

Titration of Pure TC II-R with DTNB and Labeled NEM—Pure TC II-R (50 μg), unreduced or reduced in the presence or absence of urea (4 M) in Tris-glycine buffer, pH 8.0, containing 0.2 mM EDTA was incubated with DTNB (10 μg) for 1 h at 22 °C. The absorbance of the samples was measured at 412 nm, and the spectrophotometric titrations of thiol groups were calculated based on the known extinction coefficient of DTNB (13,600 mol^{-1}/cm^{-1}) according to Habeeb (18). Titration of pure TC II-R (5 μg) was carried out using [^{3}H]NEM (760,000 dpm/100 mM) under reducing and nonreducing conditions and in the absence and the presence of urea as described above for DTNB titration. The samples were dialyzed and counted for the bound radioactivity.

SDS-PAGE and Immunoblotting—Pure native, reduced, and SH-modified TC II-R (0.2–2 μg of protein), Caco-2 cell homogenate (50 μg), or Triton X-100 (1%) extract of total Caco-2 cell membrane (200 μg), and labeled TC II-R immunoprecipitated from NEM treated and untreated cells were subjected to nonreducing SDS-PAGE (7.5%) according to Laemmli (19). In some experiments separated proteins were subjected to immunoblotting to detect either the monomer or the dimer form of TC II-R as described before (4). The bands were visualized by autoradiography at −70 °C using X-Omat plates. The bands were quantified by the AMBIS radioimaging system, and the image density was found to be in the linear range (3).

Cell Culture—Caco-2 cells (passages 76–80) were grown in DMEM (25 mM glucose) supplemented with 20% heat-inactivated fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Confluent monolayers were subcultured every 7 days by treatment with 0.05% trypsin-EDTA in phosphate-buffered saline. In some experiments, the cells were plated at a density of 2×10^6 cells on plastic tissue culture 75-cm^2 flasks and were harvested 3–12 days after plating. For the ligand uptake studies, cells were grown as epithelial layers by high density seeding (1.5×10^6 cells/filter) onto nitrocellulose membrane filter inserts (Millicell-HA, 30-mm diameter, 0.45 μM pore size). The formation and integrity of monolayers were assessed by the development of significant transepithelial resistance of 250–300 ohms/cm^2 over the resistance of filter alone. The filter grown cells that were treated with NEM were also measured for transepithelial resistance, which was found to be similar to that of cells not treated with NEM. All resistance readings were measured with a Millicell-ERS Voltohmeter (Millipore Corp.).

NEM Treatment of Postconfluent Caco-2 Cells Grown on Culture Inserts—Filter grown Caco-2 cells were incubated with NEM (10^{-12} to 10^{-6} M) for 16 h, and the cells were harvested and homogenized in 1 ml of 10 mM Tris-HCl buffer containing 140 mM NaCl and 0.1 mM PMSF. Untreated and NEM-treated cell homogenates were subjected to nonreducing SDS-PAGE and immunoblotting. Using another set of filters similarly treated with NEM, the basolateral binding of the ligand, TC II-[^{57}Co]Cbl (500 fmol), was determined as described earlier (6). Total TC II-R activity was determined in these cells by the DEAE-Sephadex method using Triton X-100 (1%) extract of the harvested cell homogenates prepared using 1 ml of 10 mM Tris-buffered saline containing 0.1 mM PMSF.

Metabolic Labeling of Caco-2 Cells—Postconfluent Caco-2 cells were first incubated with NEM (10^{-6} M) for 8 h, the medium was replaced with fresh medium containing [^{3}H]NEM (4×10^6 dpm/T-75 flask, 10^{-6} M), and the cells were incubated for an additional 8 h at 37 °C. The cells were harvested, homogenized in Tris-HCl buffer, pH 7.5, containing 140 mM NaCl and 0.1 mM PMSF (TBS). The homogenate was centrifuged for 60 min at 100,000 × *g*, and the pelleted membrane was rehomogenized in the same buffer and extracted with Triton X-100 (1%). The cellular homogenate, total membranes, cytosol, and the immunoprecipitated fraction obtained using Triton X-100 extract of the total membranes and antiserum to TC II-R or CI-MPR were subjected to nonreducing SDS-PAGE. The gel was treated with EN^{3}HANCE and dried, and the bands were visualized by fluorography. In some experiments, the cells were incubated for 8 h with NEM (10^{-6} M), and then [^{35}S]methionine (200 μCi/flask) was added and cells were incubated for an additional 8 h. Prior to labeling, the cells were incubated with methionine-free DMEM for 30 min. Further processing, extraction, and immunoprecipitation of labeled TC II-R was carried out as above.

TABLE I
Effect of SH modification on TC II-R activity

The values reported are mean ± S.D. of six separate assays under each condition using each of the SH-modifying agents. TC II-R activity was determined by the DEAE-Sephadex method of Seligman and Allen (16). Other details of the conditions are provided under "Experimental Procedures."

TC II-R treated with	SH-modifying agent		
	NEM	IAM	MMTS
	pmol ligand bound/μg TC II-R		
Nothing	14.1 ± 0.60	14.5 ± 0.50	13.8 ± 0.60
SH-reagent	14.3 ± 0.50	14.2 ± 0.60	14.0 ± 0.30
Urea	14.0 ± 0.40	14.2 ± 0.50	14.0 ± 0.60
Urea + SH reagent	13.0 ± 0.70	13.4 ± 0.60	13.5 ± 0.70
2-ME + SH reagent	3.7 ± 0.20	3.5 ± 0.40	3.5 ± 0.30
Urea + 2-ME + SH reagent	1.3 ± 0.08	1.2 ± 0.04	1.3 ± 0.02
Urea + 2-ME + SH reagent + DTT	1.3 ± 0.02	1.3 ± 0.01	12.5 ± 0.40

Pulse-Chase Labeling of Caco-2 Cells—Cells were first incubated with or without NEM (10^{-6} M) for 8 h and then with methionine-free medium for 30 min. The medium was removed, and fresh medium containing [^{35}S]methionine (200 μCi/flask) was added and incubated for 1 h in the presence and absence of NEM (10^{-6} M). The medium was removed, and the cells were washed with medium maintained at 4 °C and incubated with fresh medium in the absence and presence of NEM (10^{-6} M) but containing methionine (20 mM) for 0–16 h. Cells were harvested and extracted with Triton X-100, and the radioactivity was immunoprecipitated with TC II-R antiserum and protein A-Sepharose. The immunoprecipitated material was treated with SDS, and the liberated radioactivity was subjected to nonreducing SDS-PAGE (7.5%), and the bands were visualized by fluorography.

