Acquisition of the Functional Properties of the Transferrin Receptor during Its Biosynthesis*

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The properties of the newly synthesized and partially glycosylated forms of the transferrin receptor were examined to determine which co- and post-translational modifications are necessary for the acquisition of transferrin binding activity and transport of the receptor to the cell surface. The nascent transferrin receptor containing core-glycosylated asparagine-linked oligosaccharides does not possess complete inter-subunit disulfide bonds, sediments predominantly as a monomer in sucrose density gradients, and shows reduced binding to transferrin-agarose. Within 20–30 min after synthesis, the transferrin receptor acquires the ability to bind to a transferrin-linked affinity column. Intersubunit disulfide bond formation occurs slowly throughout the transit of the receptor to the cell surface. These results indicate that core glycosylation of the receptor may be necessary but is not sufficient for the acquisition of the ability of the receptor to bind transferrin and that intersubunit disulfide bond formation is a post-translational event. Inhibition of complex carbohydrate synthesis by either swainsonine (1 µg/ml) or deoxynojirimycin (4 mM) does not inhibit the ability of this receptor to form intersubunit disulfide bonds or to be transported to the cell surface. The partially glycosylated receptor, however, does show an approximately 3-fold reduced affinity for transferrin.

The human transferrin receptor is a cell surface protein involved in the uptake of iron into cells. Like all integral plasma membrane proteins identified to date, it is synthesized in the endoplasmic reticulum and undergoes many co- and post-translational modifications during its transit to the cell surface. The modifications include dimer formation (Enns and Sussman, 1981), intersubunit disulfide bond formation (Sutherland et al., 1981; Trowbridge and Omary, 1981; Goding and Burns, 1981), acylation with palmitate (Omary and Trowbridge, 1981), phosphorylation of a serine residue (Schneider et al., 1982), N-linked1 (asparagine-linked) glycosylation (Omary and Trowbridge, 1981; Schneider et al., 1982), and O-linked glycosylation (Do et al., 1990).

We and others have been interested in defining the roles that these modifications play in the correct folding and transport of the receptor to the cell surface. Earlier studies indicated that N-linked glycosylation is important in the correct folding of the transferrin receptor (Reckhow and Enns, 1988). In the present studies, the properties of a nascent form of the transferrin receptor and a form of the transferrin receptor with altered N-linked oligosaccharides were examined.2 These studies demonstrate that the newly synthesized receptor is a monomer and is incapable of binding to transferrin-linked Sepharose. Treatment of cells with swainsonine, an inhibitor of N-linked complex carbohydrate formation, does not affect the ability of the transferrin receptor to dimerize, form intersubunit disulfide bonds, or be transported to the cell surface. However, it does reduce the affinity of the receptor for transferrin.

EXPERIMENTAL PROCEDURES

Cell Culture and Radiolabeling—Dulbecco's modified Eagle's medium (DMEM), DMEM minus methionine, RPMI 1640 medium, RPMI 1640 minus methionine, fetal calf serum, and gentamicin were purchased from Gibco or Flow Laboratories. A431 cells, a human epidermoid carcinoma cell line, were a gift of Dr. Graham Carpenter, Vanderbilt University. They were maintained in DMEM containing 10% fetal calf serum and 0.05 µg/ml gentamicin. Subconfluent cells in 24-well plates (approximately 3–4 × 10⁵ cells/well) were washed twice with sterile PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) prior to addition of DMEM minus methionine containing 10% fetal calf serum. The cells were incubated with glycosylation inhibitors for 1 h prior to addition of label. Glycosylation inhibitors included tunicamycin (Calbiochem) 0.01–1.0 µg/ml, swainsonine (Genzyme) (0.5–4 µg/ml), and deoxynojirimycin (Genzyme) (2–8 mM). Each lot of inhibitor was titrated to determine the optimum inhibitor concentration. Metabolic labeling was effected by incubating the cells with 12.5 µCi of [35S]methionine/cysteine (Translabel, ICN) in the continued presence or absence of inhibitor for 12–24 h. K562 cells were from American Type Tissue Collection and were maintained between 1 × 10⁶ and 8 × 10⁶ cells/ml in RPMI 1640 medium, 10% fetal calf serum.

