In Migrating Fibroblasts, Recycling Receptors Are Concentrated in Narrow Tubules in the Pericentriolar Area, and then Routed to the Plasma Membrane of the Leading Lamella

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Abstract. By following the intracellular processing of recycling transferrin receptors and the selective sorting of a-2 macroglobulin in chick embryo fibroblasts, we have shown that the concentration of 60 nm diam tubules which surrounds the centrioles represents a distal compartment on the recycling pathway. In migrating cells transferrin receptor tracers can be loaded into this compartment and then chased to the cell surface. When they emerge the recycling transferrin receptors are distributed over the surface of the leading lamella.

MEMBRANE proteins are removed and returned to the cell surface continuously in a recycling process which is thought to involve a turnover of plasma membrane area equal to the entire cell surface every 60–90 min (Steinman et al., 1976). The uptake steps in this recycling pathway, which involve the concentration of membrane proteins within clathrin-coated pits on the plasma membrane and their transfer to a compartment of low pH, the endosome, have been well documented (Pearse and Robinson, 1990; Trowbridge et al., 1993) but the later stages, which occur following the removal of lysosome-directed ligands and lead to the return of the recycling proteins to the cell surface, have been difficult to analyze. This is partly because processing within endosomal compartments is too asynchronous for the later stages to be probed using conventional pulse-chased procedures and partly because endosomal compartments have an extremely pleiomorphic and plastic form. The recent demonstrations (Hopkins et al., 1990; Tooze and Hollinshead, 1991) that endosomal compartments include extensive interconnecting tubular networks is an additional complication since their positions along the recycling pathway remain to be defined.

To outline the chronology of events in the later stages of the recycling pathway we have devised methods for observing receptor trafficking in living cells which leave the endocytic pathway relatively uncompromised and which can be correlated directly with high resolution, semi-quantitative analyses of receptor distributions. We have used chick embryo fibroblasts (CEF)[1] because their migratory behavior is well defined (Thom et al., 1979) and because we have available an expression system which allows them to express high levels of human transferrin receptors (Jing et al., 1990). To define the later stages of the recycling pathway we have compared the trafficking of transferrin receptor (TfnR)–transferrin (Tfn) complexes (which recycle with an efficiency close to 99%) (Klausner et al., 1983, Jing et al., 1990) with the routing of alpha-2-macroglobulin (a-2M) since this ligand (which rapidly dissociates from its receptor within the low pH of the endosome) is delivered to the lysosome with an efficiency close to 90% (Yamashiro et al., 1989).

Materials and Methods

Preparation of Tracers

Tfn-FITC was prepared by adding 0.5 mg FITC (Sigma Chemical Co., St. Louis, MO) into 100 ml ethanol to 2 mg Tfn (Sigma, cat no. T-0519) in 0.5 ml 0.25 M $NaHCO_3$, pH 9.0, stirring rapidly and incubating 2 hr are RT before separating on Biolgel P4. Final conjugates contained 1:10 Tfn:FITC. a-2M-FITC was prepared with methylamine activated a-2M exactly as described for Tfn-horseradish peroxidase (Tfn-HRP): For preparing TFn-HRP 10 mg Tfn was conjugated to 10 mg HRP (type II Sigma Chemical Co.) using SPDP as previously described (Hopkins, 1985). For preparing a-2M-HRP, native a-2M was prepared as described previously (Harpel, 1976). It was activated by incubation with 200 mM methylamine in 50 mM sodium phosphate, 150 mM NaCl pH 7.4 for 1 h at RT, and then dialyzed extensively against 0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3. HRP was activated with SPDP (20 fold molar excess) then added in 30-fold molar excess to methylamine activated a-2M, left to react 1 h at RT, and then dialyzed against 0.1 M acetate at pH 4.5.

5 nm colloidal gold sols were made as described by Slot and Geuze

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1. *Abbreviations used in this paper*: a-2M, alpha-2-macroglobulin; CEF, chick embryo fibroblasts; Tfn-HRP, Tfn-horseradish peroxidase; Tfn-R, transferrin receptor.

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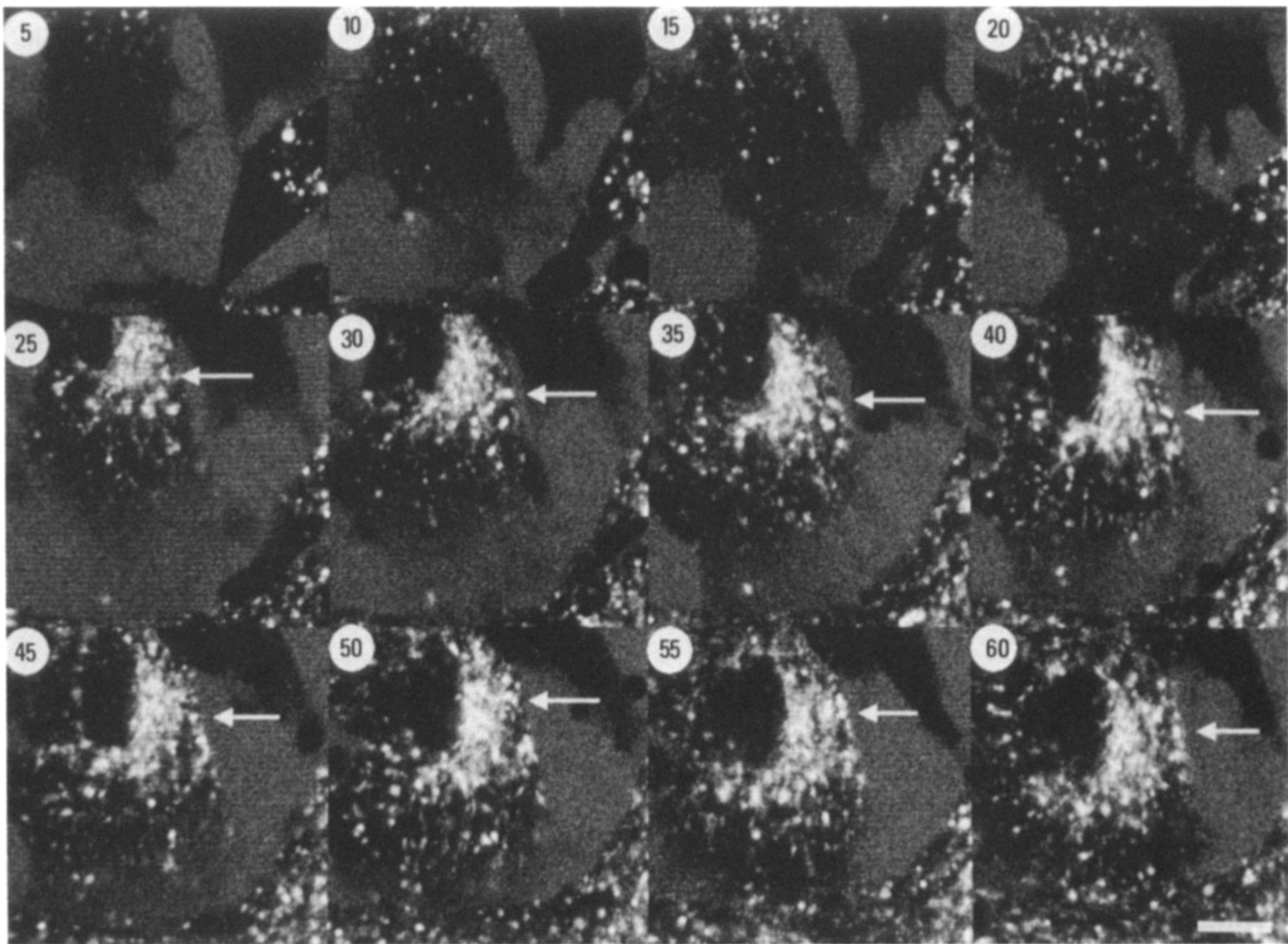