Cell Surface Biotinylation—Biotinylation of the basolateral surface of Caco-2 cells incubated with NEM (10^{-12} to 10^{-6} M) was carried out by adding 5,5′-dithiobis(2-nitrobenzoic acid), sulfosuccin imidobiotin (0.5 mg/ml) to the basolateral compartments of filter-grown monolayers (12-day growth) and was performed a total of three times for 30 min each at 4 °C in PBS containing 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$. The cells were then washed six times in PBS containing 0.1 mM $CaCl_2$, $MgCl_2$ and 50 mM glycine (5 min each) at 4 °C. The cells were then harvested and extracted with PBS containing Triton X-100 (1%). The extract was immunoprecipitated with TC II-R or CI-MPR antiserum (25 μl) and protein A-Sepharose (100 μl of 1:1 suspension in PBS). The washed immunopellet was subjected to nonreducing SDS-PAGE (7.5%), the bands were transferred to Nitrocellulose membranes and probed with ^{125}I-streptavidin (3×10^7 dpm/blot), and the bands were visualized by autoradiography as described above. In some experiments, filter-grown Caco-2 cells incubated first with NEM (10^{-6} M) for 16 h were washed with DMEM and then incubated for 15 min with DMEM containing cycloheximide (200 μg/ml) and then chased for 0–60 min with the same medium but in the absence of NEM. Basolateral surfaces of cells that were not incubated with either NEM or cycloheximide or with both were biotinylated and processed for SDS-PAGE and detection for biotinylated TC II-R as before.

Metabolic Labeling of Basolateral or Intracellular TC II-R—To study the effects of NEM and other SH-reagents on the basolateral expression of *de novo* synthesized TC II-R, filter-grown Caco-2 cells were metabolically labeled and processed as follows; NEM-treated (10^{-6} for 16 h) cells were pulsed with [^{35}S]methionine (200 μCi/filter) for 1 h at 37 °C. The cells were then washed with DMEM and chased with NEM-free DMEM containing nonradioactive methionine (10 mM) for 0–2 h. At each time interval, the cells were washed with ice-cold DMEM, followed by treatment of the basolateral surface with TC II-R antiserum (25 μl) for 45 min at 5 °C. The cells were washed with cold Tris-buffered saline to remove the excess antiserum, harvested, and extracted with Tris-buffered saline containing Triton X-100 (1%). The extract was treated with protein A-Sepharose (150 μl of 1:1 suspension), and the radioactivity precipitated was released using SDS-sample buffer. The samples were subjected to nonreducing SDS-PAGE as described before.

In some experiments, filter-grown cells were first treated with MMTS (10^{-6} M) for 16 h and then pulsed for 1 h with [^{35}S]methionine (200 μCi/filter) at 37 °C. The cells were then chased with MMTS-free DMEM containing nonradioactive methionine (10 mM) and DTT (10^{-4} M) for 0–120 min. At each time interval, the basolateral cell surface was treated with TC II-R antiserum as above. Following the separation of [^{35}S]TC II-R present in the basolateral surface by immunoprecipitation with protein A-Sepharose, the immune supernatant (representing intracellular labeled TC II-R) was reimmunoprecipitated with TC II-R antiserum and protein A-Sepharose as before. The intracellular and the basolateral labeled TC II-R immunprecipitated was subjected to SDS-PAGE, the bands were visualized by fluorography, and the image density was quantified by the AMBIS radioimaging system.

Other Methods—Protein in all samples was determined according to Bradford (20). In some experiments, [^{35}S]TC II-R (10,000–15,000 dpm) obtained from NEM-treated and untreated cells was digested with Endo H or PNGase F or with sialidase followed by treatment with *O*-glycosidase as described earlier (1, 21).

RESULTS

SH Group Modification of Pure TC II-R and Its Effect on Ligand Binding and SDS-PAGE Mobility—TC II-R reduced and treated with the sulfhydryl-modifying agents, NEM, IAM, or MMTS (Table I), was assayed for TC II-[^{57}Co]Cbl binding by the DEAE-Sephadex method (16). This method, which uses charge-based separation of receptor bound and free ligand, revealed >90% loss of ligand binding activity only when these reagents were added following the reduction of TC II-R in the presence of 4 M urea. Treatment with urea alone or with urea and the SH reagents had no effect on ligand binding by TC II-R. In the absence of urea but following reduction, all three reagents inhibited ligand binding by about 74%. Nearly 85% of the activity lost was regained following treatment of the MMTS-modified TC II-R with DTT, while treatment with DTT of TC II-R alkylated with IAM or NEM had no effect (Table I). To confirm that loss of ligand binding as assessed by the DEAE-Sephadex method was not due to an artifact of an altered charge on the modified TC II-R molecule, we carried out chemical cross-linking of the reduced and SH-modified TC II-R with ^{125}I-TC II-Cbl (Fig. 1). ^{125}I-TC II-Cbl was able to bind only to the native untreated (*UT*) receptor in the absence of excess unlabeled TC II-Cbl but not in its presence. The size of the 105-kDa band detected was consistent with the size of the TC II-R·TC II-Cbl complex (62 plus 43 kDa). No cross-linked product was noted using reduced TC II-R that was treated with SH-modifying agents, NEM, IAM, or MMTS.

