

The steady state distribution of humTGN46 is not significantly altered in cells defective in clathrin-mediated endocytosis

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SUMMARY

It has been shown previously that whilst the rat type I integral membrane protein TGN38 (ratTGN38) is predominantly localised to the *trans*-Golgi network this protein does reach the cell surface from where it is internalised and delivered back to the *trans*-Golgi network. This protein thus provides a suitable tool for the investigation of trafficking pathways between the *trans*-Golgi network and the cell surface and back again. The human orthologue of ratTGN38, humTGN46, behaves in a similar fashion. These proteins are internalised from the cell surface via clathrin mediated endocytosis, a process which is dependent upon the GTPase activity of dynamin. We thus reasoned that humTGN46 would accumulate at the surface of cells rendered defective in clathrin mediated

endocytosis by virtue of the fact that they express a GTPase defective mutant of dynamin I. It did not. In fact, expression of a dominant negative GTPase defective mutant of dynamin I had no detectable effect on the steady state distribution of humTGN46. One explanation for this observation is that humTGN46 does not travel directly to the cell surface from the *trans*-Golgi network. Further studies on cells expressing the dominant negative GTPase defective mutant of dynamin I indicate that the major recycling pathway for humTGN46 is in fact between the *trans*-Golgi network and the early endosome.

Key words: Dynamin, TGN, Endocytosis, Clathrin

INTRODUCTION

HumTGN46 is the human orthologue of the previously described rat type I integral membrane protein TGN38 (Banting and Ponnambalam, 1997; Luzio et al., 1990; Ponnambalam et al., 1996). These proteins are predominantly localised to the TGN at steady state, but have been described as constitutively cycling between the TGN and the cell surface (Banting and Ponnambalam, 1997; Luzio et al., 1990; Ponnambalam et al., 1996). TGN38 has been suggested to play roles in both the morphology of the TGN and the formation of secretory vesicles at exit sites from the TGN (Banting and Ponnambalam, 1997; Girotti and Banting, 1996; Jones et al., 1993; Ponnambalam et al., 1996). The precise mechanisms by which humTGN46 and ratTGN38 leave the TGN have yet to be determined, but there is a considerable body of evidence that these proteins are internalised from the cell surface via a clathrin-mediated mechanism (reviewed by Banting and Ponnambalam, 1997). Indeed, a conserved tyrosine-containing internalisation motif in the cytosolic domain of these proteins has been shown to interact directly with the medium chain (μ 2) of the AP2 adaptor complex which couples clathrin cages to the cytosolic domains of integral membrane proteins to be internalised from the cell surface (Ohno et al., 1995; Stephens et al., 1997). Mutations of the tyrosine-containing internalisation motif which ablate or reduce interaction with μ 2 lead to elevated levels of ratTGN38 expression at the cell

surface (Bos et al., 1993; Humphrey et al., 1993; Roquemore and Banting, 1998; Wong and Hong, 1993).

We argued that if humTGN46 and ratTGN38 do constitutively cycle between the TGN and the cell surface, then we would expect to see an accumulation of these proteins at the surface of cells in which clathrin-mediated endocytosis had been blocked. A convenient, and highly specific, way to block clathrin-mediated endocytosis is to utilise cells expressing dominant-negative mutants of dynamin I (Damke et al., 1995). Dynamin I associates with the cytosolic face of the constricting neck of invaginating clathrin coated pits prior to their excision from the cell surface (Hinshaw and Schmid, 1995; Takei et al., 1995). The hydrolysis of GTP to GDP by dynamin I is a prerequisite for coated pits becoming fully constricted and pinching off to form coated vesicles (Hinshaw and Schmid, 1995). It has been shown previously that, in cells expressing GTPase defective forms of dynamin I, clathrin mediated endocytosis is significantly inhibited (Damke et al., 1995; Herskowitz et al., 1993).

We therefore chose to study the subcellular localisation of humTGN46 in HeLa cells expressing either wild-type or a GTPase defective (K44A) mutant of dynamin I (Damke et al., 1995). In these cells expression of dynamin I (wild type or mutant) is under the control of a tetracycline regulated expression system (Gossen and Bujard, 1992), thus allowing the regulated, transient, elevated expression of potentially deleterious, dominant negative mutant proteins (such as K44A

dynamain I) in populations of cells. We anticipated that humTGN46 would accumulate at the surface of cells expressing K44A dynamain I. However, we observed no significant change in the steady state distribution of humTGN46 in these cells. The results of further investigation suggest that humTGN46 travels to the cell surface via early endosomes rather than directly. We propose that there are two overlapping, recycling pools of humTGN46 in cells, one moving between the TGN and early endosomes, the other between early endosomes and the cell surface.

MATERIALS AND METHODS

Cell culture

HeLa cells expressing either wild-type or the K44A mutant of dynamain I under the control of a tetracycline regulated expression system have been described previously (Damke et al., 1994) and were cultured as described previously (Damke et al., 1994).

