The Coated Pit and Macropinocytic Pathways Serve Distinct Endosome Populations

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Abstract. Clathrin-coated vesicle endocytosis and macropinocytosis are distinct endocytic pathways demonstrable in several cell types including human epidermoid A431 cells (West, M.A., M.S. Bretscher, and C. Watts. 1989. *J. Cell Biol.* 109:2731-2739). Here we analyze the extent of mixing of macropinocytic endosome (macropinosome) content with that of conventional endosomes served by coated vesicle endocytosis. Using laser scanning confocal fluorescence microscopy we detected very little delivery of macropinosome content to either early or late endosomes-lysosomes as defined by labeling with transferrin or with LDL. Mixing of the contents of the macropino-

somes and conventional endosomes was not induced by the addition of brefeldin A. Moreover, the morphology of macropinosomes was not grossly altered in the presence of brefeldin A, whilst in the same cells there were dramatic tubulation effects on conventional endosomes as reported by others. Although refractory to fusion with conventional endosomes, macropinosomes were nonetheless dynamic structures which sometimes exhibited vesiculo-tubular morphology in living cells and were capable of fusing with each other. We suggest that different endocytic mechanisms can give rise to distinct endosome populations.

CLATHRIN-coated pits and the endosomal processing system they serve are recognized as the major route of entry for most endocytosed material, particularly that taken up via cell surface receptors (reviewed in Gruenberg and Howell, 1989; Hubbard, 1989; van Deurs et al., 1989; Smythe and Warren, 1991). However, it is now clear that other endocytic pathways which are not clathrin mediated exist on a variety of cell types (reviewed in van Deurs et al., 1989; Watts and Marsh, 1992). Characterization of these pathways is at an early stage relative to the clathrin-mediated system but there appear to be distinct types based on vesicle morphology, size, sensitivity to inhibitors, and other criteria.

Smooth flask shaped pits often referred to as caveolae have been frequently described on several cell types, particularly endothelial cells (Palade, 1953; Fawcett, 1965) and it was suggested that cholera and tetanus toxins enter via such smooth pits (Montesano et al., 1982). In fibroblasts similar cell surface invaginations are responsible for the delivery of receptor-bound folate (Rothberg et al., 1990a,b). These caveolae feature a distinct filamentous coat which includes a 22-kD v-src tyrosine kinase substrate termed caveolin (Rothberg et al., 1992). A homologous protein, VIP 21, has now been shown to reside in MDCK cell caveoli as well as in *trans*-Golgi network–derived vesicles (Dupree et al., 1993). How-

ever, morphological and biochemical criteria indicate that caveolae may operate without complete detachment from the cell surface (Rothberg et al., 1990a,b; van Deurs et al., 1993). In this case clathrin-independent pinocytosis of bulk phase markers must be mediated by alternative means. Hansen et al., have described distinct non-clathrin–coated pre-endosomal vesicles in HEp-2 cells which are 95 nm in diameter and which continue to form under conditions that block clathrin-coated vesicle formation, e.g., K⁺-depletion (Hansen et al., 1991). Another distinct class of non-clathrin–coated endocytic vesicles are the macropinosomes. Macropinocytosis can occur constitutively but is dramatically up-regulated by growth factors or phorbol esters on some cell types (Haigler et al., 1979; West et al., 1989; Swanson, 1989; Racoosin and Swanson, 1989, 1992; Sandvig and van Deurs, 1990). Unlike clathrin-coated pits, caveolae and the 95 nm non-clathrin–coated vesicles, macropinosomes appear to be very heterogeneous in size and often form at the leading edge of cells and where membrane ruffling activity is maximal. We found that the activity of clathrin-coated pits and macropinocytotic activity could be clearly distinguished based on the sensitivity of the latter to amiloride and amiloride analogues which inhibit Na⁺/H⁺ exchange (West et al., 1989; and L. Hewlett, unpublished results). Although the mechanism of macropinocytosis and the basis of its sensitivity to amiloride is not yet established, growth factor induced membrane ruffling and pinocytosis appears to be regulated by members of the ras family (Bar Sagi and Feramisco, 1986) and particularly the rho sub-family (Ridley et al., 1992) of ras-related GTPases. Ruffling and

pinocytosis could be stimulated following microinjection of activated rac-1 (V12 racl) and growth factor–induced ruffling was inhibited by microinjection of N17 racl, which presumably inhibits endogenous rac-1 function in a dominant fashion (Ridley et al., 1992).

The existence of as many as three distinct endocytic pathways (assuming all contribute to net membrane uptake) raises several questions about the fate of the endocytic vesicles generated by these apparently diverse mechanisms. Recently, Hansen et al. (1993) have shown that markers endocytosed via 95 nm non-clathrin–coated vesicles deliver their contents into transferrin receptor positive early endosomes. Here we analyze the capacity of macropinosomes in EGF-stimulated A431 cells to communicate with these endosomes. Surprisingly, we find that while macropinosomes appear to be dynamic structures and can fuse with each other, their contents do not become mixed with markers such as transferrin and LDL delivered through coated pits.

Materials and Methods

Reagents

EGF was purchased from AMS Biotechnology (Witney, Oxon, U.K.). Cell culture media was from GIBCO BRL (Paisley, Scotland) Brefeldin A was a gift from Sandoz (Basel, Switzerland) or was purchased from Sigma Immunochemicals (St. Louis, MO). Texas red Dextran 70,000 mol wt, lysine fixable, and Texas red were purchased from Molecular Probes, Inc. (Eugene, OR). Glass coverslips and microscope slides were from BDH Chemicals Ltd. (Poole, U.K.). Tissue culture plastics were purchased from Costar (Bucks, U.K.). Citifluor was from Agar (Stansted, U.K.). All other reagents were from Sigma Immunochemicals, (Poole, U.K.).

Cell Culture

A431 cells (passage 35), were a generous gift from Professor Colin Hopkins (University College, London) or were obtained from the European Collection of Animal Cell Cultures (Porton, U.K.) and were maintained in DME, supplemented with 10% heat-denatured FCS, 100 U/ml kanamycin and 2 mM glutamine. Cells were grown in a 37°C incubator containing a 95% air/5% CO_2 atmosphere. Experiments were performed on cells that had almost reached confluency in 30 mm petri dishes following subculture 2 d previously. For microscopy, cells were seeded at low density onto sterile glass coverslips (10^5 cells per 30 mm dish) 2 d previously. Cells were not used for experiments beyond passage 45. Before an experiment the growth medium was removed and the cells were incubated for an hour at 37°C in serum-free medium buffered with 20 mM Hepes, pH 7.4, containing 2 mg/ml BSA.

Preparation of Low-density Lipoproteins

Low-density lipoproteins (LDL)[1] were prepared by serial density ultracentrifugation of human serum (Havel et al., 1955). VLDL's were removed by overlaying the serum with 196 mM NaCl, 0.27 mM EDTA (ρ = 1.0063 g/ml), and centrifuging for 20 h at 45,000 rpm at 20°C in a rotor (55.2 Ti; Beckman Instruments, Palo Alto, CA). The VLDL-containing layer was then carefully removed. LDL was isolated by resuspending the remaining solution and adding half the volume of 2.46 M NaBr, 196 mM NaCl, 0.27 mM EDTA (d = 1.1816 g/ml) and centrifuging at 45,000 rpm for 20 h at 20°C. The LDL layer was removed, dialyzed for 24 h against PBS and used for conjugation (see below). Lipoprotein-deficient serum was then obtained by removing HDL from the remaining resuspended solution. The density of the solution was increased by adding half the volume of a 7.53 M NaBr, 196 mM NaCl, 0.27 mM EDTA (d = 1.4744 g/ml), followed by centrifugation at 50,000 rpm for 24 h at 20°C. The HDL layer was discarded and the remaining solution was dialyzed extensively against DME buffered with Hepes, pH 7.4, and used as serum to supplement normal growth medium in experiments where LDL was used.

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1. *Abbreviations used in this paper*: LDL, Low-density lipoproteins.

Preparation of Labeled Conjugates

Texas red (sulphonyl chloride) was conjugated to transferrin. 1 mg of Texas red was dissolved in 50 μl of dimethylformamide and added very slowly in an ice bath to a gently stirred solution of ferri-transferrin previously dialyzed into 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.2. The bath was allowed to warm to room temperature over a period of 2 h in the dark. Hydroxylamine (0.1 M, pH 8.0) was added for 1 h to hydrolyze any tyrosine esters formed. The conjugate was then passed down a 10 ml Biogel P-10 column pre-equilibrated in PBS in order to remove unbound Texas red.

Fluorescein isothiocyanate was conjugated to LDL and transferrin. The conjugation was carried out as detailed above except that the FITC was dissolved in DMSO and the conjugation was for a period of 8 h at 4°C. 50 mM NH_4Cl was added to quench the reaction for 2 h at room temperature before passing the conjugate down the column.

Specificity of receptor binding was assessed by performing an incubation in the presence of an excess of the appropriately unlabeled protein.

