

Concentration of Transferrin Receptor in Human Placental Coated Vesicles

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Abstract. Coated vesicles were purified from human placenta by sedimentation, isopycnic centrifugation, and gel filtration. Quantitative Western blotting of the endogenous transferrin receptor (tR) demonstrated the presence, on average, of roughly one receptor per vesicle. tR appeared undersaturated with transferrin. After solubilizing vesicles in nonionic detergent, we looked for evidence of tR interactions with other pro-

teins. Solubilized tR had an unexpectedly high mobility by gel filtration, apparently resulting from its self-association. This property was not seen in purified tR or in tR from a different cell fraction. The tR complexes, though noncovalent, were largely resistant to conditions that disassemble coat proteins, and they did not appear to contain any other protein species.

An early event in receptor-mediated endocytosis is the localization of cell surface receptors to coated pits, indented regions of the plasma membrane characterized by the presence of a cytoplasmic protein, clathrin. The concentration of receptors in coated pits appears to be selective, since only a subpopulation of cell surface proteins are found in coated vesicles (Bretscher et al., 1980; Watts, 1984) and the internalization rates of individual receptor types are independently regulated (Ciechanover et al., 1983). Studies of naturally occurring and engineered mutations have shown that determinants in the cytoplasmic domain of low density lipoprotein receptor are needed for coated pit localization (Davis et al., 1986). Similarly, the transferrin receptor (tR) cytoplasmic domain is needed for internalization (Iacopetta et al., 1988).

To explore the interaction of receptors with coated vesicles and by inference coated pits, we have purified human placental coated vesicles by a combination of rate zonal and isopycnic centrifugation and gel filtration (Pearse, 1982; Altstiel and Branton, 1983). These coated vesicles have been well-characterized previously. They consist of a phospholipid vesicle surrounded by a protein coat consisting chiefly of clathrin heavy and light chains, and two groups of proteins of roughly 50 and 100–120 kD molecular mass (reviewed in Pearse and Crowther, 1987). The presence of some of these in coated pits has been established by EM and membrane

reconstitution experiments (Heuser, 1980; Moore et al., 1987).

Proteins transported via coated vesicles are present as integral proteins in the vesicle membrane or as soluble proteins in the vesicle lumen. Among the former is the tR, a disulfide-linked homodimer with 90-kD subunits (reviewed in Newman et al., 1982). The monomer structure consists of three regions: a 61-residue NH₂-terminal cytoplasmic segment, a 28-residue hydrophobic membrane-spanning segment, and a 672-residue ectoplasmic segment (Schneider et al., 1984; McClelland et al., 1984). The receptor dimer binds two molecules of transferrin (tf), an iron-transport serum glycoprotein (Enns and Sussman, 1981; Schneider et al., 1982a). Placental trophoblasts are particularly rich in this receptor because of the iron requirement of the rapidly growing fetus (Seligman et al., 1979; Anderson et al., 1986; Turkewitz et al., 1988a).

tR has previously been associated with coated vesicles in both cytological and biochemical studies (Hopkins, 1983; Booth and Wilson, 1981). We confirm those observations and quantify the average receptor occupancy of the purified vesicles. The receptors do not appear to be stably associated with other membrane proteins or with proteins in the coat structure, but they appear instead to be self-associated after detergent solubilization. This self-association is not a property of purified tR or tR–tf complexes, nor is it a property of tR solubilized from noncoated vesicle membranes.

Materials and Methods

Phenylmethanesulfonyl fluoride, DTT, deuterium oxide, Ficoll, Triton X-100, *n*-octyl glucopyranoside, Ponceau S, and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Sigma Chemical Co. (St. Louis, MO); human tf, from Calbiochem-Behring

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1. *Abbreviations used in this paper:* CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; SAND, sulfosuccinimidyl 2-(*m*-azido-*o*-nitrobenzamido)-ethyl-1, 3'-dithiopropionate; tf, transferrin; tR, transferrin receptor.

Corp. (San Diego, CA); rabbit anti-human tR IgG, horseradish peroxidase-conjugated goat anti-rabbit IgG, and rabbit anti-mouse IgG, from Accurate Chemical & Scientific Corp. (Westbury, NY); ^{125}I -labeled affinity-purified protein A, from Amersham Corp. (Arlington Heights, IL); SDS-PAGE molecular mass markers and horseradish peroxidase-color development reagent, from Bio-Rad Laboratories (Richmond, CA); cyanogen bromide-activated Sepharose 4B, Sepharose CL4B, protein A-Sepharose, Sepharose-DEAE, and Sephacryl S-1,000 SF, from Pharmacia Fine Chemicals (Piscataway, NJ); Ultrogel AcA 22, from LKB Instruments (Gaithersburg, MD); and dimethyl pimelimidate and SAND, from Pierce Chemical Co. (Rockford, IL).

Purification of Coated Vesicles

A human placenta was obtained within 1 h of delivery and placed immediately on ice. All subsequent operations were at 4°C. After removal of the cord and trimming of membranes, tissue was homogenized in 10 mM 2-(*N*-morpholino) ethanesulfonic acid (Mes), pH 6.5, 100 mM KCl, 0.5 mM MgCl_2 , 1.0 mM EGTA, 0.1 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride (Buffer 6.5). The supernatant of a 30-min 12,000-g spin was pelleted for 1 h at 100,000 g. This pellet was suspended with a homogenizer (Dounce, Vineland, NJ), and 12 ml aliquots were layered on discontinuous sucrose gradients containing 12 ml of 30% sucrose (wt/vol) and 14 ml of 10% sucrose. After centrifugation for 45 min at 85,000 g, the 10% layer was harvested, diluted, and pelleted at 100,000 g for 1 h. Pellets were suspended, cleared with a 20-min 10,000-g spin, and 10 ml aliquots layered on three 28-ml continuous Ficoll-D₂O gradients (2% Ficoll 9% D₂O-20% Ficoll 90% D₂O, in Buffer 6.5). These were centrifuged at 85,000 g for 14–16 h. Coated vesicles were present in a diffuse band near the bottom of the tube. These were diluted and spun for 10 min at 10,000 g to clear aggregates. The supernatant was pelleted for 1 h at 100,000 g, and this pellet was suspended in a small volume of Buffer 6.5. The sample of coated vesicles was used at this stage, or was applied to a 3.2 × 90-cm Sephacryl S-1,000 SF column. The variable first elution peak consisted of protein aggregates and large vesicles; this was followed by the main peak, consisting of coated vesicles, which were pelleted at 100,000 g and suspended. This final purification step did not discernibly affect the results. Yield was ~10 mg of coated vesicle protein from a single placenta.

