

# Transferrin Receptors Recycle to *Cis* and Middle As Well As *Trans* Golgi Cisternae in Ig-secreting Myeloma Cells

John W. Woods, Myriam Doriaux, and Marilyn Gist Farquhar

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

**Abstract.** The recycling itinerary of plasma membrane transferrin receptors (TFR) was charted in IgG-secreting mouse myeloma cells (RPC 5.4) by tagging surface receptors with either bound anti-transferrin receptor antibodies (anti-TFR) or Fab fragments thereof and determining the intracellular destinations of the tagged receptors by immunocytochemistry. By immunofluorescence, TFR tagged with either probe were seen to be rapidly internalized and translocated from the cell surface to the juxtannuclear (Golgi) region. When localized by immunoperoxidase procedures at the electron microscopic level, the anti-TFR-labeled receptors were detected in all cisternae (*cis*, middle, and *trans*) of the Golgi stacks as well as in

endosomes and *trans* Golgi reticular elements. There was no difference in the routing of TFR tagged with monovalent Fab and those tagged with divalent IgG. Tagged receptors were detected in Golgi stacks of ~50% of the cells analyzed. The position of the labeled cisternae within a given stack was found to be quite variable with *cis* and middle cisternae more often labeled at 5 min and *trans* cisternae at 30 min of antibody uptake. The finding that recycling plasmalemmal TFR can visit all or most Golgi subcompartments raises the likely possibility that any Golgi-associated posttranslational modification can occur during recycling as well as during the initial biosynthesis of plasmalemma receptors and other membrane proteins.

At present there is a good deal of interest in charting the pathways of recycling membrane traffic between various intracellular and extracellular (cell surface) destinations. Previous work using electron-dense tracers such as cationized ferritin and dextran indicates that there is considerable membrane traffic from the cell surface to the stacked Golgi cisternae and secretory granules or vacuoles in regulated secretory cells, such as parotid (14), lacrimal (14), anterior pituitary (7), thyroid (15), and exocrine pancreatic epithelia (16), as well as in nonregulated (constitutive) secretory cells (plasma cells and myeloma cells [29, 40]). The most plausible explanation for this plasmalemma to Golgi traffic is that much of it is connected with the recycling of membrane utilized as containers in the packaging of secretory products (see references 8 and 9). However, the studies mentioned were subject to the criticism that, since the tracers utilized bind nonspecifically (e.g., by ionic interaction) to the plasmalemma, they may not be reliable membrane markers because they can dissociate and relocate during transit through intracellular compartments.

To circumvent this objection we have studied the fate of a specific plasmalemmal membrane protein—the transferrin receptor (TFR)<sup>1</sup>—in mouse myeloma cells after tagging the receptor with anti-transferrin receptor (anti-TFR) antibodies. We report here our findings indicating that plasmalem-

mal TFR tracked by bound antibody reach all the stacked Golgi cisternae.

## Materials and Methods

### Materials

Mouse myeloma RPC 5.4 cells were obtained from the American Type Culture Collection, Rockville, MD. Cell culture media and supplies were from Gibco (Grand Island, NY). LR White resin and Epox 812 were obtained from Ernest F. Fullam, Inc. (Schenectady, NY).

### Antibodies

A rat anti-TFR monoclonal antibody (clone R17-217, subclass IgG<sub>2a</sub>) and Fab fragments prepared therefrom were kindly provided by Drs. Ian Trowbridge and Jane Lesley, The Salk Institute, San Diego, CA. This antibody has been previously shown to specifically immunoprecipitate the mouse TFR (23, 24). It does not inhibit transferrin binding to the receptor and can be used to immunoprecipitate both occupied and unoccupied receptors.

Rabbit anti-rat IgG was from Accurate Chemical & Scientific Corp. (Westbury, NY). Fluorescein isothiocyanate-conjugated rabbit anti-rat IgG was purchased from Cappel Laboratories (Cochranville, PA) and was depleted of anti-mouse IgG cross-reactivity by passage over a mouse IgG-agarose affinity column before use. Horseradish peroxidase (HRP) conjugated sheep anti-rabbit Fab was obtained from Biosys (Compiègne, France), and goat anti-rabbit IgG conjugated to 10-nm colloidal gold was from Janssen Life Sciences Products (Piscataway, NJ).

### Cell Culture

Mouse myeloma cells (RPC 5.4) were cultured at 37°C in an atmosphere of 95% air and 5% CO<sub>2</sub> in 75-cm<sup>2</sup> flasks in Dulbecco's modified Eagle's

1. **Abbreviations used in this paper:** DME, Dulbecco's modified Eagle's medium; HRP, horseradish peroxidase; PBS/OVA, PBS containing 0.1% ovalbumin; TFR, transferrin receptor(s).

medium (DME) containing 4,500 mg/l glucose, nonessential amino acids, penicillin (1,000 U/100 ml), streptomycin (10 mg/100 ml), and 10% heat-inactivated fetal bovine serum. Under these conditions, the cells typically had a doubling time of 20 h and achieved a maximum density of  $2-3 \times 10^6$  cells/ml. Cells were harvested at a density of  $4-8 \times 10^5$ /ml to ensure that they were in the exponential growth phase, and either fixed immediately (to determine the distribution of TFR at steady state), or used for anti-TFR binding studies.

### Binding and Uptake of Anti-TFR

In some experiments anti-TFR IgG (10  $\mu$ g/ml) or Fab fragments thereof (20  $\mu$ g/ml) were added directly to the culture media (DME containing fetal bovine serum), and the cells were incubated for 5–30 min at 4° or 37°C, after which they were fixed and processed for immunocytochemistry. In other experiments cells were depleted of endogenous (bovine) transferrin by washing them (three times) with serum-free DME at 37°C followed by culture in serum-free DME at 37°C for 60 min and further washing (three times) with serum-free DME at 37°C and then incubated at 37°C for 5–30 min with anti-TFR (10  $\mu$ g/ml).

### Preparation of Cells for Immunocytochemistry

Cells were fixed for 4–6 h at 25°C by adding an equal volume of periodate-lysine-paraformaldehyde fixative (27) (2% formaldehyde, 0.075 M lysine, 0.01 M NaIO<sub>4</sub>, 0.0375 M NaPO<sub>4</sub> [pH 6.2]) to the incubation media. After fixation, they were then washed three times (by centrifugation and resuspension) in protein-free phosphate-buffered saline (PBS), the final pellet was resuspended in a minimal volume of PBS, and a 30- $\mu$ l aliquot containing  $10^5-10^6$  cells was placed on a polylysine-coated glass microscope slide (25). The cells were allowed to adhere for 30 min after which the slides were extensively washed with PBS containing 0.1% ovalbumin (PBS/OVA) to remove unbound cells leaving an attached, tightly spaced monolayer.

### Immunofluorescence

Antibody incubations were carried out at room temperature on glass slides as follows: 40  $\mu$ l of antibody diluted in PBS/OVA containing 0.05% saponin (to permeabilize the cells) was added to the attached cells, and the slides were placed in a humid chamber. For visualization of TFR, cells were first incubated with rat monoclonal anti-TFR (10  $\mu$ g/ml) followed by incubation in fluorescein-isothiocyanate-conjugated rabbit anti-rat IgG (diluted 1:50). For visualization of prebound anti-TFR, the cells were incubated only with the latter reagent. Generally the cells were incubated in each antibody for 1–2 h, rinsed (five times) with PBS/OVA containing 0.05% saponin after each incubation, mounted in phenylenediamine/glycerol (30), and examined in a Zeiss photomicroscope III equipped with epifluorescence illumination. Photomicrographs were taken with the largest diameter of the cells in the plane of focus to distinguish intracellular from surface label.

