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TGN38/41 Recycles Between the Cell Surface and the TGN: Brefeldin A Affects its Rate of Return to the TGN

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TGN38 and TGN41 are isoforms of an integral membrane protein (TGN38/41) that is predominantly localized to the trans-Golgi network (TGN) of normal rat kidney cells. Polyclonal antisera to TGN38/41 have been used to monitor its appearance at, and removal from, the surface of control and Brefeldin A (BFA)-treated cells. Antibodies that recognize the lumenal domain of TGN38/41 are capable of specific binding to the surface of both control and BFA-treated cells. In both control and BFA-treated cells internalized TGN38/41 is targeted to the TGN; however, there are differences in 1) the morphology of the intracellular structures through which TGN38/41 passes and 2) the kinetics of internalization. These data demonstrate that TGN38/41 cycles between the plasma membrane and the TGN in control and BFA-treated cells and suggest that recycling pathways between the plasma membrane and the TGN exist for predominantly TGN proteins as well as those that normally cycle to other intracellular compartments. They also demonstrate that addition of BFA not only alters the morphology and localization of the TGN but also the kinetics of endocytosis.

INTRODUCTION

The trans-Golgi network (TGN)[1] is composed of a series of tubules and vesicles in which newly synthesized proteins are sorted and packaged for delivery to different post-Golgi destinations within the cell (Farquhar, 1985; Roth *et al.*, 1985; Griffiths and Simons, 1986; Griffiths *et al.*, 1989). Recycling between this compartment and the plasma membrane has been demonstrated for a number of proteins including asialo-transferrin receptors (Snider and Rogers, 1985), the 46- and 215-kDa mannose-6-phosphate receptors (Duncan and Kornfeld, 1988), and the asialoglycoprotein receptor (Stoorvogel *et al.*, 1989). These proteins, however, do not reside exclusively in the TGN but rather perform targeting functions within the endosomal/lysosomal system and consequently have different distributions under steady-state conditions.

Using normal rat kidney (NRK) cells and a polyclonal antiserum that recognizes a TGN-specific integral membrane protein, TGN38/41 (Luzio *et al.*, 1990), it has been demonstrated that Brefeldin A (BFA) treatment induces morphological changes to the TGN. These include the collapse of the TGN upon the microtubule-organizing center (MTOC) in a GTP- and microtubule-dependent manner (Reaves and Banting, 1992) and the fusion of endosomes with the TGN (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991). The effects of BFA on the TGN are fully reversible and conclusively demonstrate that the TGN is an organelle independent of the Golgi stacks.

Immunofluorescence analysis of BFA-treated NRK cells using anti-TGN38/41 polyclonal antiserum demonstrated that an early event in the BFA-induced relocalization of the TGN is a dispersal of elements of the TGN; most, but not all, of these elements eventually collapse around the MTOC (Reaves and Banting, 1992). It was not apparent from these initial studies whether the dispersed TGN38/41 remained intracellular or appeared at the cell surface. Lippincott-Schwartz *et al.* (1991) demonstrated that TGN38/41 is internalized in BFA-treated cells but failed to detect any internalization of TGN38/41 in control cells. A conclusion drawn from

[1] Abbreviations used: BFA, Brefeldin A; FITC, fluorescein isothiocyanate; MTOC, microtubule-organizing center; TGN, trans-Golgi network.

these data was that BFA induces the appearance of TGN38/41 at the cell surface. In this report we have investigated the possibility that TGN38/41 is present at the surface of control cells and that it maintains its steady-state distribution by recycling from the plasma membrane to the TGN. We have demonstrated that antibodies that recognize the lumenal domain of TGN38/41 are capable of binding to the surface of both control and BFA-treated cells and have partially characterized the kinetics and microtubule-dependence of internalization. These data suggest that recycling pathways between the plasma membrane and the TGN exist for predominantly TGN proteins, as well as those that normally cycle to other intracellular compartments, and demonstrate that BFA does not induce the appearance of TGN38/41 at the cell surface.

MATERIALS AND METHODS

Materials

BFA was purchased from Cambio (Cambridge, UK)/Epicenter Technologies (Madison, WI), stored as a 5 mg/ml stock in methanol at −20°C and used at a final concentration of 5 μg/ml in culture medium. ^{125}I-labeled anti-rabbit immunoglobulin (IgG) was purchased from Amersham (Amersham, UK). Fluorescein-labeled transferrin was obtained from Cambridge Bioscience (Cambridge, UK)/Molecular Probes (Eugene, OR). Other chemicals were obtained from Sigma Chemical Company (Poole, Dorset, UK). The concentration of nocodazole was 20 μg/ml in all incubations and was kept as a 10 mg/ml stock in dimethyl sulfoxide at −20°C.

Antibodies

The anti-TGN38/41 polyclonal antiserum has been described previously (Luzio *et al.*, 1990). The rabbit anti-TGN41 antiserum was raised to a hexadecapeptide corresponding to the C-terminus of the cytoplasmic domain of TGN41 and has been described previously (Reaves *et al.*, 1992). The sheep anti-TGN38/41 antiserum was raised against an sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) purified, plasmid-encoded, β-galactosidase fusion protein using previously published procedures (Brake *et al.*, 1991). The anti-mannosidase II monoclonal antibody (Burke *et al.*, 1982; Baron and Garoff, 1990) was the kind gift of Dr. Graham Warren (ICRF, London, UK). The monoclonal anti-lgp110 antibody was kindly supplied by Dr. Paul Luzio (Dept. of Clinical Biochemistry, University of Cambridge). The polyclonal rabbit anti-rab5 antiserum was generously provided by Dr. Marino Zerial (EMBL, Heidelberg, Germany). Fluorescein-labeled goat anti-rabbit IgG, rhodamine-labeled goat anti-mouse IgG, and fluorescein-labeled donkey anti-sheep IgG were obtained from Sigma Chemical Company, and rhodamine-labeled swine anti-rabbit IgG was from Dako (High Wycombe, Bucks, UK).

Cell Culture and Immunofluorescence Microscopy

NRK cells were grown in McCoy's medium supplemented with 10% fetal calf serum, 2 mM glutamine (GIBCO, Life Technologies Ltd., Paisley, Scotland), 100 mg/ml streptomycin (Evans Medical Ltd., Langhurst, Horsham, England) and 60 mg/ml penicillin (Glaxo Laboratories Ltd., Greenford, England) at 37°C in 5% CO_2. Cells were plated onto 22-mm glass coverslips 24–48 h before use (~60–70% confluency). After the appropriate drug treatments and incubations with antibody, cells were washed three times with phosphate-buffered saline (PBS) and then fixed and permeabilized by incubation in methanol at −20°C for 5 min. Cells to be used for staining with anti-rab5 antiserum were not methanol fixed, but were paraformaldehyde fixed and saponin permeabilized essentially as described by Gorvel *et al.*, (1991). The coverslips were then incubated in PBS/0.2% bovine serum albumin (BSA) for 5 min followed by 4× 2-ml washes in PBS. Cells were incubated at room temperature with fluorescently labeled secondary antibody for 30 min, washed, and mounted in Mowiol (Calbiochem, San Diego, CA). Cells that were double labeled were incubated in PBS/0.2% BSA for 5 min after fixation and then washed in PBS before incubation with primary antibody in PBS/0.2% BSA for 1 h at room temperature. Cells were washed in PBS (4× 2 ml) before incubation in fluorescently labeled secondary antibody and processed as above. Epifluorescence microscopy was performed on a Zeiss Universal (Thornwood, NY) microscope with a 63×, 1.4 oil immersion objective. All surface-labeling and internalization experiments were performed using a 1:200 dilution of antiserum.