Immunoprecipitation of the Transferrin Receptor—Metabolically labeled cells were washed twice with phosphate-buffered saline containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (CM-PBS) prior to solubilization in 1 ml of 10 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4, 1% Triton X-100 (NET-Triton). Staphylococcus aureus (100 µl of a 10% solution) that had been washed three times with NET-Triton was added to the cell extract to preadsorb proteins that bind nonspecifically to S. aureus. After 1 h, the S. aureus was pelleted and the supernatant transferred to a fresh tube. Goat anti-human transferrin receptor serum (1.4 µl) (Enns et al., 1981) and S. aureus (100 µl of a 1%

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1 The abbreviations used are: N-linked, asparagine-linked; O-linked, serine/threonine-linked; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; transferrin agarose, transferrin-linked Affi-Gel 15; endo H, endo β-N-acetylglucosaminidase H; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

10% solution) were added to the supernatant, and the suspension was agitated for 1 h at 4 °C. All immunoprecipitations were performed with 2-5 times excess antisem as previously determined (Reckhow and Enns, 1988). The receptor-antibody complex immobilized on S. aureus was pelleted and the pellets either washed three times with 50 mM Tris, 150 mM NaCl, pH 7.4, 0.1% sodium dodecyl sulfate, 1% Triton X-100, and 5% mercaptoethanol. The eluates of the immunoprecipitates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 8 or 6% gels. The gels were agitated in a 5% acetic acid, 25% methanol solution for 1 h or more followed by electrophoresis in Amplify (AmerhamCorp.) according to the manufacturer’s directions. The gels were dried and subjected to autoradiography at -70 °C using Kodak X-Omat AR film and Du Pont Lightning Plus intensifying screens. Quantitation of the autoradiographs was performed with a Molecular Dynamics laser densitometer. Each autoradiograph was scanned at two exposures to ensure linearity.

**Succrose Density Gradient Centrifugation**—Solubilized extracts (3-4 mg) of cells cultured in 100 ml of DMEM (Triton X-100) of 35S-labeled cells were layered on 10-30% (w/v) sucrose gradients (4.5 ml) containing 10 mM Tris-Cl, 150 mM NaCl, pH 7.4, 0.1% Triton X-100. Centrifugation was performed for 4 h at 44,500 rpm using a Beckman SW 50.1 rotor at 4 or 20 °C. Transferrin (0.1 mg) (4.9 S) and catalase (0.1 mg) (11.4 S) were subjected to centrifugation at the same rpm. At the completion of each run, fractions were collected from the top using a Haake-Buchler gradient digit tap. The fractions from tubes containing transferrin and catalase were assayed for protein by the method of Bradford (1976). Each fraction from the tubes containing the cell extract of 35S-labeled cells was immunoprecipitated and subjected to electrophoresis on SDS-8% polyacrylamide gels.

**Binding to Transferrin-linked Affinity Columns**—Transferrin (Calbiochem) was linked either to CNBr-activated Sepharose (Pharmacia LKB Biotechnology Inc.) or Affi-Gel 15-agarose (Bio-Rad) according to the manufacturers’ instructions to final concentrations of 3-7 mg/ml packed gel. Transferrin-agarose was used in later experiments as it did not precipitate the nonspecific binding occurred using this material. Subconfluent cells were treated with inhibitors or left untreated and metabolically labeled with 35S-methionine/cysteine. Cells were washed twice with CM-FBS and solubilized with NET-Triton and the nuclei and debris removed by centrifugation. The extracts were precleared by incubating with bovine serum albumin (BSA) linked to Affi-Gel 15 (BSA-agarose) for 1 h at 4 °C. The BSA-agarose was then pelleted, and the supernatants were incubated with iron-saturated transferrin linked to Affi-Gel 15 (transferrin-agarose) for 1 h at 4 °C. The transferrin-agarose was pelleted and the supernatants containing the unbound transferrin receptors were immunoprecipitated with S. aureus and goat anti-human transferrin receptor antiserum as previously described. The functional transferrin receptors bound to the transferrin-agarose pellet were washed by pelleting through 1 ml of RIPA, 15% sucrose in a microcentrifuge for 30 s. The pellets were eluted with 1 ml of RIPA, 15% sucrose in a microcentrifuge for 30 min, solubilized with 2 ml of 1 N NaOH (for 30 min at room temperature) and counted on a Beckman γ counter. All concentrations were performed in triplicate. Nonspecific binding was less than 10% of specific binding.

