A Mutated Transferrin Receptor Lacking Asparagine-linked Glycosylation Sites Shows Reduced Functionality and an Association with Binding Immunoglobulin Protein*

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The function of the transferrin receptor is to transport iron-bound transferrin into the cell. In order to function properly, this dimeric glycoprotein must be expressed on the cell surface and be able to bind transferrin. Site-directed mutagenesis was performed to abolish the three asparagine-linked glycosylation consensus sequences of the human transferrin receptor. The DNA encoding the mutated transferrin receptor was stably transfected into mouse fibroblasts. This form of the human transferrin receptor shows reduced transferrin binding, reduced intersubunit bond formation, and reduced cell surface expression, indicating that the transferrin receptor which lacks asparagine-linked glycosylation is not fully functional. In addition, the mutated form of the receptor is not processed as quickly. It shows an association with an endoplasmic reticular chaperone protein, binding immunoglobulin protein, leading to the hypothesis that the mutated transferrin receptor experiences increased retention in the endoplasmic reticulum.

The human transferrin receptor, a dimer composed of two 94-kDa subunits, is involved in the uptake of iron via the binding and endocytosis of transferrin. The transferrin receptor is synthesized and modified in the endoplasmic reticulum and is further modified as it passes through the Golgi to the cell surface. Serine 24 is phosphorylated (1), cysteine 62 is acylated (2), and asparagines 251, 317, and 727 are glycosylated (3-5). There is also at least one O-linked glycosyl group, but its position has not been established (6, 7).

It is not clear to what extent many of the modifications affect transferrin receptor function. Proper endocytosis, recycling, and membrane association occur in the absence of phosphorylation and acylation (2, 8-10). However, some evidence does exist that critical roles are played by the N-linked glycosyl groups (11). N-linked glycosylation has been postulated to have various functions in different proteins, e.g. as a signal for intracellular sorting and cell-cell interactions, promoting resistance to proteases, preventing aggregation of proteins, and maintaining proper conformation and hydrophilicity for protein transport, processing, and/or activity (reviewed in Refs. 12 and 13). If the receptor is to perform its function, it must be expressed at the cell surface, probably must form dimers, and must be able to bind transferrin.

Previous studies in this lab have indicated that in A431 cells (a human epidermoid cell line) treated with tunicamycin, an inhibitor of N-linked glycosylation, the unglycosylated transferrin receptor is not transported to the cell surface, does not bind transferrin, and does not form dimers (11). Because of the pleiotropic effects of tunicamycin, however, we have sought to confirm the putative roles of the N-linked glycosyl groups by more specific means: the oligonucleotide-directed mutagenesis of the consensus sites for N-linked glycosylation. Specific alteration of these consensus sequences to prevent N-linked glycosylation would result in forms of the receptor lacking N-linked glycosyl groups, the behavior of which could then be examined without radically altering normal cell functioning.

MATERIALS AND METHODS

Plasmids pCD-TR1 and pZipNeoSV(X) were gifts from Dr. A. McClelland, Yale University, and Dr. M. Lane, State University of New York Health Science Center, Syracuse, respectively. NIH-3T3 cells were obtained from American Tissue Culture Collection. A431 cells were from Dr. Graham Carpenter, Vanderbilt University. 125I-Labeled methionine/cysteine was from ICN. Geneticin, gentamycin, Dubesco's modified Eagle's medium and fetal bovine serum were from Gibco. Staphylococcus aureus and tunicamycin were from Calbiochem. Rat anti-binding immunoglobulin protein (BiP) culture fluid was a gift of Dr. David Bole, Yale University. Molecular weight markers (4C-labeled) were from Bethesda Research Labs (BRL).

