Functional Reconstitution of the Human Placental Transferrin Receptor into Phospholipid Bilayers Leads to Long Tubular Structures Proceeding from the Vesicle Surface†

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ABSTRACT: We have reconstituted the human placental transferrin receptor (hTfR) into phospholipid vesicles using either dialysis, dilution, or gel filtration. Several different detergents and phospholipids and a variety of lipid-to-protein weight ratios were tested. Preformed vesicles as well as detergent-solubilized phospholipids were used. Reconstituted mixtures were fractionated by sucrose density gradient centrifugation and screened for ferritransferrin binding activity, and peak fractions were analyzed by electron microscopy. The efficiency of reconstitution was strongly influenced by the choice of the phospholipids used and the reconstitution method. Best results were obtained when hTfR was dissolved with octyl-polyoxyethylene, mixed with detergent-solubilized soy bean lecithin, and reconstituted by slow dialysis. The data show that hTfR capable of binding ferritransferrin was reconstituted into vesicles with an irregular surface and many protrusions. In addition the reconstitution of hTfR resulted in the formation of tubular structures proceeding from the vesicle surface, which may serve as an *in vitro* model for future studies on the relevance of self-assembly processes for cellular endocytosis.

As in other vertebrates, the human transferrin receptor (hTfR)[1] mediates cellular iron uptake via binding and internalization of the serum iron transport protein transferrin. The complex of hTfR and ferritransferrin is rapidly internalized from the cell surface to early endosomes, where iron dissociates and is transported into the cytoplasm. The hTfR−apotransferrin complex recycles via endosomes to the cell surface (van Renswoude et al., 1982; Dautry-Varsat et al., 1983; Hopkins & Trowbridge, 1983; McGraw et al., 1991). However, the binding of transferrin is not essential for hTfR endocytosis (Watts, 1985). The hTfR is a transmembrane glycoprotein which consists of two identical subunits of 90−95 kDa (Enns & Sussman, 1981) linked via two disulfide bonds (Jing & Trowbridge, 1987). The monomer contains three N-linked oligosaccharides of the complex and hybrid types (Orberger et al., 1992). Furthermore, the hTfR is O-glycosylated (Do & Cummings, 1992; Hayes et al., 1992) and undergoes acylation (Alvarez et al., 1990) as well as phosphorylation (Davis et al., 1986). Each subunit has a 61-residue N-terminal cytoplasmic domain, a 28-residue hydrophobic transmembrane domain, and a 672-residue extracellular domain (Schneider et al., 1984; McClelland et al., 1984). Whereas the primary structure of the hTfR, its posttranslational modifications, and its intracellular trafficking [as reviewed by Maxfield and Yamashiro (1991)] have been known for many years, its three-dimensional structure and supramolecular organization are not yet determined. Although a tryptic 70-kDa fragment lacking the transmembrane and cytoplasmic domains has been crystallized (Borhani & Harrison, 1991), three-dimensional structural information could not be derived from these crystals. Previous reconstitution experiments with the hTfR (Kurrle et al., 1990; Demant et al., 1992; Di Giulio et al., 1994) have yielded little structural information about the supramolecular organization of the receptor. Therefore, as a basis for a more detailed structural analysis of the hTfR, possibly by digital imaging of two-dimensional crystals, we have systematically studied the efficiency of various methods for reconstituting the hTfR purified from placenta into phospholipid vesicles of differing phospholipid composition. The resulting structures were analyzed by electron microscopy, and their phospholipid and receptor composition was quantitatively determined. The orientation of hTfR reconstituted into lipid

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[1] Abbreviations: hTfR, human transferrin receptor; PBS, phosphate-buffered saline (150 mM NaCl and 10 mM phosphate, pH 7.5); PBST, PBS including 0.05% Tween 20; TBS, Tris/HCl-buffered saline (100 mM NaCl and 50 mM tris(hydroxymethyl)aminomethane, pH 8.0); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; LDAO, lauryldimethylamine-oxide; 8-POE, octyl(polydisperse)oligooxyethylene; β-DM, decyl β-D-maltoside; β-OG, octyl β-D-glucoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; DMPC, L-α-dimyristoyl-sn-PC (C14:0); DOPC, L-α-dioleoyl-sn-PC (C18:1,[cis]-9); DMPG, L-α-dimyristoyl-sn-PG; DOPG, L-α-dioleoyl-sn-PG; SBL, soy bean lecithin; eosinNCS, eosin-5-isothiocyanate; TLC, thin-layer chromatography; ELISA, enzyme-linked immunosorbent assay.

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bilayers was studied by protease digestion and antibody binding.

EXPERIMENTAL PROCEDURES

Materials. 8-POE was obtained from W. Kolb AG (Hedingen, Switzerland). Other detergents [CHAPS, lauryldimethylamine-oxide (LDAO), decyl β-D-maltoside, decyl β-D-glucoside], phospholipids, trypsin (EC 3.4.21.4) from bovine pancreas, and bovine serum albumin were purchased from Sigma (St. Louis, MO). Fetal calf serum was from Gibco BRL (Paisley, Scotland). Sephadex G-50 superfine, Sephacryl S-300 HR, and protein A-Sepharose were from Pharmacia (Uppsala, Sweden). Monoclonal rat anti-mouse κ-chain antibodies and 12-nm colloidal gold–AffiniPure goat anti-mouse IgG (F_c) antibodies were products of Dianova (Hamburg, Germany). 3,3′,5,5′-Tetramethylbenzidine was obtained from Merck (Darmstadt, Germany), and eosinNCS was from Molecular Probes (Eugene, OR).

Purification of the Transferrin Receptor and the Monoclonal Antibody OKT9. Human placental transferrin receptor (hTfR) was purified according to the method of Turkewitz et al. (1988) with the modification that the final elution was performed under non-denaturing conditions with 2 M KCl and 10 mM CHAPS in 50 mM HEPES, pH 7.5, in order to obtain fully functional hTfR with more than 95% rebinding activity for transferrin (data not shown). The purified receptor was dialyzed extensively against PBS in order to remove any detergent that might interfere with further detergents used for the subsequent solubilization experiments. Previous experiments had shown that hTfR remained soluble in this form for several months. Purity of the receptor preparations was checked by SDS–PAGE and by immunoblotting as described recently (Orberger et al., 1992). Monoclonal antibodies OKT9 were obtained from serum-free supernatants of the hybridoma cell line OKT9 (American Type Culture Collection, Rockville, MD) and were purified by protein A-Sepharose affinity chromatography.

Labeling of hTfR with Eosin-5-isothiocyanate. hTfR (600 μg in 1 mL of PBS) was incubated in the dark for 2 h at room temperature with eosinNCS at a molar ratio of 1:20. Unreacted educt was inactivated and removed by extensive dialysis against TBS at 4 °C.

Measurement of hTfR Aggregation and Solubilization of the Aggregates with Various Detergents. The effect of different detergents on the solubilization of hTfR was analyzed by incubating the receptor (17.6 μg) for 1 h at room temperature in 132 μL of PBS containing either of the indicated detergents (see Materials) at a concentration well above the critical micelle concentration. Large aggregates were removed by ultracentrifugation in an airfuge (Beckman) for 6 h at 130000*g*. The supernatant was examined by electron microscopy after negative staining with 1% uranyl acetate. A semiquantitative assessment of the aggregate formation was achieved by 7.5% SDS–PAGE separation of 30-μL aliquots of the detergent-solubilized receptor before and after centrifugation and of the total resuspended pellets. The gel was stained with Coomassie Blue R-250.

Gel Filtration of hTfR Preparations. Purified and detergent-solubilized hTfR (325 μg) was separated at a flow rate of 15 mL/h on a Sephacryl S-300 HR column (1.6 cm × 95 cm) equilibrated with the respective sample buffers. The column was calibrated with Blue Dextran 2000 (Pharmacia) and protein standards as indicated in the caption of Figure 1E.

