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TGN38-green fluorescent protein hybrid proteins expressed in stably transfected eukaryotic cells provide a tool for the real-time, in vivo study of membrane traffic pathways and suggest a possible role for ratTGN38

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SUMMARY

The green fluorescent protein (GFP) of *Aquorea victoria* is fluorescent when expressed as a recombinant protein in eukaryotic cells and has been used as a convenient marker of gene expression in vivo. It has also been used as a marker of the intracellular targeting of recombinant fusion proteins (part GFP, part protein of interest) which have been transiently expressed in eukaryotic cells grown in tissue culture. Thus, the use of GFP has proved a useful tool to study intracellular events in real-time. However, some transiently transfected cells fail to express, or localise correctly, the GFP-tagged protein. Therefore the production of stable cell lines expressing GFP-tagged integral membrane proteins may be essential for long-term studies. The generation of stably transfected eukaryotic cells expressing an integral membrane protein with a known, but poorly characterised intracellular trafficking pathway, would provide useful reagents for future, more precise, analysis of that pathway. TGN38 is a type I integral membrane protein which cycles between the *trans*-Golgi network (TGN) and cell surface; at steady state it is localised to the TGN. As such, TGN38 is an ideal candidate for tagging with GFP. We have generated cDNA constructs encoding ratTGN38 tagged at either the N- or C terminus with GFP. Transiently transfected rat (NRK) cells expressed active fluorophore, but failed to show correct localisation of the fusion protein. In contrast, both constructs are appropriately localised in stably transfected NRK cells and both are fluorescent. Furthermore, the recombinant GFP-tagged proteins and the endogenous TGN38 molecules show identical responses to drugs and temperature blocks known to perturb intracellular morphology and membrane traffic pathways. In fact morphological changes to the TGN induced by brefeldin A were observed at earlier time points than had been described previously using immunofluorescence analysis of fixed cells, thus validating the use of in vivo, real-time analysis of GFP-tagged proteins. In addition, we show that (in contrast to the situation in COS cells) elevated expression of ratTGN38 in NRK cells does not lead to a fragmentation of the TGN; this has implications for the role which TGN38 is playing in the maintenance of the morphology of the TGN. The data we present demonstrate that: (i) it is possible to generate stable cell lines expressing integral membrane proteins tagged with GFP; (ii) the GFP tag remains fluorescent when expressed on either the cytosolic or the lumenal side of all membranes of the secretory pathway up to and including that of the TGN; (iii) the GFP tag does not interfere with the transport of TGN38 along the secretory pathway or its retention in the TGN; (iv) GFP remains fluorescent in cells which have been processed for immunofluorescence analysis (using either paraformaldehyde or methanol fixation); and (v) TGN38 plays a role in maintaining the morphology of the TGN. Thus, stably transfected cells expressing GFP-tagged integral membrane proteins can be used as effective tools for the real-time study of intracellular morphology and membrane traffic pathways in eukaryotic cells.

Key words: Green fluorescent protein, *Trans*-Golgi network, Brefeldin A

INTRODUCTION

Recent years have seen classical morphological descriptions of membrane systems within eukaryotic cells complemented by their biochemical characterisation. Thus a growing number of integral membrane and membrane-associated proteins have been shown to be predominantly localised to specific membranes within eukaryotic cells. However, it is becoming abundantly clear that the steady state distribution of many of these proteins is a reflection of their distribution along specific recycling pathways (Luzio and Banting, 1993). So called 'resident' proteins of the ER membrane and lumen are continually being recycled from the *cis* face of the Golgi stack (Pelham and Munro, 1993) whilst the integral membrane proteins furin and TGN38, which are predominantly localised in the *trans*-Golgi network (TGN), cycle between the TGN and the cell surface. 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 (Crosby et al., 1992; Ponnambalam et al., 1996); however, the precise itinerary which it follows has yet to be elucidated.

The conventional methods for studying intracellular trafficking of integral membrane proteins have included immunofluorescence analysis of suitably fixed cells using antibodies against the protein(s) of interest