^{125}I Labeling of Cells and Immunoprecipitation

Cells were harvested using disposable cell scrapers (Costar, Cambridge, MA), washed in PBS and surface labeled by iodination with *N*-succinimidyl 3-(4-hydroxy,5-[^{125}I]iodophenyl)proprionate (Bolton and Hunter reagent, Amersham). PBS-washed cells (1×10^7) were resuspended in 200 ml 0.1 M borate-buffered physiological saline, pH 8.5, and added to 300 mCi of Bolton and Hunter reagent (which had been dried from 70 μl of dry benzene, 0.2% dimethylformamide under a gentle stream of nitrogen) in a 1.5-ml Eppendorf tube. The tube was then agitated gently on ice for 10 min. Cells were then washed extensively in ice cold PBS before being lysed by incubation at 10^7 cells/ml in CLB (1% wt/vol NP40, 0.5% wt/vol sodium deoxycholate, 10 mM tris(hydroxymethyl)aminomethane [Tris]-HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine, 50 kallikrein units/ml aprotinin, 0.05 mg/ml leupeptin, 0.05 mg/ml antipain) for 15 min on ice. Nuclear and nonsolubilized material was removed by centrifugation at 15 000 × *g* and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube.

Protein A Sepharose CL4B (Pharmacia, Piscataway, NJ) was prepared for immunoprecipitation experiments by swelling overnight at 4°C in PBS. Swollen Protein A Sepharose CL4B was then washed five times in CLB to remove any nonbound protein A and to equilibrate the Sepharose in the appropriate buffer. The final suspension was 50% vol/vol Protein A Sepharose CL4B. Proteins that might bind nonspecifically to the Protein A Sepharose were removed during a series of "pre-clearing" incubations. Seventy-five microliters of the Protein A Sepharose suspension was added to each 1 ml of labeled cell lysate and incubated, with gentle agitation, on ice for 15 min. Sepharose beads were then pelleted by a brief (5–10 s) pulse of centrifugation at 4°C in a microfuge and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube. A fresh 75-μl aliquot of the Protein A Sepharose suspension was then added and incubated, with gentle agitation, at 4°C overnight. Sepharose beads were then pelleted as before and the lysate was subjected to two more 15-min pre-clearings on ice. The final 1 ml of pre-cleared lysate was made to 1.5 ml with CLB and 500 μl used for each immunoprecipitation. One milliliter of antiserum was added to each aliquot of lysate and incubated at ambient temperature for 60 min before addition of 75 μl of Protein A Sepharose CL4B suspension and a further 30-min incubation at ambient temperature. Sepharose beads were then pelleted by a brief (5–10 s) pulse of centrifugation and washed three times in RIPA buffer (1% vol/vol Triton X-100, 1% wt/vol sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM PMSF, 0.05 M Tris, pH 7.5), once in STN pH 7.4 buffer (0.25% NP40, 0.15 M NaCl, 10 mM PMSF, 0.01 M Tris, pH 7.4) and once in STN pH 6.8 buffer (0.25% NP40, 10 mM PMSF, 0.01 M Tris, pH 6.8) (1 ml per wash). Thirty microliters of reducing sample buffer (0.0625 M Tris, pH 6.8, 10% wt/vol glycerol, 2% SDS, trace of bromophenol blue, 50 mM dithiothreitol [DTT]) were added to each final pellet, and the samples were boiled for 5 min before being loaded on a 10% SDS polyacrylamide gel with 5% stack (Laemmli, 1970).

^{14}C radiolabeled Rainbow molecular weight markers (Amersham) were included on all gels and electrophoresis continued until the 30-

kDa size marker reached the bottom of the gel. Gels were dried onto Whatmann 3MM paper and exposed to Hyperfilm (Amersham) at −70°C for the times indicated in figure legends.

Indirect Radioimmunoassay

NRK cells were grown to ~70% confluency before plating onto 10 × 10 cm petri dishes and incubating overnight at 37°C. After a 3-h incubation in the presence or absence of 5 μg/ml BFA cells were scraped, pelleted, and resuspended at 2×10^6 cells/ml in 1% BSA/PBS with 0.1% sodium azide. One hundred microliters of the cell suspension was added to each well of a V-bottomed polyvinylchloride plate (Linbro, Hamden, CT) and centrifuged for 5 min at ~400 × *g*. Supernatants were removed by aspiration, the cells were resuspended on a microtiter plate shaker, and 50 μl of a 1:250 dilution of primary antibody was added. The plates were then incubated for from 1 to 3 h at 4°C and washed in 2× 200 μl of ice cold 1% BSA/PBS/azide before addition of ~50 000 cpm of ^{125}I-labeled (goat) anti-rabbit IgG (Amersham) in 50 μl ice cold 1% BSA/PBS/azide per well. After a 90-min incubation at 4°C the plates were washed three times with 200 μl of ice cold 1% BSA/PBS/azide as described above. Individual wells were then excised and counted on a Nuclear Enterprises Technology Ltd. gamma counter (Berks, UK). Each sample was assayed in quadruplicate, and results are expressed as counts per minute per 2×10^5 cells ± SEM.

RESULTS

TGN38/41 is Expressed at the Surface of Control and BFA-treated NRK Cells

Immunofluorescence analysis of methanol-fixed NRK cells shows that anti-TGN38/41 antibody specifically binds to a juxtanuclear region of NRK cells (Luzio *et al.*, 1990; Reaves and Banting, 1992)(Figure 1A). This region has previously been shown, by immunogold electron microscopy using Vesicular Stomatitis Virus (VSV)-infected cells, to be the TGN (Luzio *et al.*, 1990). Immunofluorescence analysis of methanol-fixed NRK cells that had been incubated at 37°C in the presence of BFA (5 μg/ml) for 3 h before fixation shows a morphological change to the TGN (Figure 1B) corresponding to a collapse of the majority of the TGN around the MTOC (Reaves and Banting, 1992). In these cells there is some faint, dispersed staining over the rest of the cell (Figure 1B)(Reaves and Banting, 1992), which we considered might correspond to cell surface TGN38/41. To address this possibility, control and BFA-treated (5 μg/ml for 3 h) NRK cells were incubated in the presence of a 1:200 dilution of anti-TGN38/41 polyclonal antiserum for 2 h at 4°C before methanol fixation and incubation with rhodamine-labeled swine anti-rabbit IgG. This anti-TGN38/41 polyclonal antibody recognizes lumenal epitopes on TGN38/41 (Wilde *et al.*, 1992) and should therefore recognize the portion of TGN38/41 that is exposed at the cell surface. No staining was observed in either control or BFA-treated cells. Similar results were obtained 1) using an antibody which recognizes the cytoplasmic C-terminal domain of TGN41 (Reaves *et al.*, 1992) and 2) when nonpermeabilized paraformaldehyde-fixed cells were incubated with anti-TGN38/41 antibody before processing for immunofluorescence analysis. Although these results suggested that TGN38/41 is not present at the surface of either control or BFA-treated cells, we also considered it possible that the assay system we were using immunofluorescence detection of surface-bound antibody, might not be sufficiently sensitive to detect low level surface expression of TGN38/41. We reasoned that if cell surface TGN38/41 were internalized it would be concentrated in endocytic compartments and hence be more easily detectable by immunofluorescent microscopy. This hypothesis was supported by the finding of Lippincott-Schwartz *et al.* (1991) that internalized TGN38/41 could be detected in BFA-treated cells. Control and BFA-treated NRK cells were therefore incubated in the presence of TGN38/41 antiserum for 2 h at 4°C and then warmed to 37°C and incubated for a further 1 h at that temperature before methanol fixation and incubation with rhodamine-labeled swine anti-rabbit IgG (Figure 1, C and D). Intracellular staining of both populations of cells is observed under these conditions, implying that 1) TGN38/41 is expressed at the surface of control and BFA-treated cells and 2) that antibody-bound TGN38/41 can be endocytosed from the surface of cells that have and have not been incubated with BFA. Control experiments were performed using the same concentration of other antibodies, including 1) antibodies that recognize different lumenal domain epitopes on TGN38/41, but unlike the antibody used throughout this paper, are incapable of binding TGN38/41 at the cell surface, and 2) an antiserum that recognizes only the cytoplasmic domain of TGN41. No intracellular staining of either control or BFA-treated cells was observed with any of these antibodies, confirming the specificity of the results obtained with the anti-TGN38/41 antibody.