Cell Culture and Radiolabeling—A431 and NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and gentamycin (60 µg/ml); subconfluent cells in 24-well plates (A431, 3 x 10⁵ cells/well) or 6-well plates (NIH-3T3, 2 x 10⁶-4 x 10⁶ cells/well) were washed twice with sterile phosphate-buffered saline prior to addition of 0.5 (24-well plate) or 1.0 ml (6-well plate) of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and gentamycin (60 µg/ml); confluent cells in 1.0 ml (6-well plate) of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. The cells were labeled with adding 10-12.5 µCi per well (24-well plates) or 5-10 µCi per well (6-well plates) of [125I]-labeled methionine/cysteine for 12-24 h. When cells were treated with tunicamycin, they were incubated in medium containing 1 µg/ml of this inhibitor for 1 h prior to addition of label. In pulse-chase experiments, cells were labeled with 20 µCi of [35S]-labeled methionine/cysteine in Dulbecco's modified Eagle's medium minus methionine containing 5% fetal bovine serum. The cells were labeled by adding 10-12.5 µCi per well (24-well plates) or 5-10 µCi per well (6-well plates) of [35S]-labeled methionine/cysteine for 12-24 h. When cells were treated with tunicamycin, they were incubated in medium containing 1 µg/ml of this inhibitor for 1 h prior to addition of label. In pulse-chase experiments, cells were labeled with 20 µCi of [35S]-labeled methionine/cysteine in Dulbecco's modified Eagle's medium minus methionine containing 5% fetal bovine serum at 37 °C, 5% CO₂. After 1 h, the radioactive medium was removed, the cells washed with phosphate-buffered saline and then incubated with complete medium for 0-6 h.

Site-directed Mutagenesis—The human transferrin receptor cDNA coding region in pCD-TR1 is contained on a 2.5-kb BamHI-XbaI fragment. The HindIII site is approximately 900 bases downstream from the start codon. The 0.9-kb BamHI-HindIII fragment was subcloned

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The abbreviations used are: BiP, binding immunoglobulin protein; kb, kilobase(s); SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
into M13mp19, and the 1.5-kb HindIII-XbaI fragment was subcloned into M13mp18, for the mutagenesis procedure. Site-directed mutagenesis was performed using the Amersham oligonucleotide-directed mutagenesis system. The consensus sequence for asparagine-linked glycosylation is Asn-X-Ser/Ile where X is any amino acid except proline. Oligonucleotides were designed so that each glycosylation site and all alteration in the consensus sequence. The oligonucleotides were synthesized by B. Root (DNA/protein Core Facility, Syracuse University). The conversion of the serine codon corresponding to amino acid position 317 was made using the Amersham oligonucleotide-directed mutagenesis system. The alteration was performed using the Amersham oligonucleotide 5'TTTTAAA(;AAA('(:CT(::l'. The alteration corresponding to amino acids 317 and 253 was performed by differential hybridization to the t4 polynucleotide kinase a-3P-labeled oligonucleotide. The presence of the altered oligonucleotides was confirmed by sequencing the U. S. Biochemical Corp. Sequenase System Version 2.0 on an IRB Model STS sequencing unit.

Transfection—The 0.9-kb BamHI-HindIII fragment containing the Ser to Ala alteration at 253, and the 1.5-kb HindIII-XbaI fragment containing the Asn to Asp alteration at 317 and the Asn to Lys alteration at 727 were cut out of M13 plasmids with HindIII and BamHI and ligated into the unique HindIII site in pDSNeoSV(λ) (17). The higher molecular mass protein is the mouse transferrin receptor and antibody Western blotting on MSI Nitroplus 2000 hybridization membrane. Detection was by swine anti-goat-conjugated alkaline phosphatase (Bio-Rad) as per manufacturer's instructions.