Reconstitution of hTfR in Phospholipid Vesicles. In each reconstitution experiment 200 μg of hTfR was preincubated for 30 min in 900 μL of TBS (1.11-fold concentrated) containing 1% 8-POE. The samples were mixed with various amounts of either detergent-solubilized phospholipids or preformed phospholipid vesicles. In the first case, phospholipids were prepared by dissolving the appropriate amount of lipid in 100 μL of water containing 1% 8-POE. In the latter case the phospholipids were sonicated on ice with a Branson Sonifier B-12 (Danbury, CT) in 30-s intervals (intensity setting: 5) until the milky lipid suspension became weakly translucent. Subsequently, the solution was diluted such that the required amount of phospholipid was present in 100 μL. The protein and phospholipid solutions were then mixed and incubated for 1 h at room temperature. For screening experiments the purified hTfR was covalently labeled with eosinNCS prior to detergent solubilization. Reconstitution was performed using one of the following methods. (1) Dialysis: The samples were dialyzed at 4 °C five times for at least 3 h each against 1 L of TBS. (2) Dilution: The samples were diluted at room temperature extremely slowly by adding 100 μL of TBS dropwise every 150 s. This process was repeated 9 times. For further dilution, the whole procedure was repeated first with 10 × 200 μL of TBS, then with 10 × 400 μL and 10 × 800 μL, and finally with 8 × 1600 μL of TBS. Before centrifugation the volume was adjusted to 30 mL with TBS. The whole procedure corresponds to a 2-fold dilution every 25 min. (3) Sephadex G-50 gel filtration: The samples were applied at room temperature onto a TBS-equilibrated 1 × 50 cm Sephadex G-50 (superfine) column. The void volume and the inclusion volume had been determined with Blue Dextran 2000 (Pharmacia) and bromophenol blue (Sigma) beforehand. The gel filtration was performed with a flow rate of 8 mL/h, and reconstituted phospholipid vesicles were collected in the fractions directly following the void volume.

After reconstitution, all samples were centrifuged at 100000*g* for 2 h at 8 °C. The pellets were resuspended in 200 μL of TBS and homogenized in a precision Potter homogenizer with a Teflon pestle (Kontes, Vineland, NJ).

Sucrose Gradient Separation of Reconstituted Lipid Vesicles. The homogenate was applied to the top of a sucrose density step gradient (0.5/1.1/2.0 M sucrose in TBS) and centrifuged at 50000*g* for 22 h at 8 °C. The gradient was fractionated from the top in 1-mL fractions. Fractions used for the transferrin binding assay and for determination of protein concentrations were used directly, whereas the fractions used for the phosphate determination were first dialyzed extensively against TBS and then concentrated by a final dialysis against 20% poly(ethylene glycol) 20 000 (Fluka, Buchs, Switzerland).

Labeling of Reconstituted hTfR with 12-nm Colloidal Gold. First, 30 μg (24 μL) of the mouse monoclonal anti-hTfR antibody OKT9 was incubated for 1 h at 4 °C in TBS with 200 μL of homogenized, hTfR-containing phospholipid vesicles. Subsequently, the solution was diluted 20-fold with TBS, and the vesicles were pelleted (100000*g* for 2 h at 8 °C), resuspended, and homogenized as described above. After addition of 3 mL of TBS, vesicles were gently agitated for 30 min to wash off unbound antibody, pelleted by centrifugation, resuspended and homogenized as above. The

vesicles were then mixed with 20 μL of 12-nm colloidal gold–AffiniPure goat anti-mouse IgG (F_c) antibodies (OD_{520} = 2.0) and incubated overnight at 4 °C. The vesicles were pelleted, washed again, and finally separated by sucrose density gradient centrifugation as described above.

Partial Digestion of Reconstituted hTfR with Trypsin. Samples (about 1 μg in 100 μL of TBS) were incubated at 37 °C for 30 min with 0.4 μg of trypsin. The protease digestion was stopped by adding 3 μg of phenylmethanesulfonyl fluoride in 1 μL of dimethyl sulfoxide and 35 μL of SDS sample buffer (4× concentrated). The samples were boiled for 5 min and immediately loaded onto a 7.5% SDS–polyacrylamide gel.

Electron Microscopy. Samples (4 μg in 5 μL of TBS) were adsorbed to carbon-coated copper grids, which were then washed twice with distilled water and stained with 1% (w/v) uranyl acetate. Electron micrographs were recorded on a Zeiss 109 electron microscope at a primary magnification of 50000×.

Transferrin Binding Assay. hTfR samples were checked for the capability to bind ferritransferrin using a solid-phase *in vitro* assay (the complete details of which will be published elsewhere). This test is based on the ELISA technique and allows the quantification of functional active receptor (which here means receptor which binds ferritransferrin) in samples containing purified and nonpurified hTfR. Ferritransferrin (500 ng/well in PBS) was immobilized on Nunc Maxisorp U 16 immuno modules for 1.5 h. After coating, the wells were washed and were then blocked with a solution of 3% (w/v) bovine serum albumin and 10% (v/v) fetal calf serum in PBS for 30 min. After washing, samples containing hTfR (about 0.2–10 ng of receptor suspended in 100 μL of PBST) were added to the transferrin-coated wells and incubated for 2.5 h. Subsequently, the wells were washed and incubated for 30 min with the monoclonal antibody OKT9 in blocking buffer including 0.05% Tween 20 and then for 30 min with peroxidase-coupled monoclonal rat anti-mouse κ-chain antibodies in the same buffer. The substrate used for the peroxidase reaction was a 0.02% solution of 3,3′,5,5′-tetramethylbenzidine in a 40 mM potassium citrate buffer, pH 3.95, containing 0.01% H_2O_2 (Gallati & Pracht, 1985). The reaction was stopped after 5 min by adding 2 M sulfuric acid to each well. Absorbance was measured at 450 nm in a multichannel photometer (MR 7000, Dynatech). To minimize any light-scattering effects by the microtiter plate, the absorbance at 490 nm was subtracted. The concentration of functional active transferrin receptor was determined by using a calibration curve obtained with functionally active hTfR standards.

Receptor quantitation using this receptor ligand binding assay instead of an antibody ELISA or a standard protein assay has the advantage that only that fraction of hTfR is measured which is capable of binding transferrin. In addition, this assay is very sensitive (down to 1 pmol) and thus requires only small sample volumes.

Thin-Layer Chromatography (TLC). Phospholipids were extracted from purified hTfR or vesicle-reconstituted hTfR by thoroughly mixing 110-μL aliquots with 400 μL of methylene chloride/methanol [1:2 (v/v)] and then again after addition of 200 μL of H_2O and 200 μL of methylene chloride. After centrifugation at 13000*g* for 5 min, the upper aqueous phase was removed and extraction was repeated on this phase. The two organic solvent phases (without the interphases) were pooled and dried under a nitrogen gas stream. The dried film was dissolved in 10 μL of the TLC solvent (chloroform:methanol:water = 85:30:4) and applied to a silica gel TLC plate (No. 60777, Fluka, Buchs, Switzerland).

SDS–PAGE and Protein Determination. All samples were prepared according to Laemmli (1970) by boiling for 5 min in sample buffer containing SDS and 2-mercaptoethanol, both at a final concentration of 2%. Electrophoresis was carried out in 1.5-mm gels at constant currents of 45 mA during stacking and 65 mA (max. 400 V) during separation. Staining was with Coomassie Blue R-250 after fixing with 20% trichloroacetic acid or with silver using a modification of the method of Heukeskoven and Dernick (1988). In the latter case gels were fixed first for 30 min in 50% ethanol and 10% acetic acid and then overnight in 30% ethanol, 0.5 M sodium acetate, 0.5% glutardialdehyde, and 0.2% sodium thiosulfate pentahydrate. Subsequently, the gels were washed three times for 30 min in H_2O, stained for 60 min with 0.1% $AgNO_3$ and 0.01% formaldehyde, washed three times for 1 min with H_2O, and developed with 2.5% sodium carbonate and 0.01% formaldehyde, pH 11.2, adjusted with acetic acid. The reaction was stopped by washing the gel in 0.05 M EDTA.

Protein concentrations were determined using the Bio-Rad reagent microassay procedure (No. 500-0006), and functional active hTfR was quantified with the assay for receptor–ligand interactions described above.

Phospholipid Determination (Ames & Dubin, 1960). Ten to 100 μL of sample (containing 1–70 nmol of phosphate) was mixed with 30 μL of 10% (w/v) magnesium nitrate hexahydrate dissolved in 95% (v/v) ethanol and ashed over a flame. The residue was dissolved by boiling in 300 μL of 0.5 M HCl for 15 min. After the samples were cooled, 700 μL of a 1:6 mixture of 10% (w/v) ascorbic acid and 0.42% (w/v) ammonium molybdate tetrahydrate in 0.5 M H_2SO_4 was added, and the solution was incubated for 20 min at 45 °C. The absorption of the samples was then read at 820 nm. Concentrations were calculated using a standard curve.

RESULTS

Solubilization of hTfR in Various Detergents. A general prerequisite for the efficient reconstitution of integral membrane proteins is their efficient solubilization with a detergent to yield a homogeneous soluble species. Unexpectedly for an integral membrane protein, about 90% of the purified hTfR remained soluble after ultracentrifugation (6 h, 130000*g*) without the addition of any detergent (Figure 1A). However, when this fraction was examined by electron microscopy, we found that the hTfR does not exist as single molecules or nonspecific aggregates, but forms rosette-like structures of relatively uniform size which are termed proteoparticles according to their appearance (Figure 1B).

