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Overexpression of TGN38/41 leads to mislocalisation of γ-adaptin

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Abstract

TGN38 and TGN41 are isoforms of a monotopic integral membrane protein which recycles between the trans Golgi network (TGN) and the cell surface, but which, at steady state, is predominantly located in the TGN. Full-length and truncated versions of rat TGN38/41 have been expressed in monkey (COS) and human (Heb7a) cells under the control of the heavy metal inducible Metallothionein IIA promoter. This has allowed the regulated expression of TGN38/41 protein constructs to different levels in the transfected cells. These studies show that (i) controlled overexpression of TGN38/41 results in mislocalisation to parts of the endocytic pathway, (ii) a truncated version of TGN38/41, lacking the cytoplasmic domain, remains in the TGN, and (iii) there is a direct or indirect interaction between the cytoplasmic domain of TGN38/41 and γ-adaptin.

Key words: *trans*-Golgi network; Retention; γ-Adaptin

1. Introduction

The *trans*-Golgi network (TGN) acts as a sorting centre for proteins destined for the plasma membrane, lysosomes and regulated secretory granules and has been shown to be an organelle independent of the Golgi stack [1,2]. TGN38 and TGN41 are two isoforms of a type I integral membrane protein which has been localized to this compartment [3,4]. Although TGN38/41 is concentrated in the TGN it also recycles between the TGN and the plasma membrane [5,6]. Localization signals for the TGN have recently been investigated in four separate studies [7–10] using both chimeric proteins and deletion or point mutations of TGN38/41. A role for the cytoplasmic domain in either targetting or retention was implicated by transient transfection studies [3] in which overexpression of TGN38 or deletion of the cytoplasmic domain resulted in mislocalization to the cell surface. Transient transfectants expressing both chimeric constructs using the lumenal domain of the LDL receptor attached to the cytoplasmic tail of TGN38 as well as deletion and point mutations in the wild type TGN38 sequence [8] have been used to probe the exact sequences necessary for TGN localization. In addition, stable cell lines expressing the lumenal and transmembrane domains of either the Tac antigen [7] or glycophorin A [9] attached to the cytoplasmic domain of TGN38 gave similar results. All three studies identified the sequence YQRL, in particular the tyrosine and leucine residues, in the cytoplasmic tail as being important for targetting to the TGN and retreival from the plasma membrane. Wong and Hong [9] also identified a serine residue close to the tyrosine (SDYQRL) as an additional requirement for TGN localization, whilst Humphrey et al. found that mutation of arginine to glutamic acid in the YQRL sequence resulted in efficient internalization, but not TGN localization. This suggests that the specificity of localization signals may be contained within individual members of the generic tyrosine-containing internalization motif. This is discussed elsewhere [11,12]. Experiments using other chimeric proteins have recently shown that the transmembrane domain of TGN38/41 plays a role in retention within the TGN [10].

An important consideration in analyzing the effect of mutations and deletions on subcellular localization is the level of expression of a transfected protein. Overexpression of wild-type and chimeric proteins containing the proposed localization signal for TGN38 resulted in mislocalization to the plasma membrane and vesicles scattered throughout the cytoplasm [3,7,9] suggesting that the mechanism for retention and/or targetting is saturable. In order to investigate this further we have isolated stably transfected cell lines incorporating an inducible expression system whereby the expression of TGN38/41 can be increased incrementally. We have used this system to monitor the subcellular distribution of both wild type TGN38/41 and TGN38/41 lacking a cytoplasmic domain.

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Abbreviations: FACS, fluorescence activated cell sorter; BFA, Brefeldin A; FITC, fluorescein isothiocyanate; MTOC, Microtubule Organising Centre; TRITC, Texas red isothiocyanate; TGN, *trans*-Golgi network

2. Materials and methods

2.1. Reagents

Cell culture media (DMEM), foetal calf serum and antibiotics were from Gibco Life Technologies, Inc. (Paisley, Scotland). Hygromycin B, Boehringer Mannheim (Lewes, East Sussex, UK) was used at a final

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concentration of 200 μg/ml. Brefeldin A from Epicentre Technologies (Madison, WI) was used as previously described [1]. FITC-transferrin was from Molecular Probes, Inc. (Eugene, OR) and was used at a final concentration of 20 μg/ml. ^{125}I-Protein G was prepared with 0.75 mCi of ^{125}I (NEN, UK) and 15 μg of Protein G (Pharmacia, UK) using the Iodogen method of labelling [13]. ~10^6 cpm/ml were used per immunoblot.

2.2. Antibodies

The rabbit anti-rat TGN38 polyclonal antiserum used for the internalisation and double-labelling experiments has been previously described [3] The polyclonal antibody used in immunoblotting was raised to a β-galactosidase-TGN41 fusion protein [14]. For the majority of the immunofluorescence experiments a monoclonal antibody raised to the 13 amino-terminal amino acids of TGN38 was used [15]. The anti-γ-adaptin antibody mAb 100/3 [16] was a kind gift of E. Ungewickell. FITC-, TRITC- and phycoerytherin-labelled goat anti-mouse and goat anti-rabbit antibodies were from Sigma Chemical Co. (Poole, Dorset, UK).

2.3. Expression constructs

Unless otherwise stated, all DNA manipulation procedures were performed as described elsewhere [17]. The pMEP4-TGN38/41 constructs were made by inserting the full-length cDNA for either TGN38 [3] or TGN41 [4] into the *Bam*HI site of pMEP4 (Invitrogen, CA). pMEP4 contains a multiple cloning site downstream of the human metallothionein IIA promoter. This promoter can be induced by heavy metals. The vector also carries the hygromycin resistance gene allowing selection of transfected eukaryotic cells in medium supplemented with hygromycin B. The TGN38/41 tail deletion was constructed by PCR using a mutagenic 3′ oligonucleotide, GATCGGATCCAAAAG-CAATTCACTTTCGTTTG which substitutes a stop codon for the isoleucine 3 residues away from the end of the transmembrane domain (i.e. KRKI to KRKstop) and a 5′ oligonucleotide ATCGGATCCA-GACTACAGGATGCAGTTCCTGG corresponding to the first 24 bases of the TGN38/41 sequence [3]. Sequences of all constructs were verified by double-stranded DNA sequencing using a Sequenase Version 2.0 kit (United States Biochemicals) and [^{35}S]dATP (NEN, UK) following the manufacturer's protocol. Proteins expressed in pMEP4 are represented diagramatically in Fig. 1.