Immunoprecipitation—Metabolically labeled cells (2 x 10⁷-8 x 10⁷ cells) were washed twice with phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4), solubilized in 1 ml of NET-Triton (0.01 M Tris, 0.15 M NaCl, 5 mM EDTA, pH 7.4, 1% Triton X-100), and centrifuged for 20 min at 14,500 x g. The supernatant was preincubated with 70 μl of NET-Triton-washed S. aureus (10% solution), with gentle mixing on ice for 1 h. The S. aureus was then pelleted in a microcentrifuge for 30 s at 12,000 rpm. The supernatant was incubated with 1.5 μl of goat anti-human transferrin receptor and 35 μl of washed S. aureus (10% solution) for 1 h, on ice and pelleted as described above. The pellets were resuspended in 100 μl of RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton, 1% deoxycholate, pH 8.5) and centrifuged through 1 ml of RIPA containing 1% NP40. The pellets were resuspended in 100 μl of Laemmli buffer in the presence or absence of 10% 2-mercaptoethanol. The eluates were heated to 95°C for 5 min and subjected to electrophoresis on SDS-polyacrylamide gels (6 or 8%) overnight at 60-120 V according to the method of Laemmli (15). The gels were agitated for at least 30 min in 5% acetic acid, 25% methanol, then for 30 min in Amplify (Amersham Corp). The gels were dried and autoradiographed at ~70°C using Kodak X-OMAT AR film. Autoradiographs were quantitated using the Molecular Dynamics 3000A Computing Densitometer. Each experiment was repeated two to five times, and the autoradiographs were scanned. The averages and standard deviations of the experiments are presented under "Results." The polyclonal antibody was tested to determine if this antibody was capable of quantitatively immunoprecipitating the mutated version of the human transferrin receptor (see Fig. 1). Two methods were used to demonstrate quantitative immunoprecipitation. Different amounts of radioactivity labeled cell extracts were immunoprecipitated with the constant amount of antibody. The pellets were resuspended in 100 μl of Laemmli buffer in the presence or absence of 10% 2-mercaptoethanol. The eluates were heated to 95°C for 5 min and subjected to electrophoresis on SDS-polyacrylamide gels (6 or 8%) overnight at 60-120 V according to the method of Laemmli (15). The gels were agitated for at least 30 min in 5% acetic acid, 25% methanol, then for 30 min in Amplify (Amersham Corp). The gels were dried and autoradiographed at ~70°C using Kodak X-OMAT AR film. Autoradiographs were quantitated using the Molecular Dynamics 3000A Computing Densitometer. Each experiment was repeated two to five times, and the autoradiographs were scanned. The averages and standard deviations of the experiments are presented under "Results." The polyclonal antibody was tested to determine if this antibody was capable of quantitatively immunoprecipitating the mutated version of the human transferrin receptor (see Fig. 1). Two methods were used to demonstrate quantitative immunoprecipitation. Different amounts of radioactivity labeled cell extracts were immunoprecipitated with the constant amount of antibody. The pellets were resuspended in 100 μl of Laemmli buffer in the presence or absence of 10% 2-mercaptoethanol. The eluates were heated to 95°C for 5 min and subjected to electrophoresis on SDS-polyacrylamide gels (6 or 8%) overnight at 60-120 V according to the method of Laemmli (15). The gels were agitated for at least 30 min in 5% acetic acid, 25% methanol, then for 30 min in Amplify (Amersham Corp). The gels were dried and autoradiographed at ~70°C using Kodak X-OMAT AR film. Autoradiographs were quantitated using the Molecular Dynamics 3000A Computing Densitometer. Each experiment was repeated two to five times, and the autoradiographs were scanned. The averages and standard deviations of the experiments are presented under "Results." The polyclonal antibody was tested to determine if this antibody was capable of quantitatively immunoprecipitating the mutated version of the human transferrin receptor (see Fig. 1). Two methods were used to demonstrate quantitative immunoprecipitation. Different amounts of radioactivity labeled cell extracts were immunoprecipitated with the constant amount of antibody. The pellets were resuspended in 100 μl of Laemmli buffer in the presence or absence of 10% 2-mercaptoethanol. The eluates were heated to 95°C for 5 min and subjected to electrophoresis on SDS-polyacrylamide gels (6 or 8%) overnight at 60-120 V according to the method of Laemmli (15). The gels were agitated for at least 30 min in 5% acetic acid, 25% methanol, then for 30 min in Amplify (Amersham Corp). The gels were dried and autoradiographed at ~70°C using Kodak X-OMAT AR film. Autoradiographs were quantitated using the Molecular Dynamics 3000A Computing Densitometer. Each experiment was repeated two to five times, and the autoradiographs were scanned. The averages and standard deviations of the experiments are presented under "Results."
the selectable marker for geneticin resistance and is capable of transfecting eukaryotic cells. The wild type human transferrin receptor was also inserted into this vector to serve as a control.