Figure 1. Confocal microscopy of fibroblast migrating in medium containing Tfn-FITC and displaying the recycling pathway at steady state loading. The arrow indicates a vacuole which is continuously in view over a 35-min period. Depth of field was calculated to be ~1 μm. Bar, 20 μm.

(1988). $B_{3/25}$ a monoclonal antibody to the human TfnR was complexed to colloidal gold as described previously (Hopkins, 1985). Before incubation gold complexes were washed by centrifugation in a Beckman airfuge at 150,000 *g* for 5 min.

Transferrin (Sigma) was iodinated as described previously (Hopkins and Trowbridge, 1983).

Cell Culture and Incubations

CEF were prepared from fertilized eggs (SPAFAS Inc., Norwich, CT) and grown in DME supplemented with 1% (vol/vol) chicken serum, 1% (vol/vol) defined calf bovine serum (Hyclone Labs., Logan, UT), 2% (vol/vol) tryptose phosphate broth (Difco Labs Inc., Detroit, MI) and transfected with a viral construct containing the coding region of the human TfnR as described previously (Jing and Trowbridge, 1987). Cultures were monitored for expression of human TfnR by indirect fluorescence with $B_{3/25}$ antibody.

For light microscopy CEF expressing human TFnR were plated onto glass coverslips for 2 h, rinsed with serum-free medium and incubated with fluorescent tracer. Concentrations of tracers used were: TFn-FITC 0.1 mg/ml; a-2M FITC, 50 mg/ml; TFnHRP, 0.2 mg/ml; a-2M-HRP, 0.1 mg/ml. Albumin-FITC and free HRP when used in the range 0.5–1.0 mg/ml to assess fluid phase uptake were undetectable by light microscopy over the time periods used in our experiments.

For most experiments cells were first incubated 60 min at 37°C in TFn-FITC. To follow the loss of TFn-FITC from the cells after this preincubation the coverslips were rinsed with agitation in a large volume of DME containing 1.0 mg/ml TFn and examined at 1–2-min intervals.

To follow the intracellular processing of a-2M coverslips were transferred from medium containing TFn-FITC to dishes containing TFn-FITC plus a-2M tracer at 37°C and the incubation continued for various intervals up to 60 min.

To quench FITC-labeled tracers with cointernalized HRP tracer loaded cells were fixed with 3% formaldehyde, photographed, and then medium containing H_2O_2 and diaminobenzidine (DAB) (Graham and Karnovsky, 1966) was perfused across the coverslip. Other changes of medium and tracer are as described in the results section. The uptake and recycling of ^{125}ITFn was followed as described previously (Jing et al., 1990).

Microscopy

Cells were examined in an MRC-600 Laser scanning confocal imaging system (BioRad Labs., Richmont, CA) with an Argon/Krypton mixed gas laser. Final images were merged using Photoshop software on a Macintosh computer and photographed directly from the screen. For time lapse experiments the laser beam was attenuated to 1% transmission and each image collected over a 2-s scan. Depth of field for these experiments was calculated to be ~1 μm.

For electron microscopy, cells were plated out for 2–4 h in 3-cm petri dishes in serum-free DME and incubated with tracers as indicated in results. They were then rinsed three times in ice-cold DME, fixed in dilute Karnovsky fixative (2% paraformaldehyde 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.5) (Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* 27:137*a*.) postfixed in reduced osmium tetroxide and embedded in Epon as described previously (Hopkins and Trowbridge, 1983). Sections