Crude Membrane Preparation

Placental tissue, homogenized as above, was centrifuged for 12,000 g for 30 min. The supernatant was discarded and the pellet suspended by homogenization in a large volume of Buffer 6.5. This was repeated six times. A 10-min 1,500-g spin was done to remove connective tissue, and the supernatant used as a crude membrane fraction.

tR Purification

tR was purified from human placenta as described (Turkewitz et al., 1988a).

Preparation of Uncoated Vesicles

Coated vesicles in Buffer 6.5 were dialyzed overnight into either 0.5 M Tris (pH 7.5), 50-mM Hepes (pH 7.0), 0.5 M NaSCN, or 15 mM Hepes (pH 8.0), 0.2 mM EDTA. The sample was then applied to a Sepharose CL4B column preequilibrated with the same buffer. Sample elution was monitored by absorbance at 280 nm. The uncoated vesicles elute in the column void while clathrin and other stripped proteins migrate in the included volume.

EM

Samples were adsorbed to carbon films on copper grids and negatively stained with 1.5% uranyl acetate before visualization using a JEOL 100CXII microscope.

Protein Assay

Protein concentrations were determined using the Pierce BCA Protein Assay.

Antibodies

Rabbit antiserum against tR was generated as described (Turkewitz et al.,

1988a). The hybridoma OKT9 (Schneider et al., 1982a) was obtained from the American Type Culture Collection (Rockville, MD), and grown in ascites. Antibody was purified from ascites fluid using differential ammonium sulfate precipitation and chromatography on Sepharose-DEAE.

SDS-PAGE

Electrophoresis was performed in 1.5-mm-thick gels (Laemmli, 1970). Reduced samples were prepared by boiling for 3 min in the presence of 1% SDS and 5% 2-mercaptoethanol. Protein was visualized using the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories).

Immune Purification

tR was isolated using the monoclonal antibody OKT9 as described (Schneider et al., 1982b). Briefly, protein A-Sepharose was saturated with rabbit anti-mouse IgG, and then incubated with the mouse monoclonal antibody. These were cross-linked with 50 mM dimethyl pimelimidate to prevent antibody elution during SDS elution of the bound antigen. For immune purification using rabbit anti-human tR IgG, the antibody was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions, at a concentration of 4 mg IgG/ml gel. For immunoprecipitations using rabbit antisera against tR or the 100–120-kD proteins, coated vesicle samples were solubilized using Triton X-100 or CHAPS, cleared by centrifugation, and incubated for 2 h with antiserum. Fixed *Staphylococcus A* was prewashed first in 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and then in the appropriate solubilization buffer. After a 2-h incubation, *Staphylococcus A* was pelleted, washed briefly with Buffer 6.5 0.5% Triton X-100, and eluted with 1% SDS. Antisera against clathrin and the 100–120-kD proteins were a gift of Dr. D. Branton. We thank Dr. D. Bar-Zvi for his advice on their use.

Antisera Hybridizations

After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose. Before antibody blotting, total protein was visualized using Ponceau S according to the supplier's instructions. This stain was readily reversed and specific proteins were visualized using horseradish peroxidase-coupled secondary antibody (Turkewitz et al., 1988a). Rabbit anti-human tR serum was used at 1:100; antisera to clathrin and the 100–120-kD proteins were used at 1:500. When protein A was used instead of the secondary antibody, roughly 10^6 cpm of ^{125}I -protein A was added per blot.

Affinity Purification

Sepharose-tf was prepared as described (Turkewitz et al., 1988a).

Cross-Linking Using SAND

Sulfosuccinimidyl 2-(*m*-azido-*o*-nitrobenzamido)-ethyl-1,3'-dithiopropionate (SAND) (see Lewis et al., 1977, for a similar reagent) was dissolved in 20 mM sodium phosphate, pH 7.5, 100 mM NaCl (in a darkroom), and added to an equal volume of coated vesicles in Buffer 6.5. The final SAND concentration was 1.2 mM. These were incubated for 30 min at room temperature, after which 2-aminoethanol was added to 5 mM. After 5 min, the sample was illuminated for 75 s through a 2-mm Pyrex filter at a distance of 3 cm from a GE 275 W Sunlamp.

Gel Filtration

Analytic gel filtration was performed on a 0.8 × 45-cm AcA 22 column. Similar results were obtained using a Sephacryl S-1000 SF matrix or a FPLC Superose 6 column (Pharmacia Fine Chemicals).

Airfuge Pelleting

Coated vesicles were quantitatively pelleted in 6 min at 30 psig in an A-100 rotor (Beckman Instruments Inc.).

Results

Coated vesicles, prepared as described in Materials and Methods, appeared to be highly purified when assayed by EM, with a range of diameters between roughly 60 and 135

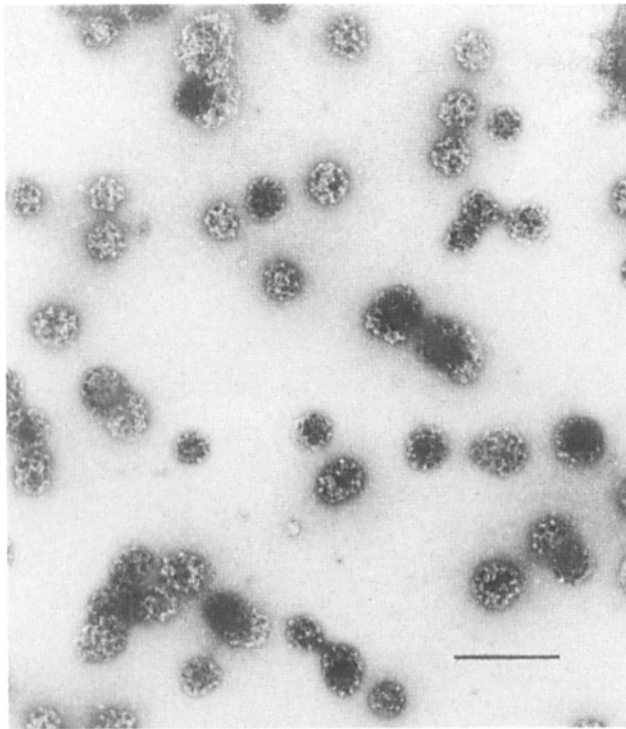
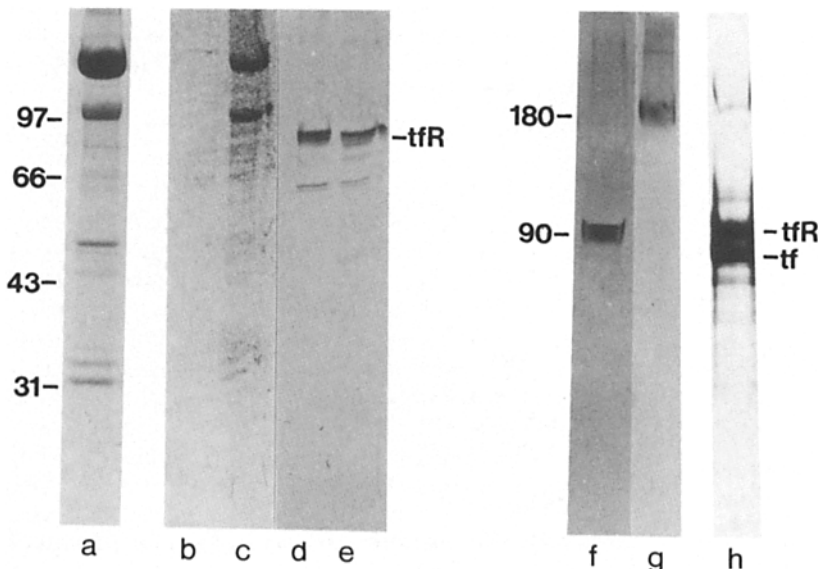