The unglycosylated human transferrin receptor, also referred to as the triple mutant or TRPL, when transfected into mouse NIH-3T3 cells, was distinguishable from the unmutated, wild type (WT), human transferrin receptor on the basis of mobility on SDS-polyacrylamide gels (Fig. 2). Unlike the human transferrin receptor from human cell lines including A431, K562, and HL 60 cells, the wild type human transferrin receptor can be resolved into a doublet when the immunoprecipitate is subjected to electrophoresis for an extended period of time (Fig. 2A). The mobility of the upper and lower bands correspond to 92 and 87 kDa, respectively. Two major bands at 83 and 78 kDa and one minor band at 97 kDa are immunoprecipitated from 3T3 cells transfected with the mutated transferrin receptor. Western analysis of whole cell extracts confirms that the 83- and 97-kDa proteins are transferrin receptor, whereas the 78-kDa protein is not detected by the anti-transferrin receptor serum. The mobility of the 83-kDa protein is consistent with the loss of three carbohydrate side chains in the mutated receptor, and the 97-kDa protein is the mouse transferrin receptor as will be demonstrated in Fig. 3. The fact that much more of the mouse transferrin receptor is precipitated with the mutated human transferrin receptor than is immunoprecipitated in 3T3 cells alone, implies that the two receptors form heterodimers. This result will be shown in Fig. 3.

The mobility of the wild type transferrin receptor from cells treated with tunicamycin was compared to the mobility of the mutated transferrin receptor: (a) to verify that the mutated transferrin receptor was not glycosylated on asparagine resi-
dues and (b) to determine if the 78-kDa protein seen associated with the mutated transferrin receptor was also associated with the unglycosylated form of the wild type receptor in tunicamycin-treated cells. When cells containing the wild type transferrin receptor are treated with tunicamycin the molecular mass of the receptor decreases to 83 kDa and protein synthesis decreases (Fig. 2). As expected, the molecular mass of the mutant receptor is not altered and remains 83 kDa upon tunicamycin treatment indicating that the receptor is not glycosylated on asparagine residues. Western analysis of whole cell extracts from tunicamycin-treated cells confirms that the 83-kDa protein is the transferrin receptor (Fig. 2B). This result indicates that the 83-kDa protein which is metabolically labeled after tunicamycin treatment is the unglycosylated transferrin receptor. In contrast, the 78-kDa protein is not immunodetected by anti serum to the transferrin receptor. This protein is only associated with the unglycosylated form of the receptor (Fig. 2A). Treatment of cells containing the mutant transferrin receptor with tunicamycin increases the amount of 78-kDa protein in the immunoprecipitates and diminishes the amount of glycosylated mouse transferrin receptor.

**Intersubunit Disulfide Bond Formation**—The mature transferrin receptor in human cells is a dimer linked by two intersubunit disulfide bonds. Two-dimensional gel electrophoresis and pulse-chase experiments using nonreducing SDS-gel electrophoresis were employed to evaluate intersubunit disulfide bond formation in the mutated and the wild type human transferrin receptor transfected into mouse 3T3 cells. Both the wild type and the mutated transferrin receptors are capable of forming intersubunit disulfide bonds but differ with respect to one another in the extent and rates of disulfide bond formation (Figs. 3 and 4). Immunoprecipitates of cells containing the wild type transferrin receptor which are labeled overnight (on) show essentially complete disulfide bond formation using nonreducing SDS-polyacrylamide gel electrophoresis (Fig. 3A). In contrast, immunoprecipitates of cells containing the mutated transferrin receptor show incomplete disulfide bond formation and three bands ranging in molecular mass from approximately 140 to 200 kDa (Fig. 3A). The 200-kDa band seen in 3T3 cells and the 190-kDa band seen in the immunoprecipitates of 3T3 cells and the cells transfected with the human wild type transferrin receptor are confirmed to be the mouse and human transferrin receptor, respectively, by Western analysis. Likewise the three bands between 140 and 200 kDa in cells containing the mutated transferrin receptor are all transferrin receptor (Fig. 3B). These bands are postulated to be (in descending molecular mobility): the mouse homodimer by comparison with the untransfected 3T3 cells, the mouse human heterodimer, and the human homodimer. These identities were confirmed by excising the bands run under nonreducing conditions, reducing the proteins in these bands with mercaptoethanol, and subjecting them to electrophoresis (Fig. 3C). Densitometric scanning of the nonreduced gels indicates that during a 16-h label, 59 ± 5% of the mutant remains in a nondisulfide-bonded form, with 58 ± 1% of the dimers as a mouse-human heterodimer, and with 42 ± 1% as a human homodimer. In contrast, over 98% of the wild type receptor in the cells is converted to a covalently dimerized form during a 16-h label.