2.4. Cell culture and transfection

COS (African Green Monkey Kidney) [18] and Heb7a (human, Hela derivative)[19] cells were grown in DMEM plus 10% FCS with 100 u/ml penicillin and 100 μg/ml streptomycin. Cells were grown to ~80% confluency, harvested by scraping using a cell lifter (Costar) scraped, and washed in ice cold PBS. After pelleting, 5–10 × 10^6 cells were resuspended in 0.8 ml HEBS buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 250 mM sucrose) containing 20 μg of plasmid and transferred to a 0.4 cm electroporation cuvette (Bio-Rad). Cells were then electroporated at 0.22 kV, 960 μF using a BioRad Gene Pulser apparatus [20]. After a 10 min incubation at room temperature, cells were plated in 140 × 25 cm tissue culture dishes. Selection with hygromycin B was started 48 h post-plating and after ~2–3 weeks colonies were isolated and transferred to multi-well plates for expansion. Positive clones were identified by immunofluorescence as described below.

2.5. FACS analysis

Transfected cells were grown to confluency in T25 tissue culture flasks, treated with varying doses of $CdCl_2$ and then processed for FACS analysis as follows. Cells were removed from the flasks using Hank's Balanced Salt Solution (Gibco Life Technologies, Inc., Paisley, Scotland) containing 10 mM EDTA (Sigma Chemical Co., Poole, Dorset, UK). After several washes in the same medium, cells were pre-incubated for 20 min on ice in PBS/0.2% BSA/0.02% sodium azide and then for a further hour with a 1:250 dilution of a rabbit anti-rat TGN38/41 [3] which recognises the N-terminal portion of the protein [14]. After 3 washes in PBS/BSA/azide the cells were incubated for 45 min on ice with a 1:40 dilution of goat anti-rabbit IgG-phycoerytherin diluted in 20% normal goat serum in PBS/azide. Immediately prior to FACS analysis, cells were washed with and resuspended in 0.2 ml of calcium/magnesium-free PBS. Analysis was carried out at Bristol University Veterinary School on an EPICS CS fluorescence activated cell sorter (Coulter Electronics, Ltd., Luton, Herts. UK) using an argon laser at 488 nm collecting log red fluorescence and linear forward light scatter.

2.6. Immunofluorescence and confocal microscopy

Immunofluorescence microscopy was carried out as previously described [1].

2.7. Protein analysis

Untransfected COS cells and cells stably transfected with pMEP-TGN41 were grown to confluency and then treated with 0–20 μM $CdCl_2$ for 18–20 h prior to processing. Cells were harvested by scraping using a cell lifter (Costar), pelleted and washed in ice cold PBS prior to lysis in CLB (10 mM Tris, pH 7.4, 0.15 M NaCl, 1% NP-40, 1 mM EDTA, 10 mM 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 non-solubilised material was removed by centrifugation at 4°C and 10,000 × *g*. Protein determinations were carried out according to the method of Bradford [21] and 100 μg of protein loaded per lane of an 8% SDS PAGE gel [22] and electrophoresis carried out until the 69 × 10^3 M_r marker reached the bottom of the gel. Following electrophoresis and electro-transfer, performed as previously described [3] the nitrocellulose was incubated with a rabbit polyclonal anti-TGN38/41 antibody binding of which was detected by incubation with ^{125}I-labelled Protein G followed by autoradiography for 18 h. Densitometry was carried out after an overnight exposure on a Molecular Dynamics Phosphoimager. Total ^{125}I is expressed relative to c.p.m. detected at the same position of a lane containing only loading buffer.

3. Results

3.1. Effects of increasing expression on TGN38/41 localisation

It has been demonstrated previously that overexpression of TGN38/41 leads to its mislocalisation to the cell surface [3]. In order to alleviate the problem of overexpression in interpreting results of mutagenesis experi-

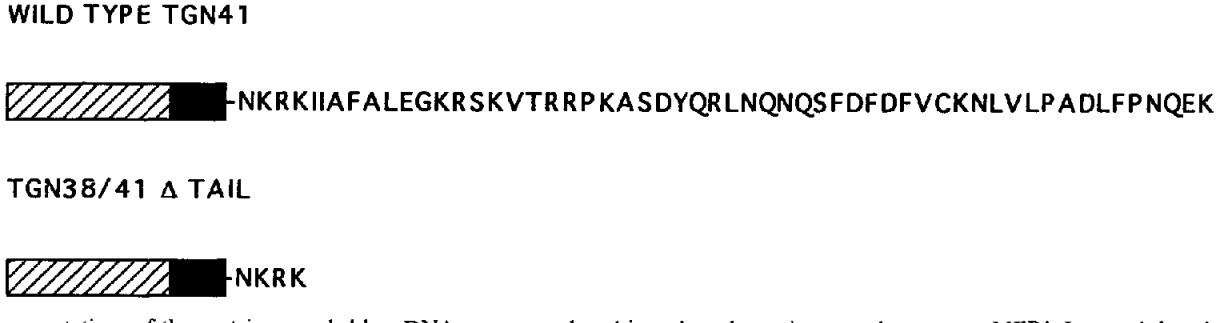


Fig. 1. Representations of the proteins encoded by cDNA sequences cloned into the eukaryotic expression vector pMEP4. Lumenal domains of proteins are indicated by a hatched box and transmembrane domains by a solid box. The entire amino acid sequence of each cytoplasmic domain is presented.