To quantify the number of free and disulfide-bonded SH groups of TC II-R, pure TC II-R was titrated with DTNB and [^{3}H]NEM (Table II). When either of these two reagents was added to TC II-R, no SH groups were revealed, and no ligand binding was lost. However, in the presence of 4 M urea, both reagents revealed the presence of two free SH groups, and modification of these two free SH groups did not result in loss of ligand binding. When the receptor was reduced with 2-mercaptoethanol and then modified, six SH groups were revealed with 75% loss of ligand binding. These results suggested that at least three disulfide bonds are accessible to the reducing agent, reduction of which resulted in nearly 75% loss of ligand binding. When TC II-R was reduced in the presence of 4 M urea and then treated with DTNB or [^{3}H]NEM, the loss of binding was complete with the modification of 10 SH groups (Table II). Taken together, these results suggested that the two free and the two SH groups that form one of the disulfide bonds are

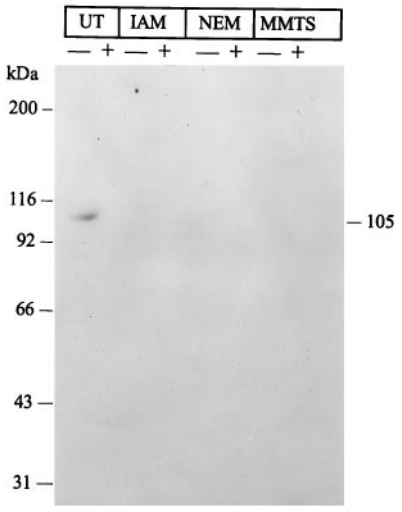

FIG. 1. **Cross-linking of native untreated and reduced and modified TC II-R with ^{125}I-TC II-Cbl.** TC II-R, native untreated (*UT*) or reduced and modified with IAM, NEM, and MMTS was cross-linked in the presence (+) or absence (−) of a 10-fold molar excess TC II-Cbl. The cross-linked products were analyzed on nonreducing SDS-PAGE (7.5%), and the bands were visualized by autoradiography. Other details are provided under "Experimental Procedures."

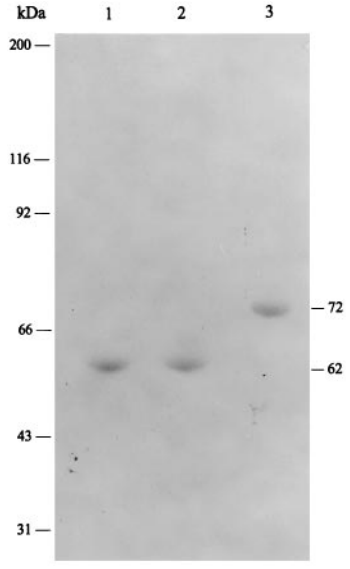

FIG. 2. **Reversal of SDS-PAGE mobility of MMTS modified TC II-R by DTT treatment.** Pure TC II-R (5 μg, *lane 1*) and reduced TC II-R treated with MMTS and then treated with DTT (*lane 2*) and without DTT (*lane 3*) are shown. The protein bands were visualized with silver nitrate staining of the gel.

TABLE II
Titration of total and free SH groups of TC II-R

Results shown are mean ± S.D. of six titrations under all conditions used. Other details are provided under "Experimental Procedures."

Conditions	DTNB	[^{3}H]NEM	Ligand bound
	SH groups/mol		*pmol/μg*
	0	0	14.1 ± 0.3
With 2-ME	6 ± 0.10	6 ± 0.16	3.6 ± 0.4
With 2-ME + urea	10 ± 0.40	10 ± 0.50	1.1 ± 0.1
With urea	2 ± 0.02	2 ± 0.01	14.4 ± 0.5

buried within the molecule. While the modification of the free buried SH groups had no effect on the ligand binding activity, the disruption of disulfide bonds by reduction altered the folding of TC II-R such that it assumed a more linear conformation as revealed by mobility changes on nonreducing SDS-PAGE. The consequence of disruption of all the disulfide bonds was complete loss of ligand binding.

Direct evidence for the increase in the apparent molecular mass of TC II-R following its reduction was obtained by determining the size of TC II-R on SDS-PAGE (Fig. 2). The size of TC II-R modified by MMTS (*lane 3*) was 10-kDa higher than the untreated TC II-R (*lane 1*) or TC II-R that was first modified by MMTS and then treated with DTT (*lane 2*). Similar reduced mobility on nonreducing SDS-PAGE with apparent increase in the molecular mass by 10 kDa was also noted following reductive alkylation with NEM or IAM (data not shown). These results clearly indicated that disruption of disulfide bonds of TC II-R and the resulting folding alterations are responsible for the loss of ligand binding. Although *in vitro* studies revealed that intramolecular disulfide bonds are important in ligand binding, we sought to examine their importance in the *in vivo* folding and trafficking of TC II-R.

Effect of NEM on TC II-R Activity and Protein Levels in Caco-2 Cells—Incubation of postconfluent Caco-2 cells grown on culture inserts with NEM (10^{-12} to 10^{-6} M) for 16 h resulted in a progressive and parallel loss of TC II-[^{57}Co]Cbl binding to the cell extracts (determined using Triton X-100 (1%) extract of the cell homogenate) and in the basolateral surface (determined by binding the ligand to the basolateral surface at 4 °C). Nearly 50 and 90% of both the total and basolateral cell surface receptor activity was lost at NEM concentrations of 10^{-8} and 10^{-6} M, respectively (Fig. 3, *left panel*). Loss of TC II-R activity was not due to loss of TC II-R protein, since quantitative immunoblot analysis using the cell homogenates from NEM-treated cells (Fig. 3, *right panel*) revealed no changes of TC II-R protein levels at any concentration of NEM. However, the physical state of TC II-R was different, being a dimer of molecular mass of 124 kDa below an NEM concentration of 10^{-8} M or a monomer of 72 kDa at NEM concentrations of 10^{-8} M or higher.

