Oligomerized Transferrin Receptors Are Selectively Retained by a Lumenal Sorting Signal in a Long-lived Endocytic Recycling Compartment

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Abstract. Cross-linking of surface receptors results in altered receptor trafficking in the endocytic system. To better understand the cellular and molecular mechanisms by which receptor cross-linking affects the intracellular trafficking of both ligand and receptor, we studied the intracellular trafficking of the transferrin receptor (TfR) bound to multivalent-transferrin (Tf_{10}) which was prepared by chemical cross-linking of transferrin (Tf). Tf_{10} was internalized about two times slower than Tf and was retained four times longer than Tf, without being degraded in CHO cells. The intracellular localization of Tf_{10} was investigated using fluorescence and electron microscopy. Tf_{10} was not delivered to the lysosomal pathway followed by low density lipoprotein but remained accessible to Tf in the pericentriolar endocytic recycling compartment for at least 60 min. The retained Tf_{10} was TfR-associated as demonstrated by a reduction in surface TfR number when cells were incubated with Tf_{10}. The presence of Tf_{10} within the recycling compartment did not affect trafficking of subsequently endocytosed Tf. Retention of Tf_{10} within the recycling compartment did not require the cytoplasmic domain of the TfR since Tf_{10} exited cells with the same rate when bound to the wild-type TfR or a mutated receptor with only four amino acids in the cytoplasmic tail. Thus, cross-linking of surface receptors by a multivalent ligand acts as a lumenal retention signal within the recycling compartment. The data presented here show that the recycling compartment labeled by Tf_{10} is a long-lived organelle along the early endosome recycling pathway that remains fusion accessible to subsequently endocytosed Tf.

THE formation of oligomeric complexes has been suggested to play role in the intracellular localization of several proteins. Machamer and colleagues have demonstrated that oligomerization of the M glycoprotein of avian coronavirus correlates with its *cis*-Golgi localization (Weisz et al., 1993). Glut-4, an insulin sensitive glucose transporter, is retained within the endocytic system in the absence of insulin by a mechanism that may involve the oligomeric state of the transporter (Bell et al., 1993; James and Piper, 1993). The sorting of soluble regulated secretory proteins can occur by self-aggregation in the *trans*-Golgi network (Kelly, 1991). Furthermore, the delivery of HLA class II molecules to a specialized antigen processing compartment (Qiu et al., 1994; Amigorena et al., 1994; Tulp et al., 1994; West et al., 1994) may be controlled by the formation of a multimeric complex $(\alpha, \beta, Ii)_3$ (Marks et al., 1990; Roche et al., 1991).

The ability of a multivalent ligand to cross-link several receptor molecules results in the oligomerization of receptor molecules. Receptor cross-linking has been demonstrated to alter the intracellular trafficking of receptor-ligand complexes. Low density lipoprotein (LDL)[1] and β-very low density lipoprotein (β-VLDL) bind the same apo-B,E, receptor (Ellsworth et al., 1987; Koo et al., 1986) either monovalently (LDL) or multivalently (β-VLDL). As a consequence of the difference in valency, these lipoproteins enter macrophages by distinct routes that diverge at the plasma membrane (Myers et al., 1993; Tabas et al., 1990, 1991). Similarly, the Fc receptor of macrophages when complexed with monovalent Fab fragments directed against the Fc receptors, rapidly recycle through the endosomal system of macrophages (Mellman et al., 1984); however, the addition of antibody directed against the Fab fragments to cross-link the Fc receptors results in the removal of the receptors from the recycling pool and degra-

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1. *Abbreviations used in this paper*: 2-IT, 2-iminothiolane; α_2-M, α_2-macroglobulin; β-VLDL, β-very low density lipoprotein; Au-F-Tf_{10}, fluorescein-labeled–multivalent transferrin-colloidal gold conjugate; Cy3-Tf, Cy3-labeled–transferrin; DAB, diaminobenzadine; DiI-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled–low density lipoprotein; F-Tf_{10}, fluorescein-labeled-multivalent transferrin; LDL, low density lipoprotein; sulfo-MBS, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; Tf, transferrin; TfR, transferrin receptor; Tf_{10}, multivalent transferrin.

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dation of the Fc receptors (Mellman and Plunter, 1984). The observed valency-induced sorting is likely to be of physiological importance since Fc receptors cross-linked by multivalent immune complexes are removed from the recycling pool and degraded (Mellman and Plunter, 1984).

Receptor redistribution has also been demonstrated indirectly through the use of antibodies and colloidal gold conjugates directed toward surface receptors (Enns et al., 1983; Kasuga et al., 1981; Larrick et al., 1985; Lesley et al., 1989; Neutra et al., 1985; Roth et al., 1983; Schwartz et al., 1986; Weissman et al., 1986). The cellular mechanism and physiological function of this sorting induced by cross-linking remains unclear as does its variable effect. For example, TfR heavily cross-linked by pentavalent IgM was aggregated on the cell surface and not internalized (Lesley et al., 1989). Divalent $F(ab)'_2$ fragments of the same antibody resulted in receptor downregulation and enhanced degradation, possibly in the lysosome (Lesley et al., 1989). In another study, a monoclonal antibody directed against TfR accumulated in an "extended" endosomal compartment after an overnight incubation with antibody (Killisch et al., 1992). Although antibody treatment generally produces a redistribution of the TfR, only a portion of the cross-linked TfR are degraded. The site of antibody-induced degradation has been suggested to be the lysosome, yet in at least one system, antibody induced degradation of asialoglycoprotein receptor was not affected by lysosomal protease inhibitors, or by incubation at 18°C (Schwartz et al., 1986). The variable effect upon receptor trafficking caused by antibody cross-linking may in part be explained by the varying extent of receptor aggregation induced by the multivalent binding of antibody. The observed variability may be explained by several factors including the number of antibody binding sites per receptor molecule, the binding affinity and/or avidity of the antibody for the receptor, and the receptor density on the cell surface. As a result it is technically difficult to determine the extent of cross-linking induced by antibody binding.

While protein oligomerization and receptor cross-linking have been shown to alter the intracellular trafficking of some proteins, the mechanisms responsible for these changes are poorly understood. To further define these mechanisms an experimental system was developed using Tf as a model ligand. Tf was chosen since its binding interaction with the TfR, as well as its intracellular trafficking pathway, has been well defined. TfR are constitutively endocytosed via coated pits and delivered to sorting endosomes (Maxfield and Yamashiro, 1991; van Deurs et al., 1989). Within the sorting endosome recycling components such as Tf, LDL-R, and bulk-phase lipids are removed with a half time of about 3 min while lysosome-directed components such as LDL and α_2M are retained (Dunn et al., 1989; Stoorvogel et al., 1991; Mayor et al., 1993; Yamashiro and Maxfield, 1987).

Tf and other recycling components are delivered to a morphologically distinct recycling compartment composed of a network of small vesicles and tubular endosomes located in the pericentriolar region of CHO cells which are not physically connected to the sorting endosome (Dunn and Maxfield, 1992; Dunn et al., 1989; McGraw et al., 1993; Yamashiro et al., 1984). The recycling compartment has been shown to be functionally separate from the sorting endosome in that the recycling compartment maintains an average pH of 6.4 while the pH of the sorting endosome has been determined to be ~6.0 (Yamashiro et al., 1984; Presley et al., 1993). Furthermore, exit from the recycling compartment has been shown to be the rate limiting step in the recycling of both Tf and bulk membrane, having a half time of ~10–15 min (Mayor et al., 1993). Similar tubular recycling compartments have been observed in many cell types although their distribution within cells varies (Hopkins, 1983; Hopkins et al., 1990; Tooze and Hollinshead, 1991; Killisch et al., 1992; Ghosh et al., 1994; Ghosh and Maxfield, 1995). These recycling compartments are identified as part of the early endosome system since they contain recycling TfR and LDL receptors (McGraw et al., 1993). However, they are a distinct part of the early endosome system since they lack LDL or α_2M which are removed from their receptors in the acidic sorting endosome and retained there. Aside from recycling receptors, the protein composition of the membranes of these recycling compartments has not been determined.

We reasoned that any changes in intracellular trafficking induced by cross-linking the TfR would be evident when compared to the well-characterized Tf recycling pathway in CHO cells. In this paper we described the synthesis of a multivalent form of Tf, composed of an average of ten Tf molecules (Tf_{10}). We show that Tf_{10} was capable of cross-linking TfR. Tf_{10} was internalized about two times slower and was retained four times longer than Tf. The internalized Tf_{10} remained accessible to subsequently endocytosed Tf for at least 60 min demonstrating that the recycling compartment of CHO cells is a long-lived compartment. Finally, we show that the mechanism responsible for the retention of Tf_{10} does not require the cytoplasmic domain of the TfR.

Materials and Methods

Reagents

2-iminothiolane (2-IT), *m*-maleimidobenzoyl-*N*- hydroxysulfosuccinimide ester (sulfo-MBS), Amino Ethyl-8 Reagent, HRP, and DAB were purchased from Pierce Chemical (Rockford, IL). Fluorescein 5-isothiocyanate (isomer 1), and Slow Fade were purchased from Molecular Probes (Eugene, OR). Glutaraldehyde, osmium tetroxide, and EMbed 812 kits were purchased from Electron MIcroscopy Sciences (Fort Washington, PA). Cy3.18 was purchased from Biological Detection Systems (Pittsburgh, PA). Balanced salt solutions and other reagents used in the preparation of media were purchased from GIBCO BRL (Gaithersburg, MD). Chromatographic matrices were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). All other reagents were purchased from Sigma Chem. Co. (St. Louis, MO).