In total, we tested five of the most commonly used detergents for their potential to efficiently solubilize hTfR. Figure 1 shows that there are large differences between the effects of those detergents on hTfR solubilization. The addition of the zwitterionic detergent CHAPS (1%) does not increase the overall solubility of hTfR (Figure 1A), but disrupts the rosette-like structures and results in the formation of many small aggregates and single particles as visualized by electron microscopy (Figure 1C). In contrast, the highly polarized detergent LDAO (0.5%) and the non-ionic deter-

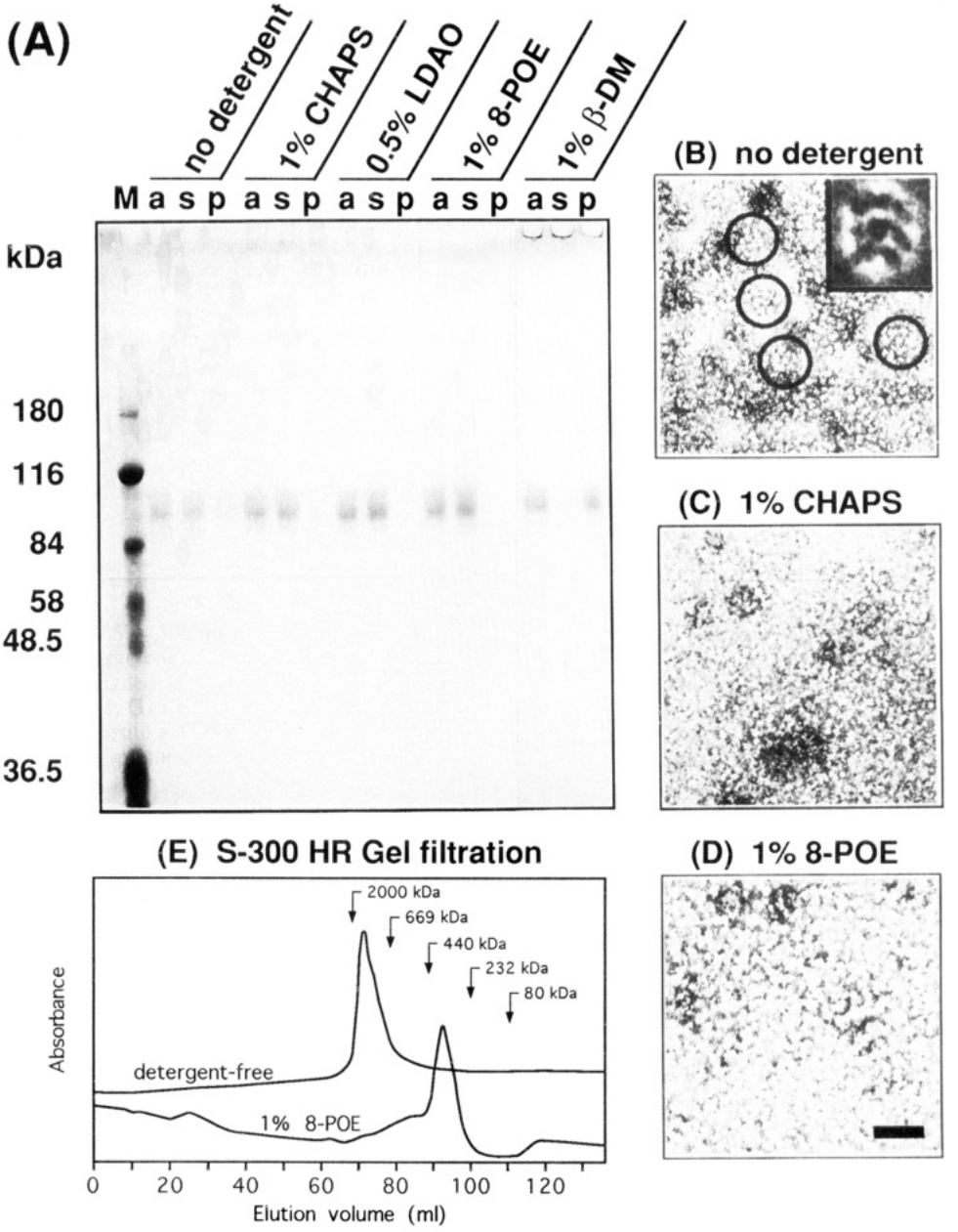


FIGURE 1: Aggregation of the human placental transferrin receptor. hTfR was either kept in PBS or was solubilized with various detergents. The samples were ultracentrifuged for 6 h at 130000g. (A) The supernatants and the resuspended pellets were separated by SDS−PAGE. Coomassie stained molecular weight marker proteins (M) and detergent-solubilized hTfR (from left to right): without detergent, with 1% CHAPS, with 0.5% LDAO, with 1% 8-POE, and with 1% decyl β-D-maltoside (β-DM). A solution of 2% octyl β-D-glucoside was also tested and found indistinguishable from the samples containing β-DM: (a) indicates the sample before centrifugation; (s), the supernatant; and (p), the resuspended pellet. (B−D) Examination of the negatively stained samples by electron microscopy. (B) Without detergent, hTfR forms typical rosette-like particles (circled). The inset shows a single proteoparticle at higher magnification (inset width: 36 nm) as recorded by transmission electron microscopy of an unstained sample [method according to Dubochet et al. (1988)]. Evaluating the size and appearance of a large number of these structures led us to the conclusion that they may consist of about 8−12 receptor dimers. (C) In the presence of CHAPS and (D) 8-POE, hTfR appears as a random pattern of single particles. Bar: 60 nm. (E) Sephacryl S-300 HR gel filtration. hTfR (325 μg) was chromatographed either detergent free (top) or solubilized with 1% 8-POE in PBS (bottom). The arrows indicate the molecular mass markers (from left to right) Blue Dextran 2000 (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and transferrin (80 kDa).

gent 8-POE (1%) lead to a complete solubilization of the receptor (Figure 1A) and produce a random pattern of single particles (Figure 1D). Two other non-ionic detergents, decyl β-D-maltoside (1%) (Figure 1A) and octyl β-D-glucoside (2%) (data not shown) have a rather opposite effect and lead to an almost complete sedimentation of hTfR with only a little protein remaining in the supernatant. The complete solubilization of the hTfR proteoparticles into isolated dimers by 8-POE was also verified by analytical gel filtration (Figure 1E). The hTfR proteoparticles eluted from the ligand-affinity column in the presence of CHAPS and dialyzed against PBS appear in the void volume of the Sephacryl S-300 gel filtration, indicating a molecular mass exceeding 1000 kDa. After solubilization with 8-POE, the large aggregates were completely dissolved and the majority of the hTfR appeared at an apparent molecular mass of about 300 kDa as expected for the receptor dimer. The apparent higher molecular mass of this 190-kDa homodimer at gel-filtration experiments may be due to the elongated shape of the receptor molecule (Turkewitz et al., 1988) and to the increased hydrodynamic

Table 1: Frequency of the Various Structures Formed by hTfR upon Phospholipid Reconstitution[a]

method	phospho-lipids	vesicles	tubules	proteo-particles	nonspecific aggregates
dialysis	solubilized	+	+	++	++
dialysis	preformed	+++	++++	+	+
Sephadex	preformed	++	++++	++	++
dilution	preformed	+	o	+++	+++

[a] Symbols: o, not observed; +, rarely; ++, moderately; +++, frequently; ++++, very frequently. These results were independent of the phospholipid composition with the exception that, after reconstitution with DOPC followed by dialysis, tubules are not observed using solubilized phospholipids and are rarely observed using preformed vesicles.

radius caused by the formation of a detergent micelle at the transmembrane domain. Assuming 100 detergent molecules per 8-POE micelle (Grabo, 1982), we calculate that one micelle alone would contribute an apparent molecular mass of 35 kDa to the receptor dimer.

Due to its excellent solubilization properties, 8-POE was used for all further reconstitution experiments. The effect of the detergents on the ligand binding capacity of the hTfR was determined by comparing the amount of functionally active receptor as measured with the receptor binding assay to that obtained by a standard protein assay. None of the detergents reduced the affinity of the hTfR to transferrin (data not shown). Thus, this functional assay proved to be a useful alternative for receptor quantification.