The molecular mass of 72 kDa noted for the TC II-R monomer when the cells were incubated with NEM concentrations $\geq 10^{-8}$ M was 10 kDa higher than the molecular mass of TC II-R monomers detected by immunoblotting using Triton X-100 extract of Caco-2 cell homogenates from cells that were not exposed to NEM (Fig. 4*A*, *left part*, *lane 2*), or pure placental TC II-R monomer (*lane 1*). This observation suggested that the monomer with a molecular mass of 72 kDa may be formed due to intracellular alkylation of TC II-R that caused it to attain a more linear extended structure with decreased mobility on SDS-PAGE. To address this issue, filter-grown Caco-2 cells were labeled with [^{3}H]NEM (Fig. 4*A*, *right part*, *lane 1*) alone or with [^{35}S]methionine (*lane 2*) in the presence of 10^{-6} M NEM. Nonreducing SDS-PAGE (Fig. 4*A*, *right part*) of immunoprecipitates obtained from cell extracts from both [^{3}H]NEM (*lane 1*) and [^{35}S]methionine (*lane 2*) labeled cells revealed a single band of molecular mass of 72 kDa. These experiments together with the observation that immunoblot analysis of NEM-treated cells revealed no 62-kDa form of the unmodified TC II-R (Fig. 3, *right panel*) suggest strongly that NEM modified newly synthesized TC II-R. It is interesting to note that under our experimental conditions only six or seven cellular proteins were labeled by [^{3}H]NEM (Fig. 4*B*, *lane 1*). The cytosol (*lane 2*) and the Triton X-100 insoluble membrane fraction (*lane 3*) demonstrated a very similar pattern of three labeled proteins, but with a higher proportion of these proteins in the insoluble fraction (*lane 3*). On the other hand, the Triton X-100 soluble fraction (*lane 4*) revealed a major protein with a molecular mass of 72 kDa and a few minor bands. This major band was immunoprecipitated with antiserum to TC II-R (*lane 5*), and no radioactivity from this fraction could be immunoprecipitated with antiserum to CI-MPR (*lane 6*). These results have shown

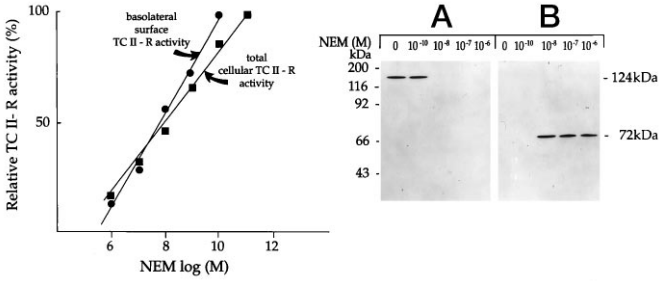

FIG. 3. **Effect of NEM on the activity and protein levels of TC II-R in postconfluent Caco-2 cells.** *Left panel*, Caco-2 cells grown on culture inserts were incubated with the indicated concentrations of NEM added to both the apical and the basolateral medium for 16 h. The medium was removed, cells were washed with NEM-free fresh DMEM and the basolateral binding of the ligand TC II-[^{57}Co]Cbl was determined at 4 °C. A separate set of filters similarly incubated with NEM was harvested, washed in PBS, and extracted with Tris-buffered saline containing Triton X-100 (1%) and assayed for ligand binding. The activities shown are an average of duplicate assays performed using three separate sets of filters exposed to NEM. *Right panel*, aliquots of the cellular homogenate (200 μg of protein) were subjected to nonreducing SDS-PAGE and immunoblot analysis to detect either TC II-R dimers (*A*) or monomers (*B*) as described earlier (1). The immunoblot analysis were carried out three times using cells from three separate experiments, and the results were the same.

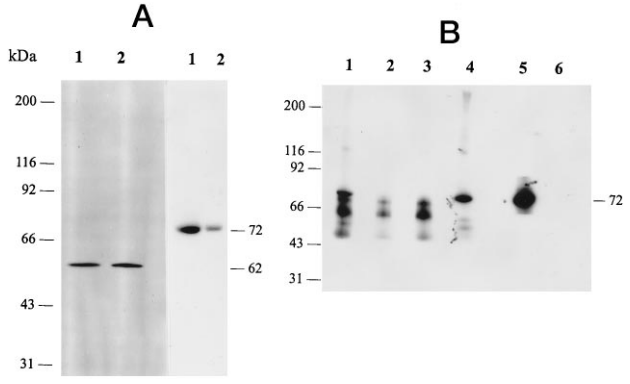

FIG. 5. **Pulse-chase labeling of TC II-R in NEM-treated and untreated Caco-2 cells.** *Left panel*, postconfluent Caco-2 cells grown on plastic (T-75 flasks) were treated with or without NEM (10^{-6} M) for 8 h. They were then pulsed with [^{35}S]methionine (200 μCi/flask) for 1 h and then chased for in the presence of 20 mM cold methionine for 1–16 h in the presence and absence of NEM (10^{-6} M). The cells were harvested at each time interval, lysed and extracted with Triton X-100, and immunoprecipitated with TC II-R antiserum and protein A-Sepharose. The immunopellets were washed and subjected to SDS-PAGE. The bands were visualized by fluorography. *Right panel*, the bands were quantified by the AMBIS radioimaging system. The data shown are an average of three separate pulse-chase experiments carried out with three separate filter-grown cells.

FIG. 4. **Relative molecular mass of TC II-R from NEM-treated and untreated labeled cells and distribution of [^{3}H]NEM-labeled protein in Caco-2 cell fractions.** *A*, *left part*, pure Triton X-100 micellar bound TC II-R (0.2 μg of protein) (*lane 1*) and Triton X-100 extract (200 μg of protein) (*lane 2*) of untreated Caco-2 cell homogenate were subjected to nonreducing SDS-PAGE and immunoblotting to detect TC II-R monomers as before. *Right part*, Caco-2 cells treated with either [^{3}H]NEM (4×10^{-6} dpm/10^{-6} M) (*lane 1*) or with [^{35}S]methionine (*lane 2*) in the presence of nonradioactive NEM (10^{-6} M) for 16 h. The harvested cells were extracted with TBS containing Triton X-100. The extract was immunoprecipitated with antiserum to human TC II-R and protein A-Sepharose. The immunopellet was washed and subjected to nonreducing SDS-PAGE. The bands were visualized by autoradiography (*left panel*) and by fluorography (*right panel*). *B*, different [^{3}H]NEM-labeled Caco-2 cells fractions, crude homogenate (*lane 1*, 16,500 dpm), supernatant (*lane 2*, 25,000 dpm), Triton X-100-insoluble (*lane 3*, 24,000 dpm), Triton X-100-soluble (*lane 4*, 20,000 dpm), and immunoprecipitated TC II-R (*lane 5*, 40,000 dpm) and CI-MPR (*lane 6*, 0 dpm) were subjected to nonreducing SDS-PAGE. The bands were visualized by fluorography.

that treatment of Caco-2 cells with low concentrations of [^{3}H]NEM results in the incorporation of the label into very few cellular proteins, and density scanning of the fluorograph (*lane 1*) revealed that labeled TC II-R represented nearly 25% of the total radioactivity incorporated into proteins.