Measurement of EGF-stimulated Pinocytosis

After a serum-free preincubation each dish was washed once with medium A (137 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM MgC_2, 2 mg/ml BSA, and 20 mM Hepes, pH 7.4), and then incubated at 37°C in 0.75 ml of medium A containing 2 mg/ml horseradish peroxidase (type 2; Sigma Immunochemicals) and 100 ng/ml EGF where indicated. After various times the cells were chased where appropriate in medium A containing 20 mM glucose for various periods of time in the presence or absence of 5 μg/ml brefeldin A. The cells were then quickly rinsed in 2 ml of medium A, immersed in two 500 ml volumes of PBS/BSA and then placed in a beaker containing ice cold PBS/BSA. Each dish was subsequently washed with PBS/BSA six times over 30 min. The cells were lysed in 0.5 ml of 0.2% Triton X-100 in PBS for 20 min at room temperature with gentle rocking. the lysates were centrifuged for 5 min at 4°C at 14,000 g.

The lysates were assayed for HRP activity as described previously (West et al., 1989).

Brefeldin A

Brefeldin A was dissolved in DMSO to a concentration of 10 mg/ml and used at a final concentration of 5 μg/ml in experiments by diluting in medium A. Cells that were not treated with brefeldin A were incubated in medium containing an equivalent amount of DMSO.

Microscopy

Variations on a standard protocol were followed. Cells were grown on 13-mm diam glass coverslips, thickness No. 2. After a serum-free preincubation the cells were incubated by carefully inverting the coverslips onto 50-μl droplets on parafilm of the appropriate fluorescently labeled endocytic marker in the presence or absence of 100 ng/ml EGF at 37°C for varying times. The coverslips were then treated appropriately, followed by washing extensively in ice cold PBS/BSA as described above. After one wash in PBS the cells were fixed for 30 min at room temperature in 4% paraformaldehyde/PBS. The cells were washed briefly in PBS and then mounted onto glass slides using Citifluor.

The samples were viewed using a conventional fluorescence microscope (Zeiss Axioskop) or a Nikon Microphot-SA attached to a confocal laser scanning microscope (MRC-600 Series, Bio-Rad Laboratories, Cambridge, U.K.) equipped with a Kr/Ar laser. Video prints of single optical sections (~1.0 μm) or projected series of sections were obtained from a Sony color video printer (Mavigraph) UP-5000P. The Zeiss Axioskop was equipped with an MC 100 camera and Kodak T-max 400 film.

Experiments on living cells were performed at 30–37°C either on cells grown in 30 mm dishes, to which the fluorescent marker was added with 100 ng/ml EGF in medium A or on cells grown on 24 mm coverslips and mounted in a Dvorak-Stotler perfusion chamber (Nicholson Precision Instruments, Gaithersburg, MD). Observations were made either on a Zeiss Axiovert 35 M inverted microscope equipped with a Contax 167 MT camera or on the systems outlined above.

Results

Dynamics of Macropinosome Formation

We and others have described the formation of macropino-

somes on human A431 epidermoid carcinoma cells in response to EGF stimulation. Large, phase bright vesicles form within minutes of EGF addition and are responsible for the (6–10-fold) transient increase in fluid phase pinocytosis observed (Haigler et al., 1979; West et al., 1989; Sandvig and van Deurs, 1990). The presence of millimolar levels of amiloride or micromolar levels of more potent analogues such as hexamethylene amiloride abolishes the stimulation of fluid phase uptake and the formation of macropinosomes without affecting coated pit mediated endocytosis (West et al., 1989; and L. Hewlett, unpublished data). The relationship between membrane ruffling and formation of macropinosomes was confirmed by video microscopy of living cells. Sites of growth factor–induced membrane ruffling activity were often, though not always, predictive of subsequent macropinosome formation (Fig. 1 a; and see also Racoosin and Swanson, 1989; Swanson, 1989; Dowrick et al., 1993). Following their formation, the large phase bright structures frequently underwent abrupt translocations following a period of stasis (Fig. 1 a). Macropinosomes were quite heterogeneous in size. The diameters of 510 randomly chosen structures present after 6 min of EGF stimulation were measured using the point to point measurement facility on the confocal microscope. 85% of macropinosomes were between 0.5 and 2.5 µm in diameter, with a modal size of 1.0–1.5 µm although a significant proportion were several microns in diameter (Fig. 2). The macropinosomes persisted in the cells for at least 60 min and also appeared to fuse with other phase bright macropinosomes (Fig. 1 a, and see Fig. 10 below).

Macropinosomes were best observed by using fluorescent markers such as FITC dextran. Although their formation was dramatically up-regulated by EGF in A431 cells, we found that macropinosomes also form constitutively in a proportion of cells which, given their large volume to surface ratio compared with the ~100 nm vesicles normally associated with endocytosis, suggests that this pathway may be responsible for a proportion of basal fluid phase pinocytosis, at least in these cells. Although we found little evidence for tubulation of macropinosomes in fixed cells, observations on living cells using the confocal microscope sometimes revealed the presence of thin tubular extensions emanating from some of the brightly stained macropinosomes (Fig. 1 b). The inherently transient nature of these extensions in living cells (Fig. 1 c) together with their tendency to photobleach prevented accurate quantitation of the proportion of macropinosomes showing tubular extensions. Nonetheless, in common with other cellular vacuolar systems and endosomes and lysosomes in particular (Hopkins et al., 1990; Knapp and Swanson, 1990; Tooze and Hollinshead, 1991), macropinosomes in A431 cells exhibit both a vesicular and tubular morphology.

Macropinosomes Do Not Fuse with "Early" Endosomes

To analyze the fate of macropinosomes we asked whether they fused with conventional "early" endosomes as judged by mixing of their contents. A431 cells were first labeled for 20 min with FITC-conjugated transferrin to allow complete labeling of early endosomes, recycling endosomes (Salzman and Maxfield, 1989) and other elements of the transferrin receptor itinerary (Killisch et al., 1992). The cells were then stimulated with EGF for 6 min in the presence of 5 mg/ml Texas red–labeled Dextran and then chased for various time periods in the absence of the fluid phase marker. FITC transferrin was present throughout the chase to maintain labeling of this endosome system. The time of exposure and concentration of fluid phase marker used was titrated to eliminate direct co-labeling with transferrin through the micropinocytic activity of coated pits (see below). Following fixation, the FITC and Texas red markers were observed independently and as merged images in the confocal microscope. In this cell type the FITC-labeled transferrin pathway appears as a punctate vesicle system uniformly distributed through the cell (Fig. 3, a, c, and e). In contrast, macropinosomes labeled with Texas red dextran after 6 min of EGF treatment, were most frequently observed close to the ruffling edges of cells, were heterogeneous in size and number from cell to cell and importantly, did not co-localize with endosomes containing transferrin (Fig. 3 b, d, and f). A minor proportion (~20%) of macropinocytic structures appeared to partially overlap with transferrin-labeled endosomes when viewed either by conventional epifluorescence microscopy or as a projection of a series of optical sections through the cell using the confocal microscope. However, detailed analysis of the individual optical sections (0.1–0.5 µm) showed that although the two markers were sometimes in close proximity, the FITC-Tf-loaded vesicles were almost always located above or below the TR-dextran-loaded macropinosomes (Figure 4). By this method of analysis only five structures out of 139 inspected (3.6%), in cells pulsed for 6 min with EGF and fluid phase marker, still showed evidence of coincidence with transferrin. During a 2-h chase the Texas red–labeled macropinosomes persisted although they became fewer in number consistent with other data indicating recycling from these structures (see below). However, we could find no evidence for co-localization of transferrin and the fluid phase marker during this chase period (Fig. 3, a–f). In contrast, when cells were preloaded with FITC transferrin and then pulsed for 6 min with TR-transferrin, doubly labeled endosomes were abundant (data not shown) as previously reported by others for various combinations of sequentially endocytosed markers (Salzman and Maxfield, 1988; Stoorvogel et al., 1989; Hopkins et al., 1990).