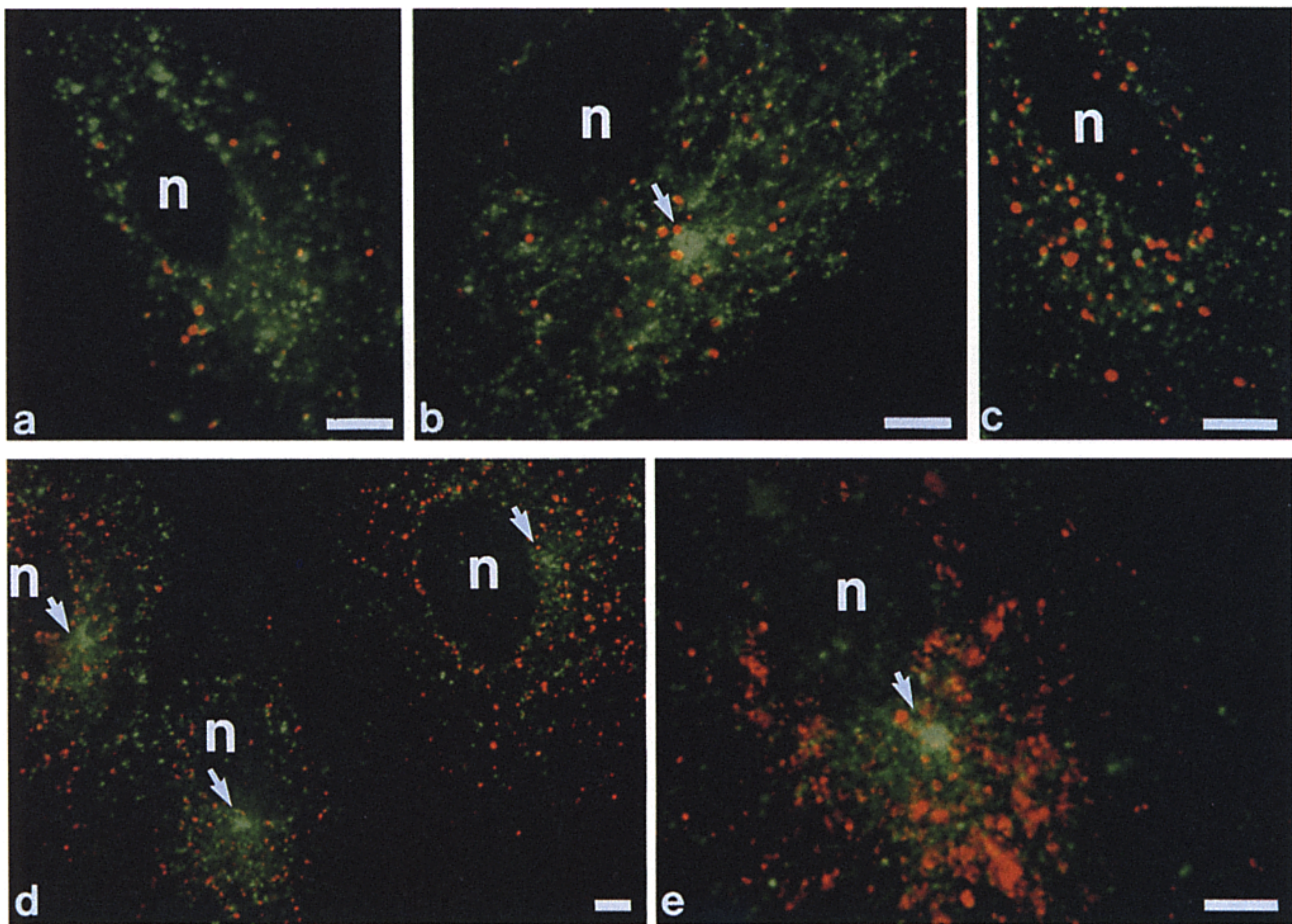

Figure 2. Cells incubated to steady state with Tfn-FITC, and then incubated with a-2M-HRP for 15 min (*a*), 45 min (*b*, *c*, and *d*), and 60 min (*e*). Cells fixed, photographed, and then treated with H_2O_2/DAB. Apple green fluorescence shows distribution of unquenched Tfn-FITC. HRP reaction product visualized by transmitted light is self colored to orange. With time, a-2M build up in the vacuoles and the pericentriolar concentrations containing recycling Tfn-FITC become increasingly apparent (*arrows*). Focal plane in all except *c* is at the level of the centrioles, in *c* the focal plane is above centrioles in order to display a-2M loaded 1–2 μm diam vacuoles *n*, nuclei. Bar, 50 μm.

were cut at either 70 nm and stained with uranyl acetate and lead citrate or up to 1 μm thick, stabilized with a thin film of evaporated carbon and viewed, unstained, in a Philips CM12 electron microscope. Whole mount and labeling with $B_{3/25}$ gold was carried out exactly as described in Hopkins (1985).

Quantitation

Cell were incubated with Tfn-HRP and $B_{3/25}$ gold for 60 min at 37°C and fixed and processed for electron microscopy as described above. In thick (dark blue interference color) sections centrioles were identified and all of the HRP-containing 60 nm diam tubules and vacuoles within a radius of 2.0 μm scored for their content of HRP reaction product and/or gold particles. 1,000 tubules were counted in each preparation. Cells incubated with a-2M HRP and $B_{3/25}$ gold for 60 min at 37°C, were processed and analyzed in the same way.

Results

Distribution of the Endosome Compartment in Migrating Cells

To outline the distribution of the entire Tfn/TfnR-containing compartment, cells were incubated for 60 min at 37°C with Tfn-FITC and viewed by confocal microscopy. The ability of the instrument to collect signal from a narrow plane of focus (i.e., to obtain optical sections of ~1 μm) allows intracellular elements to be identified with minimal interference from the tracer in the surrounding medium. Images were collected at 5-min intervals over a period of 60 min. During this time cells continued to migrate at the expected rate (~5 μm/min, Thom et al., 1979), intracellular structures continued to display saltatory movement and there was no indication that these repeated exposures to laser illumination affected endosome movement adversely. Previous work indicates that during 60 min incubation at 37°C Tfn tracers reach a steady state distribution throughout the intracellular compartments in which they traffic (Hopkins and Trowbridge, 1983) and since changing to tracer-free medium resulted in rapid export of intracellular tracer (discussed further below), we assume that a steady flux of receptor is being maintained throughout the system being displayed.

The endocytic pathway loaded to steady state with Tfn-tracer is a heterogeneous collection of vacuoles and tubulo-vesicles frequently interconnected by long (2–3 μm), thin tubules. Its detailed morphology, which is described below by electron microscopy, is essentially the same as the system described in detail in earlier studies on epithelioid cells (Hopkins and Trowbridge, 1983; Hopkins, 1985). In ac-

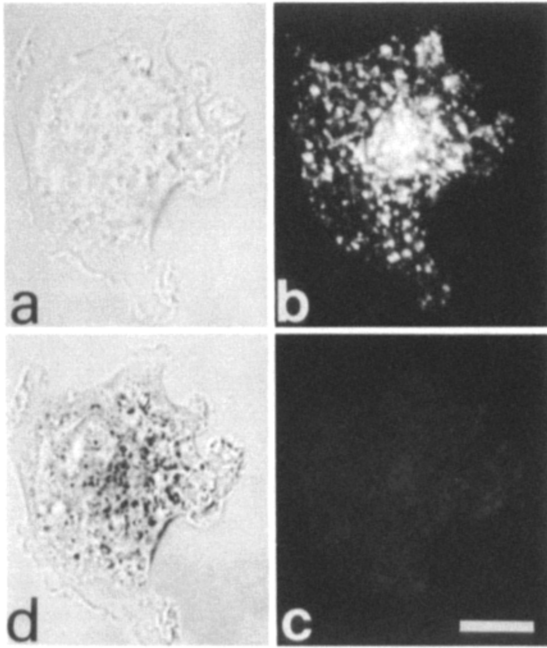

Figure 3. Peroxidase quenching of Tfn-FITC by a-2M-HRP. Cells incubated with Tfn-FITC (50 μg/ml) and a-2M-HRP (0.1 μg/ml) for 10 min. Preparation then fixed and treated with H_2O_2/DAB by perfusing fluid beneath coverslip. After a 10-min incubation with a-2M-HRP, all of the Tfn-FITC fluorescence is quenched (compare Fig. 2, where, in cells preloaded with Tfn-FITC, pericentriolar elements remain unquenched by a-2M HRP), (*a*) bright field, phase contrast (*b*) dark field displaying distribution of Tfn-FITC (*c*) dark field after H_2O_2/DAB treatment; all Tfn-FITC fluorescence quenched (*d*) bright field showing DAB reaction product coincident with Tfn-FITC tracer seen in *a*. Bar, 20 μm.