The lack of detection of the unmodified TC II-R by immunoblotting suggested that the half-life of TC II-R is considerably less than 16 h, the time period of incubation of cells with NEM. In addition, this observation also suggested that NEM alkylated the newly synthesized TC II-R before it had a chance to form its disulfide bonds. To address this issue, pulse-chase labeling of Caco-2 cells was carried out with [^{35}S]methionine in the absence and presence of NEM (Fig. 5) to determine the half-life of TC II-R. Nonreducing SDS-PAGE of [^{35}S]TC II-R immunoprecipitated from cell extracts revealed a single band with molecular mass of 72 and 62 kDa, when the cells were labeled in the presence and absence of NEM, respectively (*left panel*). Quantitation of these bands demonstrated a very similar decay pattern (*right panel*) following a chase period of up to 16 h, and the $t_{1/2}$ for both the native 62-kDa and modified 72-kDa TC II-R was 7.5 h. The lack of surface expression of TC II-R activity in NEM-treated cells (Fig. 3), despite the lack of effect on its turnover and protein levels, suggested that either the inactive extended monomer reaches the surface membrane or it is retained in the intracellular compartments due to misfolding. To test directly these possibilities, basolateral surface TC II-R protein levels were determined during the incubation of cells with various concentrations of NEM as well as during the recovery of cells from NEM treatment.

TC II-R Protein Is Not Expressed in the Basolateral Surface of NEM-treated Cells—Domain-specific biotinylation of the basolateral cell surface of filter-grown Caco-2 cells treated with various concentrations of NEM revealed that TC II-R protein gradually disappeared from the basolateral cell surface (Fig. 6*A*), and quantitation of cell surface protein levels revealed that at NEM concentrations of 10^{-8} and 10^{-6} M, the cell surface loss of TC II-R protein was 50 and 100%, respectively. To test whether the loss of cell surface TC II-R protein could be reversed, domain-specific biotinylation of the basolateral cell surface was carried out using cells that were treated with 10^{-6} M NEM for 16 h and then washed with NEM-free medium, incubated for 15 min with medium containing cycloheximide (to inhibit protein synthesis), and then chased in NEM-free medium containing cycloheximide for up to 60 min (Fig. 6*B*). Although cell surface TC II-R protein levels in cells treated with cycloheximide alone for 15 min (*lane 1*) were lower than surface levels from cells that were not treated (*lane 2*), no TC II-R could be detected on the cell surfaces of cells that were chased with NEM-free medium for 0 (*lane 3*), 30 (*lane 4*), and 60 (*lane 5*) min. Similar results were obtained following chase of cells that were treated with NEM and then metabolically pulsed with [^{35}S]methionine for 1 h in the presence of 10^{-6} M NEM (Fig. 6*C*). Labeled TC II-R noted in the basolateral cell surface in control cells (*lane 1*) was absent following chase for

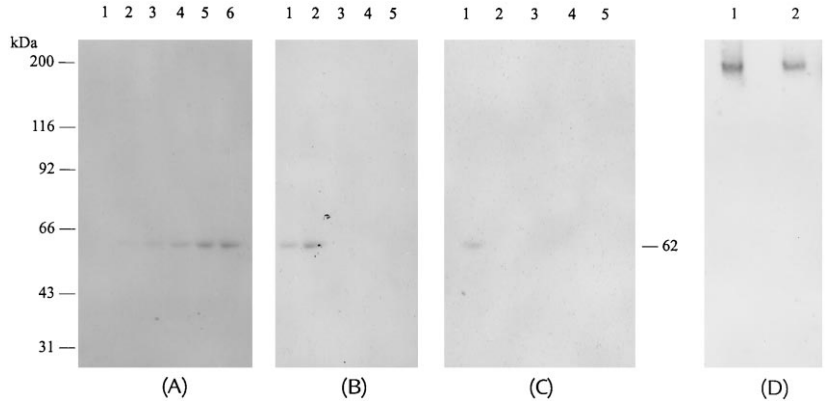

FIG. 6. **Basolateral TC II-R and CI-MPR protein levels in NEM-treated cells.** *A*, basolateral surface of filter-grown Caco-2 cells incubated without NEM (*lane 6*) and with 10^{-6} (*lane 1*), 10^{-7} M (*lane 2*), 10^{-8} M (*lane 3*), 10^{-10} M (*lane 4*), and 10^{-12} M NEM (*lane 5*) were biotinylated. Biotinylated TC II-R was immunoprecipitated, subjected to SDS-PAGE, and probed with ^{125}I-streptavidin, and the bands were visualized by autoradiography as described under "Experimental Procedures." *B*, filter-grown cells were incubated without (*lanes 1* and *2*) or with NEM (10^{-6} M) for 16 h. The cells were then washed, incubated with NEM-free medium in the presence (*lane 1* and *lanes 3–5*) or absence (*lane 2*) of cycloheximide (200 μg/ml), and chased for 0 (*lane 3*), 30 (*lane 4*), and 60 (*lane 5*) min. The cells were subjected to basolateral biotinylation and processed as before. *C*, filter-grown cells were incubated with (*lanes 2–5*) or without (*lane 1*) NEM (10^{-6} M) for 16 h. The medium was removed, and the cells were washed with fresh DMEM, pulsed for 1 h with [^{35}S]methionine, and then chased for 0–2 h in the presence of NEM-free medium. Labeled TC II-R at the basolateral surface was determined using TC II-R antiserum as described under "Experimental Procedures." *D*, same as *A* except that biotinylation was carried out in the absence (*lane 2*) and presence of 10^{-6} M NEM (*lane 1*). Biotinylated CI-MPR was immunoprecipitated and processed as before.