We were concerned that the experimental protocol and subsequent analysis might give an artefactual impression of distinct endocytic systems. Firstly, it was possible that the fluid phase marker was initially fully mixed with transferrin-positive endosomes but then segregated away into physically distinct vesicles (Salzman and Maxfield, 1989) before the first time point observed (6 min). Second, it could be argued that the two markers are simply in different parts (vacuolar versus tubular) of a continuous system (Hopkins et al., 1990) but that the concentration and time of exposure to the fluid phase marker is insufficient to reveal the connectivity. To address the first possibility we asked whether the extent of overlap of the two markers was greater in cells examined immediately after macropinosome formation. Cells were preloaded with FITC-transferrin and then, following stimulation with EGF for either 1, 2, or 3 min in the presence of TR-dextran, the cells were fixed immediately and examined using the confocal microscope. The results in Table I show that even after 1 min of EGF stimulation fewer than 4% of macropinosomes were associated with transferrin positive
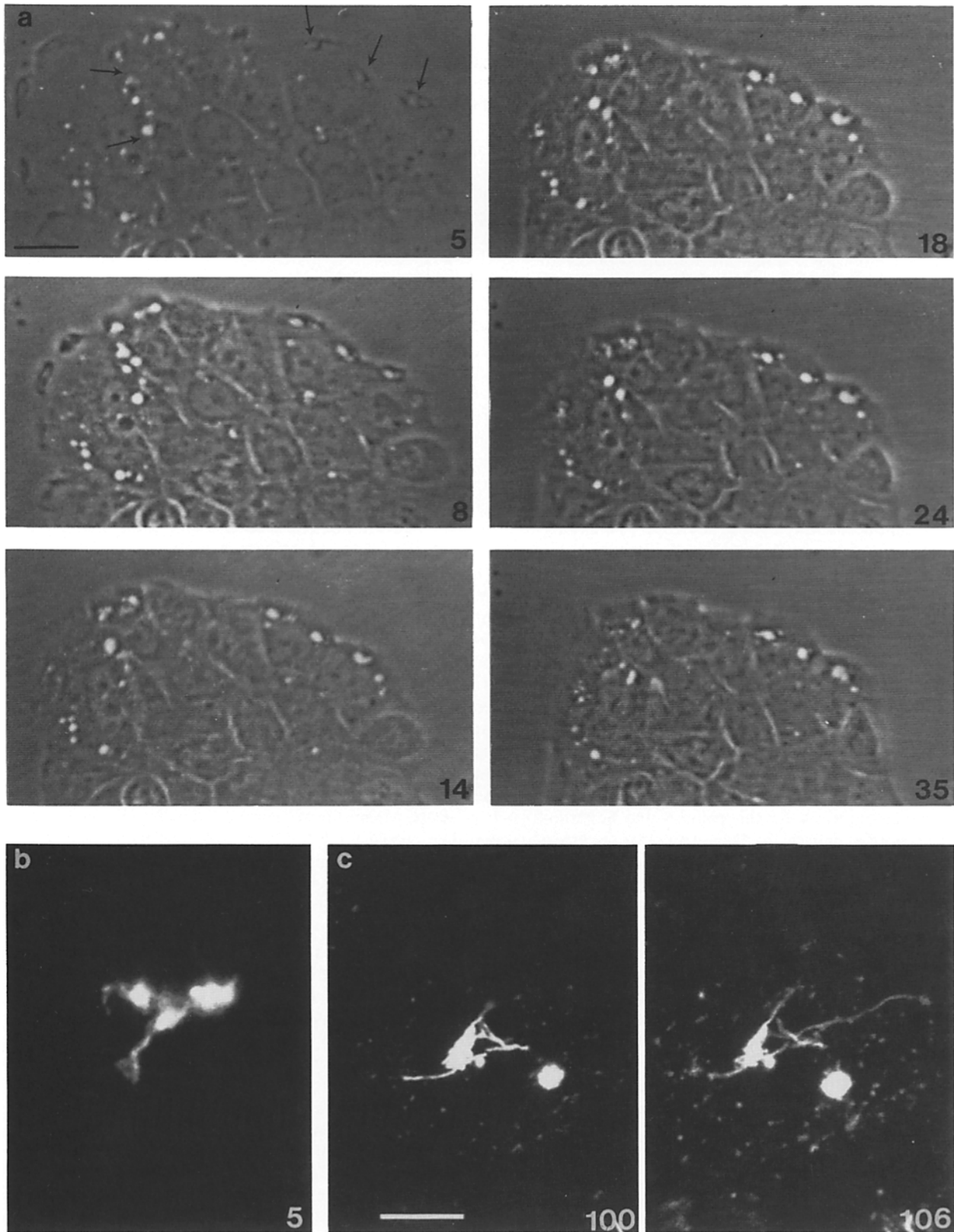
Figure 1. Dynamic behavior of macropinosomes. (*a*) After a serum-free preincubation the cells were transferred to medium A and observed on an inverted microscope maintained at 37°C. 5 mg/ml FITC-Dextran and 100 ng/ml EGF were added and the cells recorded by time-lapse video microscopy. Photographs were taken at the time intervals (minutes) shown. The sequence shown was initiated 1 min after EGF addition. Phase bright structures were confirmed as corresponding to FITC-Dextran–labeled structures by fixing and viewing by fluorescent microscopy at the end of each experiment. Note the conversion of three phase-dark membrane ruffles (*arrows, upper right*) into phase-bright macropinosomes and striking changes in the appearance of a group of macropinosomes (between two arrows at left) indicative of fusion
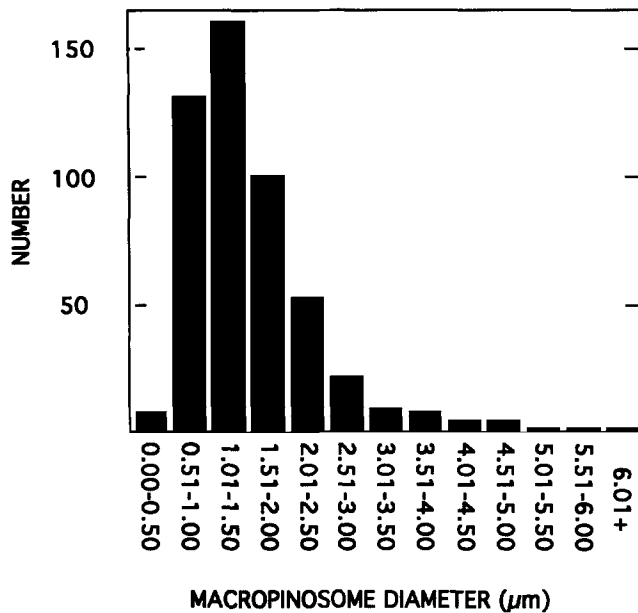
Figure 2. Macropinosomes are heterogeneous in size. A431 cells were incubated for 6 min at 37°C in the presence of 100 ng/ml EGF and 5 mg/ml TR-dextran. The cells were washed, fixed, and the diameter of all macropinosomes present in random fields was measured using the point to point measurement facility in the Cosmos software on the confocal microscope. A total of 510 structures were measured in two independent experiments.

structures, very similar to the extent of overlap indicated above after 6 min of stimulation. As expected the cells contained more macropinosomes after 2 and more still after 3 min of stimulation but the extent of overlap remained low and essentially the same at all time points (Table I). To address the second possibility we increased the concentration of fluid phase marker (FITC dextran to intensify the signal) and the time of exposure to visualize the micropinocytic activity of coated vesicles. Under these loading conditions all transferrin-positive endosomes were now also labeled with the fluid phase marker (Fig. 5). However, there were additional structures which continued to label exclusively with this marker and not with transferrin. In 134 cells examined a total of 2,510 structures labeled with the fluid phase marker were observed. 115 of these (4.6%) failed to overlap with transferrin and showed no connectivity with transferrin-labeled endosomes. Most, though not all of the latter vesicles were larger than transferrin-positive endosomes, were seen preferentially at the cell margins and corresponded to macropinosomes. Thus under conditions where transferrin receptor positive endosomes are also labeled with fluid phase tracer, macropinosomes are clearly visualized as distinct entities with characteristic size, distribution, and with no accumulation of transferrin receptors. We conclude that the failure of the macropinocytotic marker to co-localize with

transferrin under normal labeling conditions is due to its retention in a distinct endosome population which does not fuse with conventional endosomes.

Macropinosome Content Is Not Delivered to Late Endosomes

To see if macropinosome content in A431 cells was delivered to elements of the endocytic pathway not accessed by transferrin we incubated A431 cells with FITC-labeled LDL for 60 min to label late endosomes and lysosomes. Under these conditions we could identify structures in the perinuclear region of the cell which were labeled by LDL but not by transferrin which presumably represent late endosomes or lysosomes negative for recycling receptors (Goldstein et al., 1985; Mueller and Hubbard, 1986; Schmid et al., 1988; Salzman and Maxfield, 1988). A431 cells were stimulated with EGF in the presence of Texas red–labeled dextran as before and chased for various time periods in the continued presence of labeled LDL. Even after prolonged chase times following EGF stimulation we could find no evidence of co-localization of macropinocytosed marker with LDL-labeled structures (Fig. 6). We conclude that macropinosomes in A431 cells do not deliver their content to any detectable extent to the late endosome population labeled by LDL.