The rate of formation of the two higher molecular weight bands of the mutant transferrin receptor is similar to the rate of formation of the interdisulfide-linked dimer of the wild type transferrin receptor (Fig. 4, WT, 0–6 h). Disulfide bond formation is essentially complete for the wild type transferrin receptor in 4 h. The rate of formation of the lowest band in the triplet of mutated transferrin receptor is much slower and is still incomplete at the end of a 6-h chase when compared to the immunoprecipitate from cells labeled overnight (Fig. 4, TRPL, 6 h vs. on). These results indicate that in mouse cells, the triple mutant is capable of forming intersubunit disulfide bonds but does so over a much slower time frame and not nearly as efficiently as the wild type human transferrin receptor transfected into mouse cells.

**Cell Surface Expression of the Mutated Transferrin Receptor**—To examine cell surface expression, intact cells which were metabolically labeled were exposed to anti-human transferrin receptor antiserum, the cells solubilized, and the surface bound receptors isolated. The remaining transferrin receptors from the supernatant were then immunoprecipitated with anti-human transferrin receptor to determine internal receptor content (Fig. 5A). In A431 cells, approximately 15 ± 10% of the transferrin receptor appears on the cell surface (11). In mouse cells transfected with wild type human transferrin receptor, a similar proportion is expressed on the surface (18 ± 10%). Mouse cells transfected with mutant transferrin receptor, on the other hand, express at the most 1.2 ± 1% of the receptor at the cell surface (or 15 times less mutated transferrin receptors on the cell surface than wild type transferrin receptors). This method precludes preadsorption of the cell extracts with S. aureus and results in the visualization of many proteins which nonspecifically bind to S. aureus. This complication makes quantitation of the amount of unglycosylated transferrin receptor on the surface of the cells difficult. In order to verify the quantitation of cell-surface transferrin receptor, the same procedure was used on unlabeled cells. The transferrin receptors were visualized by Western analysis using the anti-transferrin receptor antibody (Fig. 5B). In addition, cell surface iodination of transfectants with Na125I and lactoperoxidase and immunoprecipitation was used to quantitate cell surface mutant and wild type transferrin receptor. This method yields 20 times less of the mutated receptor than wild type on the cell surface (data not shown).

**Ability of the Mutated Transferrin Receptor to Bind to Transferrin-Agarose**—The ability of the transferrin receptor to bind transferrin was measured to indicate if the mutated receptor has similar quaternary structure to that of the wild type transferrin receptor. Cell extracts were solubilized and incubated with transferrin-agarose (Fig. 6). After the binding step, receptors remaining in the supernatant and not capable

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**Fig. 4. Dimer formation in wild type and unglycosylated mutant human transferrin receptor.** 3T3 cells transfected with vectors carrying wild type (WT) or triple mutant (TRPL) human transferrin receptor were metabolically labeled with 5 μCi of [35S]methionine/cysteine overnight (on). In the pulse chase experiments, cells were labeled with 20 μCi of [35S]methionine/cysteine for 1 h and chased for the indicated times (0, 1, 2, 4, and 6 h). Cells were solubilized and immunoprecipitated with anti-human transferrin receptor. The immunoprecipitate was subjected to electrophoresis in a nonreducing 6% polyacrylamide gel and to autoradiography.
Fig. 5. Surface expression of wild type and unglycosylated mutant transferrin receptor. A, 3T3 cells transfected with vectors carrying wild type (WT) or mutant unglycosylated (TRPL) human transferrin receptor were labeled overnight with [35S]methionine/cysteine. The medium was replaced with Hepes-buffered Dulbecco’s modified Eagle’s medium containing anti-human transferrin receptor, and incubated for 1 h at 4°C. Cells were solubilized, and bound (external) receptors (o) precipitated with washed S. aureus. Internal receptors (i) were then immunoprecipitated from the supernatant. Eluates of immunoprecipitates were subjected to electrophoresis on SDS, 8% polyacrylamide gel, and to autoradiography. In this gel, no cell surface mutant transferrin (TRPL) receptors were detected, whereas 17% of the wild type transferrin (WT) receptors were detected on the cell surface. The results in the text are the average and S.D. of five experiments. B, the external receptors (o) and internal receptors (i) were immunoprecipitated from unlabeled cells and subjected to electrophoresis as described in A. The proteins were then transferred to nitrocellulose and immunodetected with an anti-transferrin receptor antibody by Western analysis and chemiluminescence.