**Selective Immunoprecipitation; Internal/Cell Surface Localization of the Transferrin Receptor**—Internal and cell surface transferrin receptors were isolated by differential immunoprecipitation as described previously (Reckhow and Enns, 1988).

**RESULTS**

**Properties of the Newly Synthesized Transferrin Receptor**—Earlier studies showed that an unglycosylated form of the transferrin receptor from tunicamycin-treated A431 cells is incapable of binding transferrin (Reckhow and Enns, 1988). Tunicamycin inhibits the first step in the synthesis of N-linked oligosaccharides (reviewed by Elbein (1987)). The transferrin receptor from tunicamycin-treated cells is unable to form intersubunit disulfide bonds or to bind to transferrin-linked affinity columns. The newly synthesized transferrin receptor has properties similar to those of the unglycosylated transferrin receptor from tunicamycin-treated cells. Both show reduced affinity to transferrin-linked affinity columns (Fig. 1A). Extracts from cells that had been incubated with 35S-methionine/cysteine for 12 min were divided into two aliquots and incubated either with transferrin-linked affinity columns or immunoprecipitated with anti-transferrin receptor serum. In cells that were labeled overnight in radioactive medium, the same amount of transferrin receptor (M, = 94,000) can be isolated with the transferrin-linked affinity column as can be immunoprecipitated with anti-transferrin receptor serum (Fig. 1A, lanes 4 and 3, respectively). In contrast, scanning densitometry of autoradiographs indicates that only 0-17% of the transferrin receptor from cells labeled for 12 min with radioactive medium (i.e. newly synthesized). M, = 89,000) could be isolated with the transferrin-linked affinity column (Fig. 1A, lanes 1 and 2). As reported previously, the unglycosylated transferrin receptor (M, = 80,000) from tunicamycin-treated cells shows no binding to transferrin-linked affinity columns (Fig. 1A, lanes 5 and 6) (Reckhow and Enns, 1988; Root et al., 1988).

A combination of nonreducing SDS-polyacrylamide gel electrophoresis and sucrose density gradient centrifugation was performed to determine whether the nascent transferrin receptor forms intersubunit disulfide bonds and dimers cotranslationally. The majority of the newly synthesized transferrin receptor does not possess the intersubunit disulfide bonds that are characteristic of the mature form. The electrophoretic mobility of the nascent transferrin receptor under these nonreducing conditions shows a similar mobility to that of the unglycosylated transferrin receptor in tunicamycin-treated cells (Fig. 1B, P and T). After the 12-min pulse, only a fraction (19-25%) of the newly synthesized receptors possesses intersubunit disulfide bonds by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In contrast, the mature form of the receptor migrates as a dimer under nonreducing conditions (Fig. 1B, P and T versus U). The higher molecular weight of the nascent transferrin receptor compared with that of the unglycosylated transferrin receptor is due to the co-translational glycosylation of asparagine residues as reported earlier (Omary and Trowbridge, 1981; Schneider et al., 1982) and as demonstrated by the reduction in molecular weight to nearly that of the unglyco-
FIG. 1. Structural features of the nascent transferrin receptor. A431 cells (1 × 10^5–5 × 10^5) were metabolically labeled in 24-well plates with [55S]methionine/cysteine for a short time (12-min pulse) (P); treated with tunicamycin and metabolically labeled overnight (T), or left untreated and metabolically labeled overnight (U). Each set of cells was washed and solubilized as described under "Experimental Procedures." A, binding of transferrin receptor to transferrin-Sepharose. The cell extracts were preadsorbed with BSA-linked agarose and divided into two aliquots. One aliquot (odd numbers) was incubated with anti-transferrin receptor serum and S. aureus. The other aliquot was incubated with transferrin-Sepharose. The immunoprecipitates and transferrin-agarose were washed, eluted with 2 × Laemml buffer, and subjected to SDS-polyacrylamide gel electrophoresis and fluorography. B, nonreducing SDS-polyacrylamide gel electrophoresis of anti-transferrin receptor immunoprecipitates. In this case, the entire extract was immunoprecipitated with anti-transferrin receptor serum and S. aureus, washed, and subjected to electrophoresis using SDS-6% polyacrylamide gels under nonreducing conditions. The electrophoretic mobilities of the transferrin receptor dimer (d) and the monomer (m) are indicated. C, endo H digestion of anti-transferrin receptor immunoprecipitates. The transferrin receptors from pulsed (for 10 min) cells (P), untreated cells (pulsed for 10 min and chased 2 h) (U) or tunicamycin-treated cells (labeled overnight) were incubated overnight in the presence (+) or absence (−) of endo H (5 millimiters) at 37°C. The samples were eluted with 2 × Laemml buffer and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and fluorography.