30 (*lane 2*), 60 (*lane 3*), 120 (*lane 4*), and 240 (*lane 5*) min in the presence of NEM-free medium. These results clearly established that in NEM-treated cells, cell surface expression of TC II-R is inhibited and that this inhibition is due to intracellular retention of a misfolded protein. To test whether NEM treatment affected the basolateral delivery of other proteins, steady state levels of CI-MPR (Fig. 6*D*) was determined by biotinylation of the basolateral domain of NEM-treated (*lane 1*) and untreated cells (*lane 2*). NEM treatment of Caco-2 cells did not result in the loss of steady-state levels of CI-MPR (~275 kDa) at this location.

Inhibition of Disulfide Bonding of TC II-R Affects Its Post-TGN Trafficking—TC II-R obtained from cells labeled with [^{35}S]methionine either in the presence or absence of NEM was insensitive to Endo H treatment but was equally sensitive to treatment by PNGase F, revealing a mobility shift of 2 kDa (Fig. 7, *top*). These results indicate that both the native 62-kDa and the NEM-modified 72-kDa forms of TC II-R are processed similarly with respect to the maturation of their single high mannose type of *N*-linked sugar chain to the complex type. When the native or the modified forms of TC II-R (Fig. 7, *bottom*) were digested with either sialidase alone or with sialidase and *O*-glycanase, a shift in the mobilities was also noted. However, the shift corresponding to about 4 kDa noted with sialidase treatment of the NEM-modified TC II-R was less than the shift of about 8 kDa noted by sialidase treatment of the native 62-kDa TC II-R. To test whether the intracellular retention of TC II-R in NEM-treated cells is due to its misfolding caused by disulfide bond disruption, the cells were treated with MMTS instead of NEM.

When the cells grown on culture inserts were first treated with MMTS and later withdrawn from exposure to MMTS and incubated with DTT for various intervals of time (Fig. 8, *left*, *top part*), the basolateral surface ligand binding activity rose gradually with time, and 90% of steady state ligand binding was recovered in 120 min of exposure with DTT. Upon pulse labeling of cells with [^{35}S]methionine in the presence of MMTS for 1 h, followed by chase with MMTS-free medium containing nonradioactive methionine and DTT, labeled TC II-R with a molecular mass of 72 kDa disappeared slowly from the cell interior with the appearance of the 62-kDa form of TC II-R on the basolateral cell surface. By 120 min, modified labeled TC

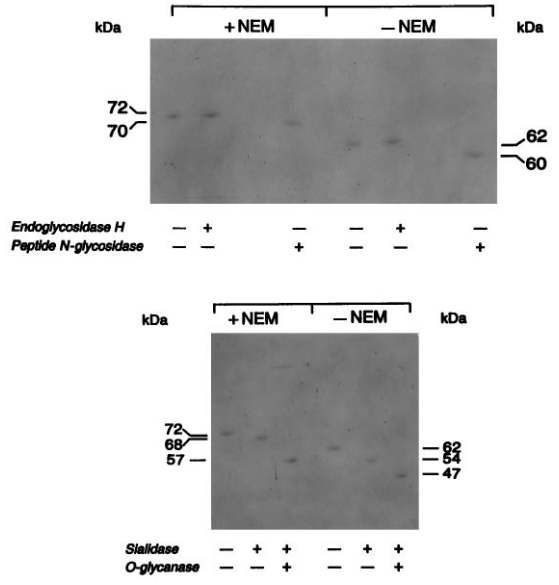

FIG. 7. **Digestion of [^{35}S]TC II-R with glycosidases.** Immunoprecipitated labeled TC II-R (25–30 × 10^3 dpm) obtained from Caco-2 cells labeled in the presence and absence of NEM were digested with Endo H or PNGase F (*top*) or with sialidase and *O*-glycanase (*bottom*), and the digested fractions were subjected to nonreducing SDS-PAGE. Other details are provided under "Experimental Procedures."

II-R present inside the cells was completely converted to the native receptor and was expressed on the basolateral cell surface (Fig. 8, *right*). Quantitation of the these radioactive bands (Fig. 8, *left*, *bottom part*) revealed that the $t_{1/2}$ for the conversion to the native fully folded form and basolateral surface expression was approximately 25 min.

DISCUSSION

The role of disulfide bond formation in the folding process and in the acquisition of transport competence has been well established for a number of proteins (22, 23). One approach to study the mechanism of disulfide bond disruption and its effect on the intracellular vesicular transport and function of cellular proteins is to expose the cells to the thiol-reducing agent DTT. When used below the concentration of 1 mM, DTT has been

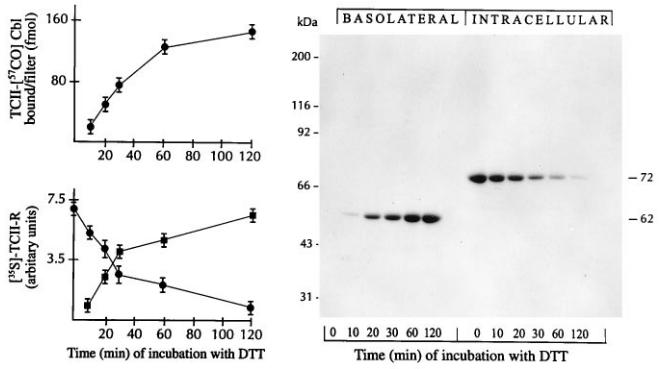

FIG. 8. **Intracellular and basolateral expression of TC II-R in MMTS-treated cells and the effect of DTT.** Caco-2 cells grown on culture inserts incubated with MMTS (10^{-6} M) for 16 h were washed with DMEM and pulsed with [^{35}S]methionine for 1 h and then chased with MMTS-free DMEM containing 10 mM methionine and DTT (10^{-4} M) for 0–120 min. The basolateral and intracellular labeled TC II-R immunoprecipitated was subjected to nonreducing SDS-PAGE, and the bands were visualized by fluorography (*right panel*) and quantified by the AMBIS radioimaging system (*left panel*, *bottom*). In some filters, the basolateral ligand binding was assessed (*left panel*, *top*). The results shown in the *left panel* are mean ± S.D. from six separate filters. Other details of antiserum treatment and separation of the intracellular and basolateral labeled TC II-R are provided under "Experimental Procedures."