### Immunoperoxidase

Incubations were carried out on cells fixed with periodate-lysine-paraformaldehyde. These cells were attached to glass slides essentially as described previously for cells attached to culture dishes (3). For visualization of TFR at steady state, fixed cells were incubated overnight in rat monoclonal anti-TFR (10  $\mu$ g/ml), followed by rabbit anti-rat IgG (1:50) and HRP-conjugated sheep anti-rabbit Fab (1:100) for 1–2 h each. For visualization of prebound anti-TFR, only the last two steps were necessary. The cells were then fixed for 30 min in 1.5% glutaraldehyde in 100 mM Na-cacodylate (pH 7.4) containing 5% sucrose, followed by extensive washing with 50 mM Tris containing 7.5% sucrose. The peroxidase reaction was initiated by covering the cells with 50  $\mu$ l of diaminobenzidine medium prepared by mixing 1 ml of 0.2% diaminobenzidine, 50 mM Tris HCl (pH 7.4), containing 7.5% sucrose and 15  $\mu$ l of 0.3% H<sub>2</sub>O<sub>2</sub> (final concentration, 0.005%). The reaction

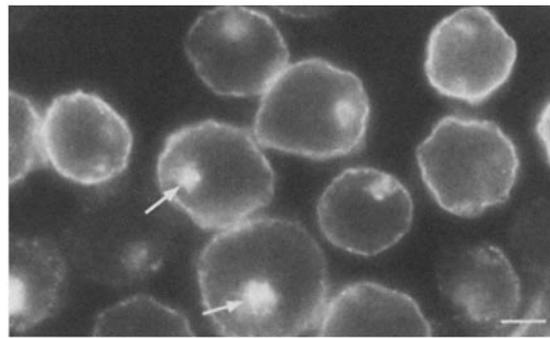


Figure 1. Indirect immunofluorescence localization of TFR in cultured RPC 5.4 myeloma cells fixed at 37°C (steady state). TFR are seen at the cell periphery and the juxtannuclear (Golgi) region (arrows). Fixed cells were permeabilized and incubated sequentially with rat anti-TFR and fluorescein isothiocyanate-conjugated rabbit anti-rat IgG. Bar, 20  $\mu$ m.

was allowed to proceed for 5–15 min (until the cells appeared brown under a dissecting microscope). The cells were then washed extensively with 50 mM Tris (pH 7.4) containing 7.5% sucrose, postfixed in a 50- $\mu$ l drop of ferrocyanide-reduced OsO<sub>4</sub> for 20 min at 4°C, and dehydrated and embedded in Epox 812 on the slide. After the final change of Epox, the slide was placed cell-side-down on a silicon rubber embedding mold and polymerized overnight at 60°C. The embedded cells were separated from the glass with a razor blade. Selected areas ( $\sim 1$ -mm square) were mounted on a support block of Epox and sectioned *en face*. Thin sections were stained with lead citrate and examined in a Philips 301 or 410 electron microscope operated at 60 kV.

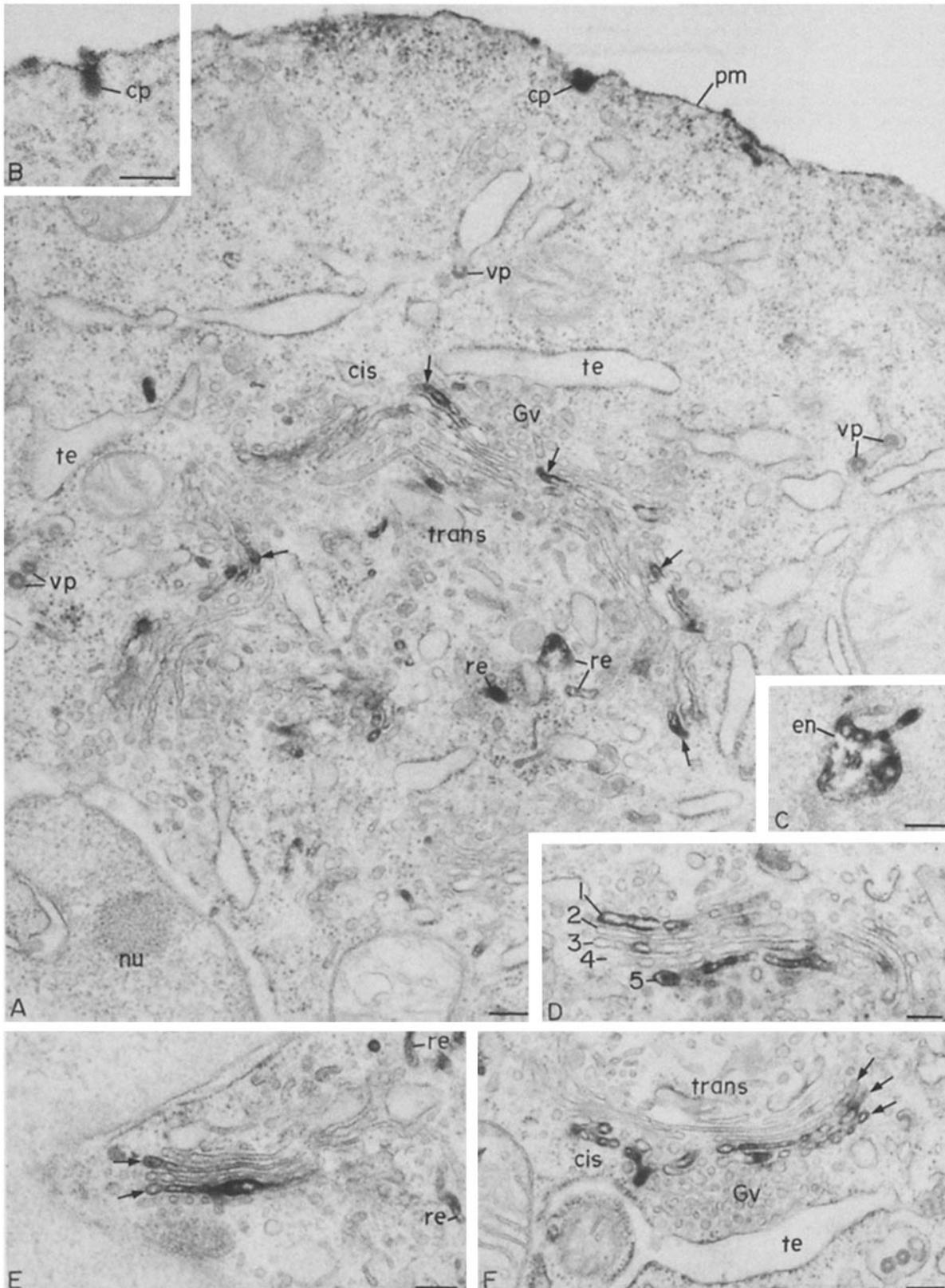
### Immunogold Localization of TFR on Ultrathin Cryosections

The basic procedures used were those of Keller et al. (20) with minor modifications. Periodate-lysine-paraformaldehyde-fixed cells were embedded in polyacrylamide (17) before freezing as follows:  $3-5 \times 10^6$  cells were resuspended in 6.5 ml PBS/OVA to which 1 ml of acrylamide/bisacrylamide (30:0.8%) in H<sub>2</sub>O and 75  $\mu$ l of 10% ammonium peroxide in H<sub>2</sub>O were added and mixed. The cells were then pelleted, resuspended in 200  $\mu$ l of the supernatant, and 2  $\mu$ l of 10% N,N,N',N' tetramethylethylenediamine in H<sub>2</sub>O was added. 250- $\mu$ l aliquots were quickly transferred to 400- $\mu$ l polyethylene tubes and centrifuged for 2 min in a Beckman 152 microfuge (Beckman Instruments, Inc., Palo Alto, CA). After polymerization, the embedded cell pellets were infiltrated with 2.3 M sucrose, frozen in liquid nitrogen, and silver sections were cut on a Reichert Ultracut microtome (Reichert Scientific Instruments, Div. Warner-Lambert Technologies, Inc., Buffalo, NY) equipped with the FC-4 cryocut attachment. Sections were incubated sequentially at room temperature with (a) anti-TFR (diluted 1:500 in 1% OVA/PBS for 60 min), (b) rabbit anti-rat IgG (1:50) for 60 min followed by (c) goat anti-rabbit IgG-gold conjugate (diluted 1:20 in 50% goat serum in PBS) for 2 h. The sections were then fixed for 5 min in 2% glutaraldehyde in PBS, postfixed in 1% OsO<sub>4</sub> in 100 mM Na-cacodylate (pH 7.4), and embedded in LR White resin (20). After polymerization overnight at 60°C, they were examined in a Philips 301 or 410 electron microscope operated at 80 or 100 kV without additional staining.