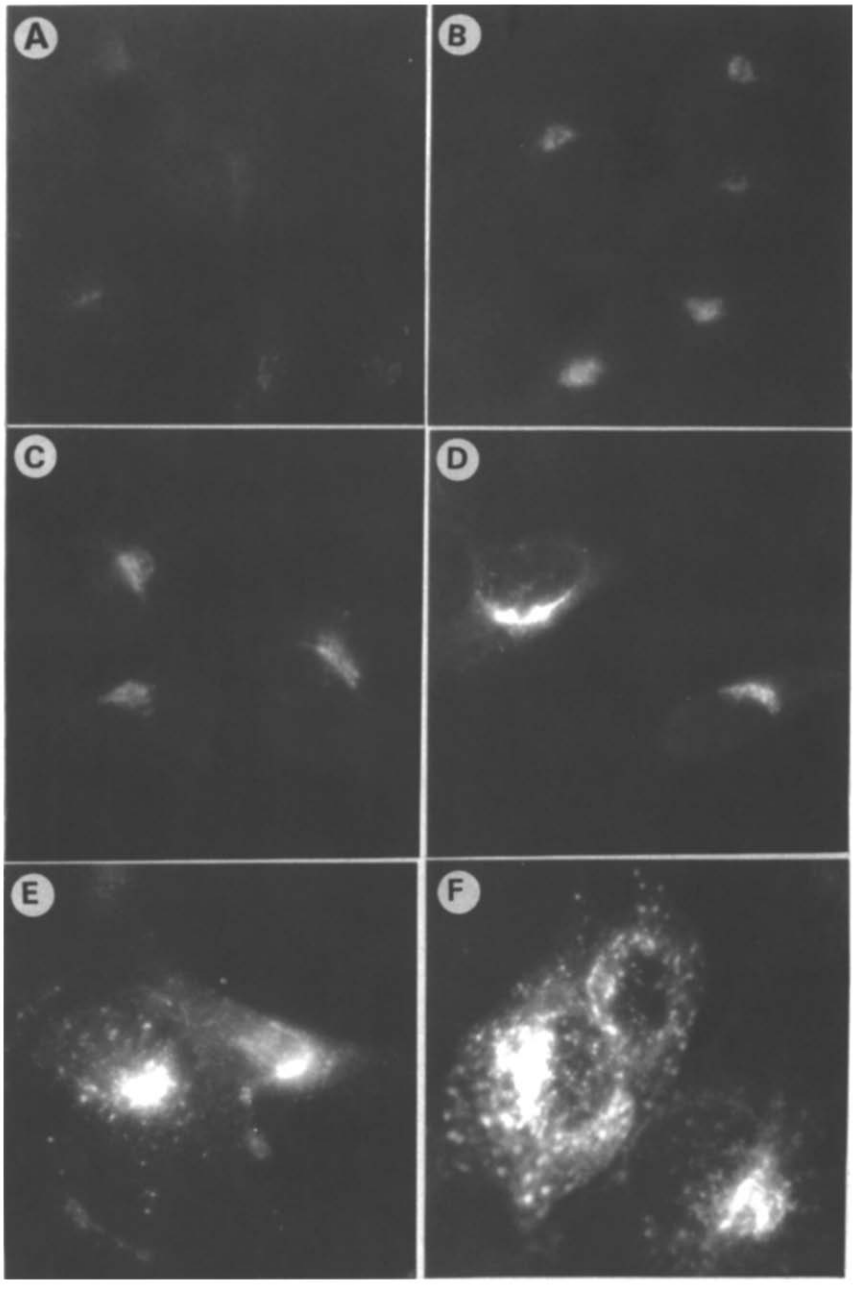


Fig. 2. Immunofluorescence analysis of TGN41 induction by varying doses of $CdCl_2$. COS41–1 cells were treated for 20 h with $CdCl_2$ prior to methanol fixation and processing for immunofluorescence microscopy using a species-specific anti-rat TGN38/41 polyclonal antibody as described in section 2. (A = 0, B = 1.25, C = 2.5, D = 5.0, E = 10.0, F = 20.0 μM $CdCl_2$).

ments designed to identify TGN localisation signals, we sought to establish stable inducible cell lines expressing rat TGN38 or rat TGN41. Polyclonal and monoclonal antibodies are available which recognise rat but not human or monkey TGN38/41 [15,21]. Monkey (COS) and human (Heb7a) cells were transfected by electroporation with pMEP4 constructs encoding either rat TGN38 or TGN41. In each case the relevant cDNA was cloned downstream of the human metallothionein IIA promoter to allow inducible expression by addition of $CdCl_2$ to the tissue culture medium. Stable cell lines were isolated as described in section 2. Clonal COS cell lines expressing TGN41 (as assayed by immunofluorescence analysis using anti TGN38/41 antibodies) were isolated. All showed a similar pattern in the immunofluorescence screen and one, COS41–1 was chosen for further study.