Fig. 6. Transferrin binding of wild type (WT) and unglycosylated mutant (TRPL) human transferrin receptor. Cells were solubilized and incubated with transferrin-agarose on a rotator at 4°C for 1 h. After pelleting the transferrin-agarose bound transferrin receptor, the supernatant was immunoprecipitated with washed S. aureus and goat anti-human transferrin receptor. Pellets were treated as indicated under “Materials and Methods,” subjected to electrophoresis on SDS-8% polyacrylamide, transferred to nitrocellulose, and detected with anti-transferrin receptor antibody by Western analysis and chemiluminescence. Lanes marked f are transferrin-agarose bound eluates; lanes marked i are immunoprecipitates.

Fig. 7. Pulse-chase of the wild type and mutated transferrin receptor under reducing conditions. Cells transfected with wild type (A) or mutated (B) transferrin receptors were labeled for 1 h with 20 μCi of [35S]methionine/cysteine. Radioactive medium was removed and replaced by 10% fetal calf serum. Dulbecco’s modified Eagle’s medium, and the cells were incubated for the indicated times before solubilization and immunoprecipitation with transferrin receptor antisera. Cells transfected with the mutated transferrin receptor were labeled with 5 μCi of [35S]methionine/cysteine overnight (on). The immunoprecipitates were subjected to electrophoresis on a reducing 8% polyacrylamide gel and to autoradiography.

Fig. 8. Coprecipitation of unglycosylated mutant transferrin receptor with BiP. NIH-3T3 cells transfected with triple mutant (TRPL) transferrin receptor were solubilized. Cell extracts (c) or anti-human transferrin receptor immunoprecipitates (i) were subjected to electrophoresis, transferred to nitrocellulose, and probed with antibody to BiP. The blot was developed using rabbit anti-rat serum, followed by alkaline phosphatase-coupled goat anti-rabbit antibody and substrate. As controls, NIH-3T3 cells containing wild type human transferrin receptor (WT) were treated with tunicamycin for 16 h or left untreated, and subjected to the same protocol as the cells carrying the mutant receptor. A431 cells were also treated with tunicamycin and subjected to the same protocol.

plasmic reticulum. In tunicamycin-treated A431 cells, unglycosylated transferrin receptor is associated with BiP, a 78-kDa endoplasmic reticular protein, and other unglycosylated proteins. We wanted to determine if the mutated human transferrin receptor was associated with BiP in the absence of unglycosylated proteins. Immunoprecipitation of the mutated transferrin receptor with transferrin receptor antisera reveals the presence of a band with variable intensity at 78 kDa that coprecipitates with mutant transferrin receptor (Fig. 7). It coprecipitates with the mutated transferrin receptor during pulse-chase experiments and does not associate detectably with the wild type human transferrin receptor during its biosynthesis (Fig. 7). To determine if the coprecipitating protein is BiP, cell extracts were immunoprecipitated with anti-human transferrin receptor serum, and the immunoprecipitates were subjected to SDS-polyacrylamide electrophoresis. Western immunodetection using a monoclonal antibody to BiP reveals a 78-kDa protein which coprecipitates with the mutated transferrin receptor but not the wild type transferrin receptor (Fig. 8). If 3T3 cells containing the wild type transferrin receptor or A431 cells are treated with tunicamycin, BiP is detected in these immunoprecipitates also (Fig. 8). It therefore appears that the unglycosylated form of

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transferrin receptor associates with BiP, and the resulting complex is stable enough to be isolated.

**DISCUSSION**

Little success has been achieved in assigning functions to the posttranslational modifications of the transferrin receptor. Acylation is required for membrane association of some proteins, but Jing and Trowbridge (2) show that membrane association was normal in transferrin receptor in which the cysteine at position 62 was altered to serine by site-directed mutagenesis, making acylation impossible. Phosphorylation has been suggested to play a role in the endocytosis and recycling of transferrin receptor, since phorbol esters increase the phosphorylation state of transferrin receptor in some cell lines and down-regulate transferrin receptor at the cell surface (20). However, other researchers have shown a decrease in phosphorylation of the receptor upon endocytosis in other cell lines (21). Furthermore, elimination of the phosphorylation site (Ser-24) by site-directed mutagenesis did not affect endocytosis and recycling (22).