Drug treatments, imaging and immunofluorescence

For immunofluorescence analysis, cells were fixed and permeabilised with methanol and processed as described previously (Reaves et al., 1993). The anti-humTGN46 rabbit polyclonal antiserum (Ponnambalam et al., 1996) was used at a final dilution of 1:500, as was the sheep anti-humTGN46 polyclonal antiserum (a generous gift from Dr S. Ponnambalam); the affinity purified rabbit polyclonal anti-clathrin antibody (a generous gift from Dr M. S. Robinson) was used at a final dilution of 1:300, and the 12CA6 monoclonal ascites (anti-haemagglutinin epitope) (a generous gift from Dr T. Nilsson) was used at a final dilution of 1:2,000. Anti-rabbit and anti-mouse FITC- or TRITC-labeled secondary antibodies (Amersham) were used at 1:500 final dilution. All images were acquired using a $\times 63$ oil immersion objective on a Leica DM-IRBE upright epifluorescence microscope attached to Leica TCS-NT confocal laser scanning system equipped

with a Kr/Ar laser. All images are presented as projected stacks of confocal images unless otherwise stated.

Antibody uptake experiments

These were performed essentially as described previously (Reaves et al., 1993). Cells grown on coverslips were incubated in 100 μ l of a 1:50 dilution (in tissue culture medium; Damke et al., 1994) of anti-humTGN46 rabbit polyclonal antiserum (Ponnambalam et al., 1996) for 60 minutes at 37°C in 5% CO₂ in a humidified incubator. The antiserum was then replaced with fresh tissue culture medium and the cells incubated for a further 15 minutes at 37°C in 5% CO₂ in a humidified incubator prior to methanol fixation and processing for immunofluorescence analysis as described above. In some cases an 'acid wash' was performed between the initial 60 minute incubation and the 15 minute chase. This was done to remove any surface-bound antibody and was performed essentially as described previously (Hopkins and Trowbridge, 1983). Cells on coverslips were rinsed in (2 \times 200 μ l), and incubated in (200 μ l) 0.2 M glycine, 0.5 M NaCl, pH 2.4, for 10 minutes at 4°C then rinsed in (2 \times 200 μ l) tissue culture medium prior to incubation in tissue culture medium for the 15 minute 'chase' period as described above.

RESULTS

Immunolocalisation of humTGN46 in cells expressing WT or mutant dynamain I

HeLa cells expressing either wild-type or K44A (GTPase defective), HA-tagged dynamain I were methanol fixed and processed for dual label immunofluorescence analysis (as described in Materials and Methods) using a rabbit polyclonal antiserum to humTGN46 and a mouse monoclonal antibody to the HA epitope tag on dynamain (12CA6) (Fig. 1). The level of dynamain I expression (both wild type and mutant), induced by the removal of tetracycline from the tissue culture medium, was

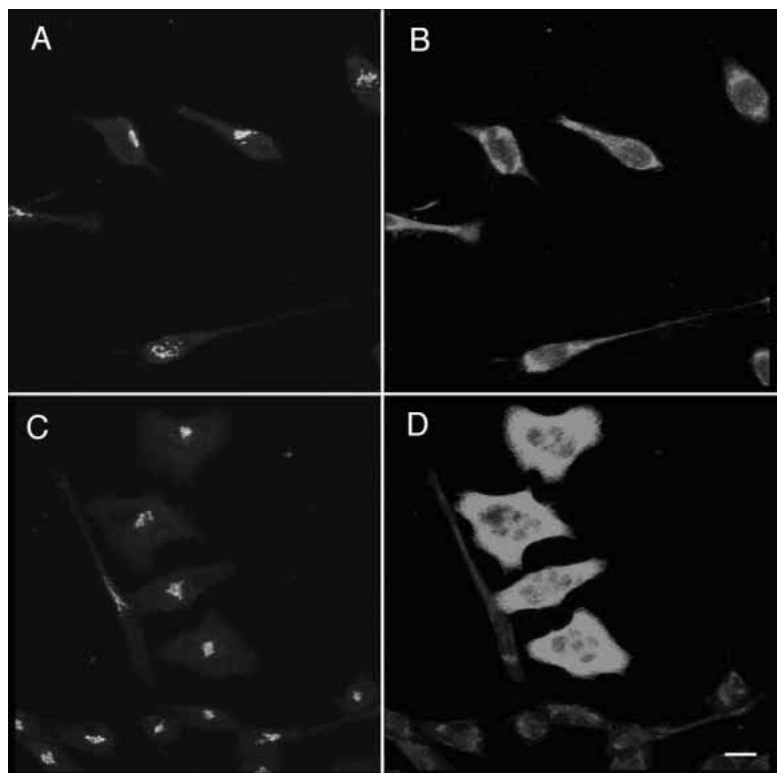


Fig. 1. humTGN46 is localised to the TGN in cells expressing K44A dynamain I. HeLa cells expressing either wild-type (A and B) or K44A (C and D) dynamain I were processed for immunofluorescence analysis using mouse monoclonal antibody 12CA6 (to detect the HA epitope tag on dynamain I) (B and D) and rabbit polyclonal antibody p12 (to detect humTGN46) (A and C). The monoclonal antibody was detected with a TRITC-conjugated sheep anti-mouse secondary antibody and the rabbit polyclonal antibody was detected with an FITC-conjugated donkey anti-rabbit secondary antibody. Bar, 10 μ m.