tively migrating fibroblasts Tfn-containing elements are distributed predominantly in the area which extends forward from the juxtanuclear area. There is usually a strong focus of fluorescence adjacent to the nucleus but within the lamellae which protrude from spreading and migrating cells fluorescently labeled elements are difficult to detect. As shown in the first four frames of Fig. 1, where the leading lamellipodium is protruding forward, most Tfn/FITC-positive structures move with the bulk of the cytoplasm. The sequence of micrographs shown in Fig. 1, also demonstrates that most vacuolar elements, and many tubules, exist for relatively long periods of time. Individual vacuoles, identifiable by their content of Tfn tracer can be identified and followed for up to 60 min.

The Limit of TfnR Migration Towards the Lysosome

To identify the stage at which ligand directed towards lysosomal compartments is sorted out of the recycling pathway, cells were labeled for 60 min at 37°C with Tfn-FITC, and then, in the continued presence of this tracer, a-2M-HRP was added to the medium. From 10 min onwards the number and size of labeled vacuolar structures increases and over the period 10–30 min the cytoplasm becomes heavily loaded with large (1–2 μm diam) vacuoles containing a-2M-HRP tracer (Fig. 2).

By double labeling with tracers conjugated to FITC and HRP, the ability of peroxidase enzyme activity to quench FITC fluorescence was exploited so that compartments which contain both tracers could be discriminated from those which contain only FITC. Thus, as shown in Fig. 3, if cells are double labeled with Tfn-FITC and a-2M-HRP for ten minutes, all of the fluorescence signal generated by the FITC is rapidly quenched by fixing and incubating in H_2O_2/DAB. The quenching process (which takes 10–15 s) can be observed directly on the microscope stage and the brown DAB reaction product generated by the peroxidase activity serves to identify the site of the enzyme for both light and electron microscopy. These studies show that the bulk of internalized Tfn and a-2M are colocalized during the first 10 min of their intracellular processing. By incubating cells with Tfn-FITC to steady state and introducing a-2M-HRP for longer periods, peroxidase quenching can also be used to follow the subsequent sorting of Tfn and a-2M into separate compartments. As shown in Fig. 2, this approach allows the identification of endosomal elements from which a-2M HRP is removed as the later stages of the recycling pathway. In most cells it clearly delineates the pericentriolar area as a focus in which recycling TfnR concentrate after the removal of a-2M.

Electron Microscopy

For correlative electron microscopy, cells were incubated for 60 min at 37°C with Tfn-HRP. As in earlier studies of the endosome compartment (Hopkins, 1985), thick sections were used in order to discriminate between small, independent vacuoles and long, thin tubules. Structures observed which contained Tfn-HRP included medium-sized vacuolar elements (0.5–1.0 μm diam) and tubules (250 nm diam) with continuities frequently being seen between the two. In addition to vacuoles and 250 nm diam tubules Tfn-HRP was also distributed throughout networks of thinner (60 nm diam) tubules. The morphology of these networks is very similar to those which have been observed in cells which had had prolonged incubation with high concentrations of fluid phase HRP (Tooze and Hollinshead, 1991). The tubules in these networks frequently branch and are sometimes seen in continuity with the 0.5–1.0 μm diam vacuoles (see Fig. 4). Branching networks of 60-nm diam tubules are found throughout the cytoplasm and are well developed just below the plasma membrane, towards the free margins of cells. However, in the main body of the cell they are especially concentrated in the pericentriolar area where they form a closely packed, interwoven mass (Fig. 4). In studies which will be reported elsewhere we have studied the relationship between this concentration of 60 nm diam tubules and *trans*-Golgi elements. The *trans*-Golgi was identified by transfecting cells with a cDNA for horse radish-peroxidase and incubating at 20°C for 60 min with anti-TfnR/gold. In these preparations HRP reaction product labeled *trans*-Golgi elements while gold conjugates label the recycling endocytic pathway. The labeled elements are closely intertwined in the pericentriolar area but colocalizations are not seen (even when the temperature is shifted to 37°C).

Double Labeling with TfnR and a-2MR

To identify the stage(s) at which a-2M ligand is sorted from, recycling TfnR cells were incubated continuously with both a-2M-HRP and TfnR antibody ($B_{3/25}$)-gold complexes. After 20 min incubation, when the larger a-2M-HRP-labeled vacu-