Iodination and Immunoprecipitation of Cell Surface TGN38/41

The presence of TGN38/41 at the surface of both control and BFA-treated cells was confirmed by immunoprecipitation of iodinated TGN38/41 from lysates of cells that had been surface labeled using Bolton and Hunter reagent (Bolton and Hunter, 1973). The anti-TGN38/41 antiserum specifically immunoprecipitates molecules of ~80–90 kDa from both lysates (Figure 2, lanes a and b) and also from purified Golgi membranes that had been similarly labeled (Figure 2, lane c). The difference in intensity of bands precipitated from Golgi membranes versus whole cells is presumably due to the inefficiency with which this method iodinates TGN38/41 in isolated membranes. In intact, isolated Golgi membranes the region of TGN38/41 accessible to label (the cytoplasmic "tail") is relatively short and contains considerably fewer potential sites for iodination by Bolton and Hunter reagent than the larger lumenal do-

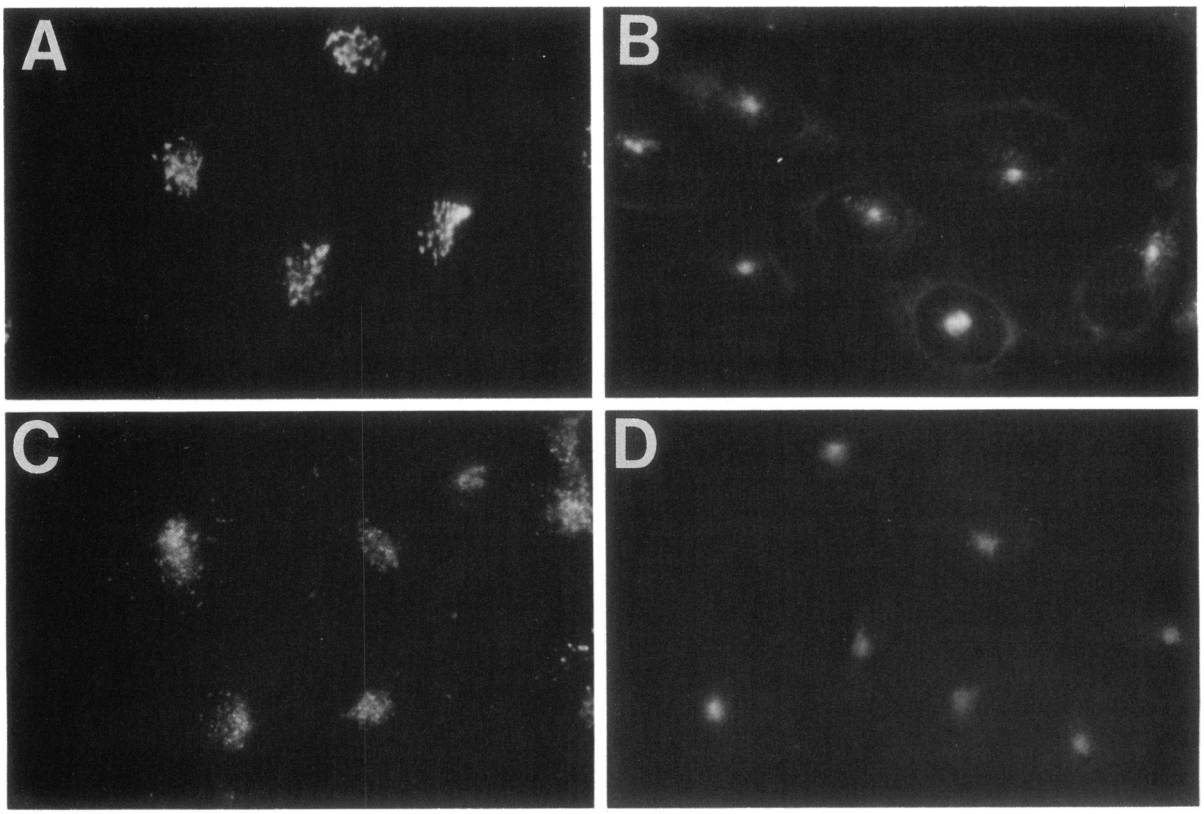


Figure 1. Immunofluorescence screen for cell surface TGN38. Cells were incubated in the absence (A) or presence (B) of BFA before fixation, followed by incubation with anti-TGN38 antibody and processing for immunofluorescence analysis or in the absence (C) or presence (D) of BFA before incubation with anti-TGN38 antibody for 2 h at 4°C and subsequent incubation at 37°C for 1 h in the continued presence of antibody before fixation and processing for immunofluorescence analysis.

main that is exposed at the cell surface. To confirm that the iodinated TGN38/41 immunoprecipitated from surface-labeled cells corresponds to surface TGN38/41, rather than intracellular TGN38/41 inadvertently labeled in damaged cells, a monoclonal antibody to mannosidase II was used in parallel immunoprecipitation experiments. This antibody would only be expected to immunoprecipitate radiolabeled molecules from surface-labeled cells if those cells were sufficiently damaged to allow intracellular labeling of Golgi membranes. The monoclonal antibody to mannosidase II fails to recognize any surface-labeled molecules (Figure 2, lanes d and e), but as expected (Burke *et al.*, 1982; Baron and Garrof, 1991), does immunoprecipitate a molecule of 135-kDa from lysate of Golgi membranes (Figure 2, lane f). Hence the 90-kDa molecule immunoprecipitated by anti-TGN38/41 antiserum from surface-labeled cells does correspond to a cell surface form of TGN38/41 and is not a contaminant due to intracellular labeling of "leaky" cells. These results confirm the presence of TGN38/41 at the surface of both control and BFA-treated cells.

The Level of TGN38/41 at the Cell Surface Does not Increase Upon BFA Treatment

Although iodination and immunoprecipitation of cell surface TGN38/41 yielded qualitative rather than quantitative results, there did appear to be a slight decrease in cell surface labeling of TGN38/41 in BFA-treated cells (cf. Figure 2, lanes a and b). A whole cell indirect radioimmunoassay (IRIA) was carried out to determine the relative amounts of TGN38/41 at the surface of control and BFA-treated NRK cells. NRK cells were incubated in the presence or absence of 5 μg/ml BFA for 3 h at 37°C before cooling to 4°C followed by incubation with either anti-TGN38/41 lumenal domain antiserum or anti-TGN41 cytoplasmic domain antibody at 4°C for a further 2 h. Bound antibody was detected with ^{125}I-labeled goat anti-rabbit IgG as described in MATERIALS AND METHODS. Incubations with primary and secondary antibody, as well as all washes, were performed at 4°C to block endocytosis. The anti-TGN41 cytoplasmic domain antibody was used as an indicator of nonspecific cell surface binding; clearly it will only bind specifically to the cytoplasmic face of the plasma membrane. This experiment was performed with

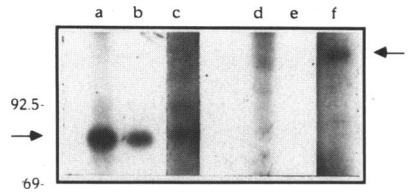


Figure 2. Immunoprecipitation of TGN38 from surface-labeled cells. Intact control (lanes a and d) and BFA-treated (lanes b and e) NRK cells were surface-labeled with ^{125}I using Bolton and Hunter reagent, as were purified rat liver Golgi membranes (lanes c and f). Proteins immunoprecipitated by anti-TGN38 antiserum (lanes a–c) or anti-mannosidase II antibody (lanes d–f) from lysates of these labeled preparations were separated by SDS-PAGE and visualized by autoradiography. Arrows indicate the positions of the immunoprecipitated molecules. The electrophoretic mobility of molecular weight standards ($M_r \times 10^{-3}$) is indicated.

quadruplicate samples on each of five separate occasions. The results of one such experiment are given in Figure 3. Anti-TGN38/41 binding to non-BFA-treated cells is approximately twice that of control antibody, whereas anti-TGN38/41 binding to BFA-treated cells is only 1.5 times that of control antibody. These data indicate that the level of TGN38/41 cell surface expression actually decreases after BFA treatment.