not uniform in all cells. It is clear that some cells in each population express elevated levels of recombinant dynamin whilst others do not (Fig. 1B and D). This provides a useful internal control, since, in each field, there are some cells expressing elevated levels of recombinant dynamin I (wild type or mutant) and some not. Thus, the effect of expression of recombinant dynamin I upon the localisation of humTGN46 can be observed. In cells expressing wild-type dynamin I, humTGN46 is predominantly localised to the TGN (as has been described previously for humTGN46 in HeLa cells) (Ponnambalam et al., 1996) (Fig. 1A,C). Contrary to what we had anticipated, we found that humTGN46 was also predominantly localised to the TGN in cells expressing the K44A mutant of dynamin I (Fig. 1C,D).

Expression of K44A mutant dynamin I does affect clathrin-mediated endocytosis

In light of the results described above, we performed the following experiments in order to confirm that the cells with which we were working were behaving appropriately (i.e.

expression of K44A mutant dynamin I was blocking clathrin-mediated endocytosis). HeLa cells expressing either wild-type or K44A dynamin I were methanol fixed and processed for dual label immunofluorescence analysis using a rabbit polyclonal antiserum to clathrin and the 12CA6 monoclonal antibody to detect dynamin I (Fig. 2). Expression of recombinant wild-type dynamin has no significant effect on the distribution of clathrin (Fig. 2A,B), but expression of recombinant K44A dynamin I leads to some accumulation of clathrin in punctate structures adjacent to the plasma membrane (Fig. 2C,D); this is discernible as a ring of immunostaining for clathrin when a single optical section is viewed (Fig. 2F). Such an observation is consistent with expression of K44A dynamin I blocking clathrin mediated endocytosis as previously reported (Damke et al., 1994, 1995; Herskowitz et al., 1993; van der Bliek et al., 1993). Such a block should also inhibit the uptake of transferrin, a process mediated by the transferrin receptor which is internalised via clathrin coated vesicles (Bleil and Bretscher, 1982; Hopkins, 1983; Hopkins and Trowbridge, 1983; Karin and Mintz, 1981; Octave et al., 1981). We