sylated form of the transferrin receptor with endo H digestion (Fig. 1C). Endo H cleaves the chitobiose core of high mannose and most hybrid oligosaccharides (Trimble and Maley, 1984).

Since the absence of intersubunit disulfide bonds does not rule out dimerization, sucrose density gradients were used to determine whether the newly synthesized form of the transferrin receptor sediments as a monomer or dimer (Fig. 2). Using transferrin (4.9 S, M_r = 80,000) and catalase (11.4 S, M_r = 242,000), it is evident that the newly synthesized transferrin receptor sediments as a monomer with a sedimentation coefficient of 5.2 S. This behavior differs from the sedimentation pattern of the unglycosylated transferrin receptor in tunicamycin-treated cells. The latter sediments as a large complex and is associated with at least two other proteins (Reckhow and Enns, 1988; Root et al., 1988). The lack of involvement of the nascent transferrin receptor in a large complex is consistent with our observation that no other proteins coprecipitate with this form of the receptor (Fig. 1A, P). Thus, the newly synthesized transferrin receptor neither binds transferrin, forms intersubunit disulfide bonds, nor dimerizes.

FIG. 2. Sucrose density gradient centrifugation of the nascent transferrin receptor. A431 cells were pulse-labeled with 100 μCi of [55S]methionine/cysteine for 12 min and solubilized. The solubilized extract of 3–4 × 10^7 cells was loaded on top of 4.5-ml 5–20% sucrose gradients. Transferrin and catalase were run in separate tubes. The samples were spun at 44,500 rpm for 6 h at 4°C in an SW 50.1 Beckman rotor. Fractions (15-drop) were collected from the top, and the pellet (P) was resuspended. Each fraction was immunoprecipitated with goat anti-transferrin receptor serum and subjected to SDS-polyacrylamide gel electrophoresis. The locations of sedimentation peaks of transferrin (tf, 4.9 S) and catalase (cat, 11.4 S) are indicated. 3H-Labeled molecular weight standards are: myosin (200,000), phosphorylase b (97,400), bovine serum albumin (68,000), ovalbumin (43,000), and carbonic anhydrase (29,000).

Processing of the Transferrin Receptor during Its Biosynthesis—Pulse-chase studies were performed to correlate the formation of disulfide bonds with the acquisition of transferrin binding activity. The acquisition of transferrin binding activity was rapid and preceded disulfide bond formation (Fig. 3, B and C). Quantitation of the time course of these events by scanning densitometry shows that virtually all of the labeled transferrin receptor attains its ability to bind transferrin within 30 min of chase, whereas only half the receptors possess interchain disulfide bonds within this time period (Fig. 3, B and C). Thus, intersubunit disulfide bond formation is not critical for the development of transferrin binding activity.