Kinetics of TGN38/41 Internalization

To investigate the kinetics of internalization of TGN38, intact control and BFA-treated NRK cells were incubated with anti-TGN38/41 antibody for various times at 37°C, washed, methanol fixed, and processed for immunofluorescence microscopy using a rhodamine-labeled second antibody. In both control and BFA-treated cells, fixed after a 5-min incubation with anti-TGN38/41 antibody, a very faint diffuse staining over the entire cell is apparent (Figure 4, A and B). The staining pattern in control cells is similar after a 15-min incubation with antibody; however, numerous, small faintly stained vesicles can be seen distributed throughout the cytoplasm (Figure 4C). In control cells fixed after a 30-min incubation with antibody, larger vesicles concentrating in the perinuclear region of the cell are detected in addition to the background of small faintly stained vesicles (Figure 4E). By 1 h the predominant pattern of staining observed in control cells is perinuclear, but a substantial number of intensely stained vesicles are still present (Figure 4G). The pattern of staining observed in similarly processed BFA-treated cells is significantly different (Figure 4, D, F, and H). By 15 min of incubation, the majority of staining is restricted to a juxtanuclear area corresponding to the "collapsed TGN" observed in cells that have been incubated with anti-TGN38/41 antibody after methanol fixation (cf. Figures 4D and 1B). The pattern of staining observed in BFA-treated cells after a 30-min (Figure 4F) or 1 h incubation (Figure 4H) with antibody is similar to that seen at the 15-min time point (Figure 4D). Incubation with antibody for 16 h before fixation and processing for immunofluorescence analysis produces no discernable change in the patterns of staining seen in either control or BFA-treated cells; i.e., these patterns are similar to those observed in cells incubated with antibody for 2 h.

A pulse-chase experiment was performed to investigate the fate of internalized TGN38/41. Control and BFA-treated cells were incubated with antibody for 4 h at 37°C; nonbound antibody was washed away, and the cells were incubated for a further 12 h at 37°C before fixation and processing for immunofluorescence analysis. In both cases only faint, diffuse intracellular staining was observed. The decrease in staining might be due to 1) a proportion of recycling antibody-antigen complexes being targeted to, and degraded in, lysosomes, or 2) antibody-antigen complexes becoming distributed around a recycling pathway between the cell surface and the TGN. Both of these possibilities would reduce the total amount of antibody-bound TGN38/41 available for detection by the rhodamine-labeled second antibody, the former due to degradation of the antibody in the lysosomes and the latter due to a loss of antibody into the extracellular medium. In either case the ultimate result would be the same, i.e., a weakening of the signal detected by immunofluorescence analysis.

Pathway of Internalization of TGN38/41

The time course data presented in Figure 4 prompted us to investigate the pathway of endocytosis for TGN38/41. Does internalized TGN38/41 pass through early endosomes en route to the TGN as implied by the diffuse staining observed in Figure 4C after 15 min of antibody uptake? To address this question we used two independent methods of labeling early endosomes: 1)

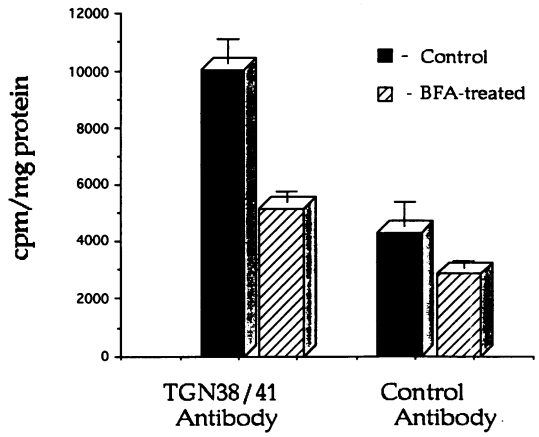


Figure 3. Detection of cell surface TGN38/41 by indirect radioimmunoassay. The experiment was performed as described in MATERIALS AND METHODS. The results are expressed as means ± SEM (n = 4).

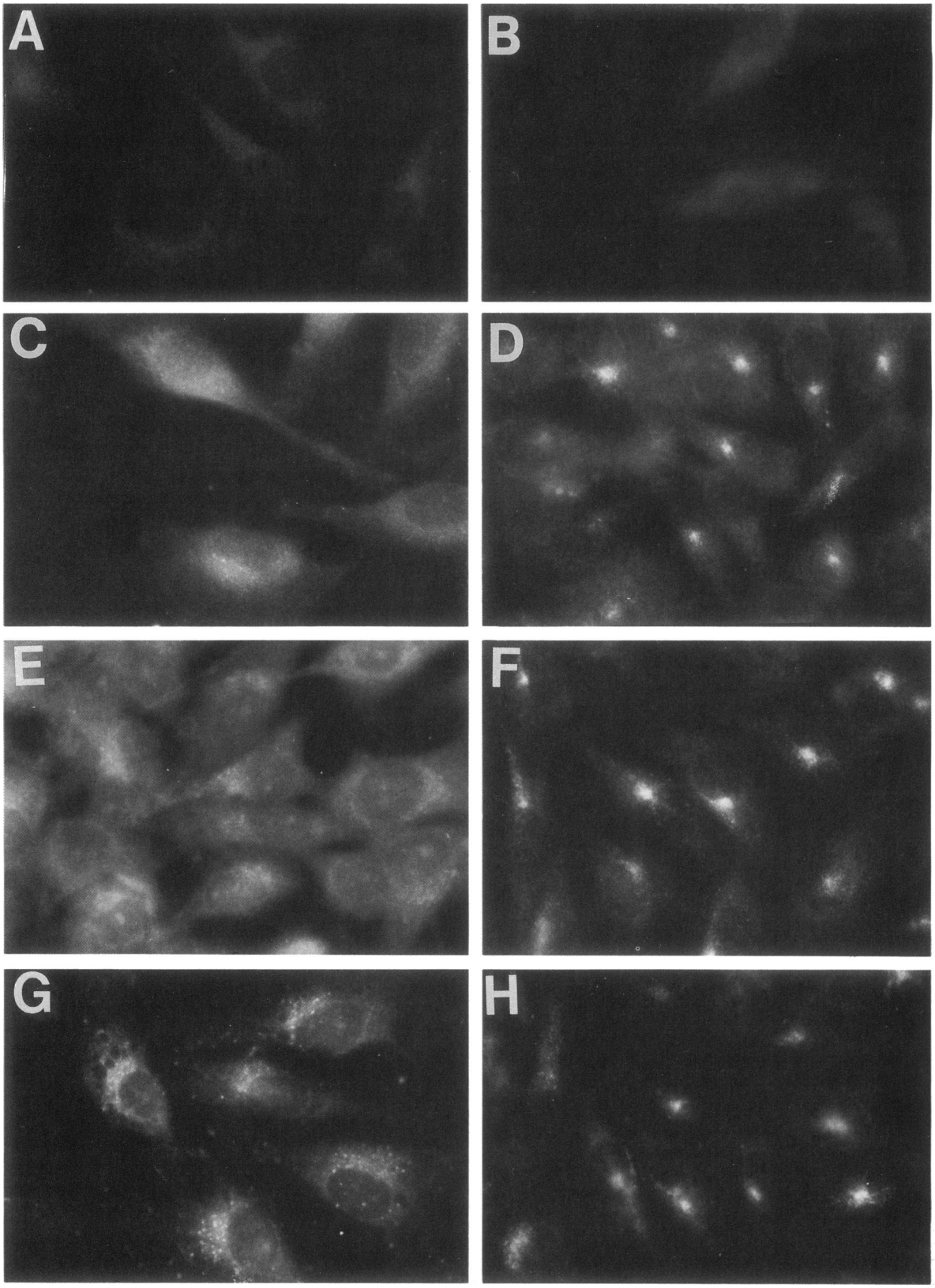


Figure 4. Time course of internalization of cell surface TGN38. Control (A, C, E, and G) and BFA-treated (B, D, F, and H) cells were incubated at 37°C for 5 min (A and B), 15 minutes (C and D), 30 min (E and F), or 1 h (G and H) in the presence of anti-TGN38/41 antibody before processing for immunofluorescence analysis.