Macropinosomes Are Resistant to the Effects of Brefeldin A

Brefeldin A has recently been shown to dramatically enhance the tubular character of the early endosomal system by promoting fusion of otherwise isolated tubular domains into a large anastomosing reticulum (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991; Tooze and Hollinshead, 1992; Klausner et al., 1992). We reasoned that brefeldin treatment of cells containing macropinosomes would therefore allow an independent assessment of their relationship to the conventional endosome system. We therefore looked to see firstly if the morphology of macropinosomes was sensitive to brefeldin and secondly whether brefeldin would induce mixing of markers taken up by coated vesicles or macropinosomes. A431 cells were labeled with either transferrin or with FITC dextran during EGF stimulation and then incubated with 5 μg/ml brefeldin A for various times. The morphology of the transferrin-loaded endocytic system changed dramatically in the presence of brefeldin such that after 10 min the scattered vesicular/tubular elements became fused to produce an extensive tubular network which collapsed at later times (Fig. 7 A), presumably around the MTOC, consistent with earlier studies (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991; Tooze and Hollinshead, 1992). In contrast the macropinosomal vesicular system, although having some tubular character detectable in living cells (Fig. 1) showed no tendency to form macroscopic tubular elements in response to brefeldin A treatment and no obvious change in distribution

and budding events. (b) Cells were incubated in the presence of 5 mg/ml FITC-Dextran and 100 ng/ml EGF for 6 min, washed briefly, and then transferred to a perfusion chamber containing medium A and 20 mM glucose for viewing over a period of time using the confocal microscope at 37°C. Time (minutes) after EGF stimulation is indicated. Note the formation of a tubular extension over a 6-min period from the macropinosome in c. Bars: (a) 10 μm; (b) 4 μm.
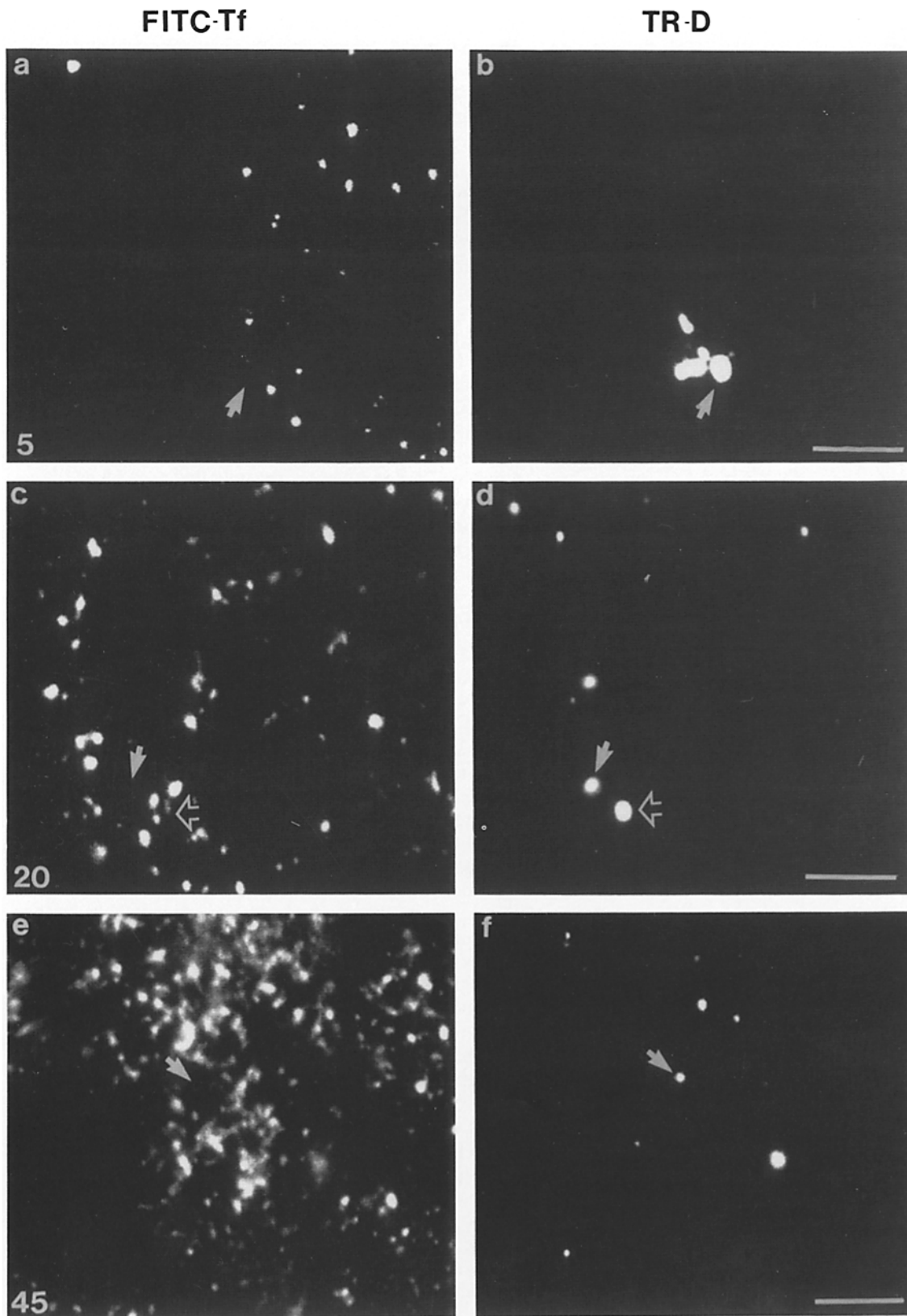
FITC-Tf   **TR-D**

Figure 3. Macropinosomes do not fuse with early endosomes. Cells on coverslips were incubated at 37°C in medium A containing 2 μg/ml FITC-transferrin for 20 min. After brief washing the cells were then stimulated for 6 min in medium containing 100 ng/ml EGF and 5 mg/ml Texas red Dextran in the continued presence of transferrin. Cells were subsequently chased for the times indicated at 37°C in medium A containing glucose, and then washed and fixed for confocal microscopy. (*a*, *c*, and *e*) represent FITC-transferrin labeling and (*b*, *d*, and *f*) represent Texas red Dextran labeling in the same cells. At each time point several macropinosomes (*arrows*, in *b*, *d* and *f*) are
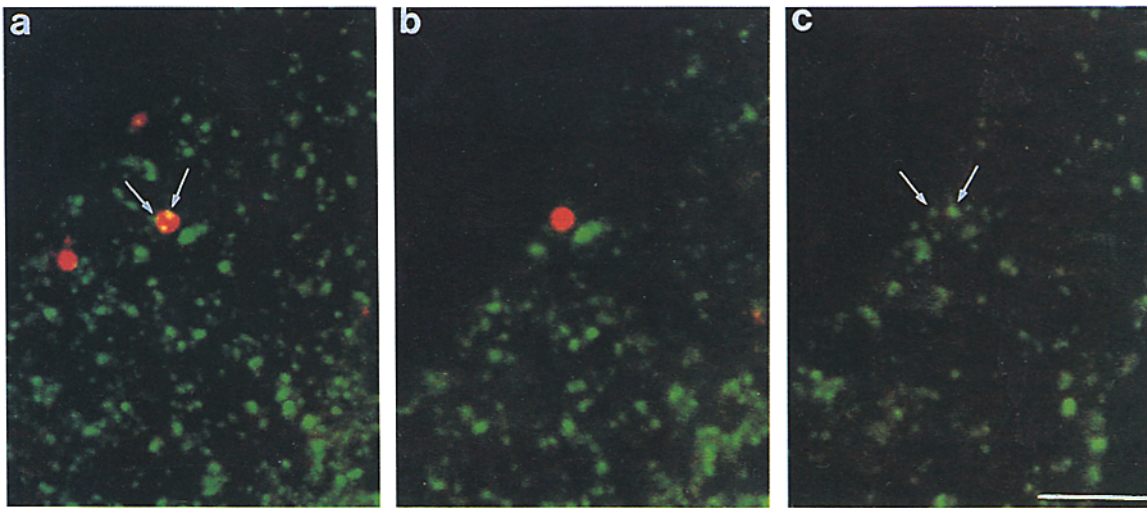
Figure 4. Apparently overlapping structures can be distinguished by confocal sectioning. Cells were pre-loaded with 2 μg/ml FITC-transferrin, stimulated for 6 min with EGF in the presence of Texas red–labeled dextran, and then chased for 5 min in the continued presence of FITC-transferrin. In this figure the merged images of the FITC and Texas red channels are shown. (*a*) Projected image comprising 22 × 0.1 μm optical sections. Arrows indicate structures (*yellow*) that appear to overlap although note that they often have distinct dimensions and shape compared with the macropinocytic vesicle (*double arrows*). The macropinocytic vesicle is in fact found in section number 6 (*b*) while the two transferrin-labeled structures are in section 8 (*c*). Bar, 5.0 μm.

even after 60 min (Fig. 7 *A*). This striking difference implied that little mixing of the two endosome types was taking place in the presence of brefeldin. To test this directly, cells were pre-labeled with transferrin, and then stimulated with EGF in the presence of fluid phase tracer and brefeldin A. In doubly labeled cells macropinosomes were observed as discrete vesicular structures sometimes adjacent to but clearly quite distinct from the fine brefeldin A induced web of transferrin-labeled endosomal tubules (Fig. 7 *B*). Observation at high power in the confocal microscope confirmed that the two markers remained in distinct structures in brefeldin treated cells even after 60 min (Fig. 8). The failure to undergo extensive tubulation and to mix with the conventional endosome system in the presence of brefeldin A provides a striking demonstration that macropinosomes constitute a distinct population of endocytic vesicles.