We also wanted to determine where these modifications occurred in the cell. The diminution in endo H sensitivity was used as an approximation of the transit of the transferrin receptor in the Golgi. The acquisition of the ability of the transferrin receptor to bind transferrin precedes the loss in sensitivity to endo H digestion (Fig. 3, A and B). Intersubunit disulfide bond formation during the biosynthesis of the transferrin receptor is a relatively slow process and appears to occur in both the endoplasmic reticulum and the Golgi (Fig. 3C). Consistent with previous reports (Omary and Trowbridge, 1981), the mature form of the transferrin receptor is only partially sensitive to endo H digestion. This partial sensitivity has led to the hypothesis that the mature transferrin receptor possesses both high mannose and complex carbohydrates (Omary and Trowbridge, 1981). Detailed carbohydrate analysis of this receptor have confirmed these results (Do et al., 1990).

Our results indicating that the newly synthesized transferrin receptor is predominantly a monomer, does not contain intersubunit disulfide bonds, and has diminished capability of binding transferrin are in conflict with previously published results of Hunt and co-workers (1989) on the transferrin
jected to electrophoresis on nonreducing SDS-polyacrylamide gel. After 1 h at 37 °C, the transferrin receptor in pulse-chase experiments. Cells were pulsed with [3sS]methionine/cysteine as described in A, pre-cleared with BSA-agarose, and incubated with transferrin-agarose. After 1 h at 4 °C, the transferrin-agarose was pelleted, and the supernatant was immunoprecipitated with anti-transferrin receptor serum. Cells were pulsed with [3S]methionine/cysteine for 10 min and chased for up to 60 min. At the indicated time points, samples were subjected to immunoprecipitation with anti-transferrin receptor serum. The washed immunoprecipitates were eluted and the eluates subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions.

Fig. 3. Time course of the acquisition of endo H resistance, transferrin binding activity, and intersubunit disulfide bond formation of the transferrin receptor. A, time course of the acquisition of endo H resistance. A431 cells were pulsed with 100 μCi of [3S]methionine/cysteine for 10 min, washed, and chased for up to 60 min. After 10, 20, 30, 45, and 60 min of chase, the cells were washed, solubilized, and the transferrin receptor immunoprecipitated with anti-transferrin receptor serum. The transferrin receptor was digested (+) or mock digested (−) overnight with 5 milliunits of endo H at 37 °C. B, time course of the acquisition of transferrin binding activity by the transferrin receptor in pulse-chase experiments. Cells were pulsed with [3S]methionine/cysteine as described in A, pre-cleared with BSA-agarose, and incubated with transferrin-agarose. After 1 h at 4 °C, the transferrin-agarose was pelleted, and the supernatant was immunoprecipitated with anti-transferrin receptor serum. Cells were pulsed with [3S]methionine/cysteine for 10 min and chased for up to 60 min. At the indicated time points, samples were subjected to immunoprecipitation with anti-transferrin receptor serum. The washed immunoprecipitates were eluted and the eluates subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions.

Role of Glycosylation in the Folding and Transport of the Transferrin Receptor to the Cell Surface—The present studies indicate that core glycosylation of the transferrin receptor appears to be necessary but not sufficient for the correct folding of the newly synthesized transferrin receptor. We wanted to determine whether complete N-linked glycosylation of the transferrin receptor was critical to its acquisition of transferrin binding activity and its transport to the cell surface. Two inhibitors of N-linked oligosaccharide processing were employed. Swainsonine inhibits α-mannosidase II, and 1-deoxynojirimycin inhibits glucosidase I (reviewed in Elbein (1987)). Incubation of cells with either of these compounds should inhibit processing of the oligosaccharide to complex forms and result in high mannose and hybrid oligosaccharides. Cells were incubated with varying concentrations of deoxy-

Fig. 4. Acquisition of transferrin binding and disulfide bond formation in untreated K562 cells. Cells (5 × 10⁶) were metabolically labeled with 50 μCi of [3S]labeled methionine/cysteine for 12 min in RPMI 1640 medium minus methionine and 10% fetal calf serum followed by incubation in nonradioactive medium containing methionine for 0, 20, 60, or 120 min. Receptors capable of binding transferrin were isolated from cellular extracts with transferrin-linked agarose (T-Binding). The remaining transferrin receptors were isolated by immunoprecipitation (Immun.). Eluates were subjected to electrophoresis on a 6% polyacrylamide gel under nonreducing conditions. M, monomer; D, disulfide-bonded dimer.