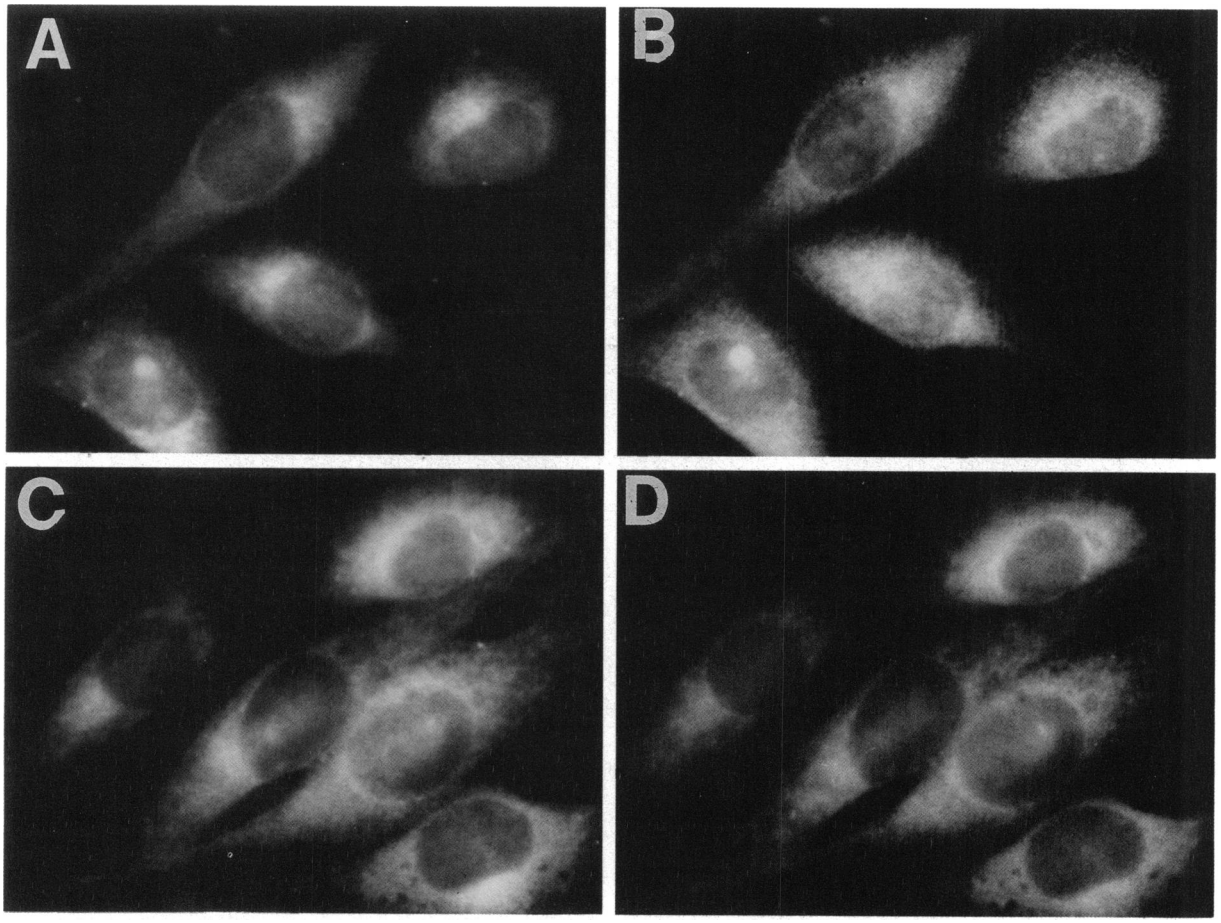
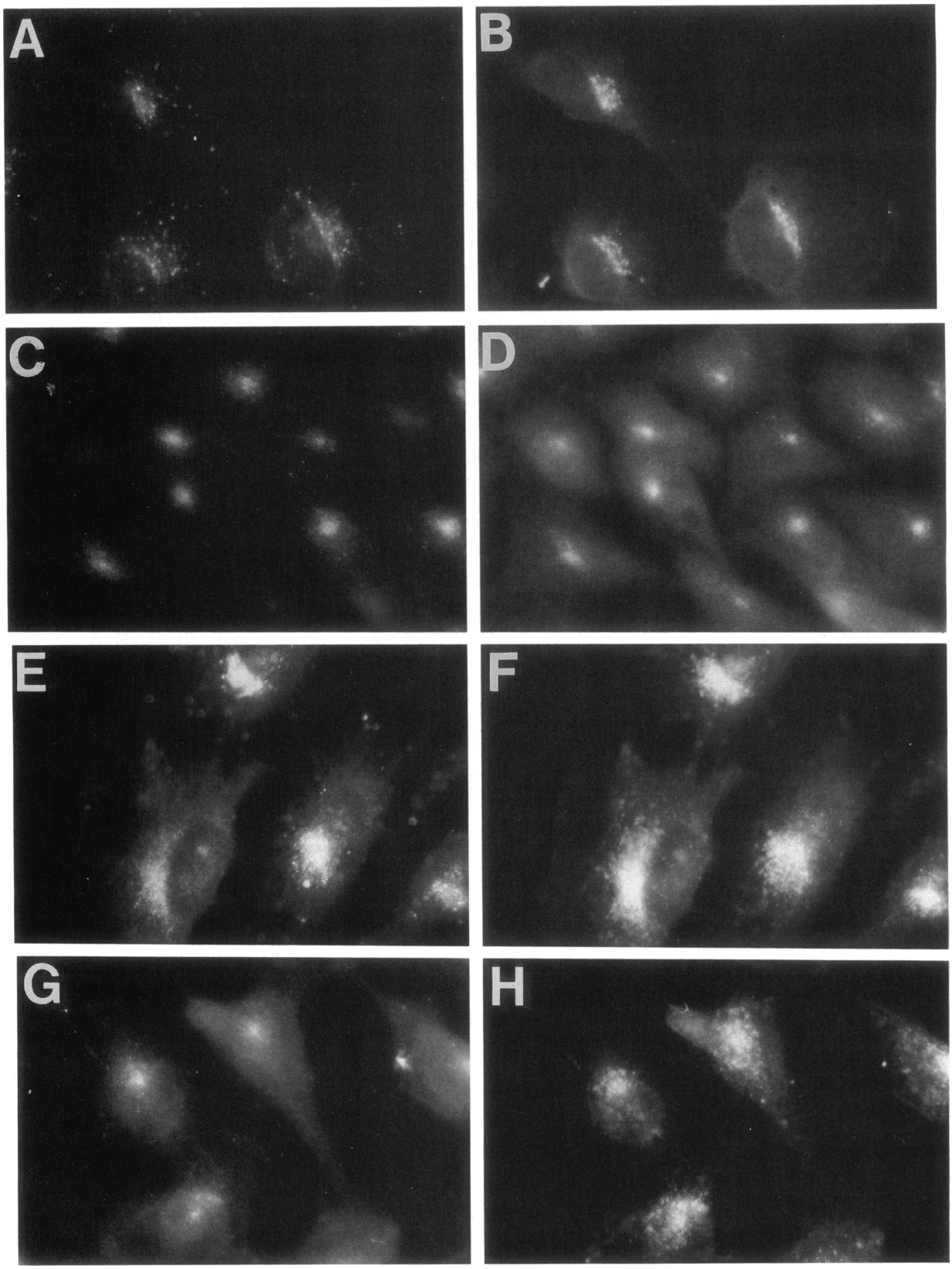