known to inhibit the disulfide bond formation of newly synthesized proteins without affecting the redox state of living cells (24). While this approach may be suitable for studying the role of disulfide bonds in the vesicular transport of secretory proteins that are secreted constitutively, it may not be suitable for studying the membrane-targeted proteins. To date, no studies are available describing the effect of SH group modification of a membrane protein and the resulting misfolding on its vectorial targeting to the plasma membrane domain in a polarized epithelial cell. We have addressed the issue of the role of intramolecular disulfide bonding of TC II-R in ligand binding and intracellular trafficking in human intestinal epithelial Caco-2 cells by exposing these cells to very low concentrations (10^{-6} M) of SH-modifying agents, such as NEM and MMTS. However, prior to studies with cells, it was important to establish the number and nature (free or disulfide-bonded) of SH groups present in human TC II-R.

Titration (Table II) of TC II-R with DTNB and [^{3}H]NEM revealed 10 half-cysteines, of which two were free and eight were involved in intramolecular disulfide bonding. Furthermore, the two free half-cysteines and the two SH groups that are involved in intramolecular disulfide bonding are buried in the molecule and could be titrated only in the presence of urea. The other six cysteines that are involved in disulfide bonding are easily accessible to the SH-modifying agents. Although modification of the free SH groups of TC II-R had no effect on its ligand binding, disulfide bond disruption of TC II-R resulted in complete loss of ligand binding (Table I and Fig. 1), and this was due to the formation of a more linear form of TC II-R. Furthermore, loss of receptor activity and the formation of an extended TC II-R molecule by all of the SH-specific reagents suggest that the same reduced SH groups are modified by all three reagents. NEM and IAM are known to modify free SH groups in proteins by forming a nonreversible thio-ether bond with the reduced thiol group (25). In contrast, MMTS introduces a much smaller group (-SCH_3) into proteins by forming a disulfide bond with the reduced cysteines. This disulfide bond is susceptible to cleavage by reducing agents like DTT, and therefore the effects of MMTS are reversible (26). When modified with MMTS, TC II-R lost activity and demonstrated an extended more linear molecule (Fig. 2), and both effects were reversed following DTT treatment of the MMTS-modified receptor. These results have demonstrated that the loss of TC II-R activity following disulfide bond disruption is not due to the nature and the size of the reactive group of the SH-modifying agent, since IAM and MMTS with smaller groups also inhibited as effectively as NEM, which contains a bulkier group. The apparent increase in molecular mass of TC II-R due to disulfide bond disruption noted in this study is not a unique situation. Other receptors such as the cation-independent mannose 6-phosphate receptor (27), neurotensin receptor (28), and β-adrenergic receptor (29) behave similarly on nonreducing SDS-PAGE following their reductive alkylation.

The loss of total and basolateral TC II-R activity without loss of total cellular TC II-R protein (Fig. 3) suggested that NEM treatment of Caco-2 cells resulted in the inactivation of ligand binding and that this inactivation was not due to the loss of cellular TC II-R protein. Immunoblotting (Fig. 3), using homogenates of Caco-2 cells treated with NEM, or SDS-PAGE (Fig. 4*A*) of the immunoprecipitates of cell extracts from cells labeled with [^{3}H]NEM or with [^{35}S]methionine in the presence of NEM revealed the presence of TC II-R monomers with a molecular mass of 72 and not 62 kDa. The detection of only the modified 72-kDa TC II-R in NEM-treated Caco-2 cells (Figs. 3 and 4*A*) clearly indicated that incubations with NEM resulted in the alkylation of TC II-R either co-translationally or soon after synthesis before it can form intramolecular disulfide bonds. These results suggested that TC II-R turns over rapidly in these cells with a $t_{1/2}$ less than 16 h, the time period that cells were exposed to NEM. Pulse-chase (Fig. 5) labeling in the presence and absence of NEM confirmed this observation and showed that the 72-kDa species of TC II-R in NEM-treated cells had the same time of decay with a $t_{1/2}$ of 7.5 h as the native labeled 62-kDa TC II-R. These results have clearly established that due to its rather fast turnover, TC II-R is selectively modified by NEM. Nearly 45–50% of the [^{3}H]NEM in the Triton X-100 extract of Caco-2 cells (which completely solubilizes membrane-bound TC II-R (1)) was on TC II-R, the rest being on three other proteins with molecular masses of 116, 60, and 55 kDa (Fig. 4*B*, *lane 4*).

Lack of a more rapid degradation of the misfolded TC II-R relative to the native TC II-R is a surprising result, since inhibition of disulfide bonding in some proteins leads them to undergo rapid degradation in the ER by a nonlysosomal pathway (30, 31). However, lack of rapid degradation of the misfolded 72-kDa TC II-R in the ER may be due to (*a*) failure of the ER quality control mechanism to detect and direct the modified (misfolded) TC II-R for degradation or (*b*) relatively rapid exit of the misfolded TC II-R from the ER. Further studies are needed to address these issues. In contrast to TC II-R, NEM treatment of Caco-2 cells did not result in either modification or the basolateral secretion of TC II, the ligand (data not shown). TC II, a nonglycoprotein Cbl binder that contains intramolecular disulfide bonds,[2] is basolaterally secreted in these cells (32). In addition, under our experimental conditions, treatment of cells with low concentrations of NEM did not affect the steady-state levels of CI-MPR at the basolateral cell surface (Fig. 6*D*). CI-MPR, a single polypeptide of molecular mass ~275 kDa and a transmembrane protein that contains several intramolecular disulfide bonds (27), is also targeted basolaterally in Caco-2 cells (33). The inability of low concentrations of NEM to modify CI-MPR and thus inhibit its basolateral targeting may be due to its longer half-life ($t_{1/2}$ = 25–27 h).[3] It is interesting to note that under our experimental conditions of

[2] N. Li and B. Seetharam, unpublished observations.
[3] N. M. Dahms, unpublished observation.

labeling, the bulk (>95%) of the labeled NEM was incorporated into six or seven proteins ranging in molecular mass between 43 and 116 kDa (Fig. 4*B*, *lane 1*).