Reconstitution of hTfR into Phospholipid Bilayers Using Dialysis, Dilution, and Sephadex G-50 Chromatography. Since preliminary studies had shown that an initial phospholipid-to-hTfR ratio of 10:1 (w/w) was usually successful in achieving reconstitution, we have employed this ratio as a starting condition for optimizing the reconstitution method. As shown previously, this ratio is also important for the production of two-dimensional crystals (Ghosh et al., 1993). In our studies, purified soybean lecithin (SBL) containing no free fatty acids was used. Three methods of reconstitution were employed:

(a) Dialysis. 8-POE-solubilized hTfR was mixed either with preformed SBL vesicles or with 8-POE-solubilized SBL, and the detergent was removed by dialysis. In both cases the reconstituted samples appeared to be quite heterogeneous after dialysis and contained at least four species which were classified as (1) vesicles, (2) tubules, (3) proteoparticles, and (4) nonspecific aggregates (Table 1; Figure 2A,B). Closer examination of the reconstituted samples formed by dialysis showed clearly that the tubules are outgrowths of and continuous with the surface of the reconstituted vesicles (Figure 2E). Reconstitution of hTfR into preformed vesicles led to a much higher yield of vesicles and tubules containing functionally active hTfR (see below) than when detergent-solubilized SBL was mixed with the purified receptor (Table 1). Thus, we employed only preformed vesicles for the subsequent reconstitution procedures.

(b) Sephadex G-50 Chromatography. This procedure led to a very high yield of tubular structures and to a lesser yield of vesicles (Table 1; Figure 2C). The amount of proteoparticles and aggregated particles was also somewhat raised in comparison to the levels observed after dialysis.

(c) Dilution. This procedure gave only a low yield of vesicles and almost no tubular structures. In contrast, large amounts of isolated and aggregated proteoparticles were observed in electron micrographs of negatively stained samples (Table 1; Figure 2D).

Reconstituted samples prepared by the above procedures were further analyzed by sucrose density gradient centrifugation, followed by hTfR activity assays and examination by electron microscopy of various fractions. To facilitate the identification of hTfR-containing fractions, the receptor was labeled with eosin-5-isothiocyanate for the initial experiments. This covalent labeling had no effect upon behavior of the protein in sucrose density gradients (Figure 3A), although the transferrin binding activity of the receptor was reduced by approximately 40%. Analysis of the hTfR binding activity of various fractions showed that, in general, three different hTfR-containing populations could be discriminated by their respective densities: The lightest active hTfR species (peak I), comigrating with free phospholipids at the top of the gradient, was confirmed by electron microscopy to consist of vesicles or, in some cases, of short tubule-like structures (Figure 4A). Peak I contained on average about 30% of the total transferrin binding activity. This fraction was increased by employing larger quantities of SBL (Table 2). Thus, at an SBL-to-hTfR ratio of 100:1 (w/w) about 65% of the total activity was associated with this vesicle-containing low-density peak (Figure 3B, bottom).

The hTfR population of intermediate density (peak II) corresponds to that of the purified hTfR proteoparticles as confirmed by electron microscopy of negatively stained samples (Table 2; Figure 4B). When hTfR was reconstituted with SBL upon dilution at a ratio of 1:10 (w/w) or 1:20 (w/w), peak II appeared to be less dense than that of the purified hTfR, although the appearance of the samples in electron micrographs was identical to the same fraction obtained from other reconstitution conditions. Peak II contained on average about 50% of the total activity (Table 2). The most dense population (peak III) appeared to consist of highly aggregated particles and some proteoparticles, as exemplified by electron microscopy (Figure 4C). These aggregates contained on average about 20% of the total transferrin binding activity (Table 2).

Effects of Fatty Acid Composition and Varying Head-Groups upon Reconstitution of hTfR into Phospholipid Bilayers. 8-POE-solubilized hTfR was also reconstituted by dialysis into preformed vesicles generated from either of two phosphatidylcholines, L-α-dioleoyl-9,10-glycerophosphocholine (DOPC) and L-α-dimyristoylglycerophosphocholine (DMPC). In contrast to SBL, which is highly heterogeneous in its content of unsaturated fatty acyl chains, DOPC contains only one type of fatty acid with a single double bond at the 9,10-position. However, the low T_m (−20 °C) of DOPC ensured that all of the reconstitution steps were performed with the phospholipid in the liquid crystalline phase. DMPC is composed of fully saturated fatty acyl chains and has a relatively high transition temperature of 23.5 °C. Thus, bilayers of this phospholipid were largely in the gel phase under the reconstitution conditions studied here.

Surprisingly, the reconstitution of hTfR into preformed bilayers of DOPC appeared to be less efficient than into preformed SBL vesicles. From the activity profiles (Figure 5A,B) obtained for two initial lipid-to-protein ratios [6:1 and 20:1 (w/w)], a large amount of nonspecific aggregates was observed in peak III, containing about 50% of the activity (Table 2). Peak II appeared to be shifted toward lower

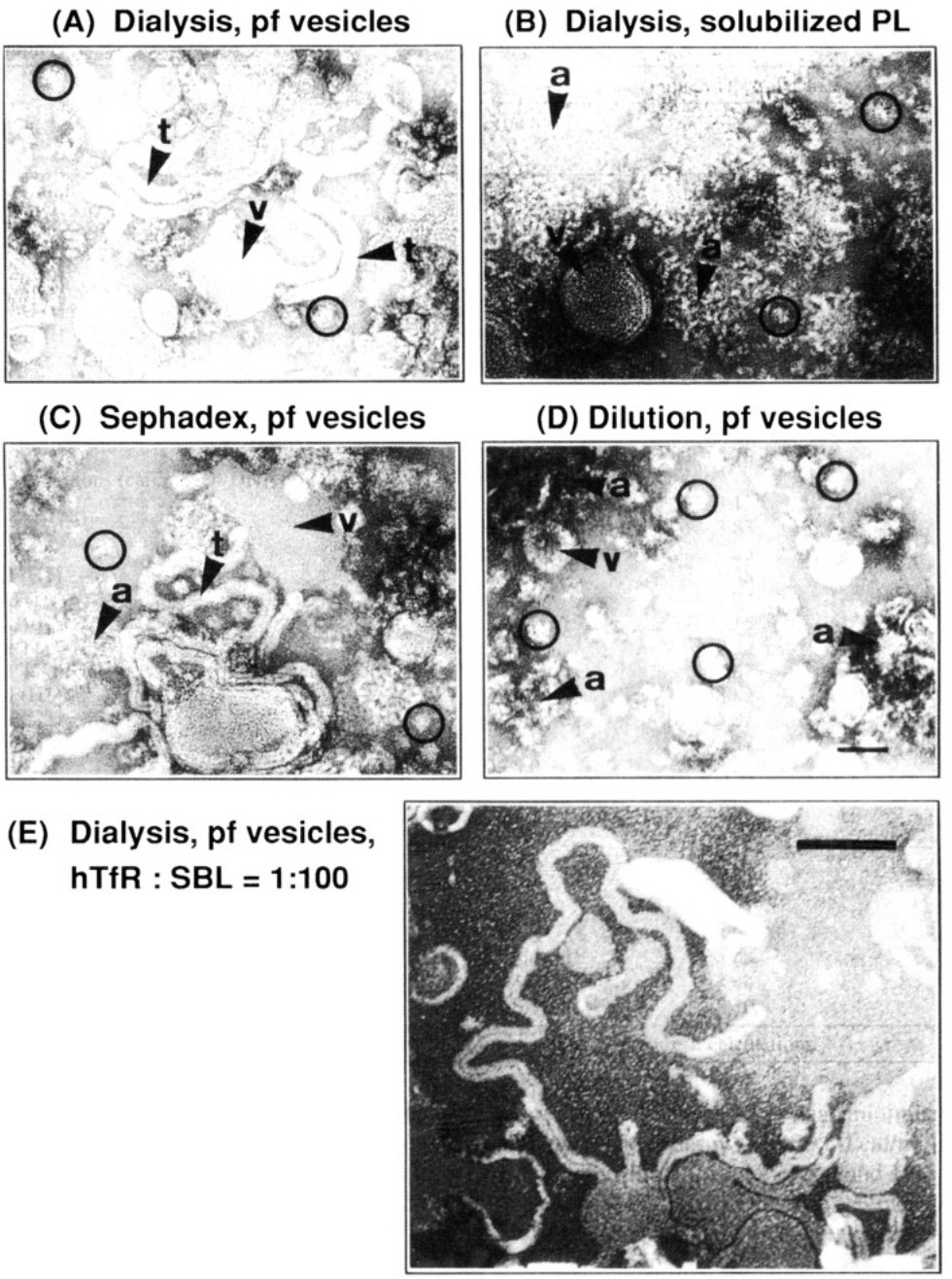


FIGURE 2: Electron micrographs of reconstituted transferrin receptor. hTfR was reconstituted with different phospholipids by dialysis, gel filtration, or dilution. The vesicles were pelleted by ultracentrifugation, resuspended, homogenized, and adsorbed to carbon-coated copper grids. The samples were negatively stained with 1% uranyl acetate and visualized by electron microscopy at a primary magnification of 50000×. SBL-reconstituted hTfR using (A, B, E) dialysis, (C) gel filtration over Sephadex G-50, and (D) dilution. Reconstitution was performed either with preformed (pf) vesicles (A, C, D, E) or with solubilized phospholipids (PL) (B). All reconstitution cocktails contained 8-POE as detergent. The longest of the observed tubules (E) has a length of 1.25 μm and a diameter of 23 nm. In this case the initial lipid-to-protein ratio was 100:1 (w/w). Arrows point to vesicles (v), tubular structures (t), and nonspecific aggregates (a). Proteoparticles are circled. Bar: 60 (A−D) or 120 nm (E).

buoyant densities than observed for the nonreconstituted hTfR. The amount of functionally active hTfR in the vesicular fraction (peak I) was considerably lower than that observed with SBL-reconstituted receptor (Table 2). The vesicular fractions appeared to contain true vesicles; no tubular structures were observed by electron microscopy (not shown).