Figure 1. Visualization of coated vesicles. Coated vesicles were prepared as described in Materials and Methods, using sucrose and Ficoll-D₂O gradients, but not gel filtration. The sample was negatively stained with 1.5% uranyl acetate. Bar, 200 nm.

nm. If no final gel filtration step was used, the final vesicle preparation (Fig. 1) also contained small ($d = 12$ nm) circular bodies, which may be ferritin. Preparations occasionally showed contamination by larger uncoated vesicles or protein



blotted with anti-tfR serum; lane e, same as lane c, blotted with anti-tfR serum. Lane f, coated vesicles (90 μ g total protein) were solubilized in 1% SDS. After SDS-PAGE, samples were transferred to nitrocellulose and incubated with rabbit anti-human tfR antiserum, as described in Materials and Methods. Lane g, same as lane f, no 2-mercaptoethanol. Lane h, coated vesicles in Buffer 6.5 (0.6 mg total protein) were solubilized in 0.5% Triton X-100, and incubated for 4 h with 20 μ l Sepharose-coupled rabbit anti-human tf IgG. Afterwards, the matrix was extensively washed with the same buffer, and bound protein was eluted using 1% SDS. Protein was visualized using silver nitrate. *tf* and *tfR* are the chief species present.

aggregates; in these cases, gel filtration was employed as a final step. SDS-PAGE of coated vesicle samples showed proteins with a molecular mass of 180 (clathrin heavy chain), 100–120, 50–55, and \sim 30 kD (clathrin light chains), as well as lesser quantities of other species (Fig. 2, lane a). Nitrocellulose transfers of coated vesicle samples, visualized with rabbit anti-human tfR antiserum showed the expected species of 90 (Fig. 2, lane f, reducing conditions) and 180 kD (Fig. 2, lane g, nonreducing conditions). tfR was also observed by SDS-PAGE and silver staining when coated vesicle samples were solubilized in nonionic detergent and incubated with Sepharose anti-tf IgG (Fig. 2, lane h). We obtained identical results when the coated vesicles were further purified by additional sucrose gradient centrifugation and gel filtration (not shown). When coated vesicles were subject to gel filtrations on a Sephacryl S-1000 column, tfR and coated vesicle structural proteins comigrated and there was no indication of the presence of tfR in other fractions (Fig. 3).

We have estimated the average tfR content per coated vesicle by comparing tfR derived from coated vesicles to known amounts of purified tfR. The tfR in coated vesicles represents roughly 1–2% of the amount isolatable from whole placental homogenate. Samples were visualized by antibody blotting on nitrocellulose after SDS-PAGE; an example is shown in Fig. 4. Four measurements on two preparations indicated that an average of 0.9–1.3 tfR dimers were present per coated vesicle. Consistent results were obtained for two other preparations using a different technique: ¹²⁵I-labeled-protein A was used to visualize antibody binding, and the autoradiograms quantified by densitometry (not shown). tf in coated vesicles was also determined using these two techniques; in four preparations, between 0.4 and 3.2 (mean = 1.2) copies of tf were present per coated vesicle (not shown).

When coated vesicles were solubilized with Triton X-100

Figure 2. Protein content of coated vesicles. Coated vesicle proteins were analyzed by SDS-PAGE (5–15% gels) and visualized directly or with antibody hybridization. Except where noted, samples contained 5% 2-mercaptoethanol. (Lane a) Coated vesicles, purified as in Fig. 1, solubilized in 1% SDS; 15 μ g protein; Coomassie-stained. SDS-PAGE molecular mass standards are indicated. (Lanes b–e) Coated vesicles in Buffer 6.5 (25 μ g total protein) were solubilized for 30 min in 0.1% Triton X-100, and the sample was divided. One half was prepared directly for SDS-PAGE; the second was centrifuged for 6 min in an airfuge, and the supernatant prepared for SDS-PAGE. After electrophoresis, samples were transferred to nitrocellulose, where total protein was visualized with Ponceau S, and tfR visualized after hybridization with rabbit anti-human tfR serum. Lane b, airfuge supernatant of detergent-treated coated vesicles, Ponceau S; lane c, whole coated vesicles, Ponceau S; lane d, same as lane b,