Ligand Preparation

Iron-saturated Tf was prepared from apo-Tf as described (Yamashiro et al., 1984) and further purified by passage over a S-300HR column (1.5 × 94 cm). Tf_{10} was prepared by the following procedure using iron-saturated Tf. Tf (10^{-3} M) was incubated with a 10-fold molar excess of 2-IT for 40 min at room temperature. The resulting thiolated-Tf was removed from excess 2-IT by passage over a PD-10 gel filtration column equilibrated in PBS. Concurrently, an equal amount of Tf was reacted with a 10-fold molar excess of sulfo-MBS for 60 min at room temperature. The sulfo-MBS–modified Tf was immediately mixed with the thiolated-Tf and allowed to react for 4 h at room temperature. Thiol-groups that did not form cross-links were blocked by incubation with Amino Ethyl-8 Reagent for 60 min at room temperature. The reaction products were size fractionated by passage over an S-300HR column (1.5 cm × 94 cm) equilibrated in PBS using

a FPLC equipped with a UV-1 monitor (Pharmacia LKB Biotechnology). Material that eluted between 60 and 80 ml of elution volume was designated as pool A, while that which eluted between 80 and 90 ml was designated pool B. Pool B was passed over the same S-300HR column, and material which eluted between 60 and 80 ml was pooled and designated as pool B_1. Pools A and B_1 were individually fractionated by passage over a S-400HR column (1.5 cm × 86 cm) which had been calibrated with Tf (79,570 D) and α_2-macroglobulin (β_2M, 820,000 D). Those fractions which eluted in the same fractions as α_2M were pooled and designated as Tf_{10}. The Tf_{10} used in all experiments, on average, is composed of 10 Tf monomers based upon hydrodynamic properties.

Fluorescein was incorporated into Tf_{10} using a previously published procedure (Yamashiro et al., 1984). 1 mg/ml of Tf_{10} was dialyzed against a 200-mg/ml solution of fluorescein 5-isothiocyanate in 50 mM borate pH 9.2 for 4 h at room temperature in the dark. The fluorescein-labeled Tf_{10} (F-Tf_{10}) was buffer exchanged to PBS by exhaustive dialysis.

Cy3-labeled Tf (Cy3-Tf) was prepared by dissolving an aliquot of Cy3.18 (the aliquot was prepared by the manufacturer and contains 80 nmol of reactive dye) in 750 ml of a 1.9-mg/ml solution of Tf in 50 mM borate, pH 9.2. The reaction was allowed to proceed for 30 min at room temperature. Excess Cy3.18 was removed by gel filtration on a PD-10 column equilibrated in PBS.

1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled LDL (DiI-LDL) was prepared according to the method of Pitas et al. (1981) and kindly provided by Drs. J. N. Myers and I. Tabas (Columbia University, NY).

Tf and Tf_{10} were iodinated using the chloramine T method as described (Yamashiro et al., 1984). The specific activities of ^{125}I-Tf and ^{125}I-Tf_{10} ranged between 200 and 800 cpm/ng.

Tf and Tf_{10} were conjugated to HRP using a modification of the multimer preparation protocol described above. Tf, 2.3×10^{-4} M in PBS, was incubated with a 10-fold molar excess of 2-IT for 40 min at room temperature. The excess 2-IT was removed by passage over a PD-10 column. Simultaneously, sulfo-MBS was added to a 10^{-3} M solution of HRP in PBS, such that there was a 10-fold excess of sulfo-MBS with respect to HRP, and incubated at room temperature for 1 h. The thiolated-Tf was added to a fivefold molar excess of MBS-HRP and allowed to react for 4 h at room temperature. HRP-Tf was isolated from free HRP and Tf by passage over a S-200HR column (1.5 × 38 cm). The mole ratio of HRP to Tf was determined using published molar absorbance coefficients at 280 nm, 403 nm, and 470 nm (de Jong et al., 1990; Paul, 1963). The HRP-Tf used in the experiments presented here had an HRP to Tf mole ratio of 0.6. A 2×10^{-5} M solution of Tf_{10} in 50 mM borate pH 9.2 was incubated with a 10-fold molar excess of sulfo-MBS for 60 min. HRP was thiolated using a 10-fold molar excess of 2-IT. The thiol-HRP was added to the MBS-Tf_{10} and incubated for 4 h. HRP-Tf_{10} was purified and assayed as above and found to have an HRP to Tf_{10} mole ratio of 0.5.

F-Tf_{10}-colloidal gold conjugates (Au-F-Tf_{10}) were prepared using 18-nm colloidal gold suspensions. The colloidal gold suspension was adjusted to pH 6.6 with 10 mM phosphate. 16 ml of the gold suspension was added to 12.8 ml of a F-Tf_{10} solution, composed of 6.25 μg/ml F-Tf_{10} and 18.75 μg/ml ovalbumin, and vortexed for 1 min. The mixture was rocked for 15 min at room temperature, brought to 2 mg/ml ovalbumin and then rocked for an additional 30 min to ensure that the gold particles were completely coated with protein. Based on data from De Roe et al. (1987), we estimate that these conditions limit the incorporation of F-Tf_{10} to approximately one per gold particle. The gold suspension was centrifuged at 4,000 *g* for 45 min at 4°C to concentrate the Au-F-Tf_{10}. The "loose" pellet of Au-F-Tf_{10} was harvested and dialyzed against 1 mg/ml ferric ammonium citrate, 50 mM $NaHCO_3$, and 20 mM Hepes, pH 7.9, for 45 min at room temperature to ensure that the Au-F-Tf_{10} was iron saturated. The Au-F-Tf_{10} was buffer exchanged to PBS containing 2 mg/ml ovalbumin by extensive dialysis. The Au-F-Tf_{10} was stored at 4°C and used within 48 h.

Cell Culture

All experiments were performed using either TRVb-1 or TRVbΔ3-59 cells. These cell lines are derived from the TRVb Chinese hamster ovary cell line, which lacks endogenous hamster Tf receptors (McGraw et al., 1987). TRVb-1 cells have been stably transfected with a wild-type human Tf receptor (McGraw et al., 1987). TRVbΔ3-59 cells were transfected with a mutant human Tf receptor which lacks amino acids 3 through 59 of the 61 amino acids which compose the cytoplasmic domain of the receptor (Johnson et al., 1993*a*). Both cell lines were grown in Ham's F-12 balanced salt solution with bicarbonate supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml G-418. Cells were plated on either 35-mm coverslip dishes (Salzman and Maxfield, 1989) or 6-well tissue culture dishes 3–4 d before an experiment so that on the day of the experiment, the cells were 50–80% confluent for light microscope experiments or 80–100% confluent for biochemical and electron microscopy experiments.

Surface Binding

The cells were washed three times with ice-cold McCoy's 5A medium with bicarbonate supplemented with 20 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM deferoxamine, pH 7.4 (McCoy's binding buffer), and kept on an ice bath throughout the duration of the experiment. 1 ml of the appropriate iodinated ligand in McCoy's binding buffer with 2 mg/ml ovalbumin (McCoy's binding buffer with ovalbumin) was added to each well. Nonspecific binding, determined in parallel wells containing the ^{125}I-ligand and a 100–200 weight excess of unlabeled Tf, never exceeded 10% of total binding in all experiments presented. The cells were incubated for 6 h on ice. Unbound material was removed with four washes of ice-cold medium 1 (150 mM NaCl, 20 mM Hepes, pH 7.45, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). The cells were solubilized with 1 N NaOH, and assayed for ^{125}I content using a LKB Clinigamma model 1272 gamma counter. Cells grown in parallel wells were trypsinized and counted.

Internalization

TRVb-1 cells were washed three times with McCoy's binding buffer at 37°C and incubated with either 3 μg/ml of ^{125}I-Tf or ^{125}I-Tf_{10} in McCoy's binding buffer with ovalbumin for varying amounts of time. Nonspecific binding was determined as before. The cells were washed three times with ice-cold medium 1, and surface-associated ^{125}I-Tf and ^{125}I- Tf_{10} were removed as before. The cells were solubilized with 1 N NaOH and assayed for ^{125}I content as above. ^{125}I contained in this fraction was designated as the internal fraction. In parallel dishes cells were incubated with either 3 μg/ml of ^{125}I-Tf or ^{125}I-Tf_{10} in McCoy's binding buffer with ovalbumin for 6 h on ice. The cells were assayed for ^{125}I content which was designated as the surface fraction.

Externalization

The cells were washed three times with McCoy's binding buffer at room temperature and incubated for 15 min at 37°C in 3 μg/ml of either ^{125}I-Tf or ^{125}I-Tf_{10} in McCoy's binding buffer with ovalbumin. Nonspecific binding was determined as above. Surface-associated Tf or Tf_{10} was removed by a wash with medium 1, followed by a 2-min incubation in mild acid wash buffer (50 mM citrate, 100 mM deferoxamine, 280 mM sucrose, pH 4.6), and three more washes with McCoy's binding buffer. The surface-stripped cells were incubated for varying amounts of time at 37°C. The supernatant was removed and assayed for radioactivity by gamma counting. The supernatant was placed on ice and brought to 1 mg/ml BSA and 10% TCA. The TCA-precipitated proteins were pelleted and assayed for radioactivity. The cells were solubilized with 1 N NaOH and assayed for radioactivity. The ^{125}I associated with the solubilized cells was designated as the cell-associated fraction, while ^{125}I associated with the TCA-precipitated proteins was designated as the released intact fraction. The release-degraded fraction was defined as the difference between the ^{125}I found in the original supernatant and that found in the released intact fraction.

Receptor Trapping

TRVb-1 cells were washed three times with McCoy's binding buffer and incubated with 60 mg/ml of either Tf or Tf_{10} for 2 h at 37°C in a 5% CO_2 incubator. The cells were washed four times with ice-cold medium 1. The chilled cells were incubated with 3 μg/ml ^{125}I-Tf for 14 h on ice to saturate surface receptors. In control experiments 80% of the surface-bound Tf_{10} or 90% of the surface-bound Tf was exchanged under these conditions. Excess unbound ^{125}I-Tf was removed by washing four times with ice-cold medium 1.

Fluorescence Microscopy

TRVb-1 cells were washed three times with Ham's F-12 balanced salt solution with bicarbonate, supplemented with 5 mM Hepes, 100 μM deferoxamine, and 2 mg/ml ovalbumin, pH 7.4 (Ham's binding buffer). The washed cells were incubated in 120 μl of Ham's binding buffer containing

the appropriate fluorescently labeled protein in a 5% CO_2 incubator at 37°C. The cells were washed four times and incubated at 37°C in Ham's binding buffer. The chase medium was removed, and the cells were placed on an ice bath. The cells were washed three times with ice-cold medium 1. Any cell surface Tf or Tf_{10} was removed by a mild acid wash for 2 min on ice and washed three times with ice-cold medium 1. The cells were fixed with 2% formaldehyde for 3 min and rinsed four times with medium 1. The cells were covered in Slow Fade before digital fluorescence microscopy. Images were collected using a Photometrics cooled charge coupled device (CCD) detector mounted on a Leitz Diavert microscope with a 63× objective lens as described (Mayor et al., 1993).