Cells were treated with 0–20 μM $CdCl_2$ for 20 h prior to processing for immunofluorescence analysis. A monoclonal antibody raised to the N-terminal 13 amino acids of rat TGN38/41 fails to recognise the endogenous simian protein thus allowing detection of rat proteins encoded by the transgene. There was some TGN41 expression without any added $CdCl_2$ (Fig. 2A), however, increasing doses resulted in a corresponding increase in

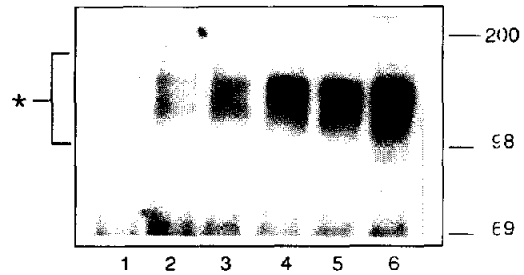
intensity of TGN41 staining (Fig. 2B–F). At low doses, 1.25 and 2.5 μM $CdCl_2$, the pattern of staining resembles that seen for the endogenous protein in NRK cells [1] (Fig. 2B,C), i.e. a tight perinuclear concentration. As is the case for the endogenous rat protein, the transfected cells varied in terms of the reticular versus vesicular nature of this staining. At 5 μM $CdCl_2$ the pattern is similar, however, the intensity of staining is increased (Fig. 2D). Starting at 10 μM $CdCl_2$ and becoming more pronounced at 20 μM $CdCl_2$, not only does the intensity increase but the pattern of staining no longer resembles the tight pattern of staining seen at lower doses (Fig. 2E,F). A number of different morphologies are present, the most common being a disruption of the concentrated staining around the nucleus accompanied by large and small vesicles scattered throughout the cytoplasm and in some cells the appearance of TGN41 at the cell surface. In some cases a large reticular network extending throughout the cell can also be seen (data not shown). 20 μM $CdCl_2$ has no effect on TGN morphology or the intracellular distribution of either rat TGN38/41 in NRK cells or of the simian TGN38/41 in COS cells (data not shown). These criteria were assayed in COS cells using an antibody which recognises the endogenous protein in these cells [14]. This dose of $CdCl_2$ has previously been shown to have no detectable toxic effects in cells in tissue culture during the time course of the experiment described [23].

To investigate the relationship between the pattern of staining and actual protein level of TGN41, COS41–1 cells were treated as above with 0–20 μM $CdCl_2$, and 100 μg of protein from either a post-nuclear supernatant or a total membrane fraction was subjected to SDS-PAGE and then immunoblotted using anti-rat TGN38/41 as primary antibody. The results are presented in Fig. 3. An increase in the level of expression of TGN41 protein is seen with increasing dose of $CdCl_2$. There was no detectable TGN41 expression in untransfected COS cells (data not shown). Quantitation by densitometry revealed a 10-fold increase in TGN41 expression in cells treated with 20 μM $CdCl_2$ compared to untreated cells. Thus, the tendency for apparent mislocalisation of TGN41 correlates with a 10-fold increase in protein expression. Similar results were obtained with (i) other clonal permanent COS transfectants expressing TGN41 (ii) permanent COS transfectants expressing TGN38 and (iii) clonal permanent Heb7a transfectants expressing either TGN38 or TGN41.

3.2. Localisation of TGN38/41 at varying levels of expression

3.2.1. Colocalisation with γ-adaptin

γ-adaptin is a coat protein which specifically associates with TGN membranes [16,24]. Double-labelling immunofluorescence experiments were performed using a



μg $CdCl_2$ added	0.00	1.25	2.50	5.00	10.0	20.0
cpm in * band	37181	75370	107201	209077	265962	396625
fold induction	1.00	2.03	2.88	5.62	7.15	10.67

Fig. 3. Immunoblotting analysis of $CdCl_2$ induced TGN41 expression. Confluent COS41–1 cells were treated with $CdCl_2$ for 20 h prior to lysis and preparation of post-nuclear supernatant. 100 μg of total protein per lane was separated on an 8% SDS-PAGE gel and immunoblotted using rabbit anti-rat TGN38/41 and ^{125}I Protein G. The presence of TGN41 protein was visualised by autoradiography. Lane 1 = 0, lane 2 = 1.25, lane 3 = 2.5, lane 4 = 5.0, lane 5 = 10.0 and lane 6 = 20 μM $CdCl_2$. The electrophoretic mobility of molecular weight standards ($M_r \times 10^{-3}$) is indicated on the right. Quantitation of the immunoblot was performed on a Molecular Dynamics Phosphorimager and the results are represented as a fold-increase over non-induced cells.

species-specific polyclonal anti-rat TGN38/41 antibody and a monoclonal antibody to human γ-adaptin [16] which also recognises simian γ-adaptin. This was in order to confirm that rat TGN41 expressed in transfected COS cells co-localised with the endogenous γ-adaptin and was therefore correctly localised to the TGN. COS41–1 cells were treated with either 2.5 μM or 20 μM $CdCl_2$ and processed for immunofluorescence as described. At low doses of cadmium TGN41 and γ-adaptin colocalise to the same perinuclear location (Fig. 4A,B). It is of note that at higher doses of cadmium, when TGN41 expression is elevated and the protein can be detected in diverse vesicles as opposed to the original perinuclear location, the pattern of anti-γ-adaptin staining is altered in a similar manner (Fig. 4C,D). These data could be interpreted to suggest that overexpression of TGN41 leads to an expansion of the TGN rather than its mislocalisation to other compartments. This would imply that TGN41 plays a pivotal role in defining the TGN as being the TGN. Alternatively they might be interpreted to show that there is a direct or indirect interaction between TGN41 and γ-adaptin, so that when TGN41 is present in significant amounts in extra-TGN structures those structures become coated with γ-adaptin. The following experiments were performed in order to help address these points.