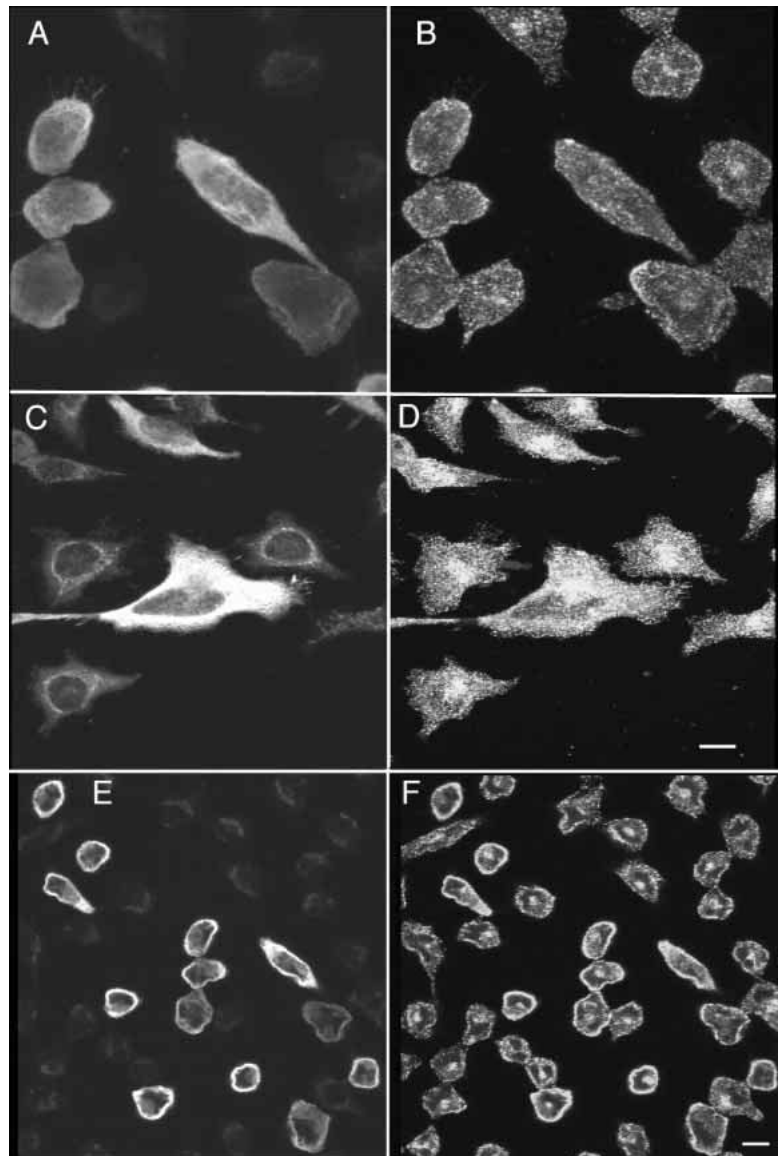


Fig. 2. Clathrin accumulates in punctate structures adjacent to the cell surface in cells expressing K44A dynamin I. HeLa cells expressing either wild-type (A and B) or K44A (C-F) dynamin I were processed for immunofluorescence analysis using mouse monoclonal antibody 12CA6 (to detect the HA epitope tag on dynamin I) (A,C,E) and a rabbit polyclonal antibody to clathrin (B,D,F). (E and F) Single optical sections through the same field of cells. Primary antibodies were detected as in Fig. 1. Bars, 10 μ m.

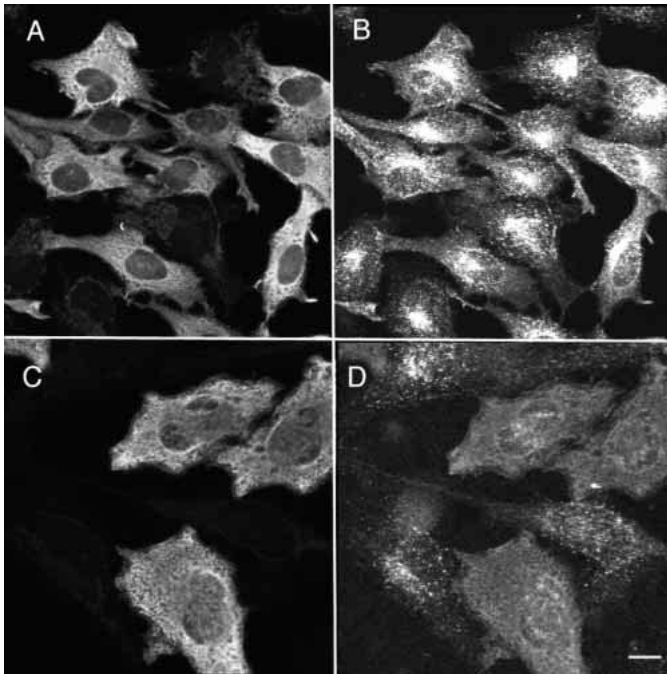


Fig. 3. TRITC-transferrin is not endocytosed in cells expressing K44A dynamin I. HeLa cells expressing either wild-type (A and B) or K44A (C and D) dynamin I were allowed to internalise TRITC-conjugated transferrin (B and D) for 30 minutes at 37°C prior to methanol fixation and processing for immunofluorescence analysis using mouse monoclonal antibody 12CA6 (to detect the HA epitope tag on dynamin) (A and C). Primary antibody was detected with an FITC-conjugated sheep anti-mouse secondary antibody. Bar, 10 μ m.

therefore performed transferrin uptake assays using TRITC-transferrin and cells expressing either wild-type or K44A dynamin I. Cells were allowed to internalise TRITC-transferrin for 30 minutes at 37°C (as described in Materials and Methods), prior to processing for immunofluorescence analysis using the 12CA6 monoclonal antibody to detect dynamin I (Fig. 3). TRITC-transferrin can be seen throughout the endocytic/recycling pathway in cells expressing wild-type dynamin I (Fig. 3A,B) whilst it is retained at the plasma membrane in cells expressing K44A dynamin I (Fig. 3C,D). It is of note that, in both the clathrin localisation experiment and the transferrin uptake experiment, those cells which fail to express K44A dynamin I have a wild-type distribution of both clathrin and transferrin (see Fig. 2C,D and Fig. 3C,D) demonstrating that it is the expression of K44A dynamin I which is leading to their redistribution. Thus, expression of K44A dynamin I does block the clathrin mediated internalisation of transferrin.