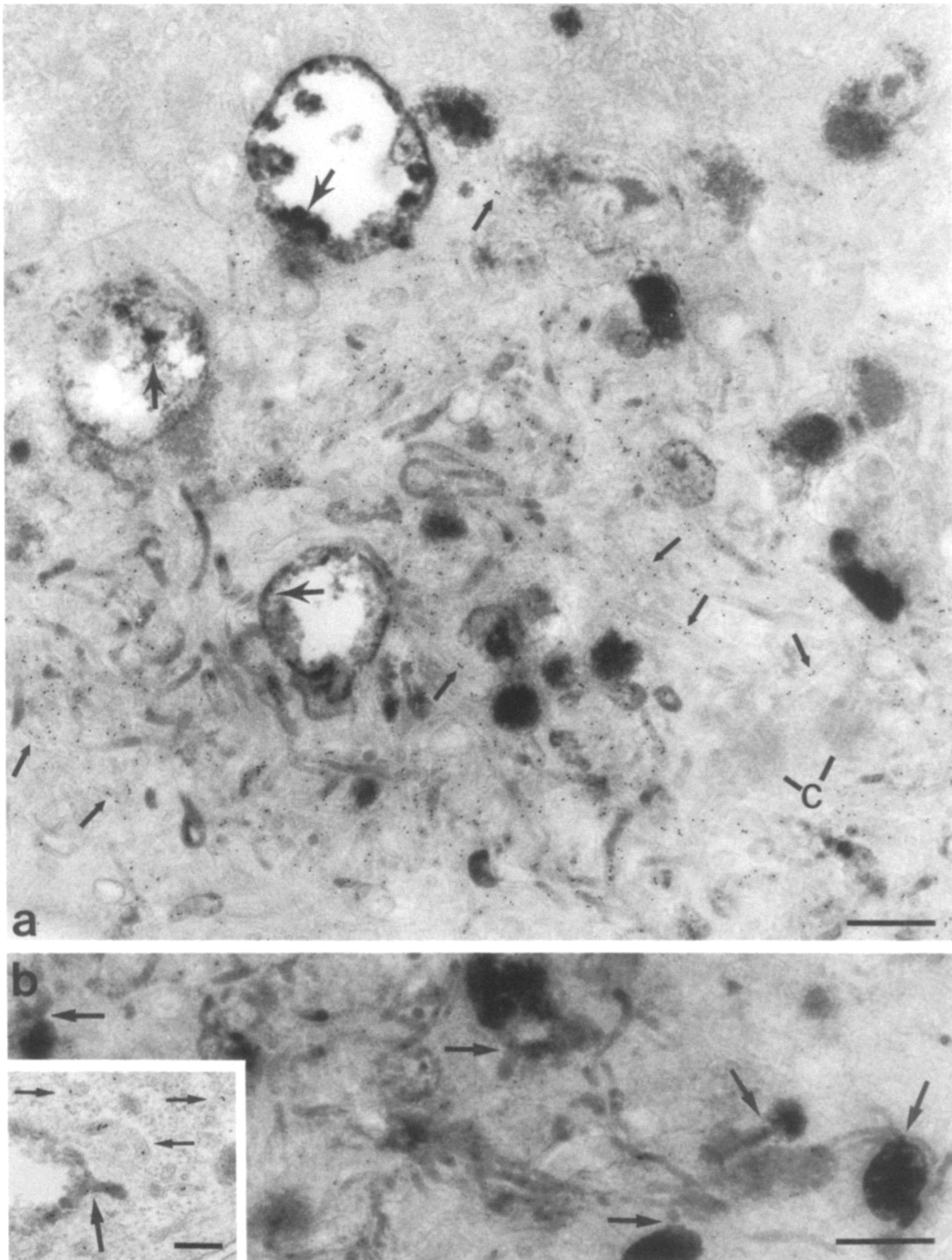

Figure 4. (*a*) Electronmicrograph of thick (1.0 μm) section of pericentriolar area of cell preloaded 60 min with a-2M HRP and $B_{3/25}$-gold. An elaborate array of 60 nm tubules and vacuoles of various sizes are shown surrounding the centrioles (*c*). Some tubules contain both tracers, others (*small arrows*) contain only $B_{3/25}$-gold. The (larger 1 to 2 μm diam) vacuoles are loaded a-2M HRP but contain a few gold particles (*large arrows*). Bar, 0.5 μm. (*b*) Similar area to *a* of cell preloaded 60 min with TFn-HRP and $B_{3/25}$-gold. 60-nm diam tubules containing both tracers are present and arrows indicate several points at which tubules and vacuoles are in close juxtaposition and probably connected. Inset shows profile in conventional thin section from same preparation as in *a* where direct continuity between a 60-nm diam tubule and a vacuole is shown (*large arrow*). Small arrows indicate gold loaded 60 nm diam tubules lacking HRP reaction product. Bar, 0.5 μm; Inset Bar, 0.1 μm.

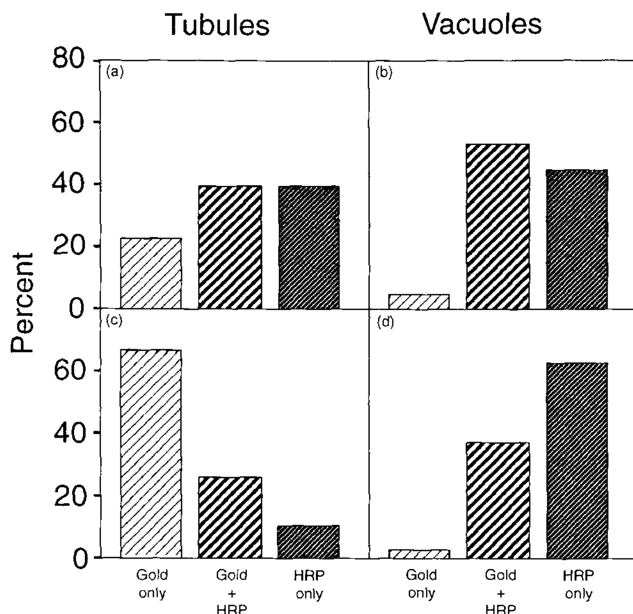

Figure 5. Quantitation of tracer separation illustrated in Fig. 4. (*a* and *b*) Cells were incubated 60 min with Tfn-HRP and $B_{3/25}$-gold. Tubules and vacuoles were analyzed for their content of one or both tracers as described in Materials and Methods. This analysis provided the baseline for the experiment shown in *c* and *d*. Thus although no separation between these Tfn-HRP and $B_{3/25}$-gold is to be expected in this preparation (i.e., they both remain bound to internalized TfnR) some doubly labeled tubules will be scored as containing only one tracer (either because gold, being particulate, will not be contained in every section plane or because the HRP reaction product, being diffuse, will not always be strong enough to allow positive identification in a grazing section). Comparing the distribution of Tfn-HRP and $B_{3/25}$-gold shows that the discrepancy arising from these potential sampling errors is of the order of 22% in underestimating the amount of HRP tracer remaining in the tubules but up to 40% in underestimating the number of gold-labeled TfnR sorted away from the HRP tracer. (*c* and *d*). Cell incubated for 60 min with a-2M HRP and $B_{3/25}$-gold, prepared and analyzed as in *a* and *b*. Despite the sampling procedure which favors the detection of HRP, there is a threefold increase in the proportion of tubules which contain only TfnR-gold tracer.

oles begin to develop, $B_{3/25}$-gold was distributed throughout the 250-nm diam tubules and on the perimeter membrane of the vacuoles, as described in previous studies on epithelioid cells (Hopkins et al., 1990; Hopkins and Trowbridge, 1983). From 30 min onwards the 60-nm diam tubules become increasingly strongly labeled with both tracers and with longer incubation times tubules containing only TfnR-gold complexes begin to appear (Fig. 4). Beyond 60 min tubules containing only gold tracer predominate. It is worth emphasizing that tubules, containing only $B_{3/25}$-gold are rarely found in preparations incubated with the tracers for less than 30 min.

To follow the separation of recycling TfnR from a-2M ligand destined for the lysosome the distribution of TfnR (labeled with $B_{3/25}$-gold) was quantified and correlated with that of tubules and vacuoles containing Tfn (Tfn-HRP) and a-2M (a-2M-HRP) tracers (Fig. 5). In cells in which tracers for TfnR and Tfn are compared (Fig. 4 *b*) (and in which separation should be negligible) 22% of the tubles appear to contain only gold (TfnR tracer) and 40% contain only Tfn-HRP.