3.2.2. Brefeldin A treatment

Brefeldin A (BFA) has been shown to cause a collapse of the TGN around the MTOC [1,5]. This is in contrast to its actions on the rest of the Golgi stack which redis-

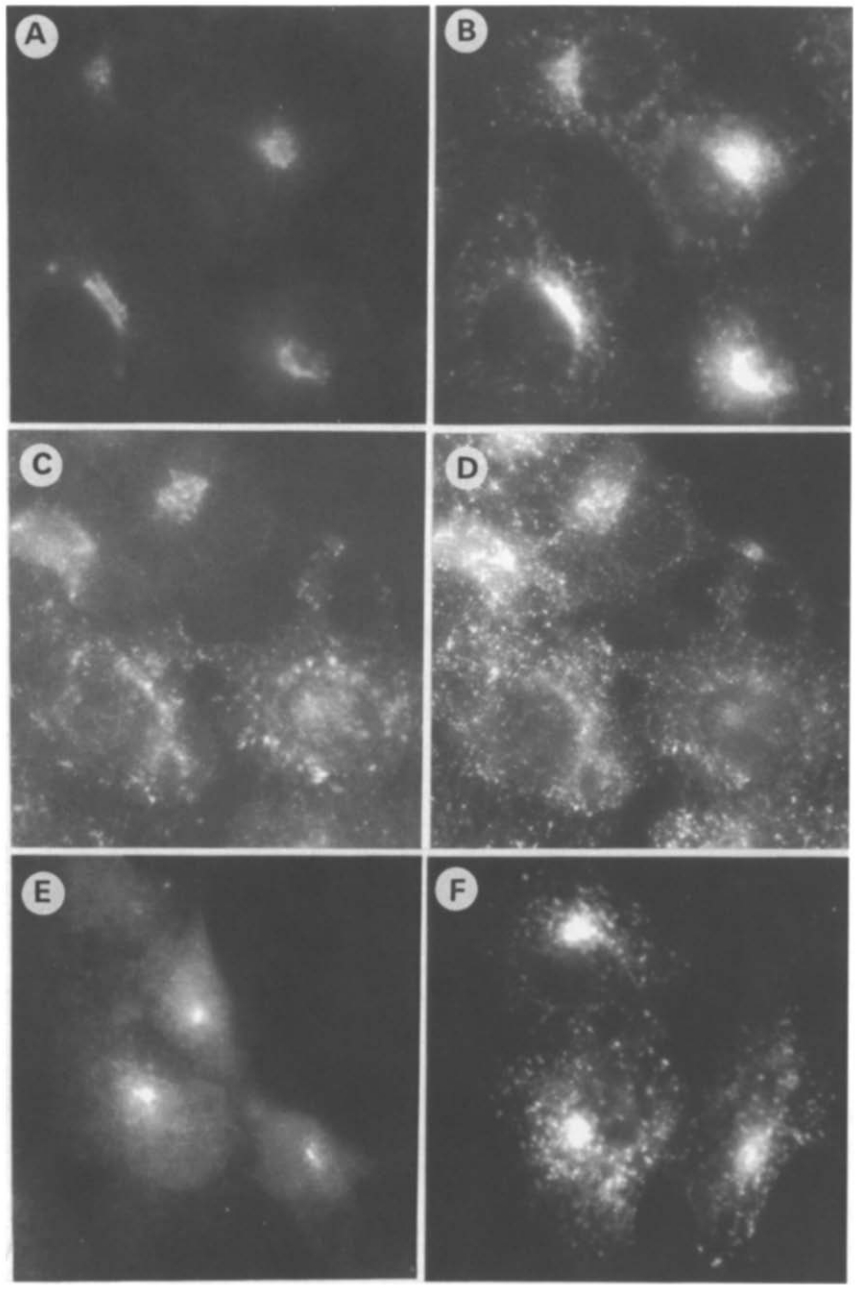


Fig. 4. Immunofluorescent localisation of recombinant TGN41 to the TGN in COS41–1 cells. (A–D) Colocalisation with γ-adaptin. COS41–1 cells were treated with either 1.25 (A and B) or 20 μM $CdCl_2$ (C and D) for 20 h prior to processing for immunofluorescence. Methanol fixed cells were incubated with a species-specific anti-rat TGN38/41 polyclonal antibody (A and C) followed by anti-rabbit IgG-TRITC or a mouse anti-human γ-adaptin monoclonal antibody followed by anti-mouse IgG-FITC (B and D). (E and F) BFA treatment of COS41–1 cells. COS41–1 cells were treated with 1.25 μM $CdCl_2$ (E) or 20 μM $CdCl_2$ (F) and then incubated in the presence of 5 μg/ml Brefeldin A for 3 h prior to processing for immunofluorescence with a rabbit anti-rat TGN38/41 antibody followed by anti-rabbit IgG-TRITC.

tributes into the ER [25]. BFA therefore provides a valuable tool for identifying the TGN. After inducing TGN41 expression in COS41–1 cells with either 2.5 or 20 μM $CdCl_2$, 5 μg/ml BFA was added to the medium and cells incubated for a further 3 h at 37°C. Immunofluorescence analysis of cells exposed to low doses of cadmium revealed the typical tight 'BFA dot' (Fig. 4E) observed for endogenous TGN38/41 in NRK cells [1]. This indicated that, at this level of expression, rat TGN41 is appropriately localised in transfected COS cells. However, at higher doses of cadmium, in addition to the concentration of staining at the MTOC, vesicles are also seen dispersed throughout the cytoplasm (Fig. 4F). These resemble those observed in highly induced, non-BFA treated cells (compare Figs. 2F and 4F). Thus, some component of the redistributed TGN41 is not susceptible to the action of BFA on the TGN suggesting that its overexpression leads to accumulation in extra-

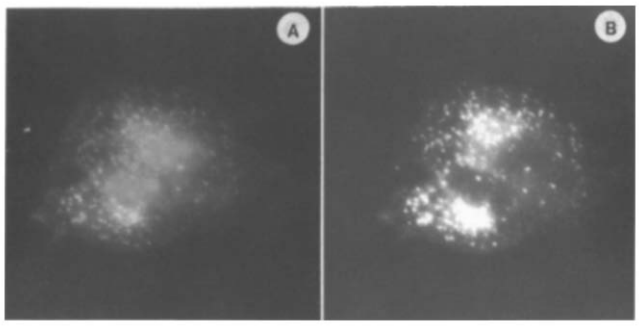


Fig. 5. Colocalisation of endocytosed FITC-transferrin and recombinant TGN41 in COS41–1 cells expressing elevated levels of the protein. COS41–1 cells were treated with 20 μM $CdCl_2$ prior to incubation in 20 mg/ml FITC-labelled transferrin for 2 h prior to fixation and processing for immunofluorescence analysis using a mouse monoclonal anti-rat TGN38/41 antibody and goat anti-mouse TRITC. Internalised transferrin is visualised in panel A, TGN41 in panel B.