Fig. 5. Differential immunoprecipitation of the transferrin receptor from inhibitor-treated and untreated cells. A431 cells were treated with deoxynojirimycin (DEOX) (4 mM), swainsonine (SWAIN) (1 µg/ml), or left untreated (UNTR) 30 min prior to the addition of [35S]methionine/cysteine. The cell surface and internal transferrin receptors were immunoprecipitated separately as described in Reckhow and Enns (1988). The eluates of the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Lane G, immunoprecipitation with nonimmune goat serum; lane W, immunoprecipitation of the transferrin receptor from whole cell extracts; lane O, immunoprecipitation of the outside (cell surface) transferrin receptors; lane I, immunoprecipitation of the internal transferrin receptors.

Fig. 6. Electrophoresis of the immunoprecipitated transferrin receptor from untreated and inhibitor-treated cells in a nonreducing gel. A431 cells were labeled with [35S]methionine/cysteine and solubilized prior to immunoprecipitation of the transferrin receptor. Eluates of the immunoprecipitate were subjected to electrophoresis on an SDS-nonreducing 6% polyacrylamide gel. Lanes U, T, S, and D are immunoprecipitates from untreated, tunicamycin-treated, swainsonine-treated, and deoxynojirimycin-treated cells, respectively. The mobilities of the monomer (m) and dimer (d) are shown.

Fig. 7. Binding of the transferrin receptor from inhibitor-treated and untreated cells to transferrin-linked Sepharose. Treated and untreated A431 cells were labeled with [35S]methionine/cysteine, solubilized, precleared with BSA-agarose, and divided in half. One aliquot was incubated with transferrin-linked Sepharose and the other was immunoprecipitated. Samples were washed and the eluates subjected to electrophoresis on a SDS-polyacrylamide gel under reducing conditions. Lanes 1, 3, 5, and 7 are immunoprecipitates of the transferrin receptor; lanes 2, 4, 6, and 8 are eluates from transferrin-linked Sepharose. Lanes D, S, U, and T are immunoprecipitates from deoxynojirimycin-treated, swainsonine-treated, untreated cells, and tunicamycin-treated cells, respectively.

control dishes contained the same number of cells. Analysis of the binding data (Scatchard, 1949) shows that although the same number of transferrin-binding sites are on the surface of swainsonine-treated cells as are on untreated cells, the affinity of transferrin for its receptor is lower by a factor of 2.5–3 (Fig. 8) (Table 1).

Fig. 8. Analysis of 125I-transferrin binding to swainsonine-treated and untreated cells. A431 cells were treated with swainsonine (○) or left untreated (□) for 4 days. See “Experimental Procedures” for details. B/F, bound/free.

### Table 1

Summary of analysis of 125I-transferrin binding to swainsonine-treated and untreated A431 cells

<table>
<thead>
<tr>
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<th>Total transferrin binding</th>
<th>ng/dish</th>
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<tr>
<td>Exp. 1</td>
<td></td>
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</tr>
<tr>
<td>Untreated cells</td>
<td>5.4 × 10^6 M⁻¹</td>
<td>56</td>
</tr>
<tr>
<td>Swainsonine-treated cells</td>
<td>1.3 × 10^8 M⁻¹</td>
<td>58</td>
</tr>
<tr>
<td>Exp. 2</td>
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<tr>
<td>Untreated cells</td>
<td>6.7 × 10^6 M⁻¹</td>
<td>44</td>
</tr>
<tr>
<td>Swainsonine-treated cells</td>
<td>2.8 × 10^8 M⁻¹</td>
<td>42</td>
</tr>
<tr>
<td>Exp. 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated cells</td>
<td>4.0 × 10^6 M⁻¹</td>
<td>40</td>
</tr>
<tr>
<td>Swainsonine-treated cells</td>
<td>1.4 × 10^8 M⁻¹</td>
<td>38</td>
</tr>
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**DISCUSSION**