Although brefeldin A did not alter the morphology of the macropinosome system we did detect a significant acceleration in the rate of recycling of fluid phase marker following stimulation of macropinocytosis (Fig. 9 *B*). In addition the rate of transferrin recycling in the presence of brefeldin was also somewhat increased (not shown). In contrast, brefeldin had no effect on the rate of pinocytosis in EGF-stimulated cells although in the absence of EGF there was a clear increase in the basal rate of uptake (Fig. 9 *A*). Consequently, the stimulation of pinocytosis by EGF was two- to threefold less in the presence of brefeldin. By microscopy there was no obvious increase in either the number of macropinosomes forming constitutively or in their size raising the possibility that there was either an increase in micropinocytosis or in the retention of micropinocytosed marker. Stimulation of apical fluid phase pinocytosis and of basolateral to apical trans-

Table I. Minimal Overlap of Transferrin Positive Endosomes with Newly Generated Macropinosomes

Pulsing time	Cells examined	Macropinosomes observed	Macropinosomes transferrin labeled	Percent transferrin labeled
min				
1	110	98	4	3.8
2	104	215	9	2.4
3	119	323	9	2.8

Cells on coverslips were incubated for 20 min at 37°C in medium A containing 2 μg/ml FITC-transferrin and then stimulated with 100 ng/ml EGF in the presence of 5 mg/ml TR-dextran for 1, 2, or 3 min in the continued presence of FITC-transferrin. The cells were washed, fixed, and data collected from at least 100 cells using the confocal microscope. Every macropinosome was assessed for labeling with transferrin and structures found in the same confocal section containing both markers were deemed to be overlapping.

shown. Equivalent positions are also arrowed in the FITC channel (*a*, *c*, and *e*) and demonstrate that no overlap with transferrin can be seen. An example of partial overlap of the two markers can be seen in *c* and *d* (*open arrows*). However, in this and most other instances examined, single optical sections revealed that the vesicles containing the two markers were in different horizontal planes (see Fig. 4). Bar, 10 μm.
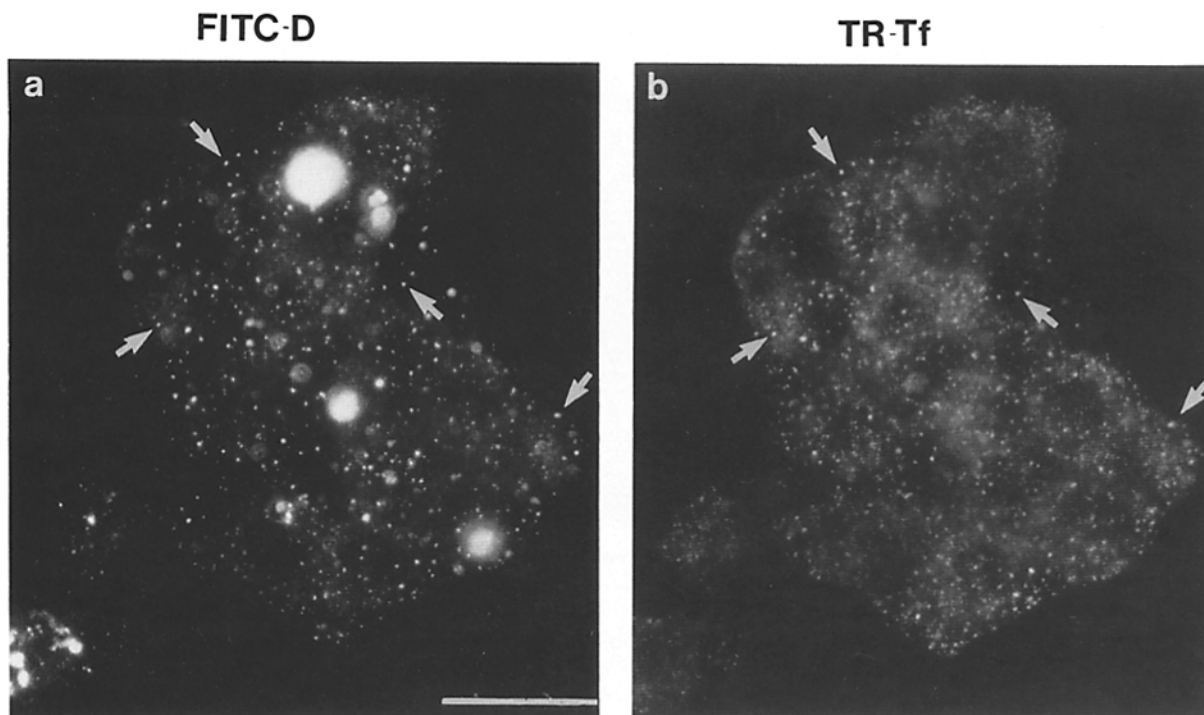
FITC-D   TR-Tf

Figure 5. Co-localization of fluid phase markers and transferrin. Cells on coverslips were incubated for 20 min in the presence of 2 μg/ml Texas red transferrin (*b*) and 20 mg/ml FITC-Dextran at 37°C (*a*) and then washed, fixed, and processed for conventional fluorescence microscopy. In this group of cells several spontaneously generated macropinosomes can be seen (*a*). Under these loading conditions there is extensive overlap of the two markers in conventional endosomes (arrows) but not in macropinosomes. Note that the size of the macropinosomes in this figure is overestimated due to "flare" from heavily labeled structures. Bar, 15 μm.

cytosis was observed in polarized MDCK cells (Prydz et al., 1992) while in other cell types brefeldin causes striking redistribution of mannose 6-phosphate receptors to the cell surface (Damke et al., 1991; Wood et al., 1991). Thus while the most dramatic effects of brefeldin are seen on internal membrane systems there is growing evidence that there are also effects on traffic to and from the cell surface. Further studies will be necessary to resolve the effects of brefeldin on the accumulation and reflux of fluid phase markers from both conventional and macropinocytic endosomes.

Macropinosomes Are Able to Fuse with Each Other

Observations of living cells undergoing macropinocytosis suggested that phase-bright macropinosomes were fusing with each other (Fig. 1). To test this directly we asked whether sequentially generated macropinosomes could fuse with each other. A431 cells were stimulated with EGF and exposed first to FITC dextran for 4 min. This was rapidly removed and replaced with Texas red dextran for a further 4-min period. The cells were maintained at 37°C throughout the pulsing and subsequent chase. Fields of cells were then inspected for singly and doubly labeled macropinosomes. We could clearly detect the presence of doubly labeled macropinosomes in cells loaded according to the above protocol. Approximately 64% of structures positive for the first marker were also positive for the second fluid phase marker as well (Fig. 10). Thus in contrast to macropinosome–endosome fusion macropinosome–macropinosome fusion was a comparatively frequent event.

Discussion

Macropinocytosis is an endocytic process distinct from both clathrin-mediated and other non-clathrin–mediated endocytic pathways and which can be up-regulated by specific stimuli in specific cell types (Haigler et al., 1979; West et al., 1989; Swanson, 1989; Racoosin and Swanson, 1989; Sandvig and van Deurs, 1990; Ridley et al., 1992). Here we have analyzed the fate of macropinosomes following their formation in EGF-stimulated human A431 cells. Specifically we have asked to what extent the content of macropinosomes mixes with endosomes labeled either with transferrin or with LDL, whether tracers taken up via macropinocytosis are transported to Brefeldin A–sensitive compartments and to what extent sequentially formed macropinosomes fuse with each other.