TGN structures, not just in highly vesiculated forms of the TGN.

3.3. *Characterisation of the TGN41-containing vesicles in transfected COS cells*

Are these structures early endosomes? To determine whether there is any localisation of overexpressed TGN41 to early endosomes, COS41–1 cells were treated with 20 μM $CdCl_2$. 20 μg/ml FITC-labelled transferrin was added to the medium and cells incubated for 2 h prior to fixation and processing for immunofluorescence analysis using the mouse anti-rat TGN38/41 antibody and goat anti-mouse TRITC. The majority of vesicles which are detected with anti-TGN38/41 antibody (Fig. 5A) also stain with FITC-transferrin (Fig. 5B) suggesting the peripheral structures containing TGN41 are predominantly early endosomes.

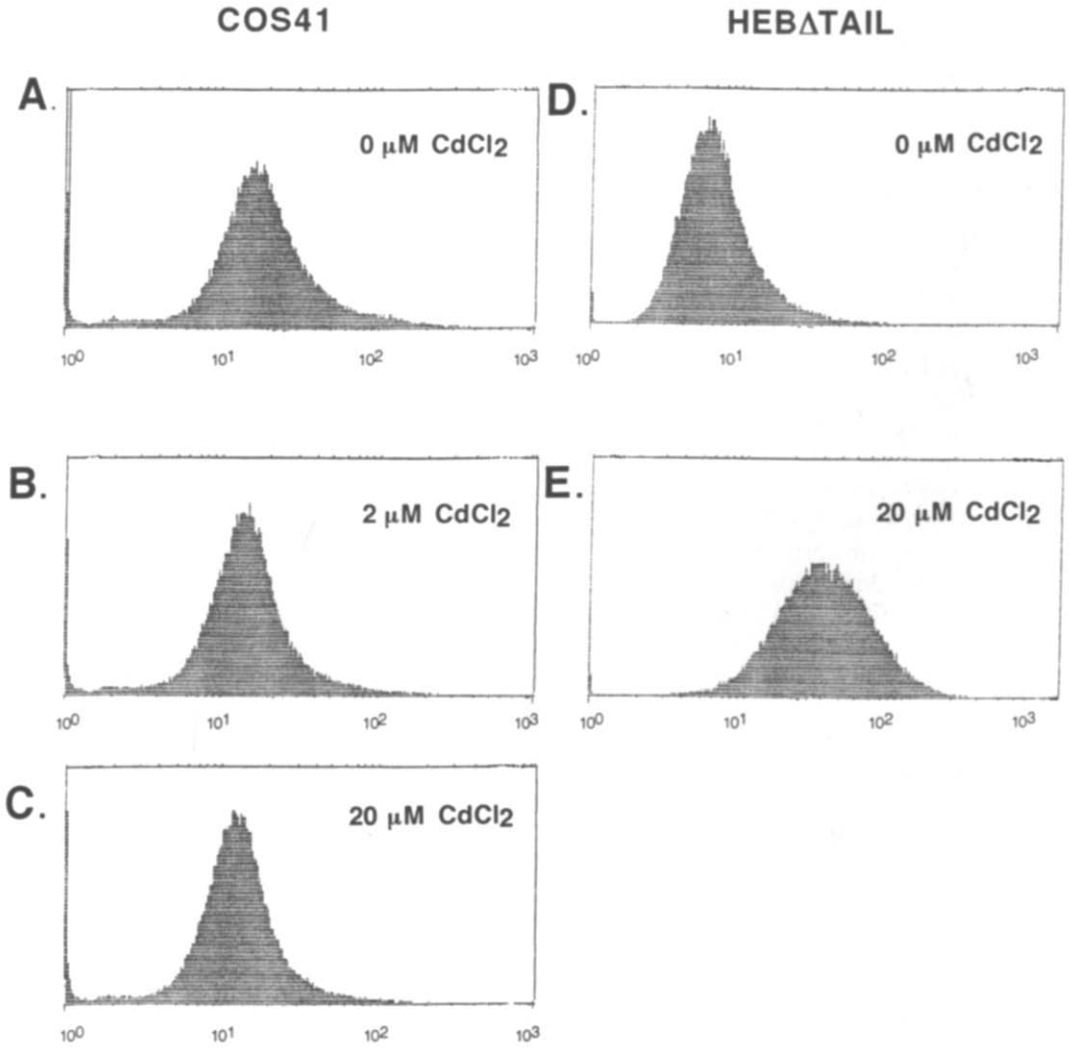


Fig. 6. FACS analysis of surface expression of full-length TGN41 in COS cells (COS41–1) and truncated TGN38/41 (lacking a cytoplasmic domain) in Heb7a cells (HebΔTail-3) using a monoclonal antibody to an amino-terminal epitope on the lumenal domain of TGN38/41. COS41–1 cells treated with 0 (A), 2 (B) or 20 (C) μM $CdCl_2$ and HebΔTail-3 cells treated with 0 (D) or 20 (E) μM $CdCl_2$ were processed as described in section 2. Log red fluorescence is plotted on the x-axis versus (forward light scatter) number of cells on the y-axis.