### Evaluation of Effect of pH on Anti-TFR Binding

Washed cells ( $1 \times 10^8$ ) were suspended in 5 ml of PBS/OVA (pH 7.4) and incubated with anti-TFR (1:1,000) at 4°C for 30 min followed by washing

Figure 2. Distribution of TFR in RPC myeloma cells as seen by indirect immunoperoxidase in cells fixed at steady state. (A) Large field showing TFR clustered in a coated pit (cp) along the plasma membrane (pm), in reticular elements (re) found on the *trans* side (*trans*) of the Golgi stacks as well as in multiple locations in the stacked Golgi cisternae (arrows). Virus particles (vp) can be recognized within several cisternae of the rough endoplasmic reticulum. The topography of the Golgi stacks is clear because the *cis* side (*cis*) is marked by the presence of multiple transitional elements (te)—part rough and part smooth—of the rough endoplasmic reticulum. (B) Portion of the plasma membrane showing TFR concentrated in a coated pit (cp). (C) TFR are present in an endosome-like structure which has two tubular extensions, one of which contains detectable TFR. (D–F) Enlargements of Golgi areas from cells fixed at steady state, demonstrating that TFR can be found in cisternae in various positions within the stack. (D) Golgi stack in which all of the cisternae (1–5) are labeled.



Cisternae 1, 3, and 5 (representing the *cis*-most, middle, and *trans*-most) are heavily labeled, whereas the intervening cisternae (2 and 4) have only traces of reaction product. (E) Golgi stack with five cisternae in which two of the five (arrows) are clearly labeled. (F) Another stack of five cisternae in which only two to three of the *cis*-most cisternae (arrows) are labeled. The polarity of this stack is particularly clear because it faces transitional elements (*te*) of the endoplasmic reticulum and clusters of Golgi vesicles (*Gv*) on the *cis* side (*cis*). Cells were fixed and incubated sequentially with rat anti-TFR, rabbit anti-rat IgG, and HRP-conjugated sheep anti-rabbit Fab. *nu*, nucleus. Bars, 200 nm.

(three times) at 4°C with PBS/OVA (pH 7.4). Cells ( $2 \times 10^6$ ) were then resuspended in 2 ml of 0.01 M  $\text{PO}_4$ /citrate buffer containing 0.1% OVA plus 0.9% NaCl which had been adjusted to a specific pH (between 6.5 and 2.0). They were incubated for 30 min at 4°C and washed (three times) with the same buffer at 4°C, washed again (two times) with PBS/OVA (pH 7.4) at 4°C, and fixed with 2% formaldehyde in PBS (pH 7.4) at 4°C for 30 min. After washing, cells were incubated sequentially with rabbit anti-rat IgG (1:50) and  $^{125}\text{I}$ -protein A (30,000 cpm) each for 45 min. Bound and free  $^{125}\text{I}$ -protein A were separated by sedimenting the cells through a phthalate-oil mixture (35). Nonspecific binding of rabbit anti-rat IgG to RPC cells was quantitated at each pH (separate samples) and subtracted from the total. Each data point is the mean of triplicate determinations.

## Results

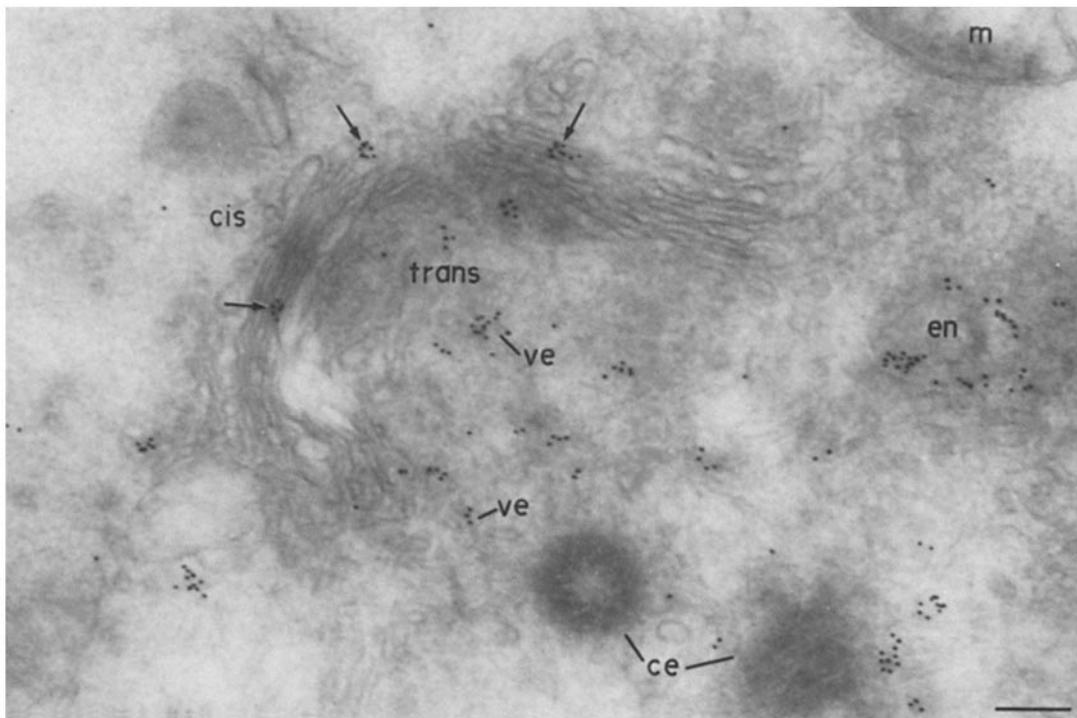
The mouse myeloma cell line (RPC 4.5) used in this study was chosen because it has been shown to constitutively secrete substantial amounts of IgG (5–7% of its newly synthesized protein/h) into the culture medium and to manifest extensive plasmalemma to Golgi vesicular membrane traffic (29). The TFR was chosen for study because it is a well characterized, integral transmembrane glycoprotein, and cells in culture and other rapidly proliferating cells are known to express many TFR (37). This is because iron is required for cell growth, and TFR serve to transport iron (bound to transferrin) into the cell (6, 28, 37). TFR traffic had not been investigated previously in these cells.