Anti-humTGN46 antibody uptake is blocked in cells expressing K44A dynamin I

We have previously shown that antibodies to the extracytosolic domain of ratTGN38 can bind to ratTGN38 at the cell surface, be internalised with ratTGN38 and be delivered back to the TGN along with ratTGN38 (Reaves et al., 1993). If expression of K44A dynamin I does block the endocytosis of humTGN46, then uptake of anti-humTGN46 antibodies would be predicted to be blocked. Antibody uptake experiments (using a rabbit

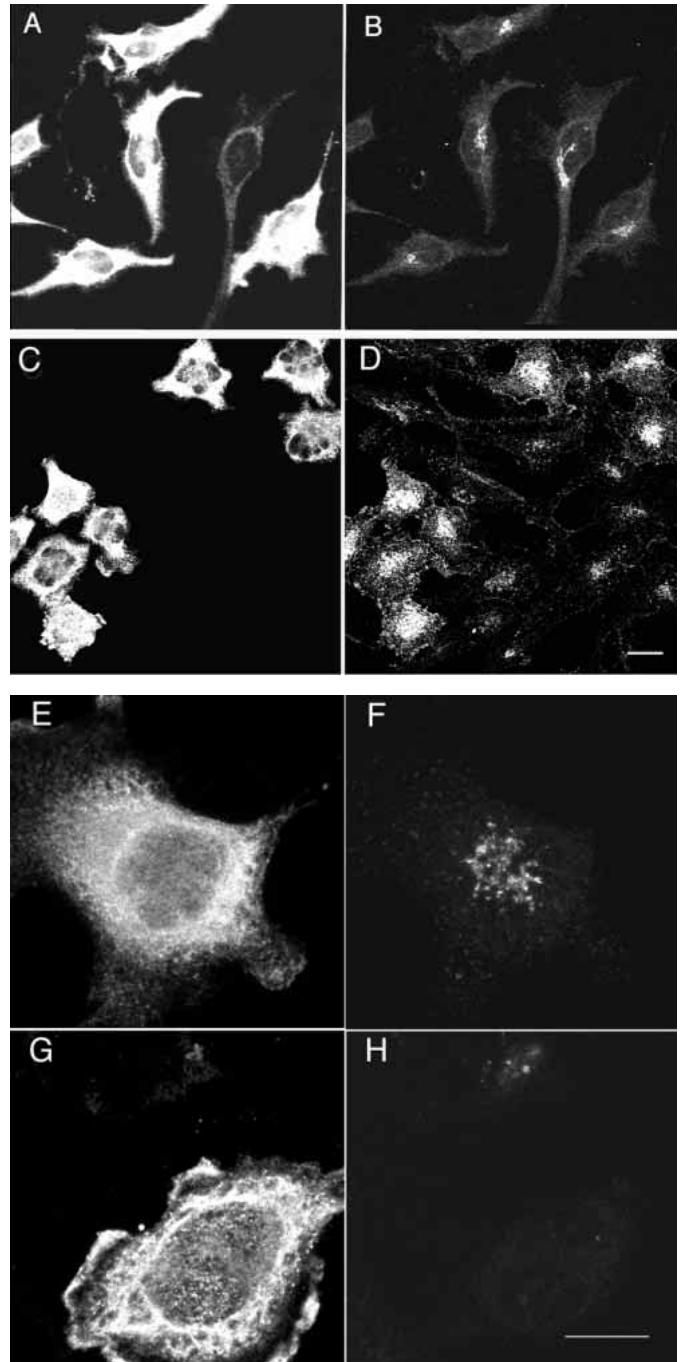


Fig. 4. Anti-humTGN46 antibody is not internalised in cells expressing K44A dynamin I. HeLa cells expressing either wild-type (A,B,E,F) or K44A (C,D,G,H) dynamin I were allowed to internalise rabbit polyclonal antibody p12 (anti-humTGN46) (B,D,F,H). Cells shown in B and D were allowed to internalise antibody for 60 minutes at 37°C prior to a 15 minute 'chase' in medium without antibody. Cells shown in F and H were treated to an 'acid wash' (see Materials and Methods) prior to the 15 minute 'chase' in order to remove any surface-bound antibody. Following the 'chase', all cells were subjected to methanol fixation and processing for immunofluorescence analysis using mouse monoclonal antibody 12CA6 (to detect the HA epitope tag on dynamin I) (A,C,E,G). Primary antibodies were detected with FITC-conjugated sheep anti-mouse and TRITC-conjugated donkey anti-rabbit secondary antibodies. Bar, 10 μ m.

polyclonal antibody to humTGN46) were performed as described in Materials and Methods on cells expressing either wild-type or K44A mutant dynamin I. Cells were then methanol fixed and processed for immunofluorescence analysis. Internalised antibody was detected with an FITC-conjugated secondary antibody (Fig. 4B and D), whilst recombinant dynamin I was detected using the 12CA6 monoclonal antibody in conjunction with a TRITC secondary antibody (Fig. 4A and C). HumTGN46 is efficiently internalised and delivered back to the TGN in cells expressing wild-type dynamin I (Fig. 4A,B), but not in cells expressing K44A mutant dynamin I (Fig. 4C,D). A modification of the antibody uptake experiment was performed in order to confirm that the anti-humTGN46 antibody is not being efficiently internalised in cells expressing elevated levels of K44A mutant dynamin I. Following incubation with antibody for 60 minutes at 37°C, cells were subjected to an acid wash at 4°C (as described in Materials and Methods) in order to remove surface bound antibody. Cells were then incubated in complete tissue culture medium for a further 15 minutes at 37°C prior to methanol fixation and processing for immunofluorescence analysis as described above. Internalised anti-humTGN46 is detected in cells expressing elevated levels of wild-type

dynamin I (Fig. 4E,F) but not in cells expressing elevated levels of K44A mutant dynamin I (Fig. 4G,H). These data are consistent with a situation in which humTGN46 is not efficiently internalised from the cell surface in cells expressing elevated levels of K44A mutant dynamin I.