Figure 5. Colocalization of internalized TGN38/41 with markers of the early endocytic pathway. NRK cells were incubated for 1 h at 37°C in the presence of FITC-transferrin, washed, paraformaldehyde fixed, saponin permeabilized, and then incubated with a rabbit polyclonal antiserum to rab5 and a rhodamine-conjugated swine anti-rabbit second antibody. (A) The pattern of staining produced by the FITC-transferrin. (B) The rab5 localization detected by rhodamine fluorescence in the same cells. A second set of NRK cells was incubated for 1 h at 37°C in the presence of FITC-transferrin. For the final 15 min of this incubation anti-TGN38/41 antibody was also present in the incubation medium. The cells were then washed, methanol fixed, and incubated with a rhodamine-conjugated swine anti-rabbit second antibody to detect internalized TGN38/41. (C) The pattern of staining produced by the FITC-transferrin. (D) The localization of internalized TGN38/41 as detected by rhodamine fluorescence in the same cells.

uptake of FITC-transferrin for 1 h at 37°C and 2) immunofluorescent labeling of paraformaldehyde-fixed and saponin-permeabilized NRK cells with antibodies to rab5 (an early endosome marker)(Gorvel *et al.*, 1991). Iron-bound transferrin binds to its receptor at the plasma membrane; these complexes are internalized via coated pits and migrate to early endosomes where the acidic pH leads to disocciation of iron from the complex (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). Transferrin and its receptor then recycle to the plasma membrane. The intracellular distribution of FITC-transferrin is therefore a convenient marker of early endosomes; a 1-h incubation in FITC-transferrin results in the efficient labeling of the early endosome population with this marker (Hopkins, 1983; Hopkins and Trowbridge, 1983). In the first of two double immunofluorescence experiments, NRK cells were incubated for 1 h at 37°C in the presence of FITC-transferrin, washed, paraformaldehyde fixed, saponin permeabilized, and then incubated with a rabbit polyclonal antiserum to rab5 and a rhodamine-conjugated swine anti-rabbit second antibody (these fixation conditions were used in place of methanol fixation, because they are the ones described by Gorvel *et al.*, 1991 as being optimal for the anti-rab5 antiserum). Figure 5A shows the pattern of staining produced by the FITC-transferrin, and Figure 5B the rab5 localization detected by rhodamine fluorescence in the same cells. FITC-transferrin, that has been internalized for 1 h (and equilibrated throughout the early endosome population) can be seen to colocalize with rab5, confirming the presence of FITC-transferrin in early endosomes. In the second double immunofluorescence experiment, NRK cells were incubated for 1 h at 37°C in the presence of FITC-transferrin. For the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final 15 min of this incubation, anti-TGN38/41 antibody was also present in the incubation medium. The cells were then washed, methanol fixed, and incubated with a rhodamine-conjugated swine anti-rabbit second antibody to detect internalized TGN38/41. Figure 5C shows the pattern of staining produced by the FITC-transferrin, and Figure 5D the localization of internalized TGN38/41 as detected by rhodamine fluorescence in the same cells. Internalized TGN38/41 can be seen to colocalize with internalized FITC-transferrin, and because the internalized FITC-transferrin colocalizes with rab5 (Figure 5, A and B), it is clear that after 15 min of uptake internalized TGN38/41 is present in early endosomes.

Localization of Internalized TGN38/41

In an attempt to determine whether internalized TGN38/41 was 1) returning to the TGN and/or 2) being diverted to lysosomes, we carried out indirect double immunofluorescence analysis. The rabbit anti-rat TGN38/41 antibody was added to BFA-treated cells in the presence or absence of BFA; the cells were then fixed, permeabilized, and incubated with either a sheep anti-rat TGN38/41 antibody to identify the TGN or a monoclonal antibody to lgp110 to identify lysosomes. Although the internalized TGN38/41 pattern in control cells was more vesicular (Figure 6A) than that seen by labeling after fixation (Figure 6B), both antibodies revealed a similar localization. In addition the intense staining of TGN38/41 around the MTOC in BFA-treated cells (Figure 6D) colocalized with the internalized TGN38/41 (Figure 6C). Parallel experiments performed with the anti-lysosomal antibody showed that, in control cells, internalized TGN38/41 partially colocalized with the lysosomal marker (Figure 6, E and F). However, it is clear that there was not complete overlap between lysosomes and internalized TGN38/41. This "lack of overlap" is particularly evident in BFA-treated cells where there are extensive areas recognized by the anti-lysosomal marker that do not contain internalized TGN38/41 (Figure 6, G and H). Thus, although this experiment cannot exclude the possibility that some of the TGN38/41 recycling from the surface may be delivered to lysosomes, it suggests that at least a portion is returning to the TGN in both control and BFA-treated cells.

Experiments were performed to determine whether the appearance of internalized TGN38/41 in the collapsed TGN was dependent on preincubation of cells with BFA. NRK cells were incubated in the presence of anti-TGN38/41 antiserum for 2 h at 37°C before the addition of BFA for a further 3 h. The cells were then fixed and processed for immunofluorescence analysis. The pattern of staining observed was identical to that seen in cells that had been incubated with BFA before extracellular addition of antibody, i.e., internalized TGN38/41 was localized to the collapsed TGN.

Microtubule-Dependence of Endocytosed TGN38/41 in Control and BFA-treated Cells

Gruenberg *et al.* (1989) have previously shown that the early steps of endocytosis are microtubule independent, whereas the latter stages are microtubule dependent. Is endocytosis of TGN38/41 in BFA-treated cells dependent on the presence of intact microtubules, or is endocytosis microtubule dependent only in control cells? We used the ability of nocodazole to inhibit tubulin polymerization (DeBrabander *et al.*, 1976) to address this question. Control and BFA-treated cells were incubated in the presence or absence of 20 μg/ml nocodazole for 2 h at 37°C before the addition of anti-TGN38/41 antiserum and a further 2-h incubation at 37°C. The cells were then methanol fixed and processed for immunofluorescence analysis. In the absence of nocodazole, TGN38 staining is observed in the TGN of control cells (Figure 7A) and the collapsed TGN of BFA-treated cells (Figure 7B). In the presence of nocodazole, the pattern of TGN38/41 staining is similar in both control and BFA-treated cells (Figure 7, C and D); it is restricted to numerous, uniformly dispersed, small vesicles. Thus in both control and BFA-treated cells lacking intact microtubules, internalized TGN38 appears to accumulate in a very early endocytic compartment, indicating that microtubules are required for transport of TGN38/41 to its final destination. The effect of BFA upon endocytosis of TGN38 must therefore be at the later, microtubule-dependent stage of the process.

DISCUSSION

The data presented here complement our earlier observation that BFA induces a redistribution of the TGN in NRK cells (Reaves and Banting, 1992). In response to BFA treatment there is an initial dispersal of elements of the TGN, as judged by immunofluorescence analysis using anti-TGN38/41 antibodies, followed by a collapse of most of the anti-TGN38/41–stained material upon the MTOC. However some diffuse, faint staining can also be observed (Figure 1B)(Reaves and Banting, 1992). We considered that this staining might correspond to a redistribution of TGN38/41 to either 1) the ER or 2)

Figure 6. Localization of internalized TGN38/41. Control (A, B, E, and F) and BFA-treated (C, D, G, and H) were incubated with rabbit anti-TGN38/41 antiserum for 2 h at 37°C before fixtion and staining with anti-rabbit IgG-RITC to visualize internalized TGN38/41 (A, C, E, and G); in addition, sheep anti-TGN38/41 coupled with anti-sheep IgG-FITC was used to localize the TGN (B and D) and a monoclonal antibody to lgp110 coupled with mouse IgG-FITC (F and H) to localize lysosomes.

the plasma membrane. The pattern of staining observed in Figure 1B could be interpreted to include ER and nuclear envelope membranes, thus supporting the first hypothesis. Previous results had suggested that TGN38/41 is not present at the surface of control cells (Luzio *et al.*, 1990), but that BFA treatment induced its appearance at the cell surface (Lippincott-Schwartz *et al.*, 1991). We have now shown that TGN38/41 is present at the surface of not only BFA-treated NRK cells, but also control, non-BFA-treated cells. We have also shown that TGN38/41 is endocytosed and that internalized TGN38/41 can return to the TGN in both control and BFA-treated cells, the movement from cell surface to TGN being demonstrably faster in BFA-treated cells than in control cells. This change in the kinetics of endocytosis in BFA-treated cells appears to be associated with a fusion between endocytic compartments and the collapsed TGN observed in BFA-treated cells that has been previously described by others (Damke *et al.*, 1991; Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991).