Glycosylation of asparagine residues appears to have the most profound effect on transferrin receptor function. In A431 cells treated with tunicamycin, transferrin binding to the unglycosylated receptor, surface expression, and dimerization are abolished (11). Tunicamycin is a heterogeneous group of uracil-based compounds which prevents synthesis of oligosaccharide moieties which are transferred from the lipid carrier dolichol phosphate to the asparagine target on the polypeptide chain (12). However, tunicamycin also may inhibit protein synthesis in many cell lines (12) and also could exert an indirect effect by perturbing the synthesis and function of proteins involved in the biosynthesis of the transferrin receptor. Consequently, there must be reservations with regard to the interpretation of results obtained from tunicamycin-treated cells. We have therefore used site-directed mutagenesis to eliminate the N-linked glycosylation sites of the human transferrin receptor. Alteration of the consensus sequence Asn-X-Ser/Thr prevents N-linked glycosylation of the initial asparagine residue. The wild type and mutated transferrin receptors were transfected into mouse 3T3 cells as previous investigators demonstrated that this receptor was functional in these cells (10). The present studies indicate that the wild type transferrin receptor is processed slower than in some human cell lines (23) and at a similar rate to that of other human cell lines (5). It does fold correctly and is transported to the cell surface. In contrast to wild type transfected transferrin receptor, the mutated form of the human transferrin receptor shows an electrophoretic mobility and insensitivity to tunicamycin treatment consistent with complete lack of N-linked glycosylation. While retaining the ability to be immunoprecipitated by a polyclonal antibody specific for human transferrin receptor, this transferrin receptor mutant has a diminished ability to form dimers and to bind human transferrin, and exhibits reduced cell surface expression. Association with BiP, an endoplasmic reticular protein which is believed to associate with improperly folded polypeptides, further suggests that a fraction of this mutated receptor is retained within the cell. Whether the altered properties of the mutant receptor are due simply to lack of the glycosyl groups, or are the result of an altered conformation due to the amino acid substitution is not easy to assess. The similarity of the results we obtained in previous studies using tunicamycin to prevent glycosylation with the site-directed mutagenesis studies strengthens the proposal that N-linked glycosylation plays an important role in the proper folding of the transferrin receptor.

While the mutated receptor shares many of the properties of the unglycosylated transferrin receptor in tunicamycin-treated cells, it differs from this form of the receptor in one respect. It appears to have more tertiary structure than the result from tunicamycin-treated cells, as it does form heterodimers with the mouse transferrin receptor and to a more limited extent it does form homodimers with itself. In earlier studies using tunicamycin-treated A431 cells, we found the unglycosylated transferrin receptor was a part of a large complex, consisting of BiP and other unglycosylated proteins (11, 17). This unglycosylated transferrin receptor did not form detectable intersubunit disulfide bonds, did not bind transferrin, and was not transported to the cell surface. Treatment with tunicamycin results in an increase in the amount of BiP immunodetected in these cells. We hypothesize and are currently examining the possibility that the increased amount of BiP and other unglycosylated proteins in these treated cells is contributing to the lack of processing of the receptor. Thus the differences we observe between the unglycosylated transferrin receptor from tunicamycin-treated cells and the unglycosylated mutated receptor could result from the fact that in tunicamycin-treated cells the unglycosylated receptor is part of a large complex with BiP and other unglycosylated proteins.

We do not know if the association of the transferrin receptor with BiP is part of the biosynthetic pathway for the wild type transferrin receptor. An association, even a transient one, between BiP and the normal transferrin receptor has not been detected, although the association may be weak and lost during immunoprecipitation. This latter phenomenon has been observed with other nascent proteins thought to be associated with BiP (24, 25). If BiP only associates with the unglycosylated form of the receptor, then during its biosynthesis it would be released when the transferrin receptor is glycosylated.

We hypothesize that association with BiP results in retention of much of the unglycosylated receptor in the endoplasmic reticulum. Our data indicate that dimerization alone is not sufficient for correct folding of the receptor and transport to the cell surface. Approximately 59% of the mutated transferrin receptors form intersubunit disulfide bonds. Given the distribution of the wild type transferrin receptor in this cell line (18 ± 10% outside) we would predict if dimerization was the only requisite for cell surface expression we should see ~9% of the mutant receptors on the cell surface. In both the immunolocalization and cell surface 125I-labeling experiments no more than 2.6% (average 1.2%) of the mutated receptor was seen on the cell surface. The intracellular location of the dimers remains to be identified.

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