Electron Microscopy

TRVb-1 cells were grown to confluence on coverslip dishes above. Cells were incubated with either HRP-Tf or HRP- Tf_{10} for 15 min at 37°C in a 5% CO_2 incubator, washed four times with Ham's binding buffer, and chased for three minutes. In dual-labeling experiments, cells were incubated with Au-F-Tf_{10} for 10 min, washed, and chased for 60 min. During the last 10 min of the chase, the cells were then incubated in HRP-Tf for 8 min, washed four times with Ham's binding buffer, and chased for 2 min. Regardless of treatment, all cells were washed four times with medium 1, fixed with 1% glutaraldehyde for 60 min at room temperature, and washed four times in medium 1. A solution containing 2.5 mg/ml DAB and 0.09% H_2O_2 in 100 mM cacodylate buffer, pH 7.4, was added for 30 min at room temperature. The cells were washed four times in 100 mM cacodylate buffer, pH 7.4, stained with 1% osmium tetroxide in 100 mM cacodylate buffer, pH 7.4, for 1 h at room temperature, and washed twice with distilled water. Cells were dehydrated by sequential 5-min incubations in 50, 75, 95%, and twice in 100% ethanol. The ethanol was removed and replaced with 1:1 mixture of ethanol and EMbed 812 and incubated overnight at room temperature. The samples were overlaid with fresh EMbed 812, degassed for 1.5 h, and baked for 72 h at 56°C. Cells were sectioned parallel to the plane of the monolayer. 500-nm sections were observed using a Phillips 400 transmission electron microscope operating at 120 KeV. For colocalization studies, 90-nm sections were stained at room temperature with a saturated aqueous solution of uranyl acetate for 15 min and with a 0.1% aqueous solution of lead citrate for 3 min. Sections were observed using a JEOL 1200EX operating at 80 KeV.

Internalized gold particles were scored for intracellular localization using the following categories: (*1*) endocytic recycling compartment: DAB-positive tubules or vesicles less than 100-nm in diameter, (*2*) early sorting endosomes: DAB-positive vesicles greater than 100 nm in diameter containing irregular, particulate electron dense material, (*3*) lysosomes: vesicles greater than 100 nm in diameter containing a relatively uniform, granular, electron-dense substance, and (*4*) other organelles: DAB-negative, membrane-bounded organelles. Sampling errors, possibly including relative geometry of organelles in the plane of the plastic section and/or variability in DAB staining, precluded positive assignment of compartment identity for 13% of internalized gold particles which were deleted from the localization analysis. Furthermore, Neutra et al. (1985) observed that clusters of gold particles were directed to lysosomes. Accordingly, we restricted our analysis to unclustered gold particles (less than three gold particles within 100 nm). Unclustered gold particles outnumbered gold particle aggregates ~4:1.

Results

Characterization of Multivalent-Transferrin

A family of multivalent-Tf was prepared by the chemical cross-linking of Tf. Unreacted Tf and small multimer-Tf, those composed of fewer than 10 Tf molecules, were removed by gel filtration chromatography. The interaction of the very large Tf-multimers with cells was assayed. Our preliminary findings demonstrated that the Tf-multimer bound the surface TfR in a saturable and competable fashion (data not shown). However, the Tf-multimers appeared to have two distinct fates. Approximately 30% of the Tf-multimers were degraded over a 3-h chase (data not shown). The majority of the Tf-multimer, however, was released intact from the cells albeit with altered kinetics with respect to native Tf. These results indicated that grossly cross-linked TfR are to some extent redirected to degradative lysosome-like compartments by a poorly described mechanism. We chose to focus our additional efforts upon the intracellular trafficking of the major fraction of multivalent-Tf which was recycled intact through the TRVb-1 cells with altered kinetics.

To further investigate the mechanism by which multivalent-Tf was retained, yet not degraded, it was reasoned that a more homogeneous preparation of Tf multimer composed of ~10 Tf molecules would be needed. Therefore, we further size fractionated the cross-linked Tf to purify a relatively homogeneous preparation of Tf_{10} whose average size was approximately the same as α_2M (820,000 D) as determined by elution from a S-400HR sizing column.

For Tf_{10} to be a useful probe it was necessary to demonstrate that Tf_{10} retained the physiological properties of Tf: release of iron when exposed to acidic conditions, specific binding to the TfR, and pH-dependent and iron-dependent binding to the TfR. Upon exposure to acidic pH, both Tf and Tf_{10} released comparable amounts of iron which was assayed by a decrease in absorbance at 470 nm (de Jong et al., 1990, data not shown). The release of apo-Tf from TfR upon exposure to neutral pH was measured. TRVb-1 cells were incubated with either ^{125}I-Tf or ^{125}I-Tf_{10} on ice. The cells were washed and incubated in a mild acid wash (pH 4.8) for 2 min. The acid wash was assayed for each ligand and found to contain less than 10% of the originally bound ligand. The acid-washed cells were then incubated in McCoy's binding buffer (pH 7.4). The McCoy's binding buffer was removed and assayed for each ligand. Greater than 90% of each ligand was released by the McCoy's binding buffer (data not shown), showing that the pH regulation of iron binding and receptor interaction for Tf_{10} are similar to Tf.

Multivalent-Transferrin Binds Multiple Transferrin Receptors

The valency of the interaction of Tf_{10} with TfR was investigated by incubating TRVb-1 cells on ice for 6 h with graded doses of either ^{125}I-Tf or ^{125}I-Tf_{10}. TRVb-1 cells bound native Tf saturably (Fig. 1 *A*). LIGAND, a ligand-binding data analysis program (Munson and Rodbard, 1980), was used to estimate a kD of 0.7 nM and 1.1×10^5 binding sites per cell, in agreement with previously reported values (McGraw et al., 1987). Tf_{10} also specifically bound the TfR of TRVb-1 cells (Fig. 1 *B*), reaching saturation at ~30 fmol of Tf_{10} per 10^6 cells, significantly less than the 180 fmol per 10^6 cells expected if Tf_{10} bound the TfR monovalently in a manner similar to Tf. This sixfold reduction in the number of particles bound at saturation suggests that each Tf_{10} particle bound approximately six TfR. The Tf_{10} binding data were analyzed using LIGAND, which estimated a kD of 0.2 nM if a single affinity was assumed. The approximately threefold increase in binding affinity of Tf_{10} compared to Tf is consistent with multivalent binding. However, the estimation of the binding affinity of Tf_{10} is complicated by an approximately fivefold reduction in the binding affinity of the MBS-Tf used in the preparation of Tf_{10} (data not shown), which would par-

tially mask the enhancement of avidity resulting from multivalent binding. The thiolation of Tf was without significant effect on the binding interaction (data not shown). Since the intrinsic affinity of MBS-Tf is less than the affinity of Tf, the increase in binding avidity as a result of multimerization is actually greater than threefold. Although methods are available to carry out a detailed analysis of multivalent binding (Perelson, 1984), we did not attempt this since the multimers were a somewhat heterogeneous mixture. Such an analysis would be very complex and difficult to interpret since the intrinsic affinity of each Tf in the multimer may vary. Despite this limitation in the analysis, the increase in affinity and the decrease in molecules bound at saturation are both strongly indicative of multivalent binding at 0°C.

To determine if Tf_{10} also formed multivalent interactions at 37°C, TRVb-1 cells were incubated with graded doses of either ^{125}I-Tf or ^{125}I-Tf_{10} for 30 min at 37°C. Control experiments showed that uptake of both ^{125}I-Tf and ^{125}I-Tf_{10} was specific and reached saturation. Analysis of this data, using 79,570 D and 795,700 D as the estimated molecular weights of Tf and Tf_{10}, respectively, revealed a 4–8-fold reduction in the number of moles of Tf_{10} which were cell associated at saturation as compared to that of Tf (Fig. 2 *A*). To confirm that the reduced Tf_{10} uptake reflected multivalent binding, the ability of Tf_{10} to compete with ^{125}I-Tf for TfR binding was compared to the ability of Tf to compete with ^{125}I-Tf for TfR binding. We found Tf_{10} and Tf to be nearly equivalent competitors of ^{125}I-Tf binding at 37°C (Fig. 2 *B*). Comparing Fig. 2 *A* to Fig. 2 *B*, it can be seen that under conditions where Tf and Tf_{10} displace an equivalent number of ^{125}I-Tf molecules (Fig. 2 *B*), there were four to eight times less Tf_{10} cell associated as compared to Tf on a molar basis (Fig. 2 *A*), indicating that

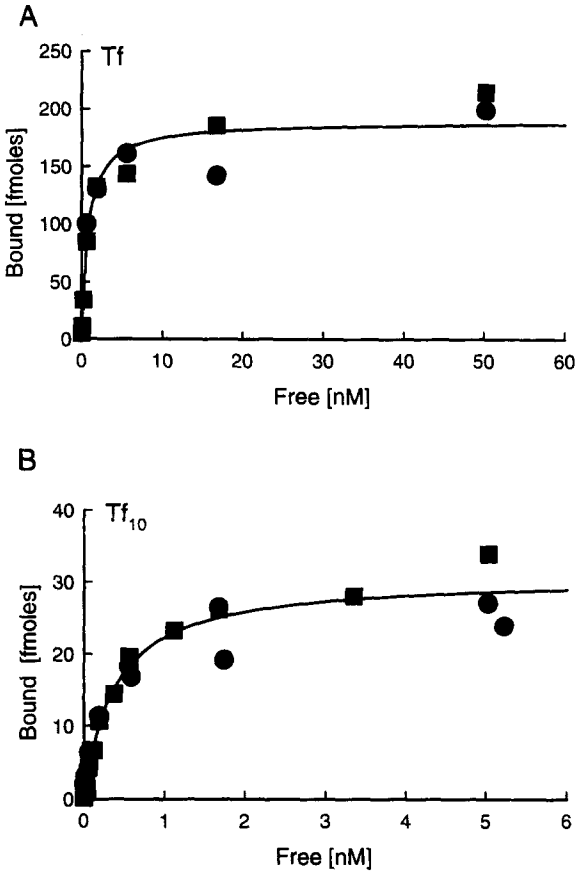

Figure 1. Multivalent-transferrin binds the transferrin receptor. TRVb-1 cells were labeled with 3 μg/ml ^{125}I-Tf (*A*) or ^{125}I-Tf_{10} (*B*) and then incubated for 6 h on ice. Unbound ligand was removed with five washes of McCoy's binding buffer with ovalbumin. The cells were solubilized with 1 N NaOH, and assayed for ^{125}I content by gamma counting. Nonspecific binding was determined as described. The data presented are from two experiments (*squares and circles*) and have been normalized to 10^6 cells/well. The amount of specific binding was calculated as the difference between total and nonspecific binding. Molar concentrations were calculated using 79,570 and 795,700 D as the molecular masses of Tf and Tf_{10}, respectively. The data have been fit to a single site hyperbolic saturation curve using Multifit, a binding data analysis program (McPherson, 1983, 1985).