### Distribution of TFR at Steady State

To determine the overall distribution of TFR in mouse RPC 5.4 myeloma cells, fixative was added directly to the culture medium, and the receptors were localized at the light and electron microscopic levels by indirect immunolocalization

procedures using a monoclonal antibody that specifically recognizes the mouse TFR. Using immunofluorescence, we found the receptors at the cell periphery and in the juxtannuclear (Golgi) region (Fig. 1) in RPC 5.4 myeloma cells as in many other cell types. When the distribution of TFR was determined at the electron microscopic level by indirect immunoperoxidase and immunogold procedures, it was clear that the surface staining seen by immunofluorescence was the result of the concentration of TFR in coated pits along the plasma membrane (Fig. 2, *A* and *B*), and that the juxtannuclear staining was the result of its localization in a variety of elements found in the Golgi region. Among the structures labeled were a number of elements—multivesicular bodies, vacuoles with tubular tails (Fig. 2 *C*), and cup-shaped bodies—assumed from their morphology to correspond to endosomes. In addition, TFR were also regularly detected in the Golgi complex where they were found in reticular cisternae associated with the *trans* side of the Golgi complex (Fig. 2 *A*), and also within the stacked Golgi cisternae themselves. The position of the labeled cisternae within the Golgi stack was quite variable: examples could be found where TFR were present in the *cis*-most (Fig. 2, *D* and *F*), the *trans*-most (Fig. 2 *D*), or the middle (Fig. 2, *A* and *E*) cisternae. Other organelles (e.g., rough endoplasmic reticulum and lysosomes) were rarely, if ever, labeled. In control experiments in which the anti-TFR antibody was omitted, no signal was observed with either the immunofluorescence or immunoperoxidase techniques.

The distribution of TFR in RPC 5.4 myeloma cells is similar to that reported for several other cell types (18, 19, 39) in that they were found in coated pits at the cell surface and



**Figure 3.** Ultrathin frozen section demonstrating localization of TFR by an indirect immunogold procedure in a cell fixed at 37°C (steady state). Colloidal gold particles are observed over the stacked Golgi cisternae (arrows) and vesicles (*ve*) associated with the *trans* side (*trans*) of the stacks. Sections were incubated sequentially with rat anti-TFR, rabbit anti-rat IgG, and goat anti-rabbit IgG conjugated to colloidal gold. *en*, endosome; *ce*, centriole; *m*, mitochondria. Bar, 200 nm.

in endosomes and *trans* Golgi reticular elements; however, the findings differ in that in myeloma cell TFR were regularly detected in the stacked Golgi cisternae as well.

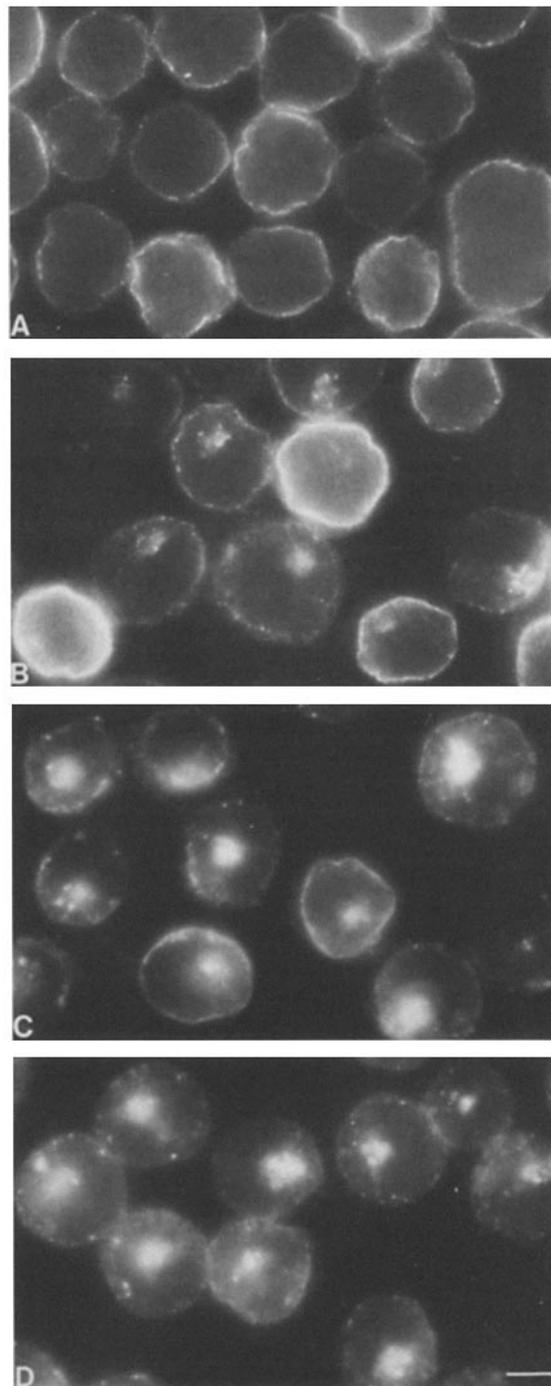
To give us some idea of the relative distribution of receptors within the stacked Golgi cisternae as compared to other Golgi-associated structures, we determined the distribution of gold particles from micrographs (e.g., Fig. 3) taken from ultrathin cryosections of five different Golgi areas. Of 261 gold particles counted, ~30% (73) were associated with the stacked cisternae and the remaining 70% (188) were associated with other structures in the Golgi region.

Thus, we obtained both qualitative and quantitative data in myeloma cells for the presence of TFR in the Golgi stacks which has not been reported previously in other cell types. However, with the approach used (cells fixed at steady state), we could not distinguish between TFR in transit through the Golgi during recycling and those in transit during biosynthesis.

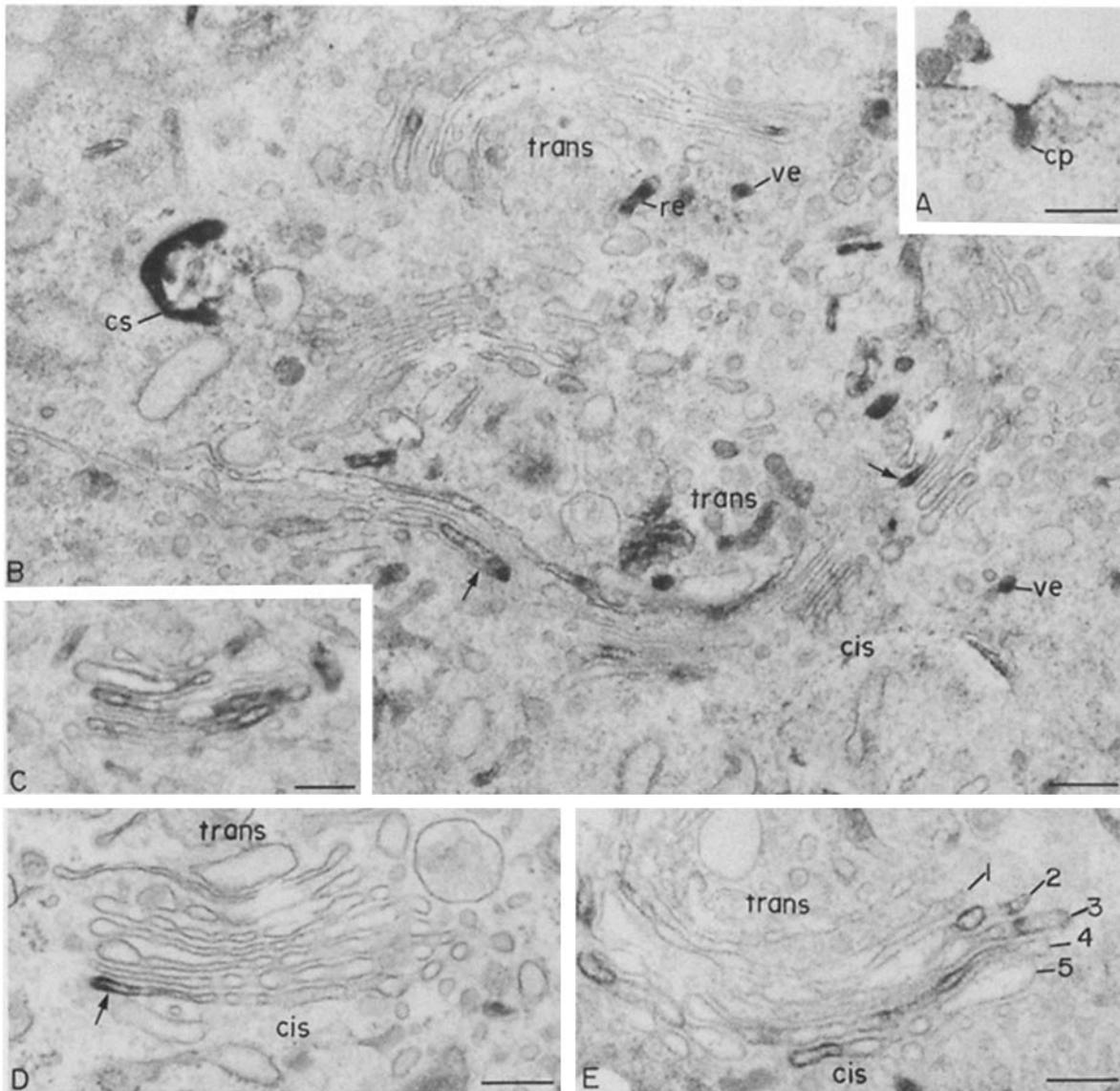
#### ***Anti-TFR IgG Bound to Plasmalemmal TFR Reaches the Golgi Stacks***