The similar apparent size alterations due to inhibition of disulfide bonding of the isolated mature 62-kDa TC II-R and TC II-R synthesized in Caco-2 cells suggested that in these cells the MMTS- or NEM-modified TC II-R is processed similarly to the native receptor. These observations were confirmed by the demonstration of insensitivity of both the native and NEM-modified TC II-R to treatment with Endo H but sensitivity to treatments with PNGase F and with *O*-glycanase. However, the mobility shift following digestion by sialidase alone was not the same for the native and the modified TC II-R, suggesting that not all *O*-linked sugars are sialylated. This difference could be due to differences in the *in vitro* affinity of sialidase toward the modified receptor or to an *in vivo* altered affinity of the sialyltranferase toward the modified TC II-R. Despite this uncertainty, the observation that both the *N*-linked and *O*-linked sugars of TC II-R are sialylated indicated strongly that the modified TC II-R is able to exit the ER and reach the trans-Golgi region, the site where sialylation of both *N*-linked and *O*-linked sugars occur (34–36).

Treatment of Caco-2 cells with NEM resulted in the loss of basolateral TC II-R activity (Fig. 3) and protein (Fig. 6, *A–C*). These results indicated that NEM-modified TC II-R, although able to travel to the trans-Golgi, was unable to undergo further trafficking to the basolateral membranes. Since the modified TC II-R, as demonstrated by enzymatic deglycosylation, contains sialic acid and thus is able to undergo sialylation of both its *N*- and *O*-linked sugars, the site of its intracellular retention is most probably the TGN. Several possibilities exist as to why the modified TC II-R does not undergo post-TGN trafficking. TC II-R, which is synthesized as a single polypeptide with a molecular mass of 45 kDa, is post-translationally modified to a mature form with a molecular mass of 62 kDa and then expressed in the plasma membranes as a homodimer with a molecular mass of 124 kDa. Following reductive alkylation of the membranes, the apparent molecular mass of membrane-bound TC II-R is 144 kDa (1). This observation suggests that both monomers of TC II-R maintain their intramolecular disulfide bonds during their dimerization. Thus, it is possible that the modified TC II-R is unable to dimerize in the plasma membranes or in the post-TGN vesicles. However, this is unlikely, since the reduced and alkylated 72-kDa TC II-R when reconstituted in egg phosphatidylcholine/cholesterol (4:1 molar ratio) liposomes was able to dimerize, like the native 62-kDa TC II-R (data not shown). Moreover, dimerization of TC II-R is due to its strong hydrophobic interactions with a rigid ordered lipid bilayer and does not appear to be affected by its folding alterations (4). A more likely possibility is that the folding alterations of TC II-R due to inhibition of disulfide bond formation are responsible for the inhibition of post-TGN trafficking to the basolateral membranes. The conversion of the 72-kDa modified TC II-R to the native 62-kDa TC II-R and the appearance of basolateral TC II-R activity and protein by DTT incubation of MMTS-treated cells support the idea that disulfide bonding is required for the final stages of TC II-R trafficking from the TGN to its location in basolateral membranes.

How does altered folding of TC II-R due to disulfide bond disruption inhibit its post-TGN trafficking to the basolateral surface without affecting its vesicular transport from the ER and through the Golgi? Sorting of basolateral membrane-destined proteins from the TGN is initiated following the interaction of the cargo protein containing the conformationally accessible sorting signal with either coat protein of *N*-ethylmaleimide-sensitive factor (NSF)-dependent coated vesicles (37–39) or with adapter proteins, such as AP-1, of clathrin-coated vesicles (40). This interaction leads to the basolateral localization of the cargo protein. Although the misfolded TC II-R is able to be transported up to the TGN independent of the vesicular pathway (NSF-dependent or clathrin-coated vesicular pathway) involved, the mechanism of its retention in the TGN is not known. It is possible that disruption of disulfide bonds of TC II-R may lead to the inaccessibility of the basolateral targeting sequence/signals to be recognized and interact with either the coat protein or with other adapter proteins. Other possibilities for the inhibition of post-TGN trafficking of TC II-R due to NEM or MMTS treatment of Caco-2 cells must also be considered. Inhibition may be due to the inactivation of the N-ethylmaleimide-sensitive factor/soluble NSF attachment protein/SNAP receptor (NSF-SNAP-SNARE) machinery, which has been shown to be important in the basolateral targeting of proteins from the TGN in polarized Madin-Darby canine kidney cells (41). Alternatively, NEM may also inactivate other chaperons such as Rab 8, a small GTPase that is thought to be involved in the post-Golgi trafficking of basolateral proteins in polarized epithelial Madin-Darby canine kidney cells (42). However, these possibilities are unlikely for the following reasons. First, in many of the *in vitro* membrane transport reconstitution assays, inactivation of NSF was achieved using 0.4–1 mM of NEM (43–45), a concentration nearly 400–1000-fold higher than the maximum NEM concentration of 0.001 mM used in this study. Second, at NEM concentrations as low as 10 nM, an effect on TC II-R trafficking could be noted, with nearly 50% of it being retained intracellularly. Furthermore, it is not known whether such low levels of NEM or MMTS used in this study can modify any of the proteins involved in vesicular budding, transport, or fusion events or whether Rab 8 is a NEM-sensitive protein. Third, the ability of DTT to reverse the misfolding and intracellular retention of TC II-R that resulted in its increased basolateral expression suggests strongly that disulfide bond disruption of TC II-R is responsible for the inhibition of its transport to the basolateral membranes. Finally, incubations with NEM had no effect on the basolateral targeting of CI-MPR in these cells, suggesting strongly that the noted effects of NEM on post-TGN trafficking of TC II-R are not due to NEM inactivation of the chaperons that are implicated in basolateral targeting of proteins in polarized epithelial cells.

In summary, the results presented in this study have shown that intramolecular disulfide bond formation by TC II-R is important for the acquisition of ligand binding and post-TGN but not for trafficking from the ER. Further studies are needed to understand how the lack of disulfide bond formation of TC II-R affects its interaction with vesicular chaperons that are responsible for its basolateral delivery.

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