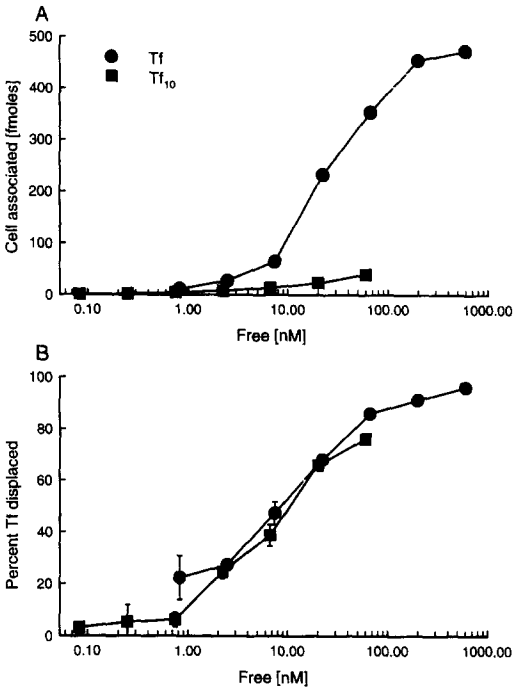

Figure 2. Multivalent-transferrin cross-links multiple transferrin receptors. TRVb-1 cells were plated as described in Materials and Methods. (*A*) The cells were washed three times with McCoy's binding buffer at room temperature. TRVb-1 cells were incubated with graded doses of ^{125}I-Tf (*circles*) or ^{125}I-Tf_{10} (*squares*) in McCoy's binding buffer with ovalbumin for 30 min at 37°C in a 5% CO_2 incubator. The cells were washed four times with McCoy's binding buffer, solubilized with 1 N NaOH, and assayed for ^{125}I content. The data presented are from a representative experiment and have been normalized to 10^6 cells/well. (*B*) Cells were plated and treated as in *A* except the cells were incubated with 70 ng/ml ^{125}I-Tf and graded doses of either unlabeled Tf (*circles*) or unlabeled Tf_{10} (*squares*). The amount of Tf displaced is the difference between the amount of ^{125}I-Tf bound in the absence of competitor and the amount of ^{125}I-Tf bound at a given dose of competitor. The percent of Tf displaced is the amount of Tf displaced divided by the amount of ^{125}I-Tf bound in the absence of competitor multiplied by 100. The standard deviation for each data point is given by the error bar.

each particle of Tf_{10} must be displacing several ^{125}I-Tf particles and by inference binding several TfR.

Multivalent-Transferrin Is Retained by TRVb-1 Cells but Not Degraded

To determine the rate at which Tf_{10} was internalized, TRVb-1 cells were incubated with ^{125}I-Tf or ^{125}I-Tf_{10} for various times at 37°C. Surface-bound ligand was removed by a mild acid wash. The amount of internalized ligand which remained after the mild acid wash was measured. The total surface binding was determined as above. The internal to surface ratio was calculated and was plotted with respect to time (McGraw and Maxfield, 1990). The internalization rate was derived from this plot (data not shown). In agreement with earlier results (McGraw and Maxfield, 1990) Tf was internalized at a rate of 0.21 min^{-1}. Tf_{10} was internalized about two times slower than Tf, at a rate of 0.09 min^{-1} (data not shown).

To compare recycling kinetics and externalization rates of Tf and Tf_{10}, TRVb-1 cells were incubated with either ^{125}I- Tf_{10} or ^{125}I-Tf for 15 min at 37°C. Surface-bound ligand was removed by a mild acid wash, and the cells were incubated at 37°C for various chase times. In agreement with previously published values (McGraw and Maxfield, 1990), Tf was released intact by the TRVb-1 cells with a half-time of ~15 min (Fig. 3 *A*). Tf_{10}, on the other hand, was released by TRVb-1 cells with a half-time of ~60 min suggesting that the Tf_{10} is trapped in the endosomal system or redirected out of the recycling pathway (Fig. 3 *B*). A small fraction of the Tf_{10} may be redirected to the lysosome, since up to five percent of the Tf_{10} was degraded (Fig. 3 *B*). While the majority of Tf_{10} was not degraded to TCA soluble fragments the possibility existed that partial proteolysis could be occurring resulting in large fragments that would be TCA precipitable. To address this, TRVb-1 cells were pulsed with ^{125}I-Tf_{10} and chased for 120 min. The supernatant from these cells was passed over a S-400HR column. The elution profile of the supernatant was indistinguishable from that of a control sample of Tf_{10} (data not shown). Thus, the extended residency of the majority of Tf_{10} within the TRVb-1 cells did not result in its degradation.

Multivalent-Transferrin Retention Is Not a Result of Recycling

The retention of Tf_{10} could have been caused if Tf_{10} were inefficiently released from the TfR after returning to the cell surface. For example, if the rate of receptor-ligand dissociation was slow in comparison to the internalization rate of the receptor, the receptor bound Tf_{10} would be reinternalized and persistently recycle. If recycling of occupied receptors occurred, a pool of surface-bound ligand would appear during externalization. TRVb-1 cells were incubated with 3 μg/ml of either ^{125}I-Tf or ^{125}I-Tf_{10} for 15 min. Surface-bound ligand was removed by a mild acid wash. The surface-cleared cells were chased for varying amounts of time, and the supernatant was removed. The cells were incubated again in a mild acid wash to release any ligand that had returned to the surface and remained bound. Radioactivity released by the second acid wash was used as a measure of recycling. No more than 1–2% of the original cell associated ^{125}I-Tf was contained in the second acid wash. While the amount of ^{125}I-Tf_{10} found in the second acid wash was approximately twice that of Tf, it never exceeded 4% of the originally bound ^{125}I-Tf_{10}. The small amount of surface Tf and Tf_{10} decreased with time (data not shown). The higher amount of Tf_{10} on the cell surface may be accounted for by the twofold decrease in the internalization rate of Tf_{10} as compared to Tf. These data indicated that the observed retention of Tf_{10} is not a result of an elevated level of recycling of Tf_{10} without release at the surface.

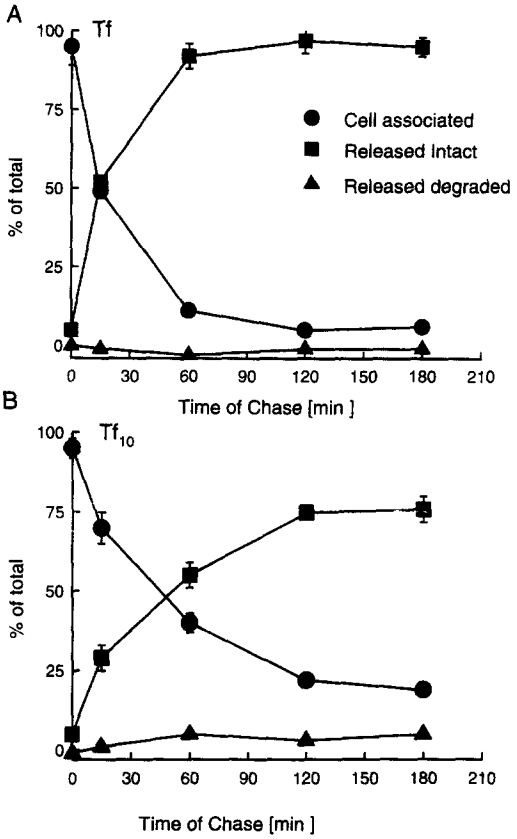

Figure 3. Multivalent-transferrin is retained by TRVb-1 cells four times longer than transferrin. TRVb-1 cells were washed three times with McCoy's binding buffer at room temperature. The cells were incubated for 15 min at 37°C in 3 μg/ml of either ^{125}I-Tf (*A*) or ^{125}I-Tf_{10} (*B*) in McCoy's binding buffer with ovalbumin. Nonspecific binding was determined as before. Surface associated Tf or Tf_{10} was removed by a 2-min mild acid wash as described in Materials and Methods. The surface-stripped cells were incubated for varying amounts of time at 37°C. The supernatant from each sample was removed, assayed for ^{125}I content and brought to 10% TCA. The resulting precipitate was assayed for ^{125}I content and defined as the released intact fraction (*squares*). The relased degraded fraction (*triangles*) was defined as the difference between the ^{125}I found in the original supernatant and that found in the precipitate. The cells were solubilized with 1 N NaOH, assayed for ^{125}I content and designated as cell associated fraction (*circles*). The data are reported as a percentage of the sum of three fractions (*Cell associated, Released Intact,* and *Released degraded*) at each time point. The sum of three fractions varied less than 5% between time points. The data presented are from a representative experiment. Error bars give the standard deviation for each data point.