To trace directly the recycling pathway taken by TFR after endocytosis, receptors were labeled at the cell surface with anti-receptor antibodies by incubating cells with anti-TFR at 4° or 37°C for various times before fixation. The distribution of anti-TFR was then determined by immunofluorescence and immunoperoxidase procedures. If cells were incubated with anti-TFR for 30 min at 4°C and fixed at 4°C, only surface staining was seen by immunofluorescence (Fig. 4 A). If, however, they were incubated with anti-TFR for 5, 15, or 30 min at 37°C, the antibody, assumed to be bound to the TFR (see below), was found not only at the cell surface, but also was found inside the cell. After 5 min anti-TFR was detected in 60% of the cells in a juxtannuclear spot corresponding to the Golgi region (Fig. 4 B), and after 15 min at 37°C, its distribution was unchanged except that a higher proportion of the cells were labeled and the juxtannuclear staining appeared to be brighter. After 30 min at 37°C, 95% of the cells displayed anti-TFR concentrated in the Golgi region (Fig. 4 C), indicating that most cells had internalized antibody-tagged surface receptors. Identical observations were obtained when cells were incubated at 4°C with anti-TFR, extensively washed, and warmed to 37°C for 5 or 30 min before fixation (data not shown). These patterns of TFR distribution were essentially the same as when TFR were localized by indirect immunofluorescence at steady state.

Immunoperoxidase localizations carried out at the electron microscopic level revealed that after a 5-min incubation with anti-TFR at 37°C, the distribution of antibody-labeled receptors was essentially the same as the distribution of TFR at steady-state: they were detected in coated pits along the plasma membrane (Fig. 5 A), in Golgi stacks (Fig. 5, B-E), in endosomes (Fig. 5 B), in vesicles, and in *trans* Golgi reticular elements (Fig. 5 B). After 30 min of anti-TFR incubation, the same structures were labeled as at 5 min, but the relative frequency with which particular Golgi-associated structures were labeled had changed: reaction product was observed less frequently in the stacked Golgi cisternae and more often in the other compartments associated with the Golgi complex (Fig. 6, A-D). Similar results were obtained in cells depleted of endogenous transferrin before incubation with anti-TFR. Similar results were also obtained in experi-



**Figure 4.** Immunofluorescence localization of either intact anti-TFR IgG or Fab fragments after binding to plasmalemmal TFR. When cells were incubated for 30 min at 4°C with anti-TFR IgG and then fixed at 4°C, permeabilized, and incubated with fluorescein isothiocyanate rabbit anti-rat IgG to detect bound antibody, only surface staining was seen (A). When cells were incubated for 5 min at 37°C in the presence of anti-TFR IgG before fixation and permeabilization (B), ~60% of the cells had internalized the anti-TFR and displayed intracellular (juxtannuclear) staining as well as staining at the cell surface. Of those remaining, 30% showed staining at the cell periphery only, and ~10% were unstained. After incubation for 30 min at 37°C with anti-TFR IgG (C) or Fab (D) before fixation (C) 95% of the cells had taken up the bound antibody which was concentrated intracellularly in the juxtannuclear region. Bar, 20  $\mu$ m.



**Figure 5.** Immunoperoxidase localization of anti-TFR in cells fixed after 5 min incubation at 37°C in the presence of the antibody. The antibody, assumed to be bound to TFR, is present in coated pits (*cp*) along the plasma membrane (*A*), and is detected within the cell in vesicles (*ve*) located on both the *cis* and *trans* side of the Golgi complex, in a cup-shaped structure (*cs*) (probably an endosome) found near the Golgi stacks, and within several of the stacked cisternae themselves (*arrows*) (*B*). *B-E*, variations in the position of the labeled cisternae in the stacks. Label can be detected in *cis*, middle, or *trans* cisternae. *C* and *E*, Golgi stacks of which at least four out of six cisternae are labeled. *D*, a stack in which a single (*cis*) cisterna is labeled. Fixed cells were incubated sequentially with rabbit anti-rat IgG and HRP-conjugated sheep anti-rabbit Fab. Bars, 200 nm.

ments in which cells were incubated with anti-TFR at 4°C, washed extensively at 4°C, and warmed to 37°C for 5 or 30 min before fixation and immunoperoxidase localization of the bound anti-TFR. Thus the distribution of internalized anti-TFR was the same under conditions in which nonspecific pinocytotic uptake of unbound antibody was ruled out.