Thus the K44A dynamin I induced block on clathrin mediated endocytosis does block the internalisation of humTGN46 from the cell surface even though it does not significantly affect the steady state distribution of the majority of the population of humTGN46 molecules. What implications does this have for the intracellular trafficking of humTGN46/ratTGN38? The implicit assumption to date has been that ratTGN38 travels directly to the cell surface in one

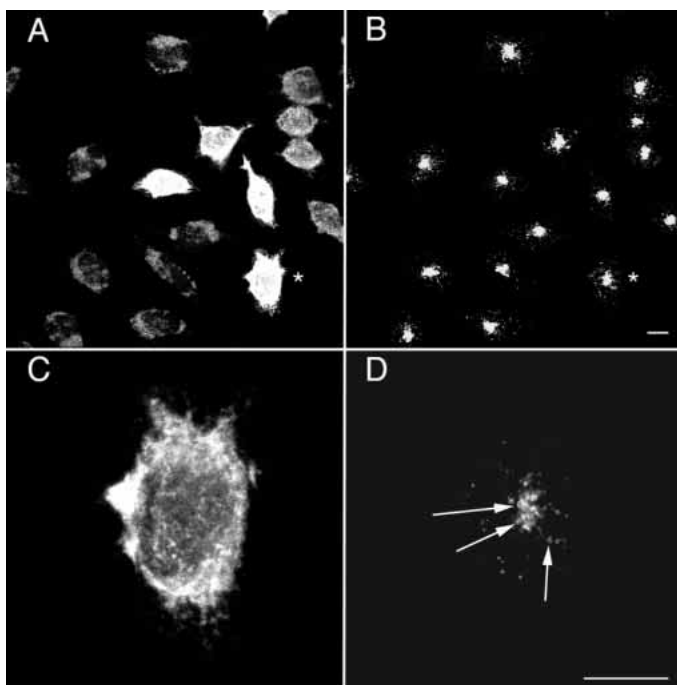
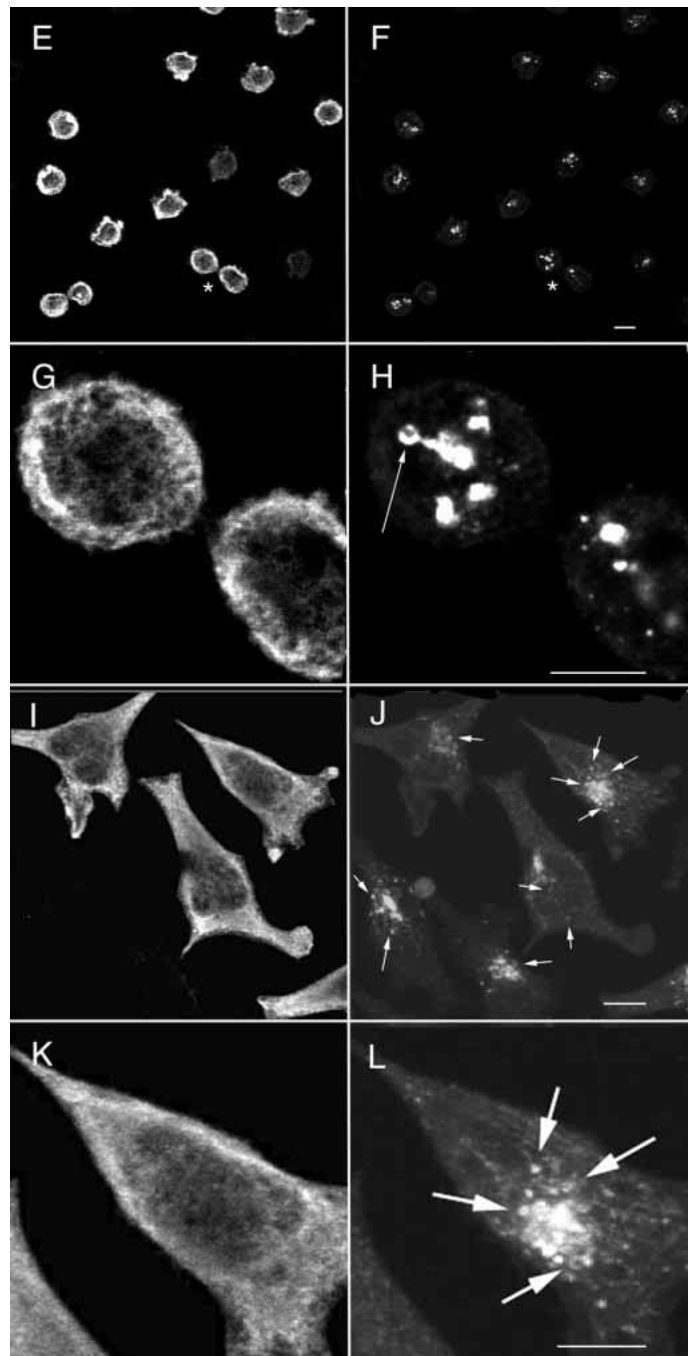