Multivalent-Transferrin Is Sorted from LDL in Sorting Endosomes, but Is Retained in the Recycling Compartment

To determine if Tf_{10} was rerouted from the Tf recycling pathway to the lysosomal pathway followed by LDL, TRVb-1 cells were allowed to cointernalize F-Tf_{10} and DiI-LDL for 2 min. The cells were washed and further incubated in the absence of both proteins for either 2 or 10 min. Previous studies (Dunn et al., 1989; Dunn and Maxfield, 1992; Mayor and Maxfield, 1993) have demonstrated that TRVb-1 cells cointernalize LDL and Tf to common sorting endosomes. The LDL and Tf rapidly sort from each other, with Tf being delivered to the pericentriolar recycling compartment and LDL retained in sorting endosomes that mature into late endosomes. If the multivalent binding of Tf_{10} caused Tf_{10} to be delivered into the late endosomes, this would be demonstrated by extensive colocalization of LDL and Tf_{10} after 10 min of chase. TRVb-1 cells were incubated with F-Tf_{10} and DiI-LDL for

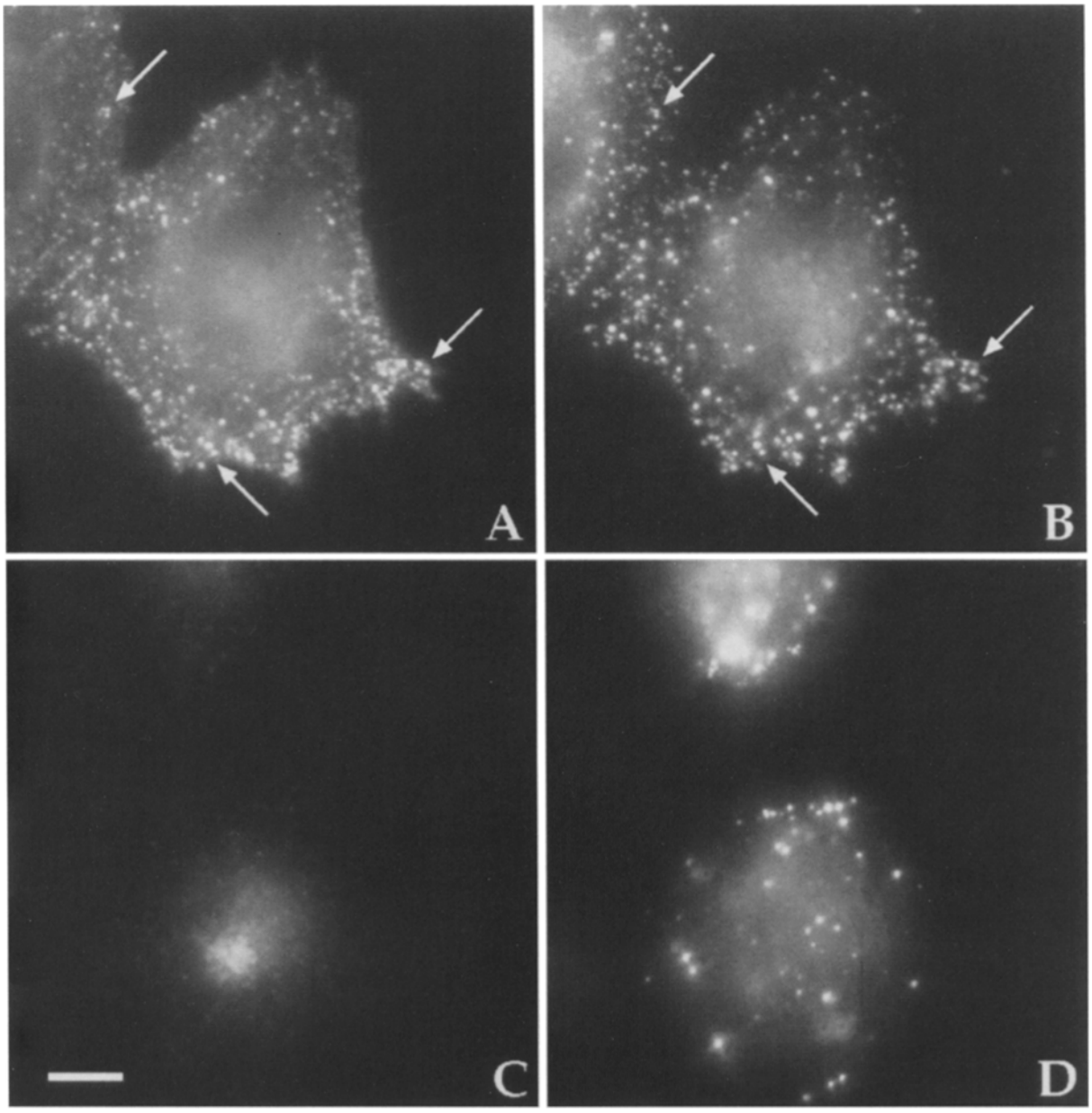

Figure 4. Multivalent-transferrin is sorted from LDL. TRVb-1 cells were grown on coverslip dishes and incubated for 2 min in 40 μg/ml F-Tf_{10} and 20 μg/ml DiI-LDL. The cells were washed four times in Ham's binding buffer and chased for either 2 min (*A* and *B*), or 10 min (*C* and *D*) at 37°C. The TRVb-1 cells were then prepared for microscopy as described in the Materials and Methods section. The focal plane was selected to optimize the F-Tf_{10} image, and the DiI- LDL image was then collected without further adjustment to focus. F-Tf_{10} images (*A* and *C*) were collected and photographed using identical settings. DiI-LDL images (*B* and *D*) were also collected and photographed using identical settings. Arrows provide examples of colocalization. Since there was a great range in the intensity of LDL-containing structures, some dim LDL spots have been lost in the presentation of *B*, and this reduces the apparent extent of initial colocalization with Tf. Bar, 10 μm.

2 min at 37°C and chased for 2 min. As shown in Fig. 4 *A* and *B*, Tf_{10} like Tf (Dunn et al., 1989; Mayor et al., 1993) is rapidly delivered to sorting endosomes which also contain LDL. Parallel cells were pulsed for 2 min as above and chased for 10 min to observe the sorting of Tf_{10} from the LDL. AFter the 10-min chase, DiI-LDL was located in widely dispersed punctate vesicles characteristic of sorting endosomes and late endosomes as previously described (Dunn et al., 1989, Fig. 4 *D*), while the majority of F-Tf_{10} was localized in the pericentriolar recycling compartment of the cell (Fig. 4 *C*). Thus, the majority of Tf_{10} was not retained in sorting endosomes or delivered to late endosomes (Fig. 4, *C* and *D*).

Since the majority of Tf_{10} sorts from lysosomally directed molecules similar to Tf, we investigated the possibility that Tf_{10} retention occurred later in the recycling pathway. TRVb-1 cells were incubated with F-Tf_{10} for 10 min at 37°C, washed, and chased for a total of 60 min. At 56, 48, or 28 min of F-Tf_{10} chase, Cy3-Tf was added for a 2-min incubation and chased for 2, 10, or 30 min, respec-

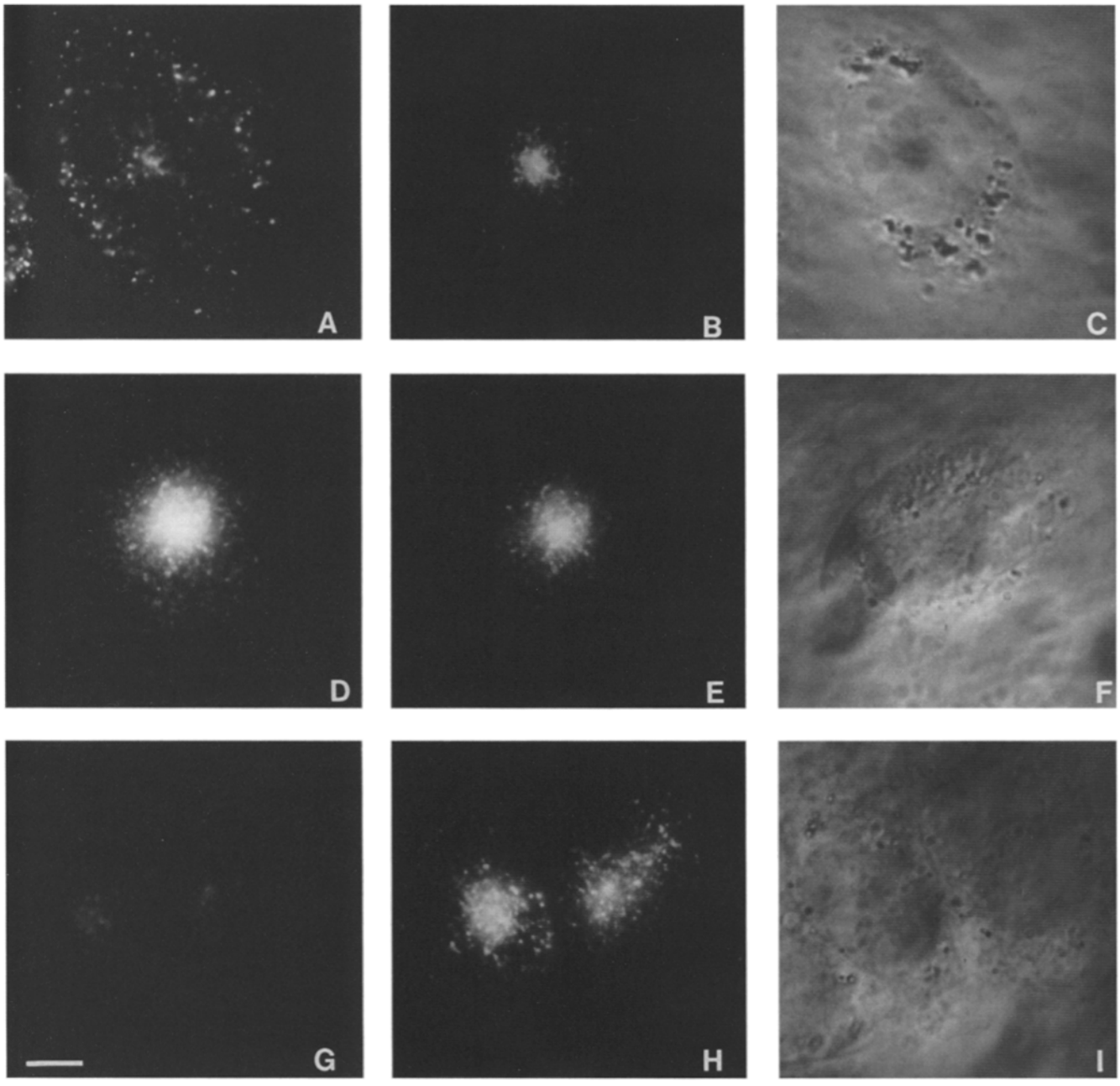

Figure 5. Multivalent-transferrin is localized in the recycling compartment of TRVb-1 cells. TRVb-1 cells were incubated in 40 μg/ml F-Tf_{10} for 10 min and then chased for a total of 60 min. At 56, 38, and 23 min of the chase, the medium was replaced with 20 μg/ml Cy3-Tf for 2 min. The cells were washed and chased for 2 (*A*, *B*, and *C*), 10 (*D*, *E*, and *F*), or 30 (*G*, *H*, and *I*) min, respectively. The cells were prepared for microscopy as described. The focal plane was selected to optimize the F-Tf_{10} image, and the Cy3-Tf image was then collected without further adjustment to focus. Cy3-Tf images (*A*, *D*, and *G*) were collected and photographed using identical settings. F-Tf_{10} images (*B*, *E*, and *H*) were collected and photographed using identical settings. Phase-contrast images are presented in *C*, *F*, and *I*. Bar, 10 μm.