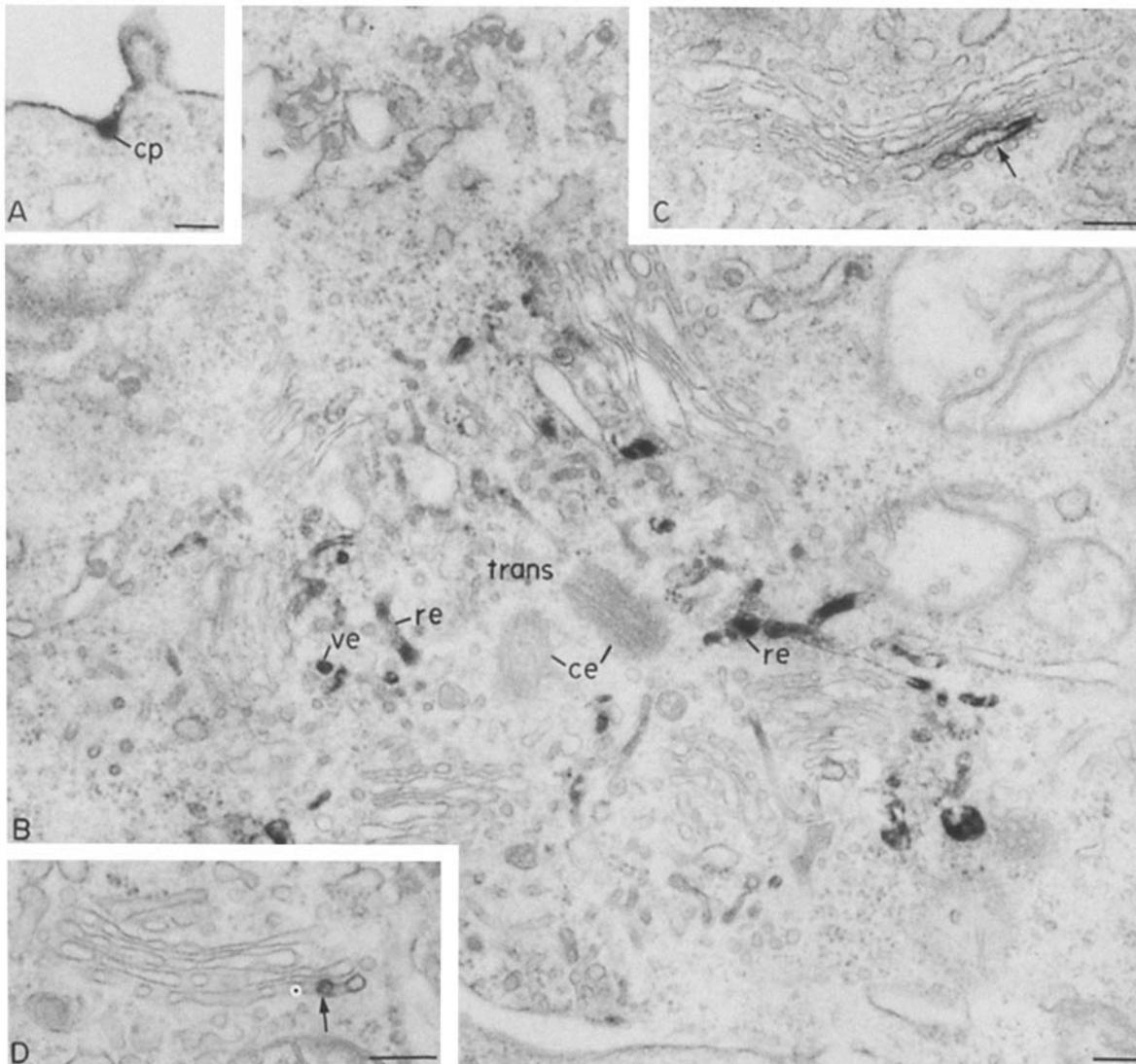
To obtain data on the frequency of uptake of anti-TFR into various Golgi elements, the distribution of anti-TFR among different Golgi subcompartments was determined. The results (Table I) indicate that after a 5-min incubation with anti-TFR at 37°C, HRP reaction product could be detected in the stacked cisternae of ~50% of the cells analyzed. Surprisingly, *cis* and middle cisternae were more often labeled than *trans* cisternae. After 30 min at 37°C, the proportion of

Golgi complexes in which the stacked cisternae contained detectable anti-TFR had decreased to 35% and reaction product was more often found in *trans* cisternae, but it could still be found in any Golgi subcompartment (*cis*, middle, or *trans*) within the stack.

It can be concluded that (a) uptake of antibody-tagged receptors into Golgi cisternae is a frequent rather than a rare event; (b) the antibody-tagged receptors reach all Golgi cisternae; and (c) *cis* cisternae are most commonly labeled at 5 min and *trans* cisternae at 30 min.

#### ***Fab* Fragments of Anti-TFR Bound to Plasmalemmal TFR Reach the Golgi Stacks**

When cells were incubated with Fab fragments of anti-TFR



**Figure 6.** Immunoperoxidase preparation similar to that in Fig. 5 except that the cells were incubated with anti-TFR for 30 min. HRP reaction product is seen in the same structures as after 5 min, i.e., in coated pits (*cp*) along the plasma membrane (*A*), in vesicles (*ve*) of various sizes and reticular elements (*re*) located on the *trans* side of the Golgi stacks (*B*), and occasionally in the stacked cisternae (*arrows*) (*C* and *D*). The only difference between the findings obtained at 30 min and those at 5 min is that labeling is less frequently encountered in Golgi stacks and is more commonly found in *trans* Golgi reticular elements at the later time point. Usually only a single cisterna within a given stack is labeled (*C* and *D*). The *trans* side of the stack is clearly marked in *B* by the presence of centrioles (*ce*). Bars, 200 nm.

instead of whole IgG, the results were very similar to those obtained using the intact antibody: after 5 min at 37°C, surface staining was visible by immunofluorescence, but was difficult to photograph, and after 30 min, anti-TFR Fab were regularly seen at the cell surface as well as concentrated in the juxtannuclear region (compare Fig. 4, *C* and *D*). By immunoperoxidase labeling, it was apparent that the Fab had been internalized into endosomes as well as into the stacked Golgi cisternae where it appeared within cisternae in all positions (*cis*, middle, and *trans*) in the stack (Fig. 7, *A-C*). Thus, there was no apparent difference in the routing of plasmalemmal TFR tagged with monovalent Fab or divalent IgG.

#### **Effect of pH on Binding of Anti-TFR to Plasmalemmal Receptors**

To assure that the antibody would remain bound to the recep-

tor at the pHs encountered in intracellular compartments (pH 4.0–7.4) the effect of pH of surface-bound <sup>125</sup>I-labeled anti-TFR was examined. The data obtained (Table II) demonstrate that the amount of anti-TFR remaining bound to the cells after incubation at pH 4–7.4 is the same. Only when the pH was lowered to <4.0 did anti-TFR dissociate from the cells: at pH 3, <5% of the IgG remained bound, and at pH 2, no bound antibody was detected. These results demonstrate that the antibody remains firmly bound to the receptor at pH 4.0–7.4.

#### **Discussion**

In these experiments, we tagged the plasmalemmal TFR of RPC 5.4 mouse myeloma cells with a monoclonal anti-TFR IgG or Fab and used the bound antibody to trace by im-

**Table I. Distribution of Endocytosed Anti-TFR within Golgi Cisternae\***

Incubation conditions	No. of cells analyzed	No. with labeled Golgi cisternae	Distribution of labeled cisternae within Golgi stack
Anti-TFR IgG, 5 min at 37°C	52	27	Cis-5 Middle-16 Trans-0 Indeterminate-0
Anti-TFR IgG, 30 min at 37°C	53	17	Cis-6 Middle-4 Trans-4 Indeterminate-3

\* Cells were incubated with anti-TFR for 5 or 30 min at 37°C after which they were fixed and processed for immunoperoxidase localization of bound anti-TFR as described in the Materials and Methods. Micrographs were taken (15–20,000 ×) of all cells in a given field that contained labeled intracellular receptor and in which the Golgi stacks were clearly visible.

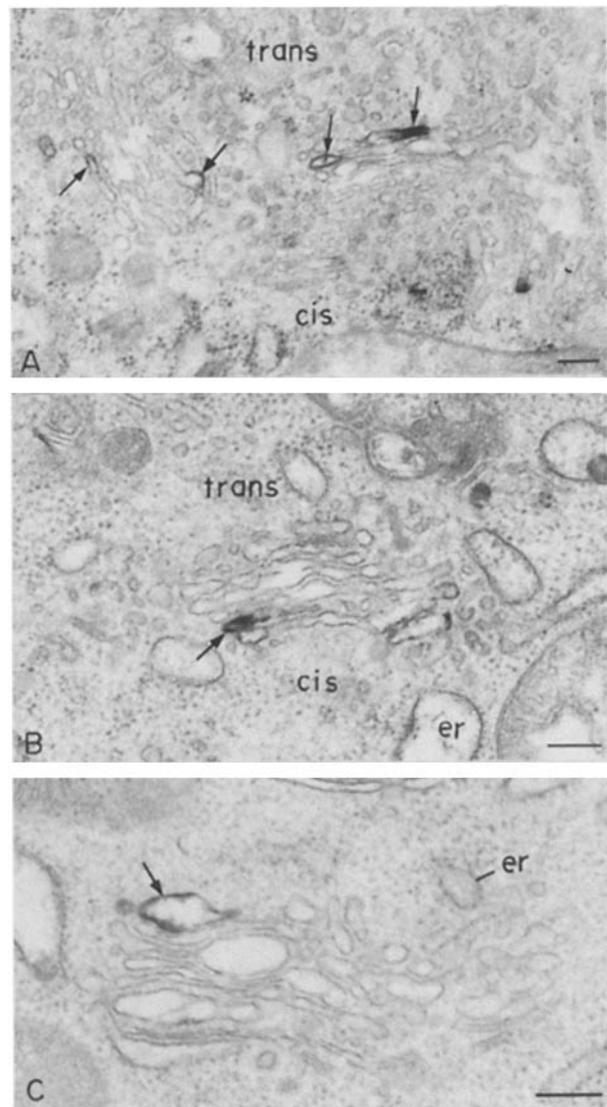
munocytochemistry the intracellular compartments reached by internalized receptors. For the antibody to be a valid marker it was necessary to rule out that it could dissociate from the receptor at the low pHs encountered in endosomes (pH 5.0) (38) or in *trans* Golgi or “para Golgi” compartments (pH 6.5) (1, 41). This was done by demonstrating that radiiodinated anti-TFR remained bound to surface receptors at the pHs (7.4 to 4.0) likely to be encountered in any intracellular compartment. Our primary finding was that internalized receptors tagged with antibody reached bona fide Golgi cisternae where they could be detected in all locations within the stacks—i.e., *cis*, middle, or *trans* cisternae—as well as in endocytic compartments. These results demonstrate that a specific integral membrane component can visit most if not all Golgi subcompartments during recycling. They also confirm and extend to a specific plasmalemmal membrane protein results obtained previously on myeloma cells after iodinating plasmalemmal constituents (40) and on myeloma cells (29) and a number of other secretory cell types (7, 14, 16) after binding electron-dense tracers to the cell surface.