Table I. Chase of Preloaded Intracellular ^{125}I Transferrin

	Percent cell associated ^{125}I Tfn		
	Intracellular	*Surface*	*Chase medium*
0′	75.7 ± 1.2	24.3 ± 1.2	–
5′	67.5 ± 2.0	11.4 ± 0.9	21.1 ± 2.3
10′	45.8 ± 3.7	7.2 ± 1.0	50.0 ± 1.7
20′	18.5 ± 0.5	5.6 ± 0.5	75.9 ± 0.8
30′	9.7 ± 0.7	2.3 ± 1.4	88.0 ± 1.9
45′	6.2 ± 0.6	1.6 ± 0.9	92.2 ± 1.2
60′	4.2 ± 0.5	3.2 ± 0.7	92.5 ± 0.8

However, in cells where TfnR and a-2M-HRP distributions are compared, tubules containing gold tracer without HRP increase threefold (to 66%) while the number of tubules containing only HRP decreases fourfold (to 10%). Of additional interest is the difference between the total number of tubules containing HRP reaction product; 80% contain Tfn-HRP whereas only 25% contain a-2M-HRP. Thus, despite the sampling errors inherent in quantifying particulate and nonparticulate tracers within thin sections of a tubular network these results clearly demonstrate that while recycling TfnR (and presumably a-2M receptors) are becoming concentrated in the 60-nm diam tubules of the pericentriolar area the amount of free, circulating ligand, as represented by a-2M-HRP, is being reduced.

The Migration of Recycling TfnR to the Cell Surface

To examine the possibility that the 60-nm tubules of the pericentriolar area concentrate recycling TfnR immediately before their return to the cell surface, a series of washout experiments was carried out in which the recycling pathway was loaded to steady state with either ^{125}I Tfn or Tfn-FITC, and then chased at 37°C with unlabeled Tfn (1 mg/ml). These experiments showed that while most of the preloaded intracellular radiolabel can be chased out of the cell within 20 min (Table I) a residual focus of tracer detectable by fluorescence usually remained in the pericentriolar area. In some cells these pericentriolar foci remained for up to 60 min and since these cells usually looked immobile, we explored the possibility that the loss of pericentriolar tracer from their neighbors was due to their migratory behavior. To examine this in detail, preparations preloaded with Tfn-FITC were recorded by video microscopy while being perfused with chase medium at 37°C. In general this is a less efficient method of chasing label out of the cells, but as shown in Fig. 6 it can be used to demonstrate directly that while cells which remain immobile retain their pericentriolar signal those which are seen to migrate rapidly lose tracer from this location.

To determine the fate of the tracer being lost from the pericentriolar area, preloaded cells were chased, fixed, and examined at high resolution. As shown in Fig. 7, when preloaded cells are chased in this way and the pericentriolar concentration of tracer is lost there is a concomitant increase in fluorescence at the cell periphery which, as judged by optical sectioning in the confocal microscope, is on the cell surface. Surface labeling is most clearly seen on cells after chases of 5–10 min when it is distributed on the leading

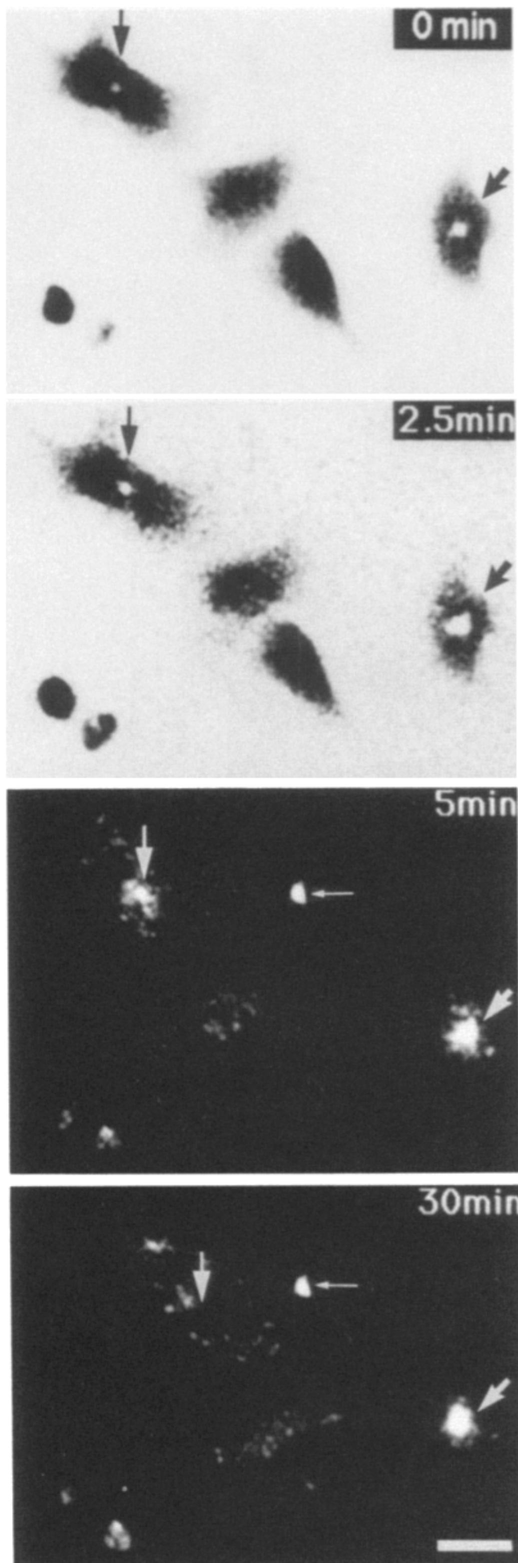

Figure 6. Confocal microscopy of living fibroblasts preloaded with Tfn-FITC, and then chased for the times indicated with medium containing unlabeled tracer (100 μg/ml Tfn). The chase is carried out by perfusion and the loss of fluorescent tracer from the medium is thus relatively slow (compare fluorescence in medium at 2.5 and 5 min). The vertical arrow indicates the pericentriolar region of a fibroblast migrating towards the lower right hand corner, a fragment of brightly fluorescent debris (*horizontal arrow*) serves as an immobile reference point. Between 5 and 30 min, tracer is lost from the pericentriolar focus of this migrating cell while it is retained within the pericentriolar focus of an adjacent immobile cell (*diagonal arrow*). Bar, 50 μm.

lamellipodia of cells with a polarized morphology (N.B the signal shown in Fig. 7, *b* and *c* is maximized to display the label on the ruffle). It is best displayed on cells which have well developed ruffle borders at their leading edge but is also well shown on cells which appear to be moving apart after cytokinesis (data not shown). Control preparations in which preloaded cells were examined immediately after the preliminary 37°C washout show cells with clearly defined lamellipodia but their fluorescence, where detectable, is punctate, and, as judged by confocal microscopy, intracellular.