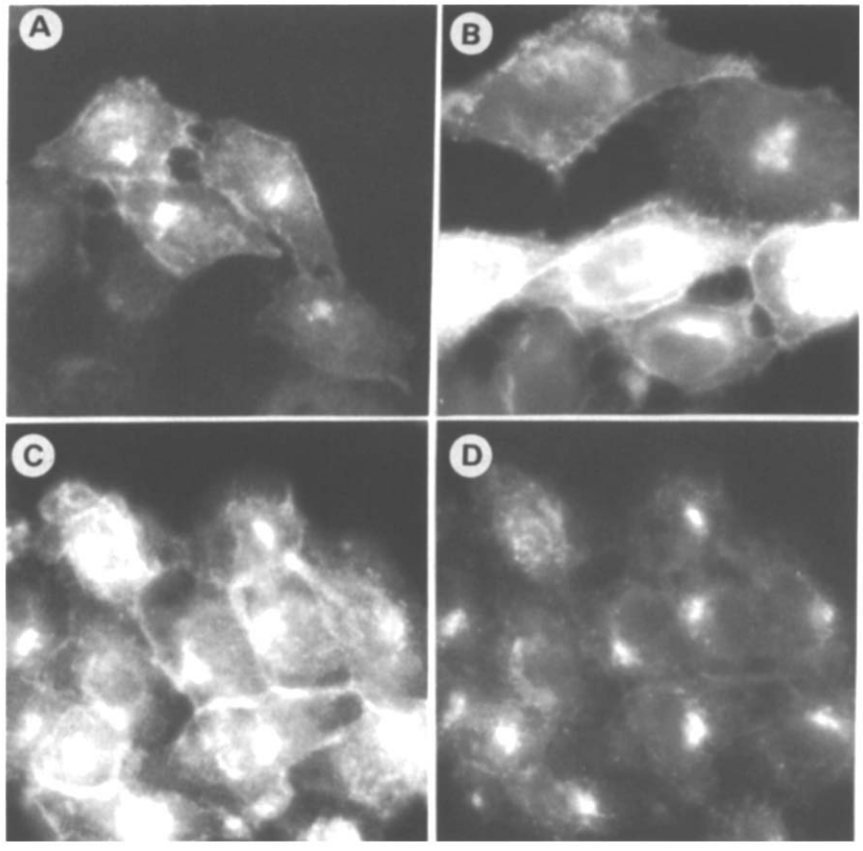


Fig. 7. Immunofluorescent localisation of truncated TGN38/41 (lacking a cytoplasmic domain) in Heb7a cells (HebΔTail-3). (A and B) Increased levels of $CdCl_2$ elevate expression of the truncated protein. HebΔTail-3 cells were treated with 2.0 (A) or 20 (B) μM $CdCl_2$ prior to methanol fixation and processing for immunofluorescence analysis using a rabbit anti-rat TGN38/41 antibody. (C and D) Co-localisation with γ-adaptin. Cells were treated with 10 μM $CdCl_2$ prior to fixation and incubation with a rabbit anti-rat TGN38/41 antibody followed by goat anti-rabbit TRITC (C) and a mouse anti-human γ-adaptin antibody followed by goat anti-mouse FITC (D).

3.4. Cell surface expression of TGN41

Is there an increased expression of TGN41 at the cell surface in cells which are expressing elevated levels of the protein? COS41–1 cells were treated with 0, 2.0 or 20 μM $CdCl_2$ and processed for FACS analysis as described in section 2. As can be seen in Fig. 6A–C there was no significant difference between the three samples. This is consistent with results from immunofluorescence analysis of non-permeabilised cells (data not shown). These data suggest that TGN41 is not accumulating at the cell surface at the levels of expression which cause its intracellular mislocalisation. The saturable sorting machinery responsible for accurate TGN localisation may therefore only come into play after internalisation has occurred. Similar results were obtained with Heb7A constructs expressing TGN41 (data not shown).

3.5. Cytoplasmic domain deletion mutants of TGN38/41 localise to both the cell surface and TGN and do not lead to mislocalisation of γ-adaptin

Having characterised the expression of full-length TGN38 and 41 in permanently transfected cells, and ascertained levels of expression which result in appropriate and inappropriate localisation of the protein, we chose to address the question of the role played by the cytoplasmic domain of the protein in its localisation to the TGN. A number of reports, using various constructs and expression systems, have demonstrated that deletion of the cytoplasmic domain of TGN38/41 results in its expression at the cell surface [3,7,8,9]. The original description [3] was of transiently transfected cells which expressed recombinant protein at very high levels. This system has been modified to give slightly lower levels of expression [8]. The other two studies [7,9] used the lumenal and transmembrane domains of other cell surface reporter proteins attached to the cytoplasmic domain of TGN38/41. In all the above studies, levels of transgene expression were unregulated, and significantly higher than those for endogenous TGN38/41 in rat cells. Since our studies with full-length rat TGN41 expressed in COS cells had shown that over-expression of the protein led to its mislocalisation, we chose to ask whether the level of protein expression affected the localisation of a truncated TGN38/41 molecule lacking the majority of the cytoplasmic domain. To address this question Heb7a cells were transfected with a pMEP4-TGN38/41 cyto-

plasmic domain deletion construct (TGN38/41 Δ TAIL in Fig. 1) and stably expressing cell lines established. One cell line designated HebΔTail-3 was chosen for further study, however, the same results were obtained with other cell lines. HebΔTail-3 cells were treated with 2.5 or 20 μM $CdCl_2$ and processed for immunofluorescence microscopy using a monoclonal antibody to the N-terminus of TGN38/41. Cell surface staining was apparent at low doses of $CdCl_2$ (Fig. 7A). The intensity of this staining increased with increasing expression levels obtained by treatment with higher doses of $CdCl_2$ (Fig. 7A,B). Interestingly, in addition to the cell surface staining a concentration of staining in the perinuclear area was also visualised at both high and low levels of expression (Fig. 7A,B). The increase in level of expression at the cell surface was confirmed by FACS analysis (Fig. 6D,E) of HebΔTail-3 cells. A 10-fold increase in $CdCl_2$ leading to a 10-fold increase in cell surface expression.