The possibility that antibody binding might perturb recep-

**Table II. Effect of pH on Elution of Anti-TFR from RPC Cells\***

pH of elution	<sup>125</sup> I-Protein A bound
	<i>cpm</i> × 10 <sup>-3</sup>
7.4	2.2 ± 0.36
6.5	1.8 ± 0.56
6.0	1.6 ± 0.18
5.5	2.1 ± 0.38
5.0	1.2 ± 0.32
4.0	1.6 ± 0.32
3.0	0.1 ± 0.12
2.0	0.0 ± 0.14

\* Plasmalemmal TFR were labeled with anti-TFR by incubating cells in the presence of the antibody for 30 min at 4°C in PBS/OVA (pH 7.4). The cells were then washed with PO<sub>4</sub>/citrate buffers of different pHs, fixed in 2% formaldehyde, incubated with anti-rat IgG followed by <sup>125</sup>I-protein A, and the amount of bound <sup>125</sup>I-protein A determined.



**Figure 7.** Golgi complexes from an immunoperoxidase preparation similar to Fig. 4 except that the cells were incubated with Fab fragments of anti-TFR IgG for 30 min at 37°C before fixation. Immunoreactive Fab is detected in a variety of positions within the stacked Golgi cisternae (arrows). *A*, HRP reaction product in the two *trans*-most cisternae in one Golgi stack (to the right) and in two cisternae on opposite sides of the stack in another (to the left). *B*, reaction product in two of the *cis*-most cisternae in another Golgi stack; and *C*, reaction product in only a single cisterna on one side of a stack in which the sidedness (*cis* vs. *trans*) is not clear. Bars, 200 nm.

tor traffic and divert it to a nonphysiological pathway cannot be ruled out completely, but seems unlikely for the following reasons: (a) similar results have been obtained with alternative labeling procedures; (b) TFR are found in exactly the same compartments in cells fixed at steady state where there has been no tagging of receptors before fixation; (c) TFR tagged with monovalent Fab fragments and those tagged with whole (divalent) IgG have the same fate. Moreover, there is no precedent for antibody binding diverting endocytic traffic to compartments other than lysosomes (26). In fact, the advantage of the approach used here to study TFR traffic is that it made use of native, unmodified anti-TFR to trace receptor

movements, whereas, most previous studies of this type have been done with anti-TFR coupled to large electron-dense tracers such as colloidal gold (18, 19) which appear to divert at least some of the receptors to lysosomes (19). In experiments in which we tagged surface TFR with anti-TFR plus a second antibody coupled to colloidal gold before fixation, the internalized gold label was found only in endosomal and lysosomal compartments and was not detected in Golgi stacks (2), suggesting that the large, multivalent gold-labeled probe diverts at least some of the receptors to lysosomes.

Previous work established that TFR bind transferrin-Fe<sup>3+</sup> at the cell surface and the TFR-transferrin complex is internalized into a low pH compartment where the Fe<sup>3+</sup> is released, thereby becoming available for cell metabolism. The resultant TFR-apotransferrin returns to the cell surface where the apotransferrin dissociates, freeing TFR to bind more transferrin (5, 21). TFR and endocytosed transferrin have been previously localized at the electron microscopic level in a number of cell types including reticulocytes (13), erythroleukemic cells (39), epidermoid carcinoma A431 cells (18, 19), and Chinese hamster ovary cells (41). In all these cell types TFR and endocytosed transferrin have been found in peripheral or *trans* Golgi endosomal compartments, but they have not been detected in the stacked Golgi cisternae.

How then do we explain our findings on the routing of antibody-tagged TFR through the Golgi stacks in myeloma cells? We believe that the differences between our findings and those of others in regard to the localization of TFR in Golgi stacks can be explained by differences in the nature of the cell types studied as well as in the methods used to study them. Most previous work was done on cell types in which the main flow of endocytic traffic is to endosomes and lysosomes, whereas we studied an IgG-secreting murine myeloma cell line, RPC 5.4, in which it had been established (29) that a considerable amount of the endocytic traffic is to the stacked Golgi cisternae. These cells constitutively secrete IgG and package it into small vesicles which are continually released by exocytosis. This type of membrane traffic from the plasmalemma to the Golgi complex, which has been documented in these as well as in a number of other secretory cells, has been assumed to be connected primarily with the recovery and recycling of membranes used as containers for secretory products. However, the present observations indicate that since the destination of membrane proteins as exemplified by TFR is not limited to *trans* cisternae where packaging occurs, there must also be considerable recycling of plasmalemmal components between Golgi subcompartments. Thus, the simplest interpretation of our findings of extensive plasmalemma to Golgi traffic of TFR in myeloma cells is that it is a reflection of the high level of membrane traffic to and through the Golgi complex that takes place in secretory cells (11) and that TFR serves as a marker for this type of traffic from the Golgi complex to the cell surface and back. It should be added, however, that we assume that similar recycling plasmalemmal to Golgi traffic also exists in other cell types because all cells utilize exocytosis for delivery of newly synthesized proteins (e.g., plasmalemmal proteins) to the cell surface, but it occurs at a much lower level than in secretory cells and is therefore more difficult to trace.

On discovering plasmalemmal to Golgi traffic a few years

ago, it was suggested (8-10) that biosynthetic repair (e.g., reglycosylation, resulfation) of plasmalemmal membrane proteins or bound ligands might occur in transit through the Golgi complex during recycling. Evidence has subsequently been obtained, indicating that resialylation of both transferrin (32, 33) and TFR (36) does occur, implying that both TFR and transferrin can visit the Golgi subcompartments where the sialyltransferase resides. Sialyltransferase has recently been localized by immunocytochemistry to 1-2 *trans* cisternae and a *trans* Golgi-tubular network in rat hepatocytes (34) comparable to the *trans* Golgi elements in which TFR (39) or transferrin (41) have been localized. Thus, the present findings indicating that surface TFR reach the *trans* Golgi reticular cisternae are compatible with both the immunocytochemical localization of sialyltransferase and the biochemical data on resialylation. The present findings in myeloma cells as well as the previous studies with electron-dense tracers indicate that recycling TFR can visit *cis* and middle cisternae where the early glycoprotein processing enzymes (GlcNAc transferases I and II,  $\alpha$ -mannosidases I and II) (4, 11, 22) and the two enzymes that add the mannose-6-phosphate recognition marker to lysosomal enzymes (12, 31) reside, and they raise the possibility that more extensive posttranslational modifications than resialylation can occur during recycling. In principle, any biosynthetic event that normally occurs in the Golgi complex—i.e., O-glycosylation, addition of glycosaminoglycans, sulfation, and proteolytic processing as well as earlier events in N-glycosylation (see references 11 and 22)—could occur in transit during recycling as well as during the initial biosynthetic passage of newly synthesized proteins through the Golgi complex. In practice it remains to be seen to what extent this repair option is exercised by different cell types.