tively. After 2 min the majority of Cy3-Tf was localized in small widely dispersed sorting endosomes (Fig. 5 *A*). By 10 min, most of the Cy3-Tf had cleared the sorting endosomes and was found primarily in the recycling compartment where it extensively colocalized with F-Tf_{10} showing that the majority of the retained F-Tf_{10} is localized in the recycling compartment (Fig. 5, *D* and *E*). After 30 min of chase the TRVb-1 cells contained barely detectable amounts of Cy3-Tf in the recycling compartment (Fig. 5, *G* and *H*). The exit of Cy3-Tf is similar to that obtained in the absence of Tf_{10} and suggests that Tf_{10} in the recycling compartment did not have a significant effect on the trafficking of subsequently added Cy3-Tf.

To observe the fine structure of the Tf_{10} containing recycling compartment, TRVb-1 cells were labeled with either HRP-Tf for HRP-Tf_{10} for 15 min at 37°C, washed, chased for 3 min, and visualized by transmission electron microscopy. DAB staining of HRP-Tf–treated TRVb-1 cells was localized by the recycling compartment, a complex network of small tubular and vesicular elements near the centrioles of the cells, and sorting endosomes, larger vesicles having granular contents that were DAB stained (Fig. 6 *A*). Sorting endosomes were observed in close proximity to the recycling compartment (Fig. 6 *A*) and throughout the periphery of the cell (Fig. 6 *A*, *inset*). This observation was in agreement with previously published data (Yamashiro et al., 1984). HRP-Tf_{10}–labeled cells contained DAB-stained vesicles and tubules similar to those of HRP-Tf–labeled cells (Fig. 6 *B*). The DAB staining of the HRP-Tf_{10}–labeled cells was not as intense as the HRP-Tf–labeled cells, since both HRP-Tf_{10} and HRP-Tf had similar molar specific activities (see Materials and Methods) but less Tf_{10} was internalized by TRVb-1 cells than Tf under comparable conditions (Fig. 2).

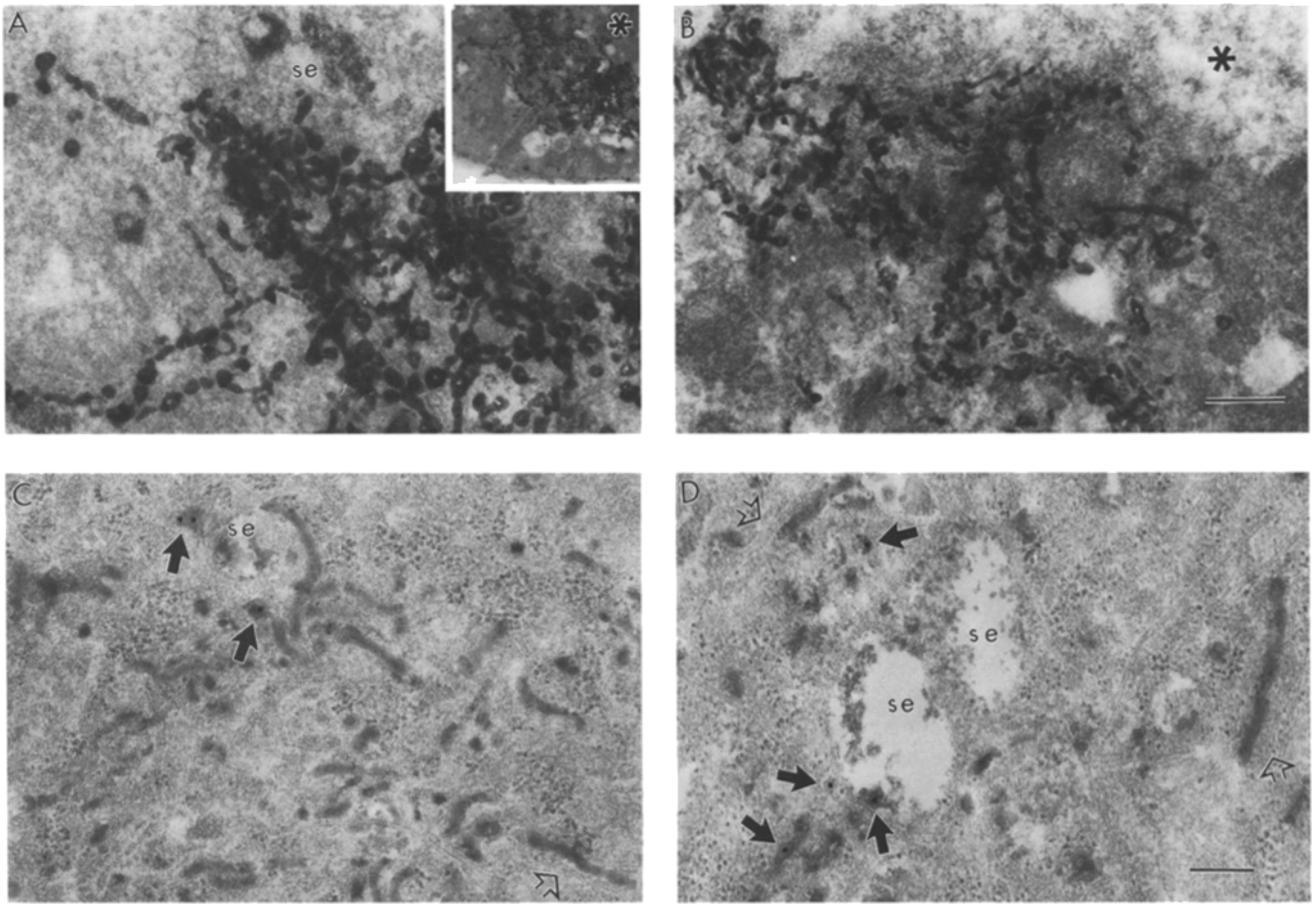

Figure 6. Endocytosed transferrin and multivalent-transferrin colocalize in pericentriolar tubules and vesicles. TRVb-1 cells were treated with HRP-Tf (*A*) or HRP- Tf_{10} (*B*) as described in Materials and Methods. After fixation, probes were localized by DAB staining, and samples were embedded in epoxy resin. Transmission electron microscopy of 500 nm sections revealed a complex network of tubular and vesicular membrane profiles adjacent to the nucleus (*asterisk*), which corresponds to the recycling compartment (compare *A*, *inset* with Fig. 5, *D* and *E*). To determine whether Tf and Tf_{10} occupied the same compartment or morphologically similar but functionally distinct compartments, TRVb-1 cells were incubated for 10 min with Au-F-Tf_{10} and chased for a total of 60 min. During the last 10 min of chase HRP-Tf was added for 8 min and chased for 2 min. After fixation HRP-Tf was localized by DAB staining and samples were embedded in epoxy resin. Transmission electron microscopy of 90-nm sections demonstrated that HRP-Tf occupied tubular and vesicular membrane profiles of the recycling compartment with diameters ranging from 50 to 75 nm (*C* and *D*). Compartments were often located along microtubules (*open arrow*). HRP-Tf was also found in sorting endosomes (*se*) visualized as 100–300-nm organelles with granular contents. Au-F-Tf_{10} particles (*filled arrow*) were observed in DAB-stained recycling compartments demonstrating that Tf and Tf_{10} occupy the same compartments. Bars: (*A* and *B*) 500 nm; (*C* and *D*) 200 nm.

To directly demonstrate that Tf_{10} was retained in Tf containing compartments and not in adjacent compartments, TRVb-1 cells were labeled with Au-F-Tf_{10} for 10 min at 37°C, and chased for 60 min. During the last 10 min of the chase the cells were labeled with an 8-min pulse of HRP-Tf, followed by a 2-min chase. The cells were then fixed, DAB stained, and visualized by transmission electron microscopy. This protocol allowed us to demonstrate the colocalization of Tf_{10} and Tf in the recycling compartment. Neutra et al. (1985) have reported the Au-Tf conjugates may be redirected to the lysosomal pathway. To ensure that our preparations of Au-F-Tf_{10} were not being redirected to the lysosomes, parallel samples were analyzed using fluorescence microscopy. TRVb-1 cells incubated with either Au-F-Tf_{10} or F-Tf_{10} for 10 min and chased for 60 min were found to have a staining pattern consistent with recycling compartment localization, indicating that the conjugation of F-Tf_{10} to the colloidal gold had not grossly altered the localization of the conjugated F-Tf_{10} (data not shown). Furthermore, the Au-F-Tf_{10} was effectively competed by excess unlabeled Tf.

Analysis of samples from three independent experiments indicated that 62% of internalized, unclustered gold particles were found in association with DAB-stained compartments, indicating a good agreement between the localization of Tf (marked by DAB) and Tf_{10} (marked by colloidal gold) at the ultrastructural level (Fig. 6, *C* and *D*; see Materials and Methods for analysis parameters). Gold particles were observed in both larger diameter vesicles indicative of sorting endosomes as well as small diameter tubules and vesicles of the recycling compartment. Frequently, tubular elements of the recycling compartment were aligned along microtubules (Fig. 6, *C* and *D*), consistent with the observation that recycling compartment morphology is disrupted by treatment nocodazole (McGraw et al., 1993).