In contrast, the vesicular fraction of DMPC-reconstituted hTfR contained only minute amounts of functionally active receptor, indicating very reduced reconstitution efficiency. This fully saturated phospholipid had no effect on the buoyant density of peak II (Figure 5C).

We have also attempted to incorporate hTfR into preformed bilayers of *Escherichia coli* PE/DOPG [80:20 (w/w)]. Similar mixtures such as PC/PG or PE/PC have been successfully used to incorporate membrane proteins (Kurrle et al., 1990). However, our attempts with hTfR were completely unsuccessful, and no active vesicular fraction was detected after density gradient centrifugation (data not shown).

Determination of Receptor Concentration in Reconstituted Vesicles. The accurate determination of proteins reconstituted into phospholipid vesicles is not trivial. The classic Lowry protein assay is not applicable to detergent-containing

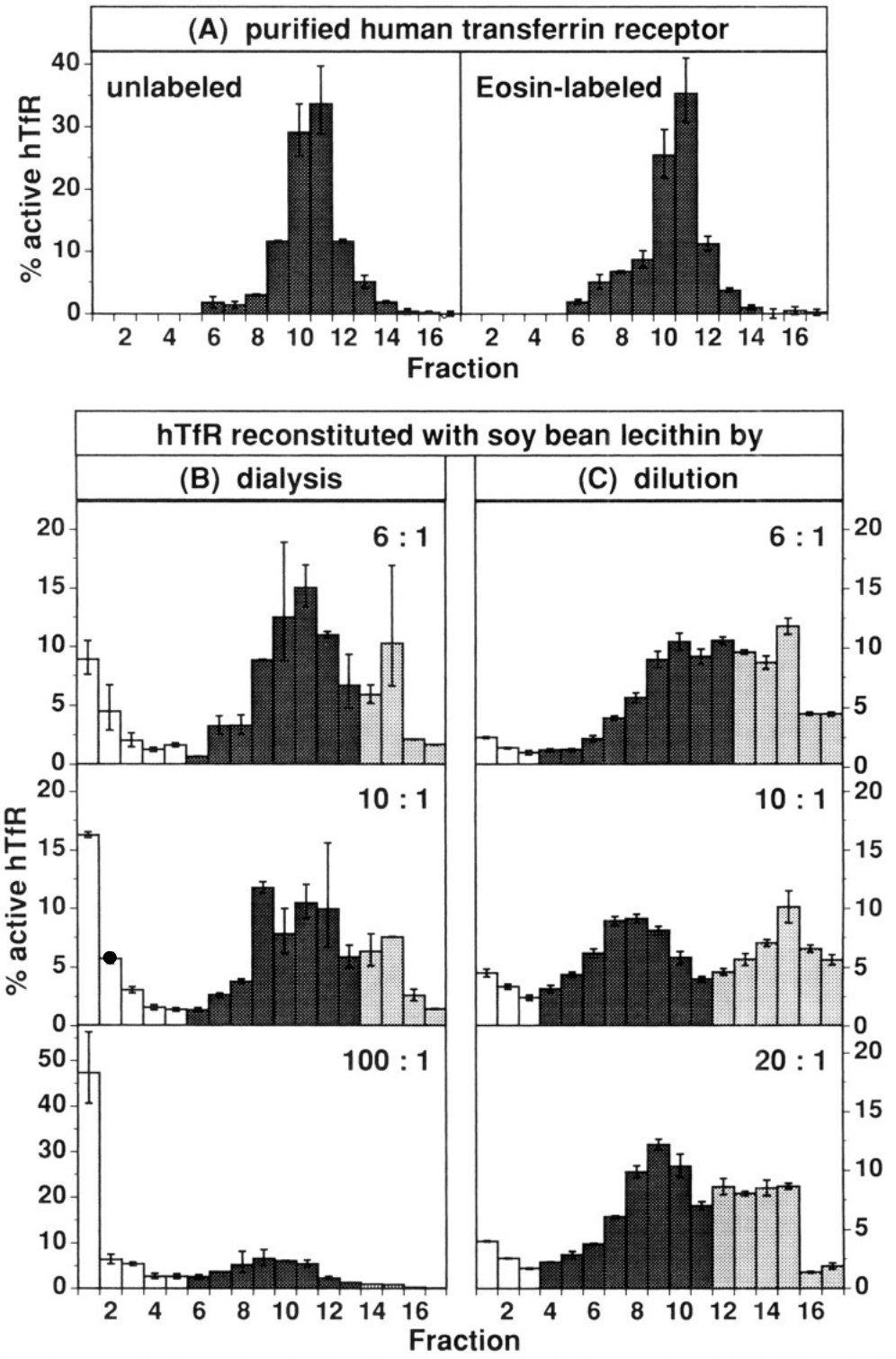


FIGURE 3: Screening of sucrose density gradient fractions for functional active SBL-reconstituted hTfR. Ten microliters of the density gradient fractions was diluted to a range of 2−100 ng/mL receptor and tested in a transferrin binding assay (see Experimental Procedures). In order to compare the results of different preparations, the amount of active transferrin receptor in each fraction was plotted as the percentage of the total amount of receptor in the gradient. The percentage of active receptor in each peak is listed in Table 2. (A) Sucrose density gradient profiles of unlabeled purified hTfR (left) and eosinNCS-labeled hTfR (right) without any phospholipids. The total amounts of functionally active receptor in the two gradients were 31.3 and 19.2 ng, respectively. (B) Profiles of functional hTfR reconstituted at lipid-to-protein ratios of 6:1, 10:1, and 100:1 (top to bottom) into preformed SBL vesicles by dialysis. (C) Profiles of functional hTfR reconstituted at lipid-to-protein ratios of 6:1, 10:1, and 20:1 (top to bottom) into preformed SBL vesicles by dilution. The different shading of the fractions denotes the assignment to peak I (light), II (dark), or III (medium).

solutions (Smith et al., 1985), and more recent methods like the related bicinchoninic acid (BCA) assay and the protein-induced Coomassie Brilliant Blue G-250 absorbance shift assay (Bradford, 1976) are both affected by the presence of phospholipids (unpublished results). We thus used an hTfR-specific solid-phase assay based on the binding of the solubilized receptor to its immobilized ligand transferrin and a classic ELISA quantitation employing an hTfR-specific antibody and a peroxidase-coupled secondary antibody. This functional assay was previously shown to be unaffected by