The evidence presented in this paper indicates that the newly synthesized transferrin receptor has not acquired its full ability to bind transferrin nor has it attained the quaternary structure of the mature receptor. That is, it has neither dimerized nor formed stoichiometric intersubunit disulfide bonds. The acquisition of transferrin binding activity is rapid. Within a 10-min pulse, 0–17% of the newly synthesized receptor is able to bind to a transferrin-linked affinity column. Optimal transferrin binding to transferrin-linked affinity columns is achieved prior to the acquisition of complex carbohydrates and partial endo H resistance. Surprisingly, intersubunit disulfide bond formation appears to be a relatively slow process with complete intersubunit disulfide bond formation not occurring until the transferrin receptor reaches the cell surface.

Previous work from our laboratory established that N-linked glycosylation appears to be necessary for the correct folding or transport of the nascent chain to a site where it is further modified to fold correctly (Reckhow and Enns, 1988; Root et al., 1988). The present studies demonstrate that although N-linked glycosylation is necessary, it is not sufficient for attaining complete transferrin binding activity. The merely core-glycosylated transferrin receptor has a much lower affinity for transferrin as demonstrated by its reduced binding to transferrin-linked affinity columns. The transferrin receptor from deoxynojirimycin- and swainsonine-treated cells is capable of forming dimers, being transported to the cell surface, and binding to transferrin-linked affinity columns. However, analysis of the transferrin binding data indicates that although the same number of transferrin-binding sites are on the surface of swainsonine-treated cells as are on untreated cells, the affinity of transferrin for its receptor is lower by a factor of 2.5–3 (Fig. 8) (Table 1).
sites are found on the surface of swainsonine-treated versus untreated cells, the affinity of transferrin for the receptor is lowered approximately 3-4-fold. Interestingly, when the gene for the human transferrin receptor is expressed in Sf9 cells, an insect cell line derived from Spodoptera frugiperda, the binding of transferrin is reduced 10-fold compared with that expressed in mammalian cells (Domingo and Trowbridge, 1988). Endo H sensitivity of the human transferrin receptor expressed in this cell line indicates that this receptor lacks complex oligosaccharides.

Some of the results presented in this paper are in direct contrast to those obtained by Hunt and co-workers (1989). In their experiments, the biosynthesis of the transferrin receptor was examined in K562 cells, a human erythroid myeloid precursor cell line. Although they approximate similar kinetics for transit through the cell, they present evidence that the nascent transferrin receptor possesses intersubunit disulfide bonds and state that the nascent receptor binds equally well to a transferrin-linked Sepharose column as the mature transferrin receptor. The basis for these discrepancies is not known. It is not likely to be a cell type difference as parallel experiments that we have performed on K562 cells demonstrate the same qualitative results obtained for A431 cells. In pulse-chase experiments where the ability of the newly synthesized transferrin receptor to bind to transferrin-linked affinity columns was examined, we also quantitated the amount of transferrin receptor that could not bind. This procedure gave a sensitive assay for binding. Two separate methods were used to assay whether the receptor formed dimers. Nonreducing SDS-polyacrylamide gel electrophoresis indicated that the newly synthesized receptor did not possess intersubunit disulfide bonds. Furthermore, sucrose density gradient centrifugation experiments indicated that this form of the receptor isolated from A431 cells sedimented as a monomer. A431 cells are exquisitely sensitive to tunicamycin. Concentrations as low as 0.01 μg/ml and treatment as short as ½ h were able to inhibit glycosylation of the transferrin receptor, whereas K562 cells are much more resistant to tunicamycin treatment. This cell line requires 5-20 μg/ml tunicamycin and pretreatment of cells 16-20 h before metabolic labeling. At these concentrations, protein synthesis is also inhibited. If Hunt and co-workers were examining a partially glycosylated form of the transferrin receptor, their experiments would be in partial agreement with our results. It is difficult to assess their results as no molecular weight standards were shown on their gels, and no endoglycosidases were used to verify that the transferrin receptor was free of all carbohydrates. Efforts are under way to understand the basis for the differences between the intersubunit disulfide formation results.

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REFERENCES