To quantitate the number of recycling receptors on the leading lamellaepodia of migrating fibroblasts, moving cells were recorded by video, the preparation was then fixed, incubated with anti-TfnR-gold complexes, and prepared for whole mount electron microscopy (Hopkins, 1985). Individual cells, which had been seen to be in the process of migrating when fixative was applied, could be identified at low magnification in the electron microscope by their shape. The density of TfnR (as indicated by the number of gold particles) was then quantitated over the surface of these cells. On leading lamellae of migrating cells, densities of 230 ± $35/\mu m^2$ were found (Fig. 7), elsewhere on the same cells surface densities were fivefold lower and in the range 43 ± $15/\mu m^2$. The increased number of TfnR seen on the surface of these leading lamellae confirms the view that the Tfn fluorescence which can be chased to this location is due to an increase in the density of surface TfnR and does not arise simply because there is a greater surface area of membrane on the ruffled lamellipodia. These studies were extended by recording cell movements over a 30 min period, fixing and staining for immunofluorescence with $B_{3/25}$/FITC. In all instances where cells had protruded a leading lamella immediately before fixation, the upper surface of the lamella showed strong fluorescence (Fig. 7 *h*).

Discussion

The Form and Stability of Endosome Compartments

The recycling pathway as outlined in this study appears to contain separate, functionally specialized subcompartments within a system of extensive structural continuity. In general our observations conform with the description by Maxfield and his colleagues of the endocytotic pathway in CHO cells which have identified a "sorting endosome" comprised of vacuoles in which lysosomally directed ligands are concentrated and separated from recycling ligands and receptors and a "recycling endosome" adjacent to the nucleus, in which recycling receptor complexes accumulate (Yamashiro et al., 1984; Dunn et al., 1989; Mayers et al., 1993). Our data do not show that all of the internalized receptors which recycle traverse the recycling endosome. Indeed, since half times for the processing of recycling receptors introduced into cells by pulse-chase procedures are in the range 10–15 min (Bliel and Bretscher, 1982; Hopkins and Trowbridge, 1983), and the