In order to determine whether the perinuclear staining corresponded to a population of TGN41 localised to the TGN, cells were incubated in 2.5 μM $CdCl_2$, prior to processing for double-label immunofluorescence analysis using the monoclonal anti-γ-adaptin antibody and a species-specific anti-rat TGN38/41 polyclonal antibody. The perinuclear concentration of TGN38/41 staining colocalised with the γ-adaptin staining (Fig. 7C,D) suggesting that some of the mutant protein was located in the TGN. It is noticeable that there is no apparent mislocalisation of γ-adaptin in cells overexpressing Δ TAIL TGN38/41 (Fig. 7D), this is in contrast to the situation in cells overexpressing full-length TGN38/41 (Fig. 4D). Similar results were obtained with COS cells transfected with the TGN38/41 Δ TAIL construct (data not shown).

4. Discussion

We have used an inducible expression system in stably transfected cell lines to investigate the targeting and retention of TGN38/41. The ability to regulate expression in stable transfectants affords a number of advantages over other systems: (i) a large population of cells expressing similar amounts of protein can be easily monitored and are available for biochemical measurements, (ii) in the absence of an inducing agent there is very little TGN38/41 being produced and therefore any deleterious effects on the cells will be minimised (iii) expression can be induced to levels which cause mislocalisation of TGN38/41 but not detachment from the substrate as had been seen previously [3] and (iv) mislocalisation due to overexpression can be dissected from mislocalisation due to alterations in amino acid sequence.

Expression of TGN41 in COS cells at high levels resulted in its appearance in vesicles of various sizes scattered throughout the cytoplasm and in a greatly extended TGN. In some cells it was completely absent from its normally perinuclear localisation and only present in the vesicular structures. Interestingly, in cells overexpressing TGN41, where TGN41 is present in a diverse array of structures, γ-adaptin is also present on the majority of those structures. This might imply that overexpression of TGN41 leads to an expansion of the TGN and hence expansion of the membrane area coated with γ-adaptin. However, many of the TGN41-containing structures observed in transfectants overexpressing TGN41 correspond to early endosomes (as ascertained by the colocalisation between internalised FITC-transferrin and TGN41; Fig. 5A,B). TGN38/41 has previously been shown to recycle between the cell surface and the TGN [5,6], with a steady state distribution whereby the majority of the protein is in the TGN. These data suggest that overexpression of TGN41 disturbs the equillibrium of this steady state distribution such that accumulation occurs along the endocytic pathway rather than in the TGN. The fact that γ-adaptin is apparently associated with these endocytic structures (Fig. 4C,D) implies some direct or indirect interaction between TGN41 and γ-adaptin, and the fact that overexpression of Δ TAIL TGN38/41 fails to lead to mislocalisation of γ-adaptin implicates the cytoplasmic domain of TGN38/41 in any such interaction. This is of note given the data which argue that TGN38/41 is required for vesicle formation from the TGN [26].

The mislocalisation of TGN38 upon overexpression has been observed previously; when a Tac-TGN38 chimaera was overexpressed in MOP-8 cells [7]. The endogenous TGN38 was seen to disappear and it was proposed that there may either be competition for a saturable localisation mechanism or that there is a complete disruption of the TGN. Overexpression of TGN38/41 may indeed result in the saturation of the retention machinery leading to its escape from the TGN; however, if TGN38/41 is important in vesicle formation from the TGN its overexpression may lead to excessive vesicle formation and hence loss of TGN structure.

Deletion of the cytoplasmic domain of TGN38/41 resulted in its expression at the cell surface but it was also demonstrated that a proportion is also retained in the TGN. In previous studies [3,8] carried out using transient transfections, expression of the same deletion mutant resulted in the detection of only plasma membrane staining. The reason for the difference in these results is not clear, however, it could be due to the levels of expression and/or transfection systems used. The levels of cell surface expression observed in immunofluorescence analysis in the earlier studies were high enough to have masked any intracellular concentration of fluorescence signal. The lower levels of recombinant protein expression obtained in this study permitted the detection of intracellular staining patterns in immunofluorescence analysis even in the presence of cell surface protein expression. There is precedence for different results being

obtained from transient and permanent transfection systems since it has been proposed that two contradictory sets of results regarding regions of the protein responsible for the targeting of the glucose transporter GLUT4 [27,28] were obtained due to one being a stable expression system and the other a transient system [29].

The presence of residual TGN38/41 in the TGN suggests that in addition to the retrieval/targeting information inherent in the cytoplasmic domain, and in particular in the residues surrounding tyrosine 333, there is also a retention mechanism functioning. The residual TGN38/41 present in the TGN, was apparent even at the highest levels of induction (Fig. 7). These data parallel those seen for many of the Golgi enzymes in that, even at expression levels several hundred-fold higher than normal, the retention system could not be saturated (reviewed in [30]). We have not determined whether it is the lumenal domain or transmembrane domain that is responsible for this retention, however, precedence in terms of the Golgi enzymes would suggest it may be the transmembrane domain and work by others has recently demonstrated that this is the case [10].

The length of the transmembrane domain has recently been put forward as a possible determining factor in retention/residence in the Golgi stacks or plasma membrane [31]. The average length of the hydrophobic domain in 17 type II plasma membrane proteins was 20 amino acids versus 15 for Golgi enzymes. Analysis of the composition of amino acids in this domain also revealed a preference for phenylalanine in the Golgi enzymes. This is consistent with the finding that polar residues are required on one face of a predicted α-helix in the transmembrane domain of E1 glycoprotein of avian coronavirus for its retention in the Golgi [32]. The transmembrane domain of TGN38/41 is 21 amino acids in length but contains two phenylalanine residues. This may be consistent with its role in recycling between the TGN and the plasma membrane, needing the length to allow incorporation upon arrival at the cell surface but also requiring some of the properties of the Golgi enzymes which would enable residence in the 'more disordered' environment of the lipid bilayer of the TGN.

The regulated expression system we have described and characterised will permit a detailed analysis, at physiologically relevant levels of expression, of sequence/structural motifs involved in TGN to plasma membrane recycling pathways.

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