Completely selective targeting of different markers to the macropinocytotic and coated pit/vesicle driven endocytic pathways is difficult to achieve since the fluid phase markers used to measure macropinocytosis also have access to coated vesicles (and other micropinocytic systems). In addition, receptors which concentrate in coated vesicles are almost certainly present to some extent on macropinosome membrane. However, in practice the enrichment of receptors in coated pits and the larger volume to surface ratio of macropinosomes allows the endocytic activity of the two systems to be distinguished and permits the extent of subsequent mixing of macropinosomes with conventional endosomes to be assessed. When cells were loaded under these conditions and either observed immediately or chased at 37°C we detected
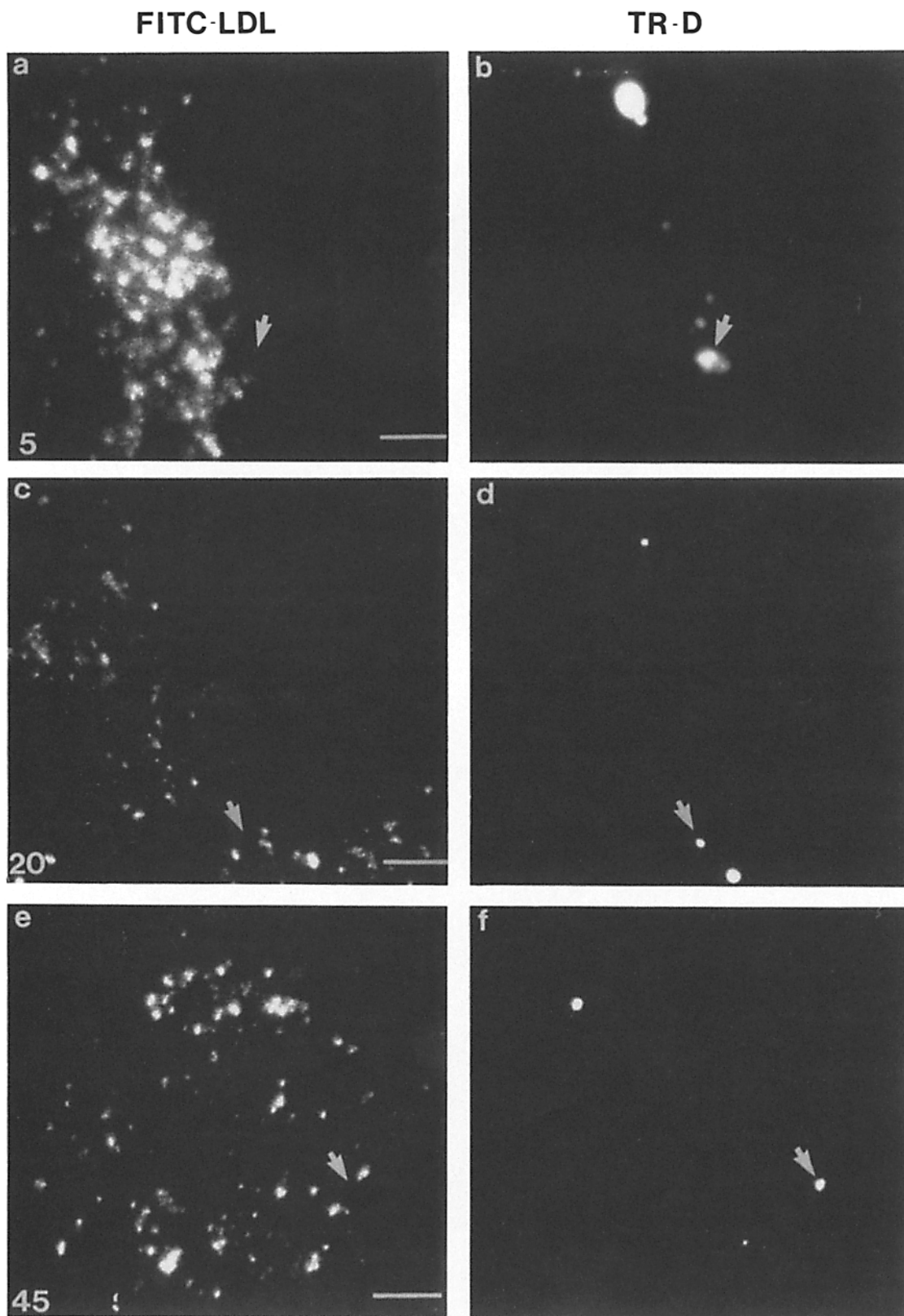
FITC-LDL   TR-D

Figure 6. Macropinosomes do not fuse with late endosomes. Cells previously grown in lipoprotein deficient medium were incubated at 37°C for 60 min with 5 μg/ml FITC-LDL and then stimulated with 100 ng/ml EGF for 6 min in the presence of 5 mg/ml Texas red Dextran and 5 μg/ml FITC-LDL. After brief washing they were chased for the times indicated in the continued presence of FITC-LDL, then washed, fixed, and processed for confocal microscopy as in Fig. 2 legend. At each time point labeled macropinosomes (*arrow, b, d,* and *f*) which do not overlap with the LDL marker (*a, c* and *e,* equivalent positions arrowed) can be seen. Bar, 10 μm.
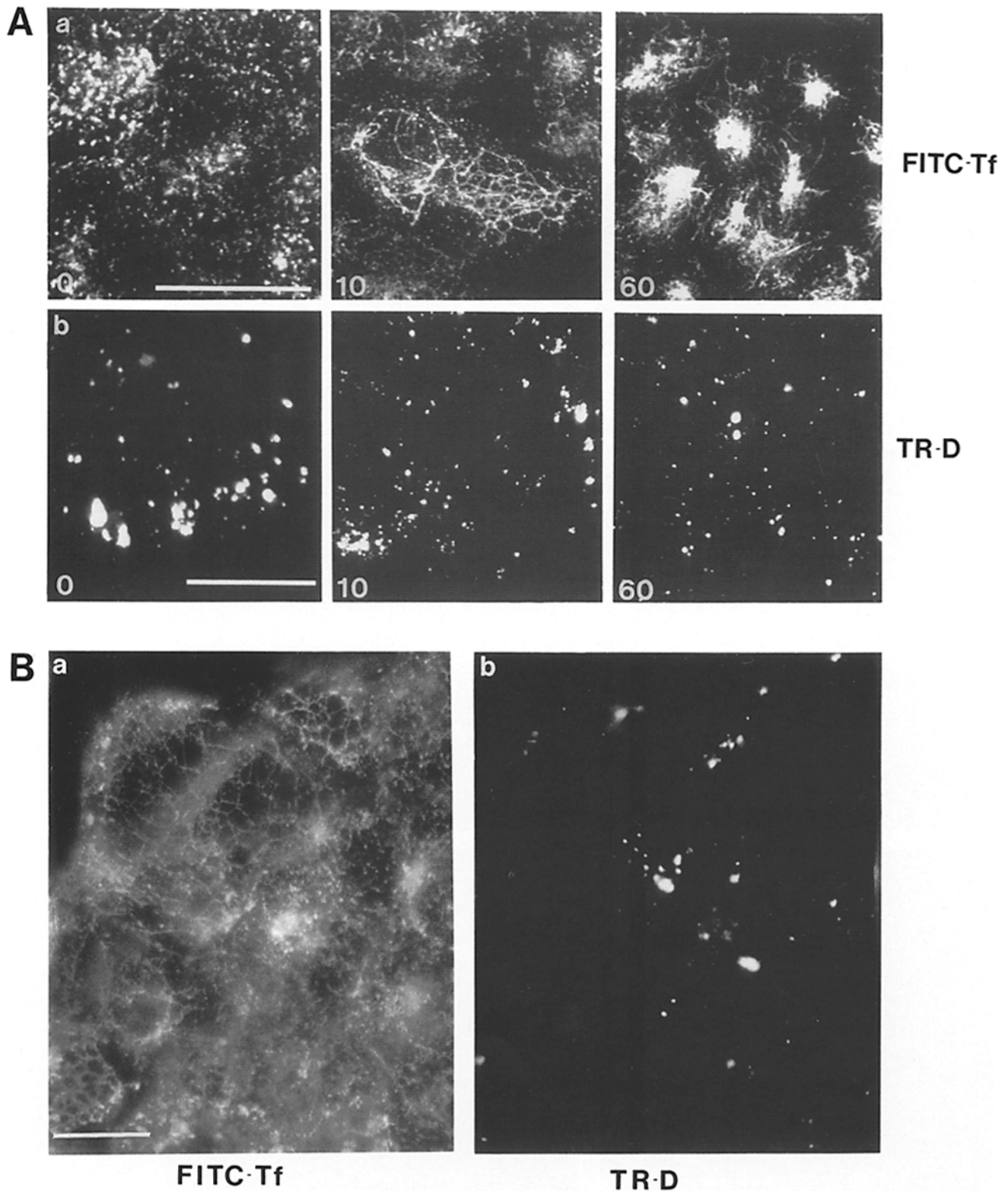
Figure 7. (*A*) Brefeldin A has differential effects on macropinosomes and early endosomes. (*a*) Cells were incubated at 37°C with 2 μg/ml FITC-transferrin in the presence or absence of 5 μg/ml brefeldin A for various times as shown before being fixed. (*b*) Cells were incubated in the presence of 5 mg/ml FITC-Dextran and 100 ng/ml EGF for 6 min and then chased for various times as shown in the presence of 5 μg/ml brefeldin A before being fixed and viewed as a projected stack of confocal sections. Extensive tubulation of the transferrin containing endosomes can be seen which then collapses while macropinosomes are unaffected over the same time course. (*B*) Cells were incubated with 2 μg/ml FITC-transferrin for 20 min, followed by a 6-min incubation with 100 ng/ml EGF, 5 mg/ml TR-dextran, and 5 μg/ml brefeldin A in the continued presence of FITC-transferrin. The cells were then fixed and observed by conventional fluorescence microscopy. Brefeldin A induces a fine network of transferrin-labeled tubules (*a*) while in the same cells macropinosomes persist as discrete vesicular structures (*b*). Bar, 10 μm.
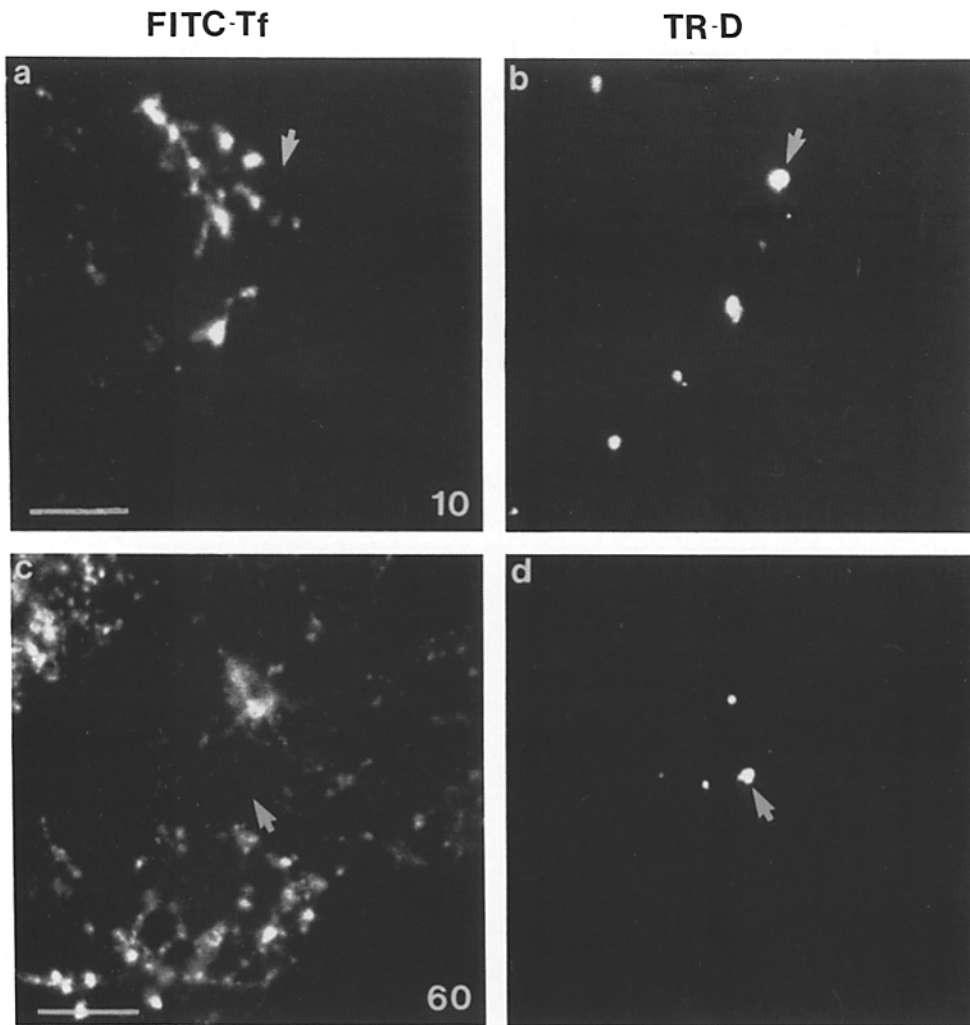
FITC-Tf