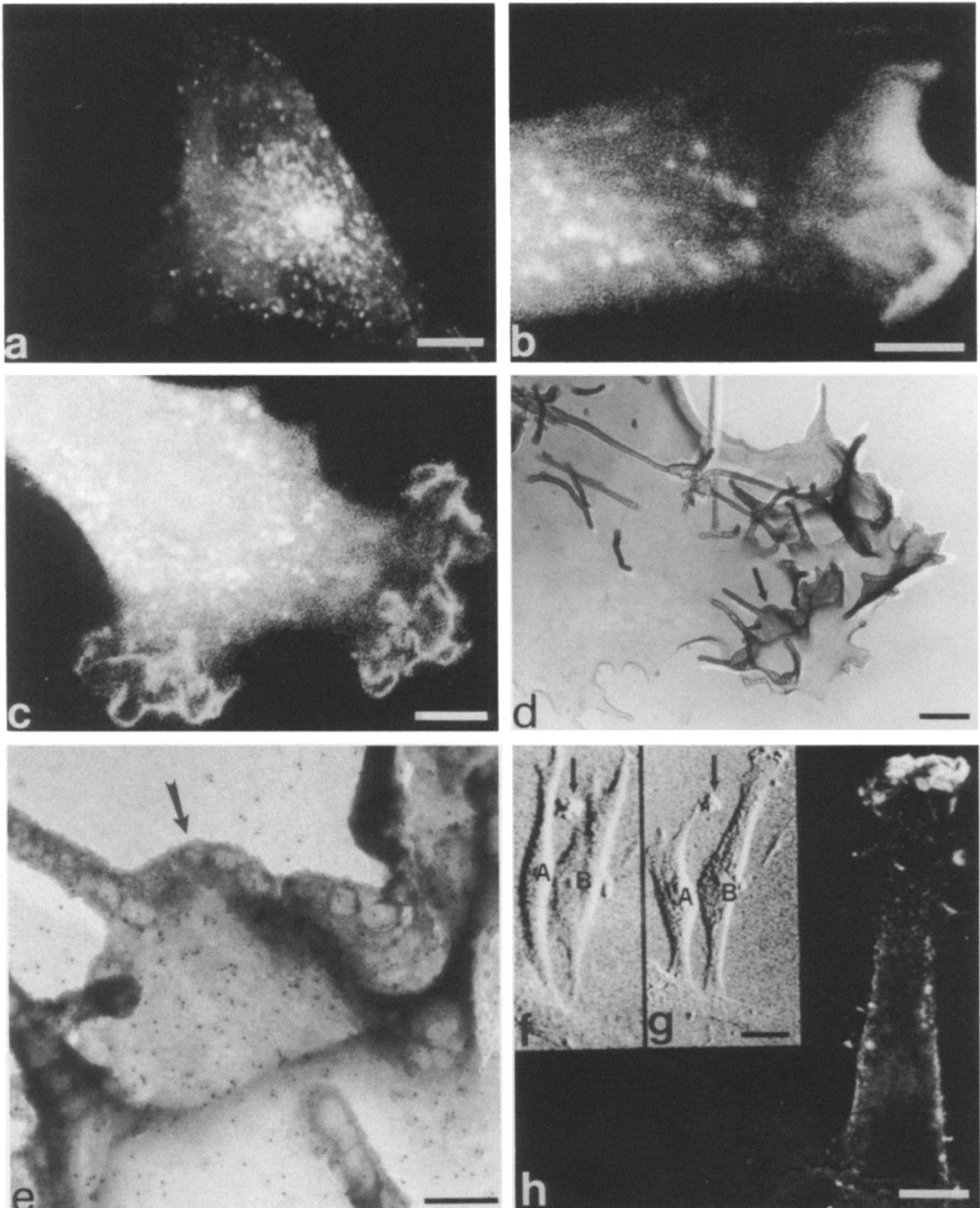

Figure 7. (*a–c*) Confocal microscopy of cells preloaded with Tfn FITC 60 min at 37°C then chased for (*a*) 0, (*b*), 5 (*c*), and 10 min. (*a*) Plane of focus at level of centrioles showing intracellular focus of tracer (*b* and *c*) plane of focus at cell surface showing fluorescence distributed over lamellipodia of cells with polarized morphology. To display the surface fluorescence on the lamella and ruffles, the signal over the body of the cell has been grossly overexposed. (*d* and *e*) Electron micrographs of a cell treated as in *a–c* fixed, incubated with TfnR antibody-gold for 30 min at 5°C, and prepared for whole mount transmission electron microscopy. In *d*, ruffles on the leading lamella are clearly displayed. The ruffle arrowed in *d* is shown at higher magnification in *e*. The 8-nm gold particles identify the TfnR and show the relatively high density of receptors on the ruffle. (*f*, *g*, and *h*) Movement recorded by video then cells fixed and stained to display surface distribution of TfnR. Comparing *f* and *g* shows that while cell *A* withdraws, cell *B* extends (compare the position of their lamella with position of debris marked with *arrow*). (*h*) Shows fluorescence on the extended lamella of cell *B*. Bars: (*a–c*) 10 μm; (*d*) 1 μm; (*e*) 0.2 μm; (*f*) 20 μm; (*h*) 10 μm.

pericentriolar compartment becomes loaded over periods of 30–60 min, it is clear (in epidermoid cells at least) that only a proportion enter this more slowly filling compartment. More than one recycling pathway must, therefore, exist, and, since all the available evidence suggests that ligand-receptor complexes internalizing via coated pits enter a common endosome compartment (Trowbridge et al., 1993), this alternative routing is presumably determined at an intracellular location. Selection for the slower rather than the faster recycling route need not depend upon a specific sorting mechanism concerned with route selection since 60 nm diam tubules are widely distributed throughout the cell and are found in the peripheral cytoplasm as well as in the pericentriolar area. The length of time recycling receptors take to return to the cell surface may, therefore, depend primarily upon how large a proportion of these tubules is oriented towards the pericentolar area. This, as discussed further below, may itself be determined by the extent to which a cell adopts a polarized form.

Our observations also show that many of the endosomal elements within the pathway have half lives significantly longer than the time taken for recycling receptors to be processed intracellularly ($t1/2$ = 10–15 min) and suggest that many endosomal elements are pre-existing, relatively stable components through which recycling receptors are fluxing continuously. There is no indication of the direction of flux from our studies but the process of "iterative fractionation" proposed for sorting TfnR and lysosomally directed ligand in CHO cells (Dunn et al., 1989) could be accomplished by circulation and recirculation of ligand-receptor complexes through the interconnecting systems of vacuoles and tubules that we and others (Tooze and Hollinshead, 1991) have described. However, our semi quantitative electron microscope studies show that the separation of recycling receptors from lysosomally directed ligand takes place within the 60-nm diam tubules of the pericentriolar area as well as in the vacuoles of the sorting endosome.

The Functional Significance of Recycling Elements in the Pericentriolar Area

The concentration of 60-nm diam tubules in the pericentriolar area, identified in previous studies (Hopkins and Trowbridge, 1983, Yamashiro et al., 1984; Tooze and Hollinshead, 1991), clearly correspond to the strong fluorescence seen in this area in cells loaded with Tfn-FITC. Similar foci of fluorescence have been described in several previous studies of recycling receptors (Hopkins and Trowbridge, 1983; Dunn et al., 1989). In the electron microscope the 60-nm diam tubules are seen to be continuous with the 1.5–2.0-μm diam vacuoles and we have shown that those in the pericentriolar area are able to sequester recycling TfnR away from lysosomally directed a-2M. Since we have also been able to show that these pericentriolar concentrations of TfnR are the last elements to contain tracer when cells labeled to steady state are chased with cold ligand, we have good evidence that these 60-nm diam tubules represent a specialized, distal segment of the recycling pathway.

The functional significance of these endosomal elements being concentrated in the pericentriolar area is not entirely clear. A concentration of 60-nm diam tubules could arise in this location because growing tubules extend in association with the microtubular cytoskeleton. However, most studies to date have associated the movement of endosomal vacuoles rather than endosomal tubules with microtubules (de Brabander et al., 1988; Matteoni and Kreis, 1987). A recent study on Brefeldin A-treated cells has described a close association of 50 nm tubules with microtubules (Tooze and Hollinshead, 1992) but all of the tubular elements in these BFA-treated cells were extensively redistributed and it is thus probable that, in this instance, their relationship with microtubules is drug related.

Alternatively the primary functional significance of the pericentriolar recycling compartment may be to direct recycling receptors along the microtubules which radiate from this area to a specific cell surface domain. In locomoting fibroblasts, migrating in a polarized fashion this is likely to be to the ruffled border at the leading edge (Singer and Kupfer, 1986). In a previous study we demonstrated the newly emerging transferrin receptors appear at free cell margins of spreading epithelial cells and that in these cells pericentriolar concentrations of 60 nm diam tubules were also a prominent feature (Hopkins and Trowbridge, 1983; Hopkins, 1985). In epithelia this kind of directed delivery may subserve other functions. In cells of polarized epithelia, for example, recycling receptors like TfnR and many newly synthesized membrane proteins are selectively routed to the basolateral border and the efficiency of this delivery also depends upon an intact microtubular cytoskeleton (Rodriguez-Boulan and Powell, 1992). It is of interest that in polarized epithelial cells microtubules are, in general, orientated with their plus ends towards the basolateral surface since the plus ends of the microtubules emanating from the centriolar area in migrating fibroblasts have a similar orientation with regard to the ruffled border (Vale, 1990).

Receptor Recycling and Cell Migration

In the past the requirement for membrane to be delivered to the leading lamellae of migrating fibroblasts has been a matter of considerable debate (Bretscher, 1989) and some recent studies have provided evidence which argues directly against this possibility (Sheetz et al., 1989). Nevertheless, other work (Altankov and Grinnell, 1993) which shows that the inhibition of endocytosis abrogates polarized migration continues to suggest a link between recycling and directed, forward movement of the cell. Our present studies strongly support the view that a proportion of recycling receptors are delivered selectively to the leading lamellae in migrating cells. The close packing of receptors which we have observed on the ruffles in this domain suggests, however, that this selective delivery of recycling receptors is likely to be more concerned with the emergence of new recognition sites in this location than the delivery of bulk membrane. Targeting receptors which adhere to extracellular matrix components or bind chemotactic ligands to the leading edge of a migrating fibroblast is an obvious requirement for polarized migration and there is recent evidence (Bretscher, 1992) to suggest that transferrin receptors in particular may directly participate in this process.

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