We thank Hans Stukenbrok for preparation of the ultrathin cryosections and M. Lynne Wootton for wordprocessing.

This work was supported by National Institutes of Health (NIH) fellowship HL 06733 (to John W. Woods) and NIH grant AM 17780 (to Marilyn G. Farquhar).

Received 31 January 1986, and in revised form 3 April 1986.

#### References

1. Anderson, R. G. W., and R. K. Pathak. 1985. Vesicles and cisternae in the *trans* Golgi apparatus of human fibroblasts are acidic compartments. *Cell*. 40:635-643.
2. Broumand, S. R. 1985. Electron microscopic study of myeloma cells utilizing lectins and antibodies as cell membrane markers. M.D. Thesis. Yale University School of Medicine, New Haven, CT. 1-74.
3. Brown, W. J., E. Constantinescu, and M. G. Farquhar. 1984. Redistribution of mannose-6-phosphate receptors induced by tunicamycin and chloroquine. *J. Cell Biol.* 99:320-326.
4. Dunphy, W. G., and J. E. Rothman. 1983. Compartmentation of asparagine-linked oligosaccharide processing in the Golgi apparatus. *J. Cell Biol.* 97:270-275.
5. Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258-2262.
6. Dautry-Varsat, A., and H. F. Lodish. 1984. How receptors bring proteins into cells. *Sci. Am.* 250:52-58.
7. Farquhar, M. G. 1978. Recovery of surface membrane in anterior pituitary cells. Variations in traffic detected with anionic and cationic ferritin. *J. Cell Biol.* 77:R35-R42.
8. Farquhar, M. G. 1981. Membrane recycling in secretory cells: implications for traffic of products and specialized membranes within the Golgi complex. *Methods Cell Biol.* 23:399-427.
9. Farquhar, M. G. 1982. Membrane recycling in secretory cells: pathways to the Golgi complex. In *Membrane Recycling*. D. Evered and G. M. Collins, editors. Pitman Books Ltd., London. 157-174.
10. Farquhar, M. G. 1983. Multiple pathways of exocytosis, endocytosis and membrane recycling: validation of a Golgi route. *Fed. Proc.* 42:2407-2413.

11. Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi Traffic. *Annu. Rev. Cell Biol.* 1:447-488.
12. Goldberg, D. E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159-3165.
13. Harding, C., J. Heuser, and P. Stahl. 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97:329-339.
14. Herzog, V., and M. G. Farquhar. 1977. Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. *Proc. Natl. Acad. Sci. USA.* 74:573-5077.
15. Herzog, V., and F. Miller. 1979. Membrane retrieval in epithelial cells of isolated thyroid follicles. *Eur. J. Cell Biol.* 19:203-215.
16. Herzog, V., and H. Reggio. 1980. Pathways of membranes retrieved from the luminal surface of exocrine cells of the pancreas. *Eur. J. Cell Biol.* 21:11-15.
17. Heusen, P., and C. Dreyer. 1981. The use of polyacrylamide as an embedding medium for immunohistochemical studies of embryonic tissues. *Stain Technol.* 56:287-293.
18. Hopkins, C. R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell.* 35:321-330.
19. Hopkins, C. R., and I. S. Trowbridge. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97:508-521.
20. Keller, G. A., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1984. An improved procedure for immunoelectron microscopy: ultrathin plastic embedding of immunolabeled ultrathin frozen sections. *Proc. Natl. Acad. Sci. USA.* 81:5744-5747.
21. Klausner, R. D., G. Ashwell, J. Van Renswoude, J. B. Harford, and K. R. Bridges. 1983. Binding of apotransferrin to K562 cells; explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. USA.* 80:2263-2266.
22. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.
23. Lesley, J. F., and R. J. Schulte. 1984. Selection of cell lines resistant to anti-transferrin receptor antibody: evidence for a mutation in transferrin receptor. *Mol. Cell. Biol.* 4:1675-1681.
24. Lesley, J. F., and R. J. Schulte. 1985. Inhibition of cell growth by monoclonal anti-transferrin receptor antibodies. *Mol. Cell. Biol.* 5:1814-1821.
25. Mazia, D., G. Schatten, and W. Sale. 1975. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. *J. Cell Biol.* 66:198-200.
26. Mellman, I., and H. Plutner. 1984. Internalization and degradation of macrophage Fc receptors bound to polyvalent immune complexes. *J. Cell Biol.* 98:1170-1177.
27. McLean, J. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077-1083.
28. Newman, R., C. Schneider, R. Sutherland, L. Vodinelich, and M. Greaves. 1982. The transferrin receptor. *Trends Biochem. Sci.* 1:397-400.
29. Ottosen, P. D., P. J. Courtoy, and M. G. Farquhar. 1980. Pathways followed by membrane recovered from the surface of plasma cells and myeloma cells. *J. Exp. Med.* 152:1-19.
30. Platt, J. L., and A. F. Michael. 1983. Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylenediamine. *J. Histochem. Cytochem.* 31:840-842.
31. Pohlmann, R., A. Waheed, A. Hasilik, and K. von Figura. 1982. Synthesis of phosphorylated recognition marker in lysosomal enzymes is located in the cis part of Golgi apparatus. *J. Biol. Chem.* 257:5323-5325.
32. Regoeczi, E., P. A. Chindemi, M. T. Debanne, and P. A. Charlewood. 1982. Partial resialylation of human asialotransferrin type 3 in the rat. *Proc. Natl. Acad. Sci. USA.* 79:2226-2230.
33. Regoeczi, E., P. A. Chindemi, and M. T. Debanne. 1984. Partial resialylation of human asialotransferrin types 1 and 2 in the rat. *Can. J. Biochem. Cell Biol.* 62:853-858.
34. Roth, J., D. J. Taatjes, J. M. Lucocq, J. Weinstein, and J. C. Paulson. 1985. Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell.* 43:287-295.
35. Segal, D. M., and M. Hurwitz. 1977. Binding of affinity cross-linked oligomers of IgG to cells bearing Fc receptors. *J. Immunol.* 118:1338-1347.
36. Snider, M. D., and O. C. Rogers. 1985. Intracellular movement of cell surface receptors after endocytosis: resialylation of asialo-transferrin receptor in human erythroleukemia cells. *J. Cell Biol.* 100:826-834.
37. Trowbridge, I. S., R. A. Newman, D. L. Domingo, and C. Sauvage. 1984. Transferrin receptors: structure and function. *Biochem. Pharmacol.* 33:925-932.
38. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing  $\alpha_2$ -macroglobulin. *Cell.* 28:643-651.
39. Willingham, M. C., and I. Pastan. 1985. Ultrastructural immunocytochemical localization of the transferrin receptor using a monoclonal antibody in human KB cells. *J. Histochem. Cytochem.* 33:59-64.
40. Wilson, P., D. Sharkey, N. Haynes, P. Courtoy, and M. G. Farquhar. 1981. Iodinated cell membrane components are transported to the Golgi complex. *J. Cell Biol.* 91(2, Pt. 2):417 a. (Abstr.)
41. Yamashiro, D. J., B. Tycko, S. R. Fluss, and F. R. Maxfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell.* 37:789-800.