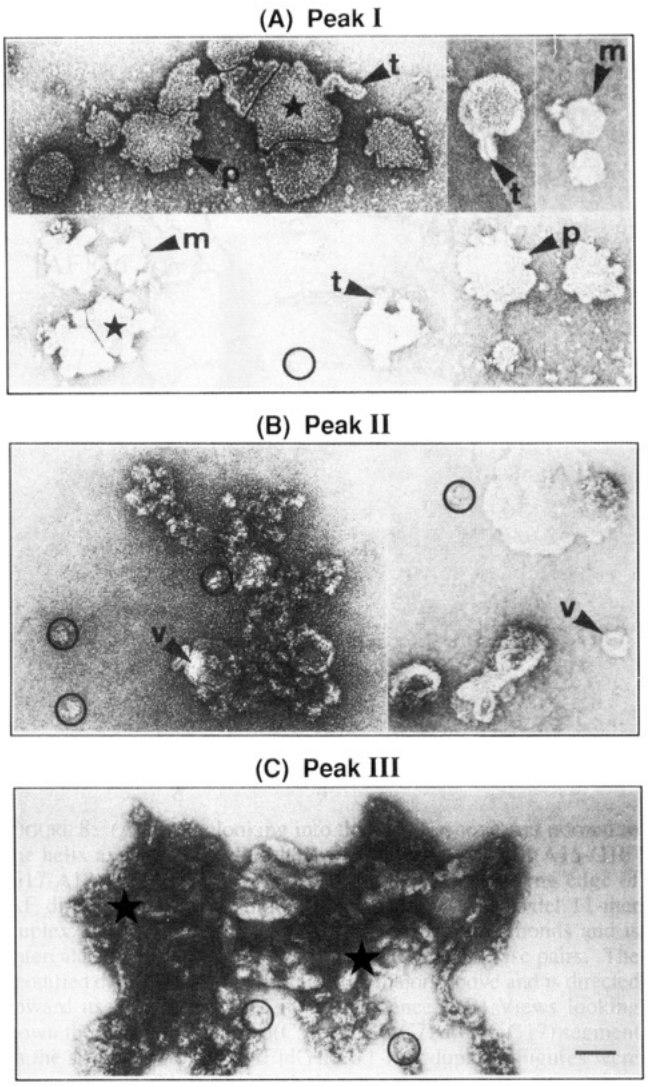


FIGURE 4: Electron micrographs of the low-, intermediate-, and high-density hTfR populations after densi ıt centrifugation. 8-POE-solubilized hTfR was reconstitute formed SBL and DOPC vesicles by dialysis or dilution lensity gradient centrifugation, samples corresponding to peaks in Figure 3 were analyzed by electron microscopy. Since the same structures were found for all different reconstitution conditions in the same peaks, only one or a few representative electron micrographs are displayed for the structural populations found in each peak. (A) Peak I contains vesicles (two of them are marked with stars) with protrusions (p) and short tubules (t); single hTfR molecules (m) and some proteoparticles (circled) can also be seen. (B) Peak II consists largely of proteoparticles (circled) and traces of phospholipid vesicles (v). (C) Peak III shows nonspecific aggregates (stars) of hTfR and some proteoparticles (circled). Bar: 60 nm.

Triton X-100 concentrations of up to 2% and sucrose concentrations of up to 7% (data not shown). In order to demonstrate that lipid-reconstituted hTfR remains fully active and that detergent-solubilized phospholipids have no effect on the binding of hTfR to immobilized transferrin, lipid-reconstituted hTfR was incubated overnight with 1% Triton X-100 and quantified with the binding assay. The recovered transferrin binding activity always corresponded to 90−100% of the initial amount determined prior to the reconstitution with the same assay. In order to eliminate any potential influence of high sucrose concentrations on the assay, all samples derived from sucrose gradients were diluted between 1:40 and 1:200 before protein determination, reducing the maximal sucrose concentration to less than 2%.

Table 2: Density Distribution of Phospholipid-Reconstituted hTfR[a]

hTfR sample	phospho-lipid (PL)	PL/hTfR ratio (w/w)	relative distribution of hTfR (%) peak I	peak II	peak III
purified			0	97	3
purified, eosin-labeled			0	98	2
reconst. by dialysis	SBL	6:1	19	61	20
reconst. by dialysis	SBL	10:1	28	54	18
reconst. by dialysis	SBL	100:1	65	33	2
reconst. by dilution	SBL	6:1	6	55	39
reconst. by dilution	SBL	10:1	10	50	40
reconst. by dilution	SBL	20:1	8	55	37
reconst. by dialysis	DOPC	6:1	10	29	61
reconst. by dialysis	DOPC	10:1	14	34	52
reconst. by dialysis	DOPC	20:1	18	31	51
reconst. by dilution	DMPC	10:1	13	59	28

[a] Percentages were calculated by integrating the transferrin binding activity of the designated gradient fractions as indicated by the shading in Figure 3.

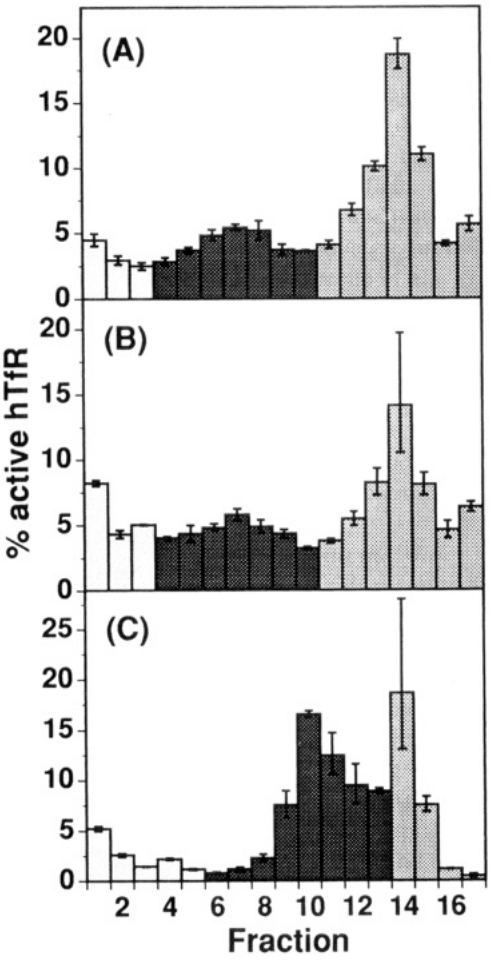


FIGURE 5: Screening of sucrose density gradient fractions for functionally active DMPC- and DOPC-reconstituted hTfR. Purified hTfR was reconstituted either by dialysis into preformed DOPC vesicles at lipid-to-protein ratios of 6:1 (A) and 20:1 (B) or by dilution into preformed DMPC vesicles at a lipid-to-protein ratio of 10:1 (C). The percentage of active receptor in each peak is listed in Table 2. The assignment of the individual fractions to the three different density populations of peaks I, II, and III is as described in Figure 3.

Phospholipid Analysis of the Reconstituted Fractions. Phospholipids were quantitatively determined for a number of representative fractions from various reconstitution mixtures. In Table 3 the lipid-to-protein ratios of the vesicles and tubules containing low-density fractions (peak I) are

Table 3: Ratio of hTfR to Phospholipid[a]

fraction	peak	phospho-lipid (PL)	PL/hTfR ratio (w/w) initial	final
	purified hTfR			0.007:1
1	I	SBL	6:1	7.7:1
1	I	SBL	10:1	21:1
7	II	SBL	10:1	3.9:1
14	III	SBL	10:1	0.3:1
1	I	SBL	100:1	70:1
1	I	DOPC	10:1	4.9:1

[a] The number of the density gradient fraction refers to the profiles shown in Figures 3 and 5. Reconstitution was performed by dialysis.

correlated with the respective initial ratios for the best reconstitution method (dialysis). This correlation clearly indicates, in conjunction with the results displayed in Figure 3B, that at lipid-to-protein ratios of up to 10:1 a large fraction of hTfR sediments at an intermediate density (peak II) containing mainly the rosette-shaped structures seen in Figures 1B (purified receptor) and 4B (after lipid reconstitution, peak II). Thus the lipid-to-protein ratio of peak I is larger than the initial ratio under these reconstitution conditions. The opposite effect is observed with a large initial excess of lipids over protein (100:1). Although two-thirds of the receptor was found in the low-density fractions (peak I) under these conditions, this does not account for the apparent decrease of the lipid-to-protein ratio. However, some lipid might have been lost during the initial steps of dialysis or by adsorption to the dialysis membrane and vial surface.

For one reconstitution experiment, the lipid-to-protein ratio was also determined for the more dense fractions found in peaks II and III (Table 3). Although the ratio decreases as expected with increasing density of the fractions, a considerable amount of lipid is taken up by the structures found in peak II. Since the proteoparticles that are also typical for the purified receptor (Figure 1B) seem to be morphologically unchanged, the additional lipid must have been incorporated into the multilamellar vesicles that are also present in peak II (Figure 4B). In contrast, very little lipid is found in the large protein aggregates of peak III.

The purified receptor that has the same density as the structures found in peak II after reconstitution (Figure 3A) contains only minute amounts of lipid that seem not to be extractable by the detergents during the purification process (Table 3). This is consistent with the assumption that this structure represents a very stable association of receptor molecules and is therefore present in peak II of all reconstitution experiments.

Thin-layer chromatography (TLC) analysis of extracted phospholipids from samples corresponding to peaks II and III revealed that in all cases examined peak II contained small amounts of the PC employed in the reconstitution, whereas with one exception Peak III contained no PC. Only peak III obtained from the reconstitution with DMPC contained traces of phospholipid. The TLC analysis also revealed that the purified receptor preparation contains small quantities of two other phospholipids as well as traces of free fatty acids (data not shown) presumably derived from placental membranes. From a determination of total phosphate we estimate a molar ratio of phospholipid to purified hTfR monomer of about 1.7:1.