Fig. 5. The location of humTGN46 is affected by BafA1 in cells expressing K44A dynamin I. HeLa cells expressing either wild-type (A-D) or K44A (E-L) dynamin I were incubated in 10 μ M bafilomycin A1 for 60 minutes at 37°C prior to methanol fixation and processing for immunofluorescence analysis using mouse monoclonal antibody 12CA6 (to detect the HA epitope tag on dynamin I) (A,C,E,G,I,K) and rabbit polyclonal antibody p12 (to detect humTGN46) (B,D,F,H,J,L). Primary antibodies were detected as in Fig. 1. (C and D) A higher magnification of the cell marked with an asterisk in A and B; (G and H) A higher magnification of the cells marked with an asterisk in E and F; (K and L) a higher magnification of an individual cell shown in I and J. Arrows indicate bafilomycin A1 induced swollen endocytic structures. Bar, 10 μ m.



vesicular transport step (Banting and Ponnambalam, 1997). It has also been suggested that the total population of ratTGN38 molecules traffics via the cell surface every 45-60 minutes (Banting and Ponnambalam, 1997; Reaves and Banting, 1994). This cycling time was based upon the observation that, in cells incubated in the presence of bafilomycin A1 (BafA1) (an inhibitor of vacuolar ATPase which leads to swelling and inhibition of fusion of early endosomes) (Clague et al., 1994) the total population of ratTGN38 molecules appears in 'doughnut'-like swollen endosomes within 60 minutes (Reaves and Banting, 1994). However, if all ratTGN38/humTGN46 molecules were to traffic via the cell surface every 60 minutes they would disappear from the TGN and appear at the cell surface in cells expressing K44A dynamin I. This clearly does not happen with humTGN46. Others have shown that not all proteins go directly to the cell surface from the TGN (Futter et al., 1995; Leitingner et al., 1995). Might this be the case for humTGN46/ratTGN38?

Immunofluorescence analysis of cells preincubated in bafilomycin A1

We argued that if humTGN46 goes directly to the cell surface from the TGN it would not accumulate in swollen early endosomes in BafA1 treated cells expressing K44A dynamin I. Cells expressing wild-type or K44A dynamin I were therefore incubated in the presence of 5 μ M BafA1 for 1 hour at 37°C prior to methanol fixation and processing for dual label immunofluorescence analysis using a rabbit polyclonal antiserum to humTGN46 and the 12CA6 monoclonal antibody to detect dynamin I (Fig. 5). The anti-humTGN46 antibody shows a similar pattern of reactivity in all cells (Fig. 5B,D,F,H,J,L). Incubation of cells expressing either wild-type (Fig. 5A-D) or K44A mutant dynamin I (Fig. 5E-L) in the presence of BafA1 has clearly led to a change in the steady state localisation of humTGN46. The presence of humTGN46 in swollen 'doughnut-like' structures in a cell expressing K44A mutant dynamin I can be seen more clearly in Fig. 5H and L (these panels represent higher magnifications of parts of the images presented as Fig. 5F and J, respectively).

DISCUSSION

We have demonstrated that inhibition of clathrin-mediated endocytosis by the expression of a GTPase defective form of dynamin I fails to significantly alter the steady state distribution of the majority of humTGN46 molecules in HeLa cells. This was an unexpected result. We had anticipated that humTGN46 would accumulate at the surface of such cells given the previous suggestions that ratTGN38 constitutively cycles between the TGN and the cell surface (reviewed by Banting and Ponnambalam, 1997). However, humTGN46 at the surface of cells expressing K44A dynamin I is not internalised efficiently, as shown by the antibody uptake experiments (Fig. 4). Thus the block on endocytosis does have some effect on the trafficking of this protein; but why does the humTGN46 in the TGN not relocate to the surface of cells expressing K44A dynamin I? The experiments performed on cells pre-incubated in BafA1 address this question. HumTGN46 ends up in the same structures in BafA1 treated cells expressing either wild-type or K44A mutant dynamin I.