It has not proved possible to detect TGN38/41 at the surface of the cells by indirect immunofluorescence analysis; however, IRIA analysis and surface iodination followed by immunoprecipitation have clearly shown that TGN38/41 is present at the surface of both control and BFA-treated cells. These experiments also show that there is no gross difference in the amount of TGN38/41 present at the surface of either population of cells (Figures 2 and 3) and imply that little, if any, of the TGN migrates to, and remains at, the cell surface in response to BFA treatment. Thus the faint, diffuse TGN38/41 staining seen in immunofluorescence analysis of BFA-treated, methanol-fixed cells is presumably due to small, dispersed, intracellular, vesicular elements derived from the TGN.

Recycling from the plasma membrane through the TGN has been demonstrated for a number of receptors including the asialoglycoprotein receptor (ASGPR), transferrin receptor (TfR) and mannose-6-phosphate receptor (MPR)(Woods *et al.*, 1986; Geuze *et al.*, 1988; Stoorvogel *et al.*, 1989); however, in this report we present evidence that a protein predominantly localized to the TGN can also follow this pathway.

Although it proved impossible to detect TGN38/41 at the surface of cells by immunofluorescence analysis, its presence at the cell surface, demonstrated by IRIA and immunoprecipitation analyses, can also be inferred from the results of a series of "time course" experiments. In cells incubated with anti-TGN38/41 antibody at 37°C for various times before fixation and processing for immunofluorescence analysis a "wave" of stained material passes from the surface to the TGN (Figure 4). However, no intracellular staining is observed in either control or BFA-treated cells when equivalent concentrations of control antibodies are used. In these experiments antibody is incubated with intact cells; any subsequent intracellular staining must therefore be due to the detection of internalized antibody. Intracellular staining is observed in control and BFA-treated cells from early time points when the antibody used is directed to the lumenal domain of TGN38/41. It is therefore clear 1) that endocytosis of TGN38/41 is a specific event and 2) that TGN38/41 must initially be present at the surface of both control and BFA-treated cells. It is of note that endocytosed TGN38/41 is primarily targeted to the TGN, not lysosomes and that although endocytosed TGN38/41 returns to the TGN of both control and BFA-treated cells, it does so more rapidly in the latter. After 15 min incubation with antibody the majority of endocytosed TGN38/41 in BFA-treated cells has returned to the TGN (Figure 4D), whereas most of the endocytosed TGN38/41 in control cells is associated with small vesicular structures (Figure 4C). Tooze and Hollinshead (1992) have demonstrated that BFA causes an increase in the rate at which fluid phase markers enter the early endosomes. Wood *et al.*, (1991) also demonstrated an increased uptake of Lucifer yellow in cells treated with BFA. We have now demonstrated an increase in the rate at which TGN38/41 reaches the interior of BFA-treated cells. This would be in keeping with the model of BFA-induced interactions between the TGN and early endosomes (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991) in which tubulation allows more rapid access to intracellular compartments. Our data from 1) immunoprecipitation experiments (Figure 2), 2) whole cell immunoassay (Figure 3), and 3) immunofluorescence analysis of nocodazole-treated cells (Figure 7) suggest that there may be a slight decrease in cell surface TGN38/41 in BFA-treated cells. One possible explanation for this would be that in the presence of BFA there is a modification to the recycling kinetics of TGN38/41 such that the steady-state distribution of TGN38/41 is altered. Thus, if TGN38/41 leaves the TGN at the same rate in control and BFA-treated cells, but returns to the TGN via the cell surface faster in BFA-treated cells than in control cells, the steady-state levels of cell surface TGN38/41 would be lower in BFA-treated cells than in control cells. Our observations are consistent with this model, which would imply an enhanced rate of endocytosis in BFA-treated cells and is at variance with an earlier report that BFA has no effect on the rate at which ^{125}I-asialofetuin is internalized and degraded (Misumi *et al.*, 1986). However it should be noted that the internalized ^{125}I-asialofetuin is destined for degradation in lysosomes, whereas internalized TGN38/41 returns to the TGN. This suggests that the sorting of endocytosed molecules en route for lysosomes, from those returning to the TGN, occurs before the site of action of BFA.

We attempted to define the intracellular compartment to which TGN38/41 is internalized by performing double-labeling experiments. Because other antibodies to integral membrane proteins of the TGN are not readily available, we raised antibodies to TGN38/41 in sheep

for use in these experiments. Internalized TGN38/41 does colocalize with the intact TGN in both control and BFA-treated cells (Figure 6). Internalized TGN38/41 also shows significant colocalization with a lysosomal marker in control cells (Figure 6). This might be due to 1) internalized TGN38/41 returning to lysosomes (possibly due to clustering of antigens due to antibody binding), 2) antibody disocciating from antigen in the early stages of the endocytic pathway and being routed to lysosomes, or 3) the TGN and lysosomes occupying the same region of the cell. Experiments with BFA-treated cells support the latter explanation. If internalized TGN38/41 were being targeted solely to lysosomes, one would expect that the pattern of internalized TGN38/41 staining would be the same before and after BFA-treatment, because BFA does not cause lysosomes to condense around the MTOC (Lippincott-Schwartz *et al.*, 1991; Reaves and Banting, 1992). This is not the case. BFA-treatment causes internalized TGN38/41 to accumulate at the MTOC while the lysosomes remain unaffected (Figure 6). This same pattern is observed irrespective of the order of addition of BFA, i.e., whether BFA is added before or after antibody addition. Experiments were designed to monitor the possible degradation of TGN38/41 using chloroquin to block the function of lysosomal enzymes. However, unexpectedly, although chloroquin had no effect on the morphology of the Golgi stack (as determined by immunofluorescence analysis using an anti-mannosidase II antibody), it did cause vesicularization of the TGN. Similar observations have been made by others (Munro, personal communication; Stanley, personal communication). Significantly, internalized TGN38/41 is also located in these large irregularly shaped vesicles, once again suggesting that it preferentially returns to the TGN.