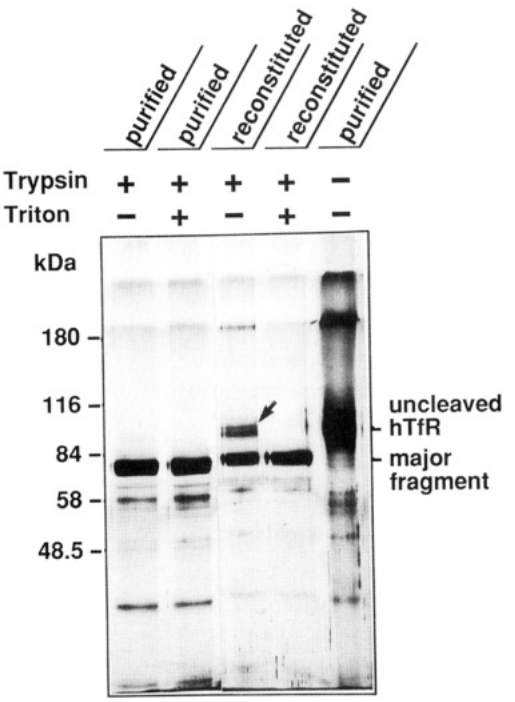


FIGURE 6: Trypsin digestion of purified and phospholipid-reconstituted hTfR. Purified hTfR, 1.6 μg, and 100 μL of DOPC-reconstituted hTfR (dialysis; phospholipid-to-hTfR ratio, 20:1) containing about 1 μg of protein were digested with trypsin in the presence and in the absence of 1% Triton X-100. Undigested hTfR was taken as a control. The samples were separated by 7.5% SDS−PAGE, and the gel was stained with silver. The arrow points to DOPC-reconstituted hTfR that is protected against digestion, indicating an outside-in orientation. The faint high molecular weight bands are dimers and multimers of the hTfR, which could be identified by OKT9 in a Western blot (not shown). The major trypsin fragment observed for the DOPC-reconstituted hTfR migrates slightly more slowly than that from purified hTfR. This might be due to preferential cleavage of the reconstituted hTfR at arginine 100, generating the serum hTfR (Shih et al., 1990), and almost no cleavage at arginine 121, the major trypsin cleavage site of the purified receptor (Turkewitz et al., 1988).

Determination of hTfR Accessibility in Reconstituted Vesicles by Trypsin Digestion and Monoclonal Antibody Binding. It has been shown previously that trypsin cleaves the purified hTfR predominantly at arginine 121, in the region connecting the transferrin binding site to the transmembrane domain (Turkewitz et al., 1988). Figure 6 shows the results of digesting vesicle-reconstituted hTfR with trypsin in the presence and absence of 1% Triton X-100. Trypsin treatment of intact DOPC−hTfR vesicles revealed that approximately one-third of the receptor was shielded against proteolytic cleavage of the extracellular domain. However, when the vesicles were solubilized with 1% Triton X-100, complete digestion was observed. The same was found for purified hTfR, even in the absence of Triton X-100. Having in mind that after reconstitution some hTfR remains in proteoparticle structures or as single molecules susceptible to trypsin digestion (Figure 4A), these results are consistent with the assumption that about 50% of the *reconstituted* hTfR is present in an outside-in orientation.

This assumption was supported by studies using the monoclonal antibody OKT9 (Reinherz et al., 1980; Sutherland et al., 1981), which binds to the extracellular domain of hTfR (Schneider et al., 1982). When OKT9 binding to hTfR reconstituted with SBL at a lipid-to-protein ratio of 10:1 (w/w) was visualized by electron microscopy after gold

labeling and negative staining, gold particles appeared only sparsely distributed at the membrane surface. The largest distribution of gold particles was detected at the surface of membrane sheets or broken vesicles, of which two examples are shown in Figure 7A. OKT9 binding was significant as OKT9−F_c-gold-labeled vesicles increased in density when analyzed by density gradient centrifugation (Figure 7B). No binding was noted in vesicles not containing hTfR employed as controls (not shown).

DISCUSSION

In the present study we have systematically examined reconstitution procedures and conditions in an attempt to optimize the incorporation of hTfR into phospholipid bilayers. The efficient reconstitution of integral membrane proteins is an important first step for achieving a homogeneous dense distribution of receptor molecules, which is desirable for a number of reasons: (1) There are many reports that the kinetic properties of reconstituted membrane proteins resemble those of the natural environment more closely than those observed for the purified protein in detergent (Miyamoto & Racker, 1980; McIntosh & Ross, 1985; Anholt, 1988; Wiedmann et al., 1988). (2) The reconstituted system is useful for examining physical or biochemical properties associated with the functional process of endocytosis and physiological signaling events such as phosphorylation (Benovic et al., 1988). (3) The incorporation of purified receptor into lipid bilayers is a prerequisite for structure determination methods such as two-dimensional crystallization and neutron diffraction or methods to determine the rotational diffusion of the functional unit (Cherry, 1979). hTfR is particularly suitable for structural analysis by biophysical methods since it is readily obtainable in relatively large quantities and remains conformationally stable under a variety of conditions.

Strategies for the reconstitution of integral membrane proteins into phospholipid bilayers require the protein to be solubilized with detergent and then allowed to associate with phospholipids present either in detergent-solubilized form or as preformed vesicles. After mixing, the detergent is removed, causing the protein and phospholipids to spontaneously assemble into mixed bilayers. The observation of an unusual soluble form of the receptor, termed here the hTfR proteoparticle, which seems to contain about 8−12 dimeric units of the receptor, suggests that strong protein−protein interactions will probably complicate the above-mentioned reconstitution strategy due to competing association events, leading to either protein-rich proteoparticles or protein−phospholipid reconstituted bilayers.

The experimental findings presented here confirm the expected effects of protein−protein interactions upon the reconstitution of hTfR into phospholipid bilayers. First, several mild detergents (CHAPS, β-OG, β-DM) commonly used for the solubilization of membrane proteins not only are unable to produce a homogeneous population of solubilized hTfR but appear to actively promote the formation of large nonspecific aggregates (Figure 1A), possibly by exposing hydrophobic regions of the receptor or by promoting delipidation (see below). This aggregating effect of CHAPS and β-OG most likely explains the inhomogeneous distribution of hTfR crowding up in dense clusters when using CHAPS and β-OG for reconstitution (Demant et al., 1992). The importance of specific detergent−protein interactions is illustrated by the solubilization of hTfR by LDAO and 8-POE; the latter is apparently able to solubilize the receptor efficiently and to suppress most protein−protein

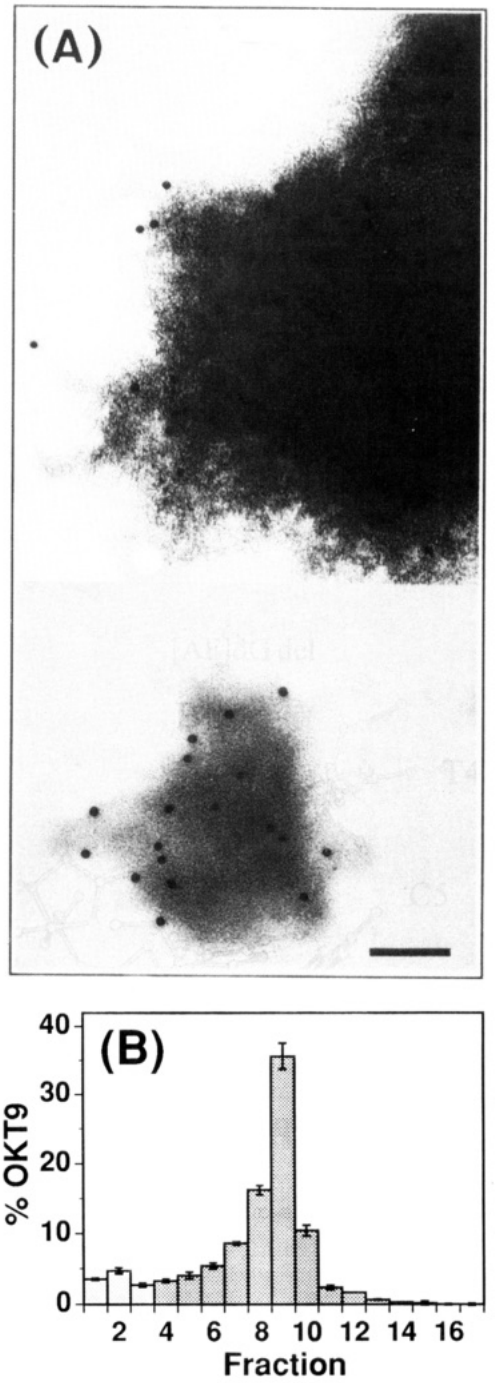
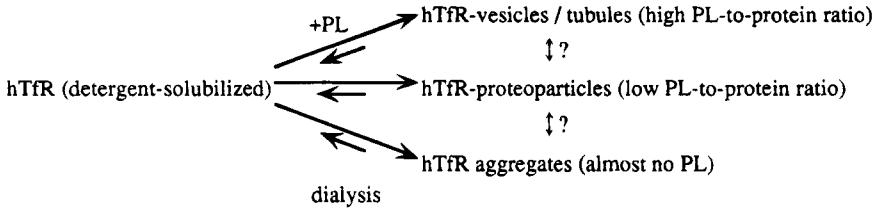