Others have shown that the effect of BafA1 is to inhibit fusion of early endosomes (Clague et al., 1994). The fact that humTGN46 ends up in these BafA1 sensitive structures in cells expressing K44A mutant dynamin I implies that humTGN46 traffics to the cell surface via early endosomes. We and others have already shown that ratTGN38 returns to the TGN directly from early endosomes (Reaves et al., 1996); it now appears that it can also travel from the TGN to early endosomes. The fact that there is not significant accumulation of humTGN46 at the surface of cells expressing K44A dynamin I suggests that humTGN46 molecules which reach early endosomes from the TGN are not committed to moving on to the cell surface, but might return to the TGN from the early endosome without ever having reached the cell surface. Thus, there appear to be two, overlapping, recycling pools of humTGN46/ratTGN38; one trafficking between the TGN and the early endosome and the other between the early endosome and the cell surface. That there is exchange between these two pools is borne out by the fact that anti-humTGN46 antibody internalised from the cell surface is delivered back to the TGN (Fig. 4; Ladinsky and Howell, 1993; Reaves et al., 1993). If there were rapid exchange between these two pools the TGN localised population of humTGN46 would accumulate at the surface of cells expressing K44A dynamin I. It does not (Fig. 1). Thus, it may be that the functional cycling pathway of humTGN46 is between the TGN and the early endosome with some 'leakage' to the cell surface from where it is retrieved by its highly efficient tyrosine-based internalisation motif. So, why is it that mutants of ratTGN38 which lack the cytosolic domain, or in which the critical tyrosine residue in the 'SDYQRL' internalisation motif has been mutated, accumulate at the cell surface (Bos et al., 1993; Humphrey et al., 1993; Luzio et al., 1990; Wong and Hong, 1993) whereas there is limited change in the steady state distribution of humTGN46 in cells expressing elevated levels of K44A dynamin I (see Fig. 1)? Both the mutations in the cytosolic domain of ratTGN38 (which lead to failure to interact with the AP2 adaptor complex (Ohno et al., 1995; Stephens et al., 1997) and expression of elevated levels of K44A dynamin I would be expected to give rise to the same phenotype, i.e. elevated cell surface expression of ratTGN38/humTGN46. The fact that this does not occur suggests that, in addition to being inefficiently internalised from the cell surface, the previously described ratTGN38 cytosolic domain mutants are also inefficiently sorted for delivery back to the TGN from the early endosome. Thus, we propose that whilst endogenous, wild-type humTGN46 cycles efficiently between the TGN and the early endosome in HeLa cells expressing elevated levels of K44A dynamin I (with a small amount 'leaking' to the surface and failing to be re-internalised due to the defective dynamin I, see Fig. 4), the ratTGN38 cytosolic domain mutants are inefficiently returned to the TGN from the early endosome and move on to the cell surface where they accumulate by virtue of their failure to interact with the AP2 adaptor complex. This model would imply that there is important sorting information in the cytosolic domain of ratTGN38/humTGN46 which is decoded at the early endosome and is consistent with the recent observation that the hydroxyl group on the serine residue in the 'SDYQRL' motif in the cytosolic domain of ratTGN38/humTGN46 is important for efficient return of

ratTGN38 from the early endosome to the TGN (Roquemore and Banting, 1998).

Another explanation for the observation that expression of elevated levels of K44A dynamin 1 does not give rise to elevated cell surface expression of humTGN46 is possible. If certain vesicle components are required for early endosome to cell surface traffic, and if those components are rapidly 'locked' at the surface in cells expressing elevated levels of K44A dynamin 1, then early endosome to cell surface traffic would be prevented in cells expressing elevated levels of K44A dynamin 1 but early endosome to TGN traffic would remain functional. In such a scenario the bulk of humTGN46 would not accumulate at the cell surface, but would by-pass the early endosome/cell surface circuit altogether and simply cycle between the TGN and the early endosome.

Results from experiments involving incubation of cells in the presence of bafilomycin A1 (see Fig. 5) demonstrate that expression of K44A dynamin 1 does not prevent trafficking of humTGN46 to early endosomes and therefore indicates that dynamin 1 is not involved in the cycling of humTGN46 between the TGN and the early endosome. This observation is consistent with the recent findings of Jones et al. (1998) who have shown a role for another dynamin isoform, dynamin II (Dyn2) in the formation of exocytic transport vesicles at the TGN. These authors make the comment that, on the basis of their observations 'of a preferential association of the Dyn2 protein with the Golgi, it is not surprising that an overexpressed mutant Dyn1 isoform would have little effect on the formation of vesicles from the TGN.' However, it would clearly be of interest to investigate the localisation and trafficking of humTGN46 in cells expressing a K44A mutant of dynamin II.

The cytosolic domains of ratTGN38 and humTGN46 are highly conserved, as are the same regions of other orthologues (Banting and Ponnambalam, 1997). This region contains the critical tyrosine residue required for interaction with the $\mu 2$ subunit of the AP2 adaptor complex and flanking residues which have been shown to be important for the faithful intracellular trafficking of ratTGN38 (Ohno et al., 1995; Roquemore and Banting, 1998; Stephens et al., 1997). What determines which post-TGN recycling pool ratTGN38/humTGN46 resides in? What signal(s) switches the molecules from one recycling pool to the other? Further study of the cytosolic domain of these proteins will undoubtedly shed light on the complexities of trafficking in the endomembrane system.

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