TR·D

Figure 8. Macropinosome contents do not mix with early endosomes in brefeldin A–treated cells. Cells were incubated for 20 min at 37°C in the presence of 2 μg/ml FITC-transferrin followed by a 6-min incubation with 5 mg/ml Texas red Dextran, 100 ng/ml EGF and 5 μg/ ml brefeldin A. Cells were then chased in the continued presence of brefeldin A for the times indicated before being fixed. *a* and *c* represent FITC-transferrin labeling and *b* and *d* represent Texas red Dextran labeling in the same cells. Each marker remains in distinct structures (*arrows*) which are viewed in 0.4 μm optical sections. Bar, 10 μm.

very little mixing of the fluid phase and ligand markers. In contrast, mixing of sequential pulses of transferrin labeled with different fluorochromes was readily detectable as reported by others. Most importantly macropinosomes labeled with one tracer became extensively labeled with a second

tracer taken up during a second round of macropinocytosis (Fig. 10). Thus vesicle fusion events, including those involving macropinosomes, could be readily detected under our experimental conditions.

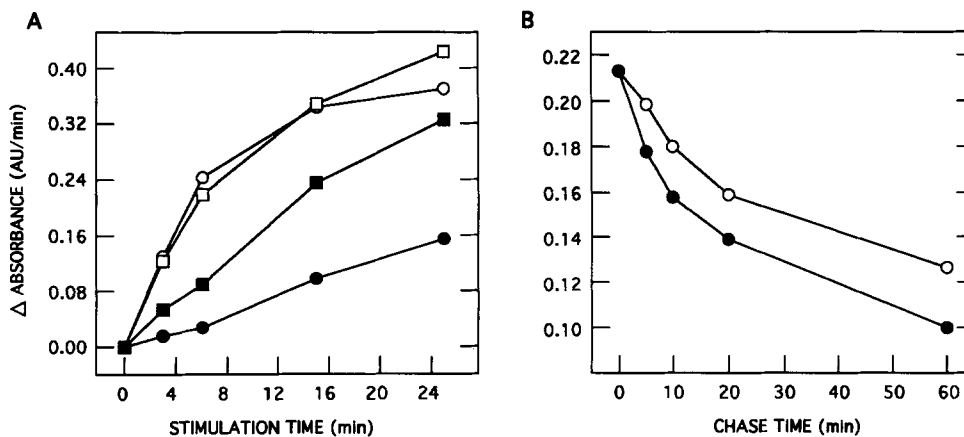Delivery of small amounts of fluid phase marker from

Figure 9. Effects of brefeldin A on flux of fluid phase markers. (*a*) After a serum-free incubation cells were incubated at 37°C in DME/Hepes with or without 5 μg/ml brefeldin A for 30 min. The cells were then stimulated for various periods of time with (□,○) or without (■,●) 100 ng/ml EGF in the presence of 2 mg/ml HRP with (□,■) or without (○,●) 5 μg/ml brefeldin A. HRP was measured as in Materials and Methods. (*b*) Cells were stimulated at 37°C with 100 ng/ml EGF in the presence of 2 mg/ml HRP. After washing briefly the cells were chased in the presence (●) or absence (○) of 5 μg/ml brefeldin A.
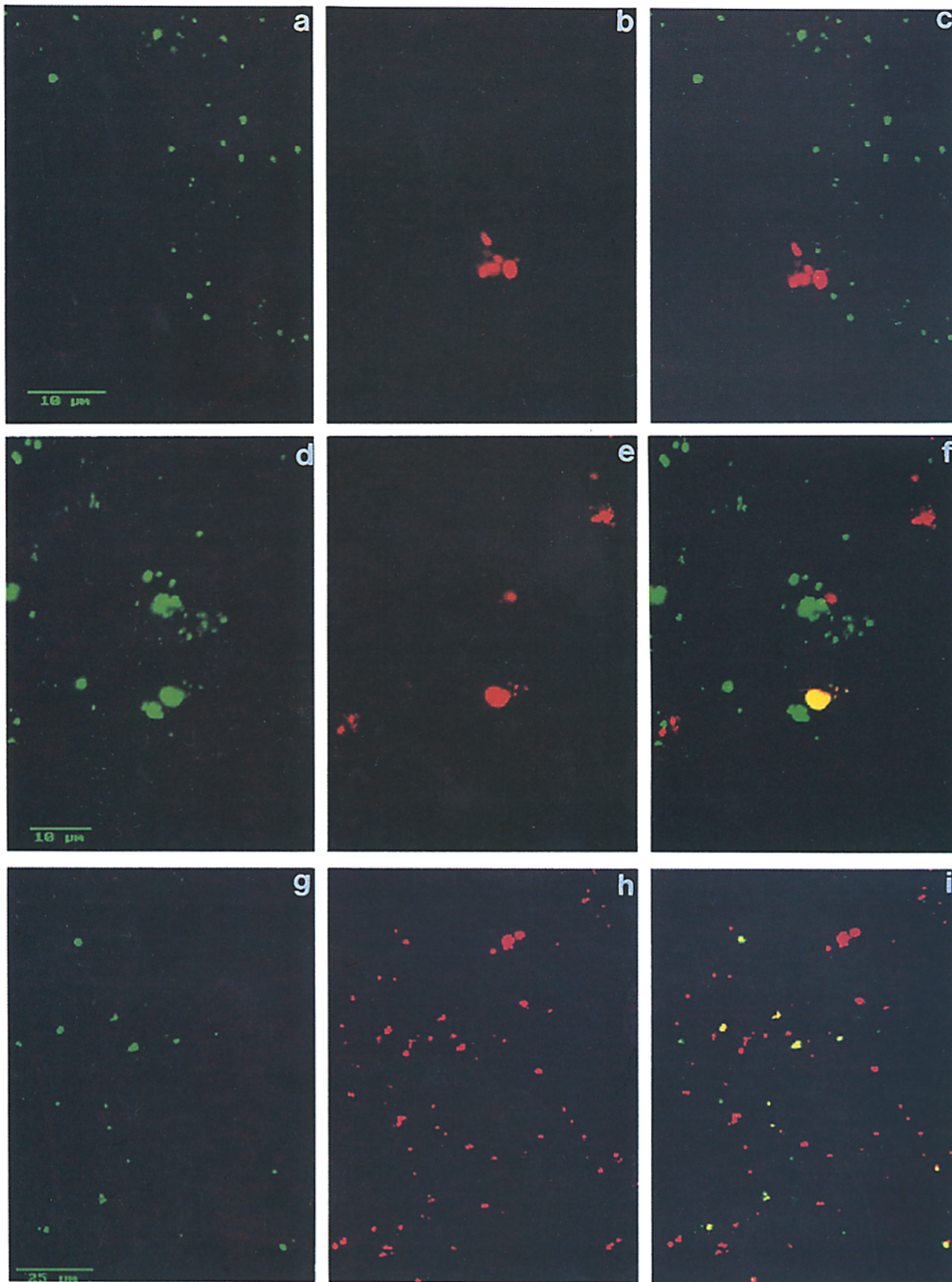
Figure 10. Sequentially formed macropinosomes can fuse with each other. (*a*) FITC-transferrin, (*b*) Texas red Dextran in the same cells. Note lack of colocalization of the two markers in the merged image (*c*). Cells were incubated in 5 mg/ml FITC-Dextran and 100 ng/ml EGF for 4 min, washed briefly, and then incubated in 5 mg/ml Texas red Dextran and 100 ng/ml EGF for 4 min before chasing for 5 min (*d–f*) and 20 min (*g–i*). The cells were then washed and fixed. (*d* and *g*) FITC-Dextran labeling, (*e* and *h*) Texas red Dextran labeling in the same cells. Merged images show that some macropinosomes contain both markers (*yellow* structures in *f* and *i*).