Intracellular Multivalent-Transferrin Retention Reduces the Number of Transferrin Receptors on the Cell Surface

We expected that retention of Tf_{10} in the recycling compartment resulted from interactions with multiple TfR within the recycling compartment. If Tf_{10} remains receptor-bound within the cell, it would be expected that Tf_{10}-treated cells would have fewer surface receptors at steady state. To test this, TRVb-1 cells were incubated with 60 μg/ml of either Tf or Tf_{10} for 2 h at 37°C and washed. The cells were rapidly chilled to 4°C and incubated with 3 μg/ml ^{125}I-Tf for 14 h on ice to allow for surface exchange of Tf and Tf_{10} with ^{125}I-Tf. The amount of ^{125}I associated with TRVb-1 cells that were preincubated with Tf-free medium was defined as 100% (Fig. 7). Cells preincubated with Tf bound an equivalent amount of ^{125}I-Tf, confirming that the surface exchange was complete after 14 h (Fig. 7). Preincubation with Tf_{10} resulted in a 30–40% decrease in the amount of ^{125}I-Tf bound, an amount consistent with fourfold decrease in externalization rate (Fig. 3) and a twofold decrease in the rate of internalization (data not shown). These results confirm that Tf_{10} causes intracellular retention of TfR and that slow release of Tf_{10} from cells is not due to poor release of Tf_{10} from recycled receptors at the cell surface.

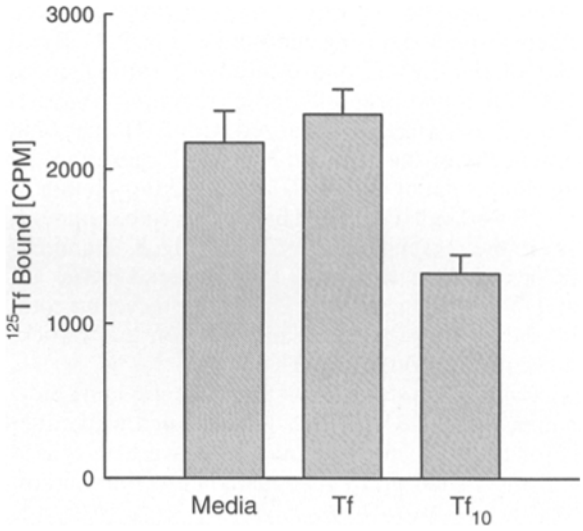

Figure 7. Multivalent-transferrin retains transferrin receptors within the recycling compartment. TRVb-1 cells were washed three times with McCoy's binding buffer and incubated with either McCoy's binding buffer with ovalbumin, 60 μg/ml Tf or 60 μg/ml Tf_{10} for 2 h at 37°C in a 5% CO_2 incubator. The cells were washed four times with ice-cold medium 1 and maintained on ice throughout the remainder of the experiment. ^{125}I-Tf (3 μg/ml) was added to each well. The cells were incubated for 14 h to allow for surface ligand exchange. Nonspecific binding was determined as before. The cells were washed four times with ice-cold medium 1, solubilized, and assayed for ^{125}I content. Error bars give the standard deviation.

The Cytoplasmic Domain of the Transferrin Receptor Is Not Involved in Multivalent Transferrin Retention

To determine if the cytoplasmic domain of the TfR contributed to the retention of Tf_{10} in the recycling compartment, we employed TRVbΔ3-59 cells which express an altered form of TfR that contains only four of the 61 amino acids that comprise the cytoplasmic domain of the receptor (Johnson et al., 1993*a*). Although these cells internalize Tf slowly, they externalize Tf at a rate comparable to that of the TRVb-1 cells (Johnson et al., 1993*a*). If the cytoplasmic domain were required for the retention of Tf_{10}, it would be expected that the TRVbΔ3-59 cells would efflux Tf_{10} at the same rate as Tf.

TRVbΔ3-59 cells internalized Tf_{10} with a rate of 0.07 min^{-1} (data not shown). To measure the rate of recycling, TRVbΔ3-59 cells were incubated with either ^{125}I-Tf or ^{125}I-Tf_{10}, washed and allowed to externalize the labeled ligand. TRVbΔ3-59 cells externalize ^{125}I-Tf with a half-time of ~15 min, but ^{125}I-Tf_{10} was released with a half-time of 60 min (Fig. 8). To demonstrate that in TRVbΔ3-59 cells, like TRVb-1 cells, Tf_{10} is retained in the recycling compartment without affecting Tf trafficking, a protocol similar to that described in Fig. 5 was used. To accommodate the slower internalization times of Tf and Tf_{10} in TRVbΔ3-59 cells, longer incubation times were used for labeling cells. TRVbΔ3-59 cells were incubated with F-Tf_{10} for 50 min at 37°C, washed, and chased for a total of 60 min. At 48, 38, or 18 min of F-Tf_{10} chase, Cy3-Tf was added for a 10-min incubation and chased for 2, 10, or 30 min, respectively. Similar to Fig. 5, much of the Cy3-Tf was localized in

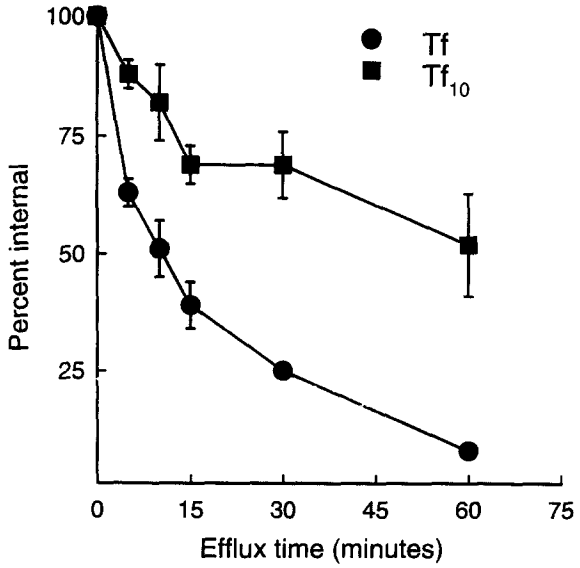

Figure 8. The cytoplasmic domain of the transferrin receptor is not involved in the retention of multivalent-transferrin. TRVbΔ3-59 cells were plated as described. The cells were washed three times with McCoy's binding buffer at room temperature. The cells were incubated for 15 min at 37°C in 3 μg/ml of either ^{124}I-Tf (*circles*) or ^{125}I-Tf_{10} (*squares*) in McCoy's binding buffer with ovalbumin. Nonspecific binding was determined as before. Surface-associated Tf or Tf_{10} was removed as described in Materials and Methods. The surface-stripped cells were incubated for varying amounts of time of 37°C. The supernatant was removed and the cells were solubilized with 1 N NaOH. The amount of cell-associated ^{125}I was determined. The data presented are the mean values of three experiments. The amount of ^{125}I-Tf or ^{125}I-Tf_{10} contained in the time zero sample is defined as 100% bound. Error bars give the standard deviation.

small, widely dispersed sorting endosomes after a 2-min chase (Fig. 9 *A*) while some Cy3-Tf was found in the recycling compartment marked by F-Tf_{10} (Fig. 9 *B*). After a 10-min chase of internalized Cy3-Tf, the majority of monomeric Tf colocalized with F-Tf_{10} in the recycling compartment (Fig. 9, *D* and *E*). The intensity of Cy3-Tf staining was significantly reduced in the recycling compartment after a 30-min chase relative to F-Tf_{10} which had been chased for 60 min (Fig. 9, *G* and *H*). Consistent with the results in TRVb-1 cells, F-Tf_{10} was retained in the recycling compartment of TRVbΔ3-59. Furthermore, passage of subsequently added Cy3-Tf was unhindered by the presence of F-Tf_{10}. The comparable rates of Tf_{10} efflux in TRVb-1 and TRVbΔ3-59 cells show that the cytoplasmic domain of TfR is not required for the retention of Tf_{10} in the recycling compartment.

Discussion

The Recycling Compartment Is a Long-lived Fusion Accessible Organelle

The intracellular trafficking of endocytosed proteins is mediated by organelles including coated vesicles, sorting endosomes, recycling endosomes, and late endosomes (Maxfield and Yamashiro, 1991; van Deurs et al., 1989). Within the sorting endosome, recycling components such as Tf, LDL-R, and bulk-phase lipids are rapidly sorted from lysosome-directed components like LDL and α_2M with a half time of ~2–3 min (Stoorvogel et al., 1991; Mayor et al., 1993; Yamashiro and Maxfield, 1987). The recycling components are delivered to a morphologically distinct recycling compartment composed of a network of small vesicles and tubular endosomes located in the pericentriolar region of CHO cells which are not physically connected to the sorting endosome (Dunn and Maxfield, 1992; Dunn et al., 1989; McGraw et al., 1993; Yamashiro et al., 1984).

An important issue is whether the various endocytic organelles are stable or transient. Clearly, coated vesicles are transient, and they fuse rapidly with endosomes after pinching off from the plasma membrane. Evidence has also been presented showing that sorting endosomes are transient organelles that mature into late endosomes (Dunn et al., 1989; Dunn and Maxfield, 1992; Stoorvogel et al., 1991). The lifetime of the recycling compartment has not been examined in detail. It has been shown that recycling Tf remains in a fusion-accessible compartment for as long as it can be detected within the cell (Salzman and Maxfield, 1989). However, the rapid loss of Tf limited measurements to ~15 min chase times. In this paper, we have shown that Tf_{10} enters the same recycling compartment of Tf with nearly normal kinetics but leaves with a half time of 60 min. This lengthy retention of Tf_{10} indicates that the recycling compartment is a stable, long-lived organelle. Furthermore, we have shown that the recycling compartment labeled with Tf_{10} remains fusion accessible to newly endocytosed material for at least 1 h. We cannot strictly rule out the possibility that labeling with Tf_{10} has induced the formation of a long-lived portion of the recycling compartment. However, we note that Tf enters and leaves this compartment at the same rate whether or not it has Tf_{10} in it. Furthermore, the morphology of the compartment is the same whether it contains Tf_{10}, Tf, or both (Figs. 5 and 6).