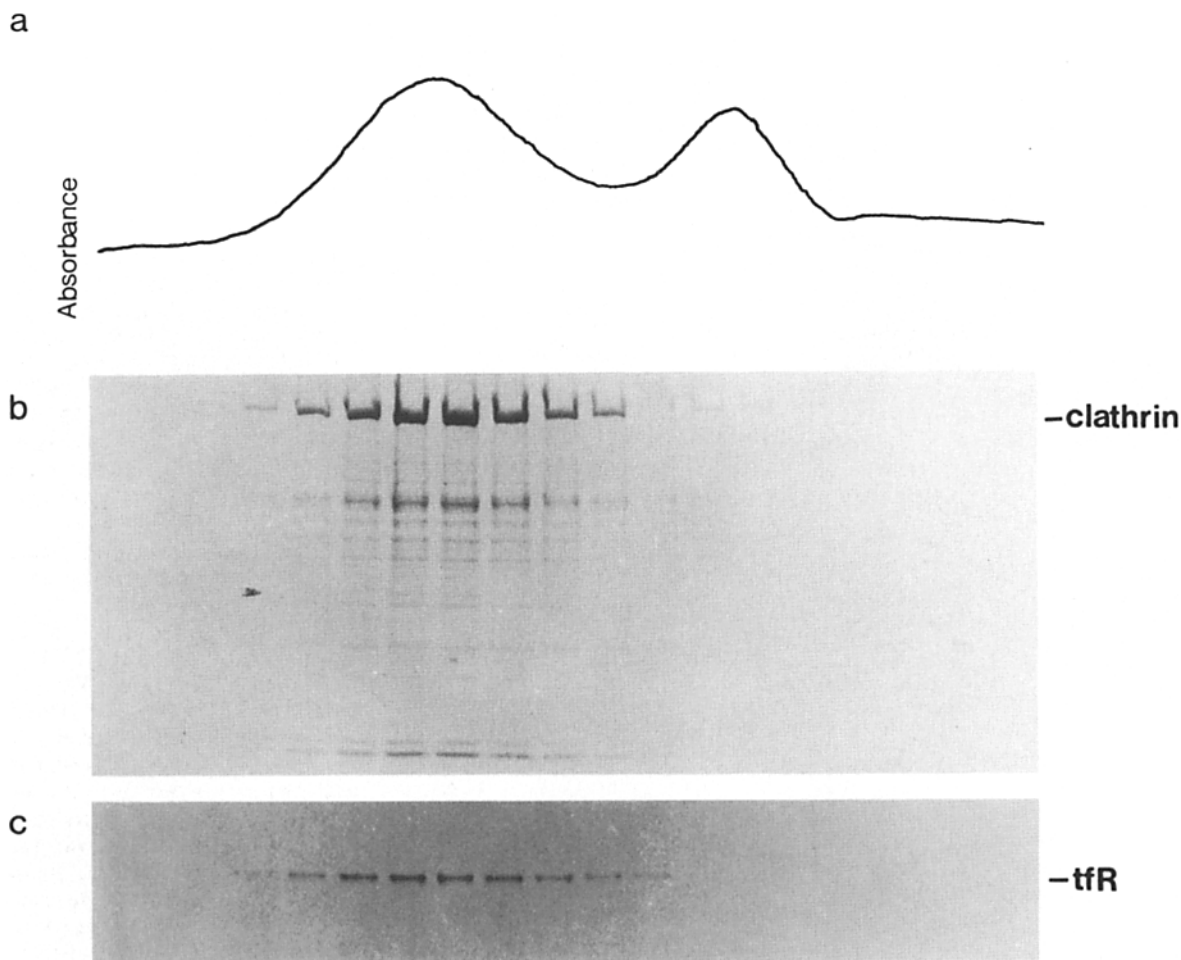


Figure 3. Gel filtration of intact coated vesicles. Coated vesicles (0.8 mg total protein), purified as in Fig. 1, were applied to a 0.8×60 -cm Sephacryl S-1000 SF column in Buffer 6.5, and run at 0.8 ml/h. Protein elution was monitored by absorbance at 280 nm; 0.6-ml fractions were collected. 50- μ l aliquots of the fractions were subjected to SDS-PAGE and transferred to nitrocellulose. (a) Elution profile. The first peak corresponds to coated vesicles; the second represents contamination by smaller species. (b) Ponceau S staining of nitrocellulose transfer. Clathrin and associated cage proteins are found in the first peak. (c) Antibody staining (rabbit anti-human tfr antiserum) of nitrocellulose.

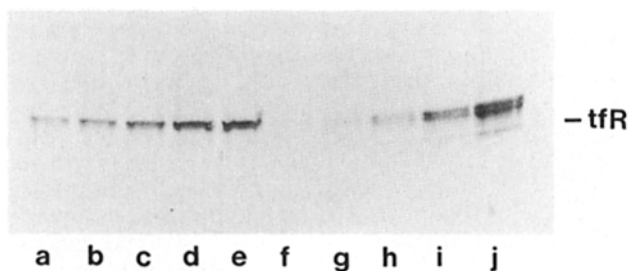


Figure 4. tfr content of coated vesicles. Coated vesicles and placental tfr were purified as described in Materials and Methods, and their protein concentrations determined using BCA. Aliquots were diluted in 0.1% SDS 1 mg/ml ovalbumin, and analyzed by SDS-PAGE (reducing conditions; 5–15% gel). tfr was visualized by antibody hybridization. Molar equivalents were calculated assuming a molecular weight of 180,000 for tfr and 66×10^6 for coated vesicles (as determined for liver coated vesicles in Steven et al., 1983). (Lanes a–e) Coated vesicles: lane a, 4.4 μ g, 6.6×10^{-14} mol; lane b, 6.5 μ g, 9.9×10^{-14} mol; lane c, 9.9 μ g, 1.5×10^{-13} mol; lane d, 14.7 μ g, 2.2×10^{-13} mol; lane e, 22.0 μ g, $3.3 \times$

in Buffer 6.5, clathrin and the associated 100–120-kD proteins pelleted in an airfuge, while tfr remained in the supernatant (Fig. 2, lanes b–e). The same result was obtained using CHAPS or *n*-octyl glucopyranoside (not shown). Consistent with these observations, gel filtration of detergent-solubilized coated vesicles showed that clathrin and associated cage proteins comigrate as a large species, eluting near the column void (Fig. 5 a). However, gel filtration of the solubilized tfr from coated vesicles showed that some fraction had an unexpectedly high mobility, close to that of the cages themselves. This was seen using either CHAPS (Fig. 5, b and c) or Triton X-100 (Fig. 5 d), and was more pronounced with the former. A second pool of tfr in the

10^{-13} mol. (Lanes f–j) Purified tfr: lane f, 1.2 ng, 6.7×10^{-15} mol; lane g, 3.7 ng, 2.0×10^{-14} mol; lane h, 11.0 ng, 6.0×10^{-14} mol; lane i, 33.3 ng, 1.8×10^{-13} mol; lane j, 100 ng, 5.5×10^{-13} mol.