macropinosomes into conventional endosomes or transferrin receptors into macropinosomes might be difficult to detect due to dilution of the incoming marker. For this reason we could not exclude the possibility that low level exchanges occur. Consequently, we sought independent evidence that macropinosomes remained fundamentally distinct from the conventional endosome system. Cells were pre-labeled with FITC-transferrin and then exposed to EGF, brefeldin A, and TR-dextran in the continued presence of labeled transferrin. In other words brefeldin-induced tubulation and mixing of early endosomes was occurring simultaneously with the formation of macropinosomes. Clearly if macropinosomes have significant capacity to fuse with conventional "early" endosomes we would expect extensive labeling of the tubulated system by the macropinocytosed marker. In fact there was no mixing of the two markers: the macropinocytosed marker remained in discrete vesicular structures of various sizes sometimes closely apposed to but distinct from the dramatically tubulated transferrin-labeled endosome system (Figs. 7 and 8). Taken together the inability of macropinosomes to fuse with conventional endosomes and their apparent resistance at the morphological level to the effects of brefeldin A argues strongly that they have a fundamentally distinct character.

We have only examined the extent to which macropinosomes in A431 cells interact with endosomes. Our studies do not rule out the possibility that macropinosomes fuse with other vacuolar systems such as lysosomes or with vesicles derived from the secretory pathway. However, a major proportion of macropinocytosed marker appears to be recycled out of the cells and it is difficult to detect labeling of the cells after 2 h of chase. Preliminary experiments indicate that macropinocytosed markers in A431 cells are not delivered to acidic compartments as defined by acridine orange retention.

A recent analysis of macropinosomes in mouse bone marrow macrophages stimulated with MCSF indicated significantly greater co-localization of macropinocytosed markers with transferrin and LDL (Racoosin and Swanson, 1992). We found that the majority of structures apparently labeled with both markers in A431 cells could be shown to be distinct structures by confocal microscopy. The differential sensitivity of the two endosome populations to brefeldin A has not to our knowledge been tested in the macrophage system. Interestingly, while this manuscript was in preparation it was reported that macropinosomes in macrophages acquired and then lost markers such as transferrin receptor and rab 7, eventually acquired Igp-A and finally fused with pre-existing tubular lysosomes (Racoosin and Swanson, 1993). It is possible that the fate of macropinosomes in professional versus non-professional phagocytic cells may differ.

Various factors may account for the failure of macropinosomes to fuse with the rest of the endosome system. In vitro and in vivo studies on endosome–endosome fusion have shown that it is a carefully regulated process requiring specific cytosolic and membrane-associated factors and only occurs efficiently between endosomes of the same type. Early endosomes fuse preferentially with other early endosomes and not directly with late endosomes (Gruenberg and Howell, 1989; Woodman and Warren, 1988; Colombo et al., 1991) while in polarized cells apical and basolateral endocytosis give rise to distinct populations of early endosomes (Bomsel et al., 1989; Hughson and Hopkins, 1990). Endo-

some–endosome fusion events both in vitro (Gorvel et al., 1991) and in intact cells are regulated by specific members of the rab family of GTP-binding proteins (Bucci et al., 1992; van der Sluijs et al., 1992).

Taken together a large body of work documents the specificity of endosome–endosome fusion. In this light the failure of macropinosomes to fuse with endosomes may be less surprising since macropinocytic vesicles differ from incoming coated vesicles in several important respects. Firstly, they are generated by a completely different mechanism activated by growth factors, phorbol esters, or members of the rho sub-family of ras-like GTPases (Haigler et al., 1979; Bar Sagi and Feramisco, 1986; Ridley et al., 1992). Secondly, they are extremely heterogeneous in size and thirdly, the lack of an obvious "coating" protein indicates a distinct membrane composition compared with clathrin-coated vesicles. In situ radioiodination with macropinocytosed lactoperoxidase suggests that "average" plasma membrane is internalized (not shown) supporting the suggestion that compared with conventional endosomes macropinosomes are "receptor poor" (Racoosin and Swanson, 1992). Downstream fusion decisions may be pre-determined by the mechanisms involved in coated vesicle formation. We suggest that in A431 cells macropinosomes do not efficiently recruit the fusion machinery needed for interaction with the pre-existing endosome system. If macropinosomes could freely fuse with the conventional endosome system there might be severe disruption to its normal sorting and processing function as a result of the sudden input of vesicles which have a volume/surface ratio 10–30 times greater than that of incoming coated vesicles. This would not apply to other clathrin-independent endocytic pathways which involve the formation of uniformly sized (95-nm diam) micropinocytic vesicles (Hansen et al., 1991). A recent study of the fate of such vesicles in K^+-dependent HEp-2 cells demonstrated delivery of a membrane bound marker to transferrin receptor–positive endosomes (Hansen et al., 1993). Thus different endocytic mechanisms, which are not clathrin mediated, can apparently be distinguished based on their fusogenic properties and the size of the vesicles produced. Nonetheless, macropinosomes can fuse with each other and probably with the cell surface as judged by recycling of their content. The requirements for macropinosome–macropinosome fusion will be interesting to define.

The fate of phagocytic vacuoles seems to depend on the types of receptor engaged during uptake (reviewed in Falkow and Isberg, 1992). For example, antibody-coated *toxoplasma gondii* taken up via Fc receptors localizes to phagosomes which acidify and fuse with lysosomes whereas bacteria taken up in the absence of antibody or via truncated Fc receptors (Joiner et al., 1990) enter a host vacuole which fails to acidify and does not fuse. Conceivably, the fate of macropinosomes may also depend on which receptors are stimulated during their formation. Perhaps this accounts for the somewhat different behavior of macropinosomes in EGF-stimulated A431 cells versus MCSF-stimulated macrophages (Racoosin and Swanson, 1992). Both phagocytosis (Greenberg et al., 1993) and macropinocytosis (Ridley et al., 1992; Dowrick et al., 1993) are driven by rearrangements of the actin cytoskeleton although in A431 cells macropinocytosis could not be blocked by perturbation of the cytoskeleton with microtubule and microfilament destabiliz-

ing drugs (Haigler et al., 1979; and L. Hewlett, unpublished data), treatments which abolish the analogous process in macrophages (Racoosin and Swanson, 1989).

A recent report demonstrated that invasive *salmonella typhimurium* induced EGF receptor tyrosine phosphorylation in cultured cells whereas non-invasive mutants did not (Galan et al., 1992). Uptake of the latter could however be rescued by addition of EGF. It is possible that these bacteria are taken up into EGF-stimulated macropinosomes.

In summary, in growth factor–stimulated A431 cells distinct endocytic mechanisms give rise to distinct endosomal vesicle populations. Fusion between macropinosomes and conventional endosomes was virtually undetectable and in the same cells macropinosomes and conventional endosomes showed a marked differential sensitivity to the effects of brefeldin A. Macropinosomes were nonetheless able to fuse with each other and to recycle their content to the cell surface. The results reported here may be useful for future studies aimed at analyzing the requirements for membrane fusion along the endocytic pathway.

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