FIGURE 7: Electron micrographs and density gradient profile of SBL-reconstituted transferrin receptor after labeling with monoclonal antibody OKT9 and colloidal gold. Transferrin receptor was reconstituted into SBL vesicles by dialysis at a lipid-to-protein ratio of 10:1 (w/w). The receptor was labeled with the hTfR-specific monoclonal mouse antibody OKT9 and stained with a secondary gold-conjugated antibody against mouse IgG (F_c). (A) Electron micrographs of samples after density gradient centrifugation (peak I). Bar: 100 nm. (B) Sucrose density gradient profile of OKT9 bound to functionally active receptor. Fractions from the density gradient were analyzed by binding the Tween-solubilized receptor to immobilized transferrin on ELISA plates. Bound receptor−OKT9 complex was quantified using an HRP-conjugated secondary antibody against OKT9. Receptor−OKT9 complexes that contain inactive receptor were *not* visualized in this assay.

Scheme 1



interactions, as indicated by its apparent molecular mass determined by gel filtration (Figure 1E).

As a second consequence of the strong protein−protein interactions, *all* of the reconstitution methods employed lead to the production of three discrete populations of hTfR in the sucrose gradient, corresponding to reconstituted hTfR vesicles and tubules (peak I), proteoparticle structures (peak II), and aggregated receptors (peak III). The relative ratio of these three populations most probably reflects the effect of the reconstitution procedure upon the sum of the competing reactions shown in Scheme 1.

The finding of the phosphatidylcholine employed for reconstitution in all of the proteoparticle fractions (peak II) examined suggests strongly that the purified hTfR proteoparticle had been successfully solubilized in detergent and was thus able to exchange with phospholipid. The increased level of phospholipid in the proteoparticles which also contain endogenous phospholipids (as observed by TLC analysis) presumably from placental membranes is consistent with the lower buoyant density of peak II observed in some cases [Figure 3C (10:1, 20:1); Figure 5A,B]. In the cases where no effect upon the buoyant density upon peak II was observed, only traces of PC were incorporated. The lack of reconstituted phospholipid in peak III suggests that this peak represents an hTfR population which rapidly aggregates, thus preventing efficient incorporation into phospholipid mixed micelles. By implication it is tempting to suggest that the aggregate structures in peak III have in fact been partially delipidated by detergent, thus inducing nonspecific aggregation, and that the presence of a low quantity of endogenous phospholipid in the proteoparticle (1.7 mol of PL/mol of hTfR; see Table 3) prevents the formation of such structures during purification and stabilizes the proteoparticle.

The experimentally determined phospholipid-to-protein ratios suggest that the apparent loss of activity of reconstituted hTfR, also observed by Di Giulio et al. (1994), is probably an artifact of the method of protein determination and that the transferrin binding assay employed to measure active hTfR is capable of determining the total amount of active hTfR even in reconstituted vesicles. The success of the assay procedure for this purpose probably lies with the effectiveness of 0.05% Tween 20 in solubilizing any remaining low quantities of membrane vesicles in the assay.

The influence of the reconstitution procedure upon formation of vesicles and tubules (peak I) appears to be correlated with the extent of detergent removal. Thus, dilution reduced the initial solubilizing concentration of 8-POE from 1% to 0.033%, i.e., an apparent reduction of 96.7%, and led to low quantities of vesicles and tubules. Sephadex G-50-mediated detergent removal, which has been reported to remove 99% of the detergent (Brunner et al., 1978), resulted in a higher yield of vesicles and tubules, and dialysis, which leads to complete removal of 8-POE (Grabo, 1982), produced the highest yields of vesicles and tubules. We have not examined the effect of temperature upon the various aggregation phenomena described, although the different amounts of vesicles and tubules in peak I obtained by Sephadex G-50 or dilution, both of which were performed at room temperature, suggest that this factor is not decisive in maximizing reconstituted hTfR−phospholipid bilayers. In addition, hTfR is quite stable at room temperature (unpublished data), so that temperature-dependent nonspecific aggregation events due to denaturation are unlikely.

The observation that the yield of reconstituted hTfR−vesicles and −tubules is dramatically increased when using preformed vesicles for reconstitution is interesting and not easily explained. Possibly the amount of detergent present in the reconstitution mixtures employing preformed vesicles is sufficient to cause extensive insertion of hTfR into the bilayers, and the subsequent high local concentration of phospholipids stabilizes the receptor and suppresses protein−protein interaction.

The importance of protein−protein interactions is strongly supported by the observed formation of tubular structures which apparently grow out of the surface of reconstituted hTfR vesicles. Although the total population of reconstituted vesicles appears to contain hTfR integrated to the same extent in either orientation in the phospholipid bilayer, as judged by the trypsin susceptibility, it is likely that the tubular structures arise from interactions of hTfR molecules present in the same orientation, most probably with the extracellular domain facing into the tubular lumen, leading to a relative concentration of outside-out-oriented hTfR molecules in the nontubular regions of the vesicles. As has been suggested previously, self-association might be one possible means of concentrating membrane proteins without requiring specific interactions with other regulatory proteins (Kelly, 1985). In this context it is interesting to note that the morphology of the tubular structures of reconstituted hTfR is similar to tubular structures observed in cells during early events of hTfR internalization (Hopkins et al., 1994), suggesting the possibility that specific receptor−receptor interactions of hTfR may play a role in endocytosis. We note, however, that the diameter of the tubules arising in the reconstituted system is somewhat smaller (23 nm) than that observed *in vivo* (60 nm; Hopkins et al., 1994). The tubular structures were only observed for reconstitutions employing soy bean PC, which contains highly unsaturated and heterogeneous fatty acyl chains, and never for reconstitutions employing DOPC, which contains only a single type of unsaturated fatty acyl chain. As both SBL and DOPC are present in the liquid crystalline phase at all temperatures employed here for reconstitution, the formation of tubules probably does not depend upon the fluidity of the bilayer but rather depends upon the order of the fatty acyl chains; i.e., the fatty acyl chains of SBL, which are highly disordered due to their heterogeneity, more readily allow receptor aggregates to assume a geometrically strained tubular structure than the

more ordered homogeneous unsaturated fatty acyl chain of DOPC. The efficient incorporation of hTfR into bilayers does depend upon the fluidity of the bilayer, as evidenced by the low yield of vesicles obtained with reconstitution involving DMPC, which is in the gel phase at the temperatures employed here. Similar results for reconstitution of other integral membrane proteins with phospholipids below their T_m have been described previously (Heyn & Dencher, 1982). The results of the present study are difficult to extrapolate to the study of Kurrle et al. (1990) reconstituting hTfR from Molt-4 cells into bilayers of DMPC and DMPC/DMPG. These authors observed a single reconstituted band on metrizamide density gradients, which was subsequently shown to contain active hTfR preferentially oriented to the outside (convex) side of the vesicle. Decreasing lipid-to-protein ratios suppressed the phospholipid thermal pretransition and broadened the main transition, as observed previously for other membrane proteins (Alonso et al., 1982). Moreover, aggregation properties of the cell-line-derived hTfR may differ significantly from that derived from human placenta, possibly by reason of different glycosylation.

In summary, our results indicate that although the efficiency of hTfR incorporation into bilayers of PC containing a population of highly unsaturated fatty acids is high, the resulting reconstituted membranous structures are heterogeneous. This might be due to extensive protein−protein associations that can be explained by specific interactions between the large extracellular domains (Borhani & Harrison, 1991). Reconstituted vesicles produced using a PC with a single species of unsaturated fatty acyl chain appear more suitable for biophysical studies, and future optimization of this system may facilitate the formation of two-dimensional crystals for the structural elucidation of hTfR. Most surprising has been the observation that hTfR reconstituted into bilayers containing heterogeneously unsaturated fatty acyl chains spontaneously forms tubular outgrowths from the vesicle outer surface. These structures may reflect receptor−receptor interactions which play a role in the initial events of endocytosis, and they may be useful as an *in vitro* assay system for the influence of hTfR structural determinants upon this fundamental process of cell biology.

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