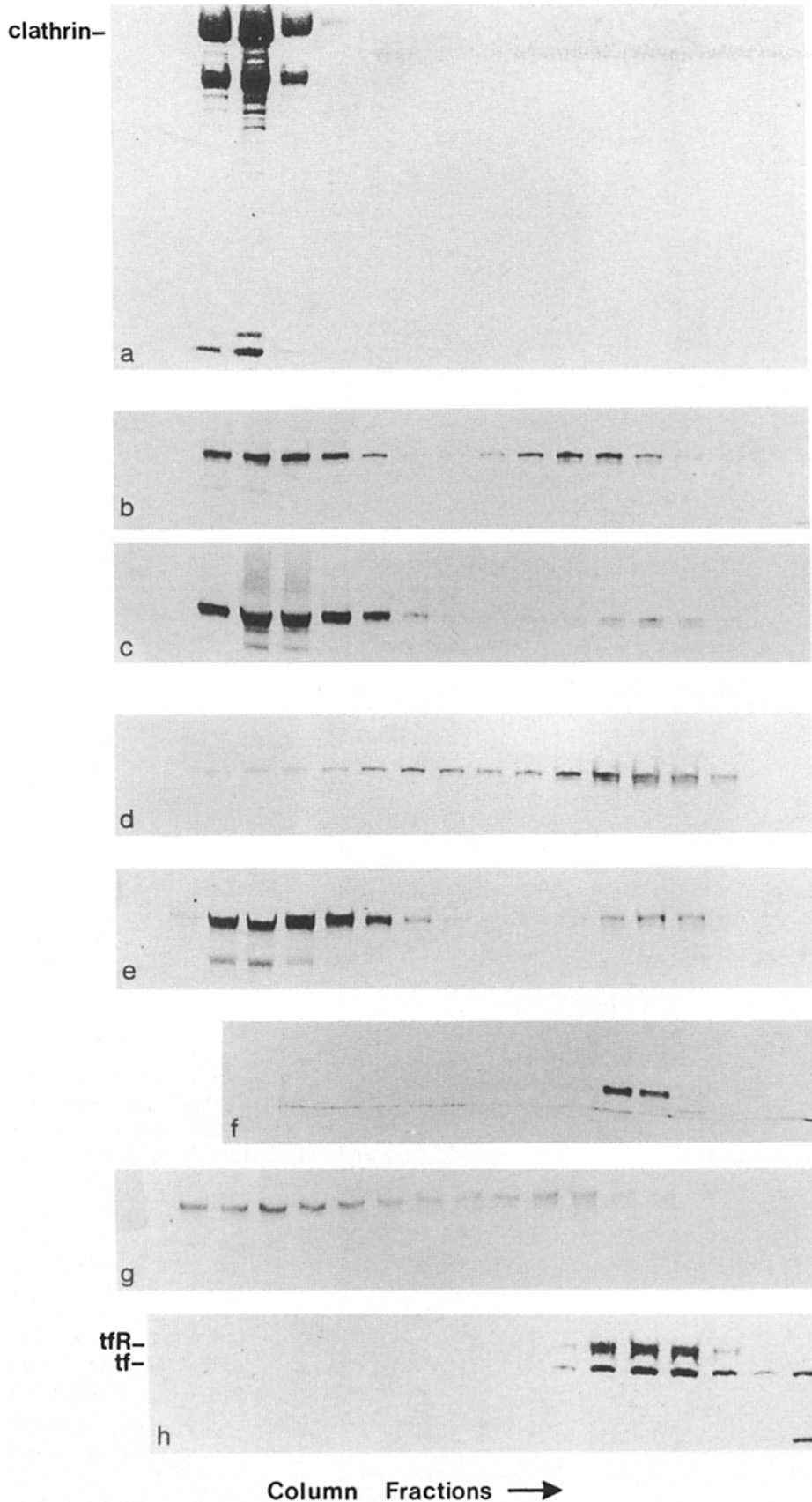


Figure 5. Gel filtration of detergent-solubilized coated vesicles. Samples were subjected to gel filtration on an ACA 22 column, as described in Materials and Methods. 0.6-ml fractions were collected, and 50- μ l aliquots were withdrawn for SDS-PAGE. After transfer to nitrocellulose, protein was visualized with Ponceau S and then with rabbit anti-human tFR antiserum hybridization. Successive column fractions are shown. (a) Coated vesicles (0.8 mg total protein) were solubilized in 15 mM CHAPS for 8 h; gel filtration was performed in 15 mM CHAPS, Buffer 6.5. Total protein was visualized using Ponceau S. (b) Same as a; tFR was visualized using antiserum hybridization. (c) Same as a, using a different preparation of coated vesicles; tFR was visualized using antiserum hybridization. (d) Coated vesicles (0.8 mg total protein) were solubilized in 0.1% Triton X-100 for 1 h; gel filtration was performed in 0.1% Triton X-100, Buffer 6.5. tFR was visualized using antiserum hybridization. (e) Same as d, but coated vesicles were first cross-linked using SAND, as described in Materials and Methods. After gel filtration, cross-link reversal was with 50 mM sodium sulfite for 30 min at room temperature, followed by boiling for 3 min in 1% SDS, 120 mM DTT. (f) Coated vesicle-depleted crude membrane fraction (0.1 mg total protein) was prepared as described in Materials and Methods. The sample was solubilized in 15 mM CHAPS; gel filtration was in 15 mM CHAPS, Buffer 6.5. tFR was visualized using antiserum hybridization. (g) Coated vesicles (0.4 mg total protein) were dialyzed overnight against 0.5 M Tris, pH 7.5, and then solubilized in 15 mM CHAPS. Gel filtration was performed in 15 mM CHAPS, 0.5 M Tris. tFR was visualized using antiserum hybridization. (h) Purified tFR (0.1 mg) and tf (0.12 mg) were incubated together in 0.1% Triton X-100, Buffer 6.5. Gel filtration was performed in 0.1% Triton X-100, Buffer 6.5. Total protein was visualized using Ponceau S.

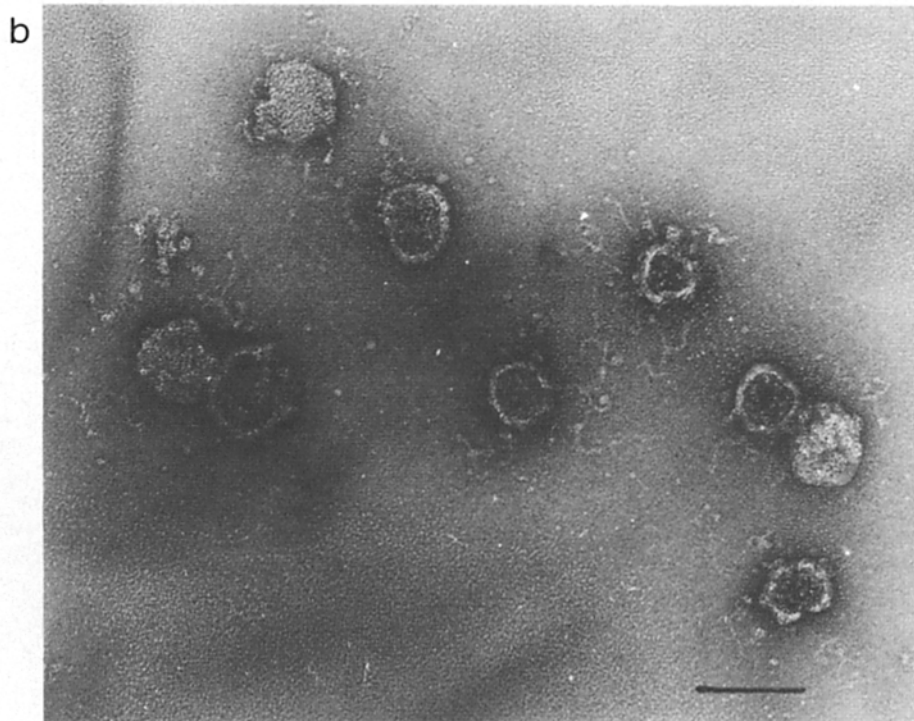
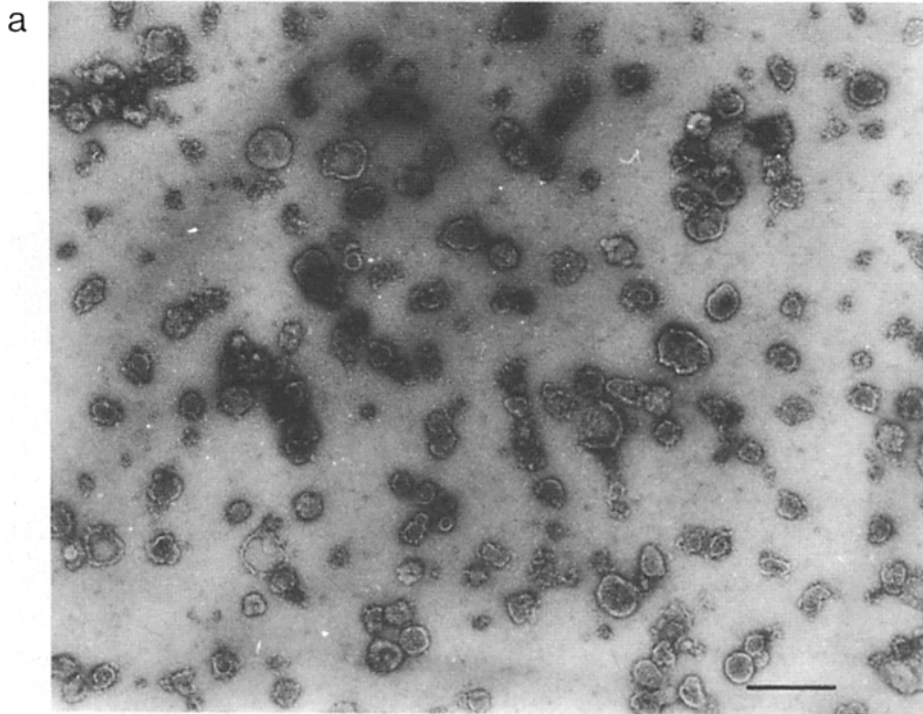


Figure 6. Visualization of stripped vesicles. Stripped coated vesicles were prepared with 0.5 M Tris, pH 7.5, as described in Materials and Methods. Samples were negatively stained with 1.5% uranyl acetate. In *b*, note that each vesicle retains a few partially disengaged clathrin trimers. Bars: (a) 250 nm; (b) 100 nm.

same samples was seen to migrate with a mobility characteristic of a dimer or of a tfR–tf complex (Fig. 5 *h*). The proportion of tfR in the two pools varied between preparations of coated vesicles; compare Fig. 5, *b* and *c*. In addition, a variably small amount of tfR migrated with intermediate mobility; this is clearly seen in Fig. 5 *d*. Changes in detergent concentration did not perceptibly alter the distribution of tfR. Triton X-100 was used between 0.05 and 1%; CHAPS between 8 and 15 mM; *n*-octyl glucopyranoside between 1 and

2%. Similar species were obtained when the intact cages were removed from the solubilized sample by airfuge centrifugation before gel filtration, showing that the large apparent molecular mass was not dependent upon interaction with the intact cage structure (not shown). Only a small fraction (<10%) of the tf comigrates during gel filtration with the large molecular mass complexes, while the rest appears as monomeric tf or bound to dimeric tfR (not shown).

Gel filtration fractions containing the high mobility form

of solubilized tFR were incubated with an immune-affinity matrix containing OKT9 as described in Materials and Methods. After washing, the matrix was eluted with SDS, and this sample was analyzed by SDS-PAGE and silver-staining. No species was detectable except tFR (not shown). We performed immunoprecipitations from detergent lysates using rabbit antisera against clathrin, the 100–120-kD proteins, and tFR. The first and second antigens, but not the third, coprecipitated under a wide range of conditions (not shown). We have also tried exhaustively to cross-link the cytoplasmic domains of tFR in intact coated vesicles, using a variety of membrane-impermeant reagents, without obtaining clear evidence for their interaction with specific proteins (not shown). tFR cannot be solubilized from vesicles which have been heavily cross-linked (not shown). Cross-linking of intact coated vesicles with SAND shifted the apparent mass of tFR after solubilization in Triton X-100 in favor of the larger species (Fig. 5 *e*). This, however, may be due to entrapment after cross-linking.

The apparent self-association of tFR derived from a coated vesicle fraction does not appear to arise from spontaneous aggregation of receptors in detergent solution. Under our conditions for solubilizing coated vesicles in Triton X-100, purified tFR migrates as a monodisperse dimer with a small amount of putative tetramer (Turkewitz et al., 1988*a*). Neither did tFR–tf complexes, under the same conditions, show any tendency to aggregate (Fig. 5 *h*). The same result was obtained for the tFR–tf complex in CHAPS (not shown). Addition of exogenous tf to the coated vesicle lysates before gel filtration had no discernible effect on the large molecular mass complexes (not shown). To rule out further the possibility of nonspecific aggregation, we prepared a crude membrane fraction from placenta in a manner designed to deplete coated vesicles. SDS-PAGE and silver staining showed no detectable clathrin (not shown), but tFR could be visualized by antibody hybridization in a detergent extract of this fraction, and gel filtration in CHAPS indicated no aggregation and only the dimeric form (Fig. 5 *f*). This result was identical in Triton X-100. This experiment was repeated with such a membrane fraction that was allowed to sit for 1 wk before solubilization, to see whether aggregates developed with time, and the same result was obtained (not shown).

We have tested the ability of a number of conditions, known to affect cage stability, to influence the large molecular mass tFR complexes. The complexes are not covalent, as demonstrated by their dissociation when analyzed by SDS-PAGE under reducing or nonreducing conditions. A number of agents have been described for removing the clathrin coat and other proteins from intact coated vesicles (reviewed in Keen, 1985). We have used several of these, including 0.5 M Tris, pH 7.5; 0.5 M NaSCN 50 mM Hepes, pH 7.0; and 15 mM Hepes, pH 8.0, 0.2 mM EDTA. The resulting uncoated vesicles appeared intact by EM, and a small quantity of protein was visible at the vesicle periphery (Fig. 6 *a*). In particular, all vesicles had a corona of partially disengaged clathrin trimers (Fig. 6 *b*). SDS-PAGE analysis showed that while a small fraction of the original clathrin and other cage proteins remained with the vesicles, the tFR content was unaffected (not shown). When such vesicles were solubilized in detergent, the tFR still appeared to be associated in large complexes, though somewhat more disperse than those derived from untreated vesicles (Fig. 5 *g*). Further dispersal occurred

at higher NaSCN concentration, but no condition was found which completely dissociated the tFR assemblies.

Discussion

The results presented here suggest that a subpopulation of placental coated vesicles are enriched in tFR, and that the receptor is self-associated within the vesicles. Our estimate of one tFR dimer per coated vesicle is likely to be a minimum based on the following considerations. Empty coats (i.e., protein coats without an enclosed vesicle) are a frequent contaminant of coated vesicle preparations (Crowther et al., 1976). We have not tried to quantify their presence in our preparation, knowing of no simple and unambiguous way of doing so, but some coats, particularly the smaller ones, appear empty by EM. Since these empty coats retain a substantial fraction of the protein content of an intact coated vesicle, their presence leads to overestimation of the number of vesicles and hence underestimation of tFR occupancy. More importantly, coated vesicles from trophoblasts are probably derived from multiple intracellular sources with an uneven distribution of tFR. The coated vesicles also contain a more variable amount of tf. Our quantitation suggests that the vesicles contain insufficient tf to occupy fully the available receptors.

Our inability to detect other proteins associated with tFR in coated vesicles presents a paradox, since selection of specific proteins for inclusion in coated pits would seem to imply protein recognition and association. tFR does not appear to be stably associated with any major or minor coated vesicle proteins, which should be detected by immune affinity purification of coated vesicle-derived tFR and SDS-PAGE/silver staining. This technique might not detect associated proteins present in low stoichiometric quantities or small polypeptides. We have also attempted to bind some component of solubilized coated vesicles to a matrix containing concentrated purified tFR, without success (not shown). In designing these experiments, we have assumed that the protein complement of coated vesicles should reflect that of coated pits, since the latter are assembly precursors of the former.

Pearse (1988) has presented evidence for recognition of some receptor cytoplasmic domains by the 100–120-kD proteins. Although no sequence similarities have been noted among such cytoplasmic domains, their colocalization to coated pits implies the existence of biochemical signals that are ultimately recognized by a common structure.

While the identity of a recognition structure has eluded us, we have detected an apparent self-association of tFR derived from coated vesicles. The tFR assemblies are detected after solubilization of the phospholipid vesicle with nonionic detergent. We cannot exclude the possibility that the self-association we observe occurs during detergent solubilization, because of the uniquely high local protein concentration in coated vesicles. We note, however, that the tFR assemblies can be isolated with three different detergents and that preparations with the same detergents from other cell fractions contain only dispersed tFR dimers. tFR assemblies are detected after the coats themselves are removed by sedimentation or disassembly, demonstrating that the high mobility does not result from entrapment within intact cages. The large apparent Stokes radius of tFR must be reconciled, how-

ever, with our observations that tFR can be solubilized from an intact cage. In a clathrin icosahedral lattice, the diameter of a facet is roughly 32 nm (calculated from Crowther and Pearse, 1981), which limits two dimensions of any tFR cluster. The large gel filtration radius suggests that the third dimension is more extended. This may correspond with lateral packing of proteins in a membrane, producing a highly asymmetric complex. Given this consideration, it is difficult to assess the number of tFR dimers needed to form a complex of the observed size. The difference between solubilization in Triton X-100 and CHAPS or *n*-octyl glucopyranoside suggests that the complexes are partially disrupted by the first. Our attempts to characterize the clusters by rate zonal centrifugation have been frustrated by their apparent instability in sucrose gradients (not shown).

We have recently shown that the extracellular domain of human tFR self-associates reversibly in solution as the result of a conformational change at approximately pH 6 (Turkewitz et al., 1988b). The lumina of coated vesicles are thought to acidify rapidly after formation, due to the presence of proton-translocating ATPases (Forgac et al., 1983; Yamashiro and Maxfield, 1987). The tFR assemblies in coated vesicles may therefore be the result of similar self-association for the intact protein. This phenomenon cannot account for the initial concentration of tFR on the cell surface in coated pits. The absence of other proteins in the clusters suggests that this self-association is quite specific and potentially a mechanism for sorting receptors in intracellular compartments after internalization. Work with the soluble extracellular domain also showed that its self-association could be inhibited by tf binding (Turkewitz et al., 1988b). Consistent with that result, our experiments indicate that tf in coated vesicle preparations is largely or entirely absent from the high mobility fractions after solubilization in nonionic detergent. Unlike the oligomers formed by the soluble domains, however, the clusters of intact tFR isolated from coated vesicles do not dissociate at neutral pH.

Interactions between receptors and components of coated vesicles are likely to involve recognition of cytoplasmic determinants. Our inability to detect such interactions suggests that they are either transient or unstable after detergent addition. Instability may be a consequence of the change in organization upon solubilization. Receptors and other vesicle components are restricted by the membrane to two dimensions. In the presence of detergent or after cage disassembly, diffusion in a third dimension will decrease effective protein concentrations and increase the degrees of freedom of those components. Significantly, the relative orientation of membrane-associated components is no longer fixed. These factors would be expected to have strong negative effects on protein interactions (Grasberger et al., 1986). Association constants that are adequate for recognition between membrane and membrane-associated proteins may be insufficient to maintain those contacts in solution. Unnecessarily elevated binding constants may be disfavored as limiting the flexibility of a biological system in which many association and dissociation events are required.

Since weak interactions are sufficient to retain receptors in coated structures, localization can involve less specific contacts than precise "lock-and-key" complementarity. The absence of any obvious consensus among the sequences of receptor cytoplasmic domains also suggests that coated pit

structures may recognize some general characteristic of these segments rather than a unique folded surface. Examples of such nonspecific qualities would be overall charge density or tendency to form an amphipathic helix. A concentration of negative charges, even in random sequences, has been shown to be the essential characteristic for activation domains of eukaryotic transcriptional regulatory proteins (Hope and Struhl, 1986; Ma and Ptashne, 1987). The quite weak interactions are sufficient because the "target" of these activation domains are spatially restricted DNA-bound proteins. Similar considerations may apply to membrane-associated structures. The localization of receptors in coated pits might be more analogous to the retention of proteins on an ion-exchange chromatography column than to their recognition by an antibody combining site.

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References

- Altstiel, L., and D. Branton. 1983. Fusion of coated vesicles with lysosomes: measurement with a fluorescence assay. *Cell*. 32:921-929.
- Anderson, G. J., A. Mackerras, L. W. Powell, and J. W. Halliday. 1986. Improved purification of the human placental transferrin receptor and a novel immuno-radiometric assay for receptor protein. *Biochem. Biophys. Acta*. 884:225-233.
- Booth, A. G., and M. J. Wilson. 1981. Human placental coated vesicles contain receptor-bound transferrin. *Biochem. J.* 196:355-362.
- Bretscher, M. S., J. N. Thomson, and B. M. F. Pearse. 1980. Coated pits act as molecular filters. *Proc. Natl. Acad. Sci. USA*. 77:4156-4159.
- Ciechanover, A. J., A. L. Schwartz, and H. F. Lodish. 1983. The asialoglycoprotein receptor internalizes and recycles independently of the receptors for transferrin and insulin. *Cell*. 32:267-275.
- Crowther, R. A., and B. M. F. Pearse. 1981. Assembly and packing of clathrin into coats. *J. Cell Biol.* 91:790-797.
- Crowther, R. A., J. T. Finch, and B. M. F. Pearse. 1976. On the structure of coated vesicles. *J. Mol. Biol.* 103:785-798.
- Davis, C. G., M. A. Lehrman, D. W. Russell, R. G. W. Anderson, M. S. Brown, and J. L. Goldstein. 1986. The J. D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. *Cell*. 45:15-24.
- Enns, C. A., and H. H. Sussman. 1981. Physical characterization of the transferrin receptor in human placenta. *J. Biol. Chem.* 256:9820-9823.
- Forgac, M., L. Cantley, B. Wiedenmann, L. Altstiel, and D. Branton. 1983. Clathrin-coated vesicles contain an ATP-dependent proton pump. *Proc. Natl. Acad. Sci. USA*. 80:1300-1303.
- Grasberger, B., A. P. Minton, C. DeLisi, and H. Metzger. 1986. Interaction between proteins localized in membranes. *Proc. Natl. Acad. Sci. USA*. 83:6258-6262.
- Heuser, J. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* 84:560-583.
- Hope, I. A., and K. Struhl. 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell*. 46:885-894.
- Hopkins, C. R. 1983. Intracellular routing of transferrin and transferrin receptor in epidermoid carcinoma A431 cells. *Cell*. 35:321-330.
- Iacopetta, B. J., S. Rothenberger, and L. C. Kuhn. 1988. A role for the cytoplasmic domain in transferrin receptor sorting and coated pit formation during endocytosis. *Cell*. 54:485-489.
- Keen, J. H. 1985. The structure of clathrin-coated membranes: assembly and disassembly. In *Endocytosis*. I. Pastan and M. C. Willingham, editors. Plenum Publishing Corp., NY. 85-130.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-682.
- Lewis, R. V., M. F. Roberts, E. A. Dennis, and W. S. Allison. 1977. Photoactivated heterobifunctional cross-linking reagents which demonstrate the aggregation state of phospholipase A₂. *Biochemistry*. 16:5650-5654.
- Ma, J., and M. Ptashne. 1987. A new class of yeast transcriptional activators. *Cell*. 51:113-119.
- McClelland, A., L. C. Kuhn, and F. H. Ruddle. 1984. The human transferrin

- receptor gene: genomic organization, and the complete primary structure of the receptor deduced from a cDNA sequence. *Cell*. 39:267-274.
- Moore, M. S., D. T. Mahaffey, F. M. Brodsky, and R. G. W. Anderson. 1987. Assembly of clathrin-coated pits onto purified plasma membranes. *Science (Wash. DC)*. 236:558-563.
- Newman, R., C. Schneider, R. Sutherland, L. Vodinelich, and M. Greaves. 1982. The transferrin receptor. *Trends Biochem. Sci.* 7:397-400.
- Pearse, B. M. F. 1982. Coated vesicles from human placenta carry ferritin, transferrin and immunoglobulin G. *Proc. Natl. Acad. Sci. USA*. 79:451-455.
- Pearse, B. M. F. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3331-3336.
- Pearse, B. M. F., and R. A. Crowther. 1987. Structure and assembly of coated vesicles. *Annu. Rev. Biophys. Biophys. Chem.* 16:49-68.
- Schneider, C., R. Sutherland, R. Newman, and M. Greaves. 1982a. Structural features of the cell surface receptor for transferrin that is recognized by the monoclonal antibody OKT9. *J. Biol. Chem.* 257:8516-8522.
- Schneider, C., R. A. Newman, D. R. Sutherland, U. Asser, and M. F. Greaves. 1982b. A one-step purification of membrane proteins using a high efficiency immunomatrix. *J. Biol. Chem.* 257:10766-10769.
- Schneider, C., M. J. Owen, S. Banville, and J. G. Williams. 1984. Primary structure of human transferrin receptor deduced from the mRNA sequence. *Nature (Lond.)*. 311:675-678.
- Seligman, P. A., R. B. Schleicher, and R. H. Allen. 1979. Isolation and characterization of the transferrin receptor from human placenta. *J. Biol. Chem.* 254:9943-9946.
- Steven, A. C., J. F. Hainfeld, J. S. Wall, and C. J. Steer. 1983. Mass distributions of coated vesicles isolated from liver and brain: analysis by scanning transmission electron microscopy. *J. Cell Biol.* 97:1714-1723.
- Turkewitz, A. P., J. F. Amatruda, D. Borhani, S. C. Harrison, and A. L. Schwartz. 1988a. A high-yield purification of the human transferrin receptor and properties of its major extracellular fragment. *J. Biol. Chem.* 263:8318-8325.
- Turkewitz, A. P., A. L. Schwartz, and S. C. Harrison. 1988b. A pH-dependent reversible conformational transition of the human transferrin receptor leads to self-association. *J. Biol. Chem.* 263:16309-16315.
- Watts, C. 1984. In situ ¹²⁵I-labeling of endosome proteins with LPO-conjugates. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1965-1970.
- Yamashiro, D. J., and F. R. Maxfield. 1987. Kinetics of endosome acidification in mutant and wild-type chinese hamster ovary cells. *J. Cell Biol.* 105:2713-2721.