The Recycling Compartment Has Sorting Functions

The ability of the recycling compartment to retain Tf_{10} while simultaneously recycling Tf reveals a sorting function of the recycling compartment not previously recognized. The mechanism which underlies the observed sorting is not understood, but we have shown that the cytoplasmic domain of the TfR is not required. Our findings indicate that the oligomerization of the TfR by Tf_{10} forms a lumenal sorting signal resulting in the selective retention of Tf_{10} and TfR in the recycling compartment.

Recycling compartment sorting has also has been demonstrated in the mutant CHO cell line 12-4. 12-4 cells like other END2 mutants exhibit a partial acidification defect (Johnson et al., 1993*a*). 12-4 cells recycle bulk membrane at rates similar to that of parental cells indicating that there is no gross recycling defect in these cells (Presley et al., 1993). However, Tf exits the recycling compartment of 12-4 cells two times slower than bulk membrane (Presley et al., 1993), indicating that the recycling compartment has a sorting function which is dependent upon the intravesicular pH. Similar sorting has been induced in TRVb-1 cells treated with bafilomycin A_1, an inhibitor of the vacuolar proton ATPase that acidifies the recycling compartment. In contrast to the effects of oligomerization, bafilomycin

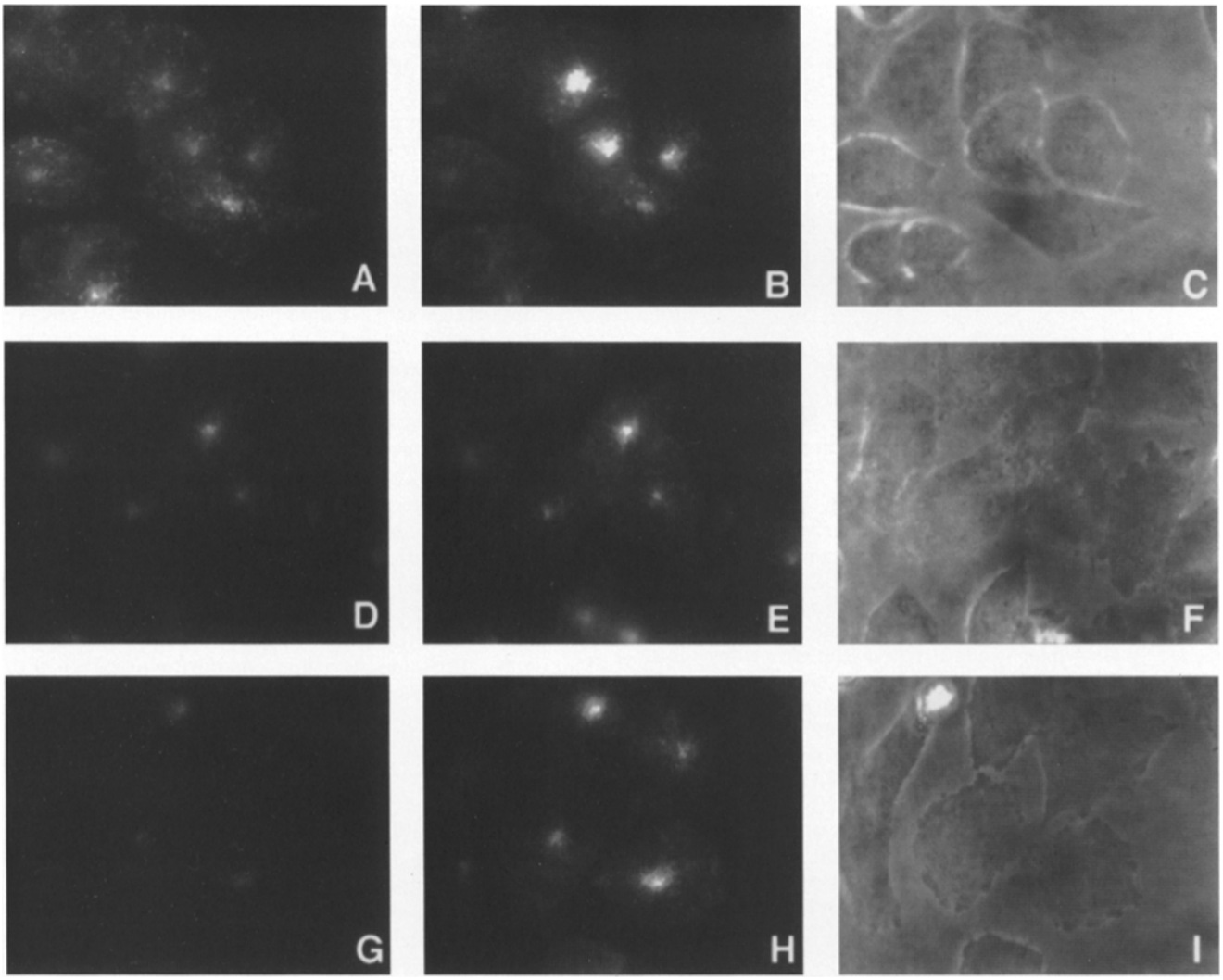

Figure 9. Multivalent-transferrin is localized in the recycling compartment of TRVbΔ3-59 cells. TRVbΔ3-59 cells were incubated in 40 μg/ml F-Tf_{10} for 50 min and then chased for a total of 60 min. At 48, 38, and 18 min of chase, the medium was replaced with 20 μg/ml Cy3-Tf for 10 min. The cells were washed and chased for 2 (*A–C*), 10 (*D–F*), or 30 (*G–I*) min, respectively. The cells were prepared for microscopy as described. The focal plane was selected to optimize the F-Tf_{10} image, and the Cy3-Tf image was then collected without further adjustment to focus. Cy3-Tf images (*A*, *D*, and *G*) were collected and processed using identical settings. F-Tf_{10} images (*B*, *E*, and *H*) were collected and processed using identical settings. Phase-contrast images are presented in *C*, *F*, and *I*.

A_1 induced retention is dependent upon the internalization motif of TfR encoded in the cytoplasmic tail of the receptor (Johnson et al., 1993*b*).

Protein sorting in the recycling compartment may occur by other mechanisms as well. Recently, it has been shown in polarized MDCK cells that transcytosed IgA is sorted from TfR, which recycles to the basolateral membrane, in a common apical recycling compartment (Apodaca et al., 1994). The insulin-dependent glucose transporter GLUT-4, in the absence of insulin, is sequestrated in a transferrin-accessible compartment, possibly as a result of oligomerization (Bell et al., 1993; James and Piper, 1993). Addition of insulin results in the redistribution of GLUT-4 to the cell surface. The trafficking of GLUT-4 appears to be controlled by at least two cytoplasmic domains, one of which lies in the amino terminus of the protein and functions as an internalization signal (Garippa et al., 1994). The second signal is localized in the carboxy terminus and has been suggested to be important for intracellular localization (Czech et al., 1993; Verhey et al., 1993). Taken together these studies suggest that the recycling compartment may actively control the rate of receptor recycling to the cell surface.

Note that in contrast to antibody cross-linking studies (Enns et al., 1983; Kasuga et al., 1981; Larrick et al., 1985; Lesley et al., 1989; Roth et al., 1983; Schwartz et al., 1986; Weissman et al., 1986), Tf_{10} was released from TRVb-1 cells intact. However, extremely large forms of multivalent-Tf, those composed of greater than 10 Tf molecules per particle, were redirected out of the recycling pathway and degraded, presumably by the same mechanism responsible for the degradation induced by anti-receptor antibodies. This suggests that grossly cross-linked receptors are targeted to the lysosomal pathway.

While the mechanism by which lumenal cross-linking slows TfR trafficking is not understood, it has been sug-

gested that changes in receptor mobility may influence receptor trafficking (Linderman and Lauffenburger, 1988). Tf_{10} binding may induce the oligomerized TfR to directly interact with the cytoskeleton in a manner similar to that of cross-linked receptor bound IgE (Menon et al., 1986). Our experiments using the TRVbΔ3-59 cells argue against a direct cytoskeletal interaction of this nature, since the cytoplasmic domain of the TfR was not required for Tf_{10} retention. We cannot rule out the existence of an additional factor(s) that could interact with the membrane-spanning domain and/or the lumenal domain of the cross-linked TfR and tether the Tf_{10}-TfR complex to the cytoskeleton, similar to the mechanism proposed for the sorting of regulated secretory proteins (Kelly, 1991).

The trafficking of TfR oligomerized by Tf_{10} may be hindered as a result of membrane microstructure. The membranous structures of cells are crowded with proteins having a variety of mobilities varying from essentially stationary to freely diffusing (Edidin, 1987; Jacobson et al., 1987). Those proteins may act as barriers to mobile proteins forming dynamic maze-like networks through which mobile proteins must travel (Kusumi et al., 1993; Zhang et al., 1991, 1993). The binding of Tf_{10} to several TfR forms a complex with several membrane-spanning domains which must move in concert. As a result, the Tf_{10}-TfR complexes are more likely to interact with other membrane components which may slow the trafficking of the bound Tf_{10}.

Tf_{10} has proved to be very useful in the further characterization of the pericentriolar recycling compartment. The long residency of Tf_{10} ($t_{1/2}$ = 60 min) in the recycling compartment has provided direct evidence that the recycling compartment is a long-lived organelle that remains able to accept newly endocytosed Tf from the sorting endosome for at least 1 h. The retention of Tf_{10} further demonstrates that the recycling compartment has sorting functions which may be physiologically important. While the mechanism of Tf_{10} retention remains an area of active research, we have shown that it is lumenal in nature and may represent a general sorting mechanism that could be employed by other surface receptors that are oligomerized as a result of ligand binding.

We would like to thank Drs. Ira Tabas and Jeffrey Myers for their gift of DiI-LDL, Dr. Timothy E. McGraw and members of the Maxfield lab for their thoughtful and careful reading of this manuscript, and Dana Gelman and the MICROMED staff for their excellent technical support.

This work has been supported by National Institutes of Health (NIH) grant number DK27083 given to F. R. Maxfield, Aaron Diamond Foundation grant number HRI 871-9332E to F. R. Maxfield and P. L. Leopold. E. W. Marsh is supported by NIH grant number AI08671-02.

Received for publication 15 August 1994 and in revised form 17 March 1995.

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