The results of immunofluorescence analysis of cells that had been incubated with anti-TGN38/41 antibody in the presence of nocodazole before fixation support the immunoprecipitation-derived model that BFA treatment does not increase the steady-state level of cell surface TGN38/41. Gruenberg *et al.*, (1989) have shown that the early stages of endocytosis do not require microtubules whereas the latter stages do. The depolymerization of microtubules induced by nocodazole means that any internalized TGN38/41 becomes trapped in early endosomes. The results of these experiments also provide a clue as to the reason for the enhanced kinetics of endocytosis in BFA-treated cells. The existence of the large vesicles observed in control cells after a 1-h incubation with anti-TGN38/41 antibody in the absence of nocodazole (Figure 7A) is clearly microtubule dependent; these therefore correspond to an endocytic compartment downstream of early endosomes (Gruenberg *et al.*, 1989). Hence 1) BFA treatment does not affect the formation of early endosomes (Figure 7C) and 2) subsequent transfer of material to the TGN is microtubule dependent in both control and BFA-treated cells (Figure 7, C and D). However, in BFA-treated cells incubated with anti-TGN38/41 antibody in the absence of nocodazole, there is no staining of the large vesicular structures observed in similarly treated control cells (Figure 7B). These observations beg the double question: If the microtubule-dependent phase of the endocytosis of TGN38/41 involves large vesicular structures and these large vesicular structures are not observed in BFA-treated cells, then why is the post early endosome phase of endocytosis still microtubule-dependent in these cells, and how does endocytosed material reach the TGN from the early endosomes? A simple answer would be that microtubule-associated endosomes have fused with the collapsed TGN of BFA-treated cells as suggested by Lippincott-Schwartz *et al.*, (1991) and Wood *et al.*, (1991). Such a system would still require the association of fused early endosomes with microtubules (Gruenberg *et al.*, 1989), otherwise nocodazole would have no inhibitory effect on endocytosis. It would also imply that once this association had taken place there would be direct fusion between the microtubule-associated, fused, early endosomes and the collapsed TGN, instead of endosomal maturation (reviewed in Murphy, 1991) or vesicular transport between pre-existing endosomal compartments (reviewed in Griffiths and Gruenberg, 1991). In such a model the endosomes would become tubular structures aligned along the microtubules with the collapsed TGN focused on the MTOC at the base of these microtubules.

Orci *et al.*, (1991) have proposed a model to explain some of the effects of BFA on the morphology and functioning of the Golgi stacks. They suggest that BFA upsets the usual balance between anterograde (vesicular) and retrograde (tubular) transport within the Golgi by inhibiting the formation of anterograde coated vesicles and allowing retrograde transport to predominate. This eventually leads to a redistribution of Golgi components into the endoplasmic reticulum. In the case of the effect of BFA on endocytosis, we are also suggesting the predominance of tubular association over vesicular transport. A significant difference between transport within the Golgi stack and endocytosis is that in the former situation vesicular and tubular transport move molecules in opposite directions, whereas in endocytosis the two proposed modes of transport are in the same direction. However, a balance between vesicular and tubular transport in endocytosis may exist in non-BFA–treated cells because evidence exists for the presence of both endocytic vesicles (Griffiths *et al.*, 1988; Gruenberg *et al.*, 1989; Bomsel *et al.*, 1990) and tubular components of the endocytic pathway (Hopkins *et al.*, 1990) in such cells. If BFA does inhibit vesicular transport within the Golgi stack by preventing the incorporation of coat subunits (Orci *et al.*, 1991), then it seems reasonable to propose that it might do the same in the endocytic pathway where at least some of the coat components are the same as those used within the Golgi stack (Diaz

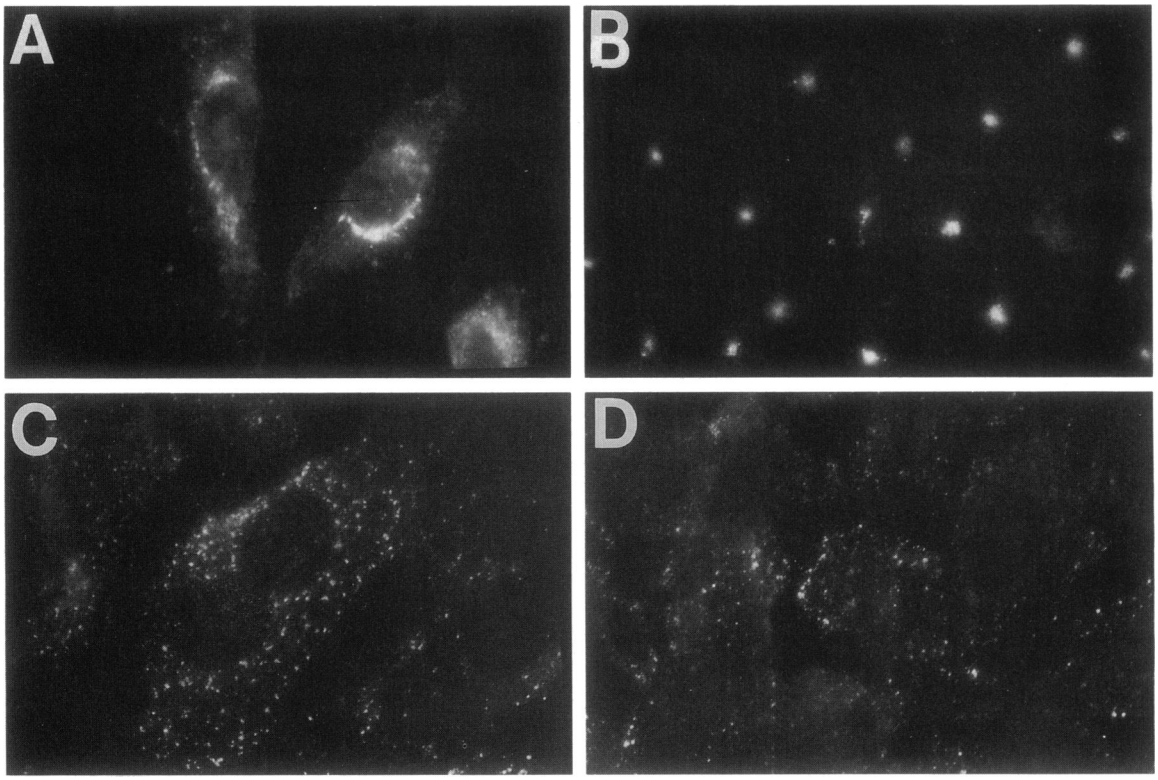

Figure 7. Microtubule dependence of TGN38 endosytosis. Control (A and C) and BFA-treated (B and D) NRK cells were incubated at 37°C with anti-TGN38 antiserum for 2 h in the absence (A and B) or presence (C and D) of nocodazole before processing for immunofluorescence analysis.

et al., 1989). In control cells, the balance between tubule formation and vesicle formation might provide a means to sort endocytozed molecules to different final intracellular locations.

Surface iodination followed by immunoprecipitation has clearly shown that TGN38/41 is present at the surface of both control and BFA-treated cells and whole cell immunoassay has shown that the level either remains the same or can actually be slightly decreased by BFA treatment. In contrast, Damke *et al.*, (1991) report a twofold and Wood *et al.*, (1991) a greater than fivefold, BFA-induced increase in the MPR at the surface of NRK cells. Wood *et al.*, (1991) postulate that this increase is due to the fusing of the TGN and early endosomes, which allows more ready access to the endosome-cell surface pathway. Although the reason for this discrepancy is not clear, the answer may reside in the nature of the steady-state localization of these two proteins; i.e., the MPR is distributed relatively evenly between the endosomal/prelysosomal compartment (PLC) and the TGN (Geuze, 1988), whereas TGN38/41 is localized primarily to the TGN (Luzio *et al.*, 1990). Because the normal pathway for MPR is most probably early endosome to PLC to TGN (Stoorvogel *et al.*, 1989) and then to the surface, the fusion of the early endosomes with the TGN may indeed provide a short-circuiting of the system with the end result being an increase in cell surface expression. TGN38/41, on the other hand, may be cycling only from TGN to cell surface to early endosome and back to the TGN; thus its surface expression would not necessarily be expected to increase in response to BFA treatment. It has been proposed that different areas of tubular endosomes may be responsible for sorting receptors and ligands (reviewed in Smythe and Warren, 1991) and perhaps in this case predominantly TGN proteins, destined for different places in the cell. The tubular endosomes may therefore play a role in segregating proteins returning directly to the TGN from those recycling via later compartments.

Note added in proof. Some of the data recently published by Ladinsky and Howell [Ladinsky, M.S., and Howell, K.E. (1992). The trans-Golgi Network can be dissected structurally and functionally from the cisternae of the Golgi-complex by Brefeldin A. Eur. J. Cell. Biol. *59*, 92–105.] complement some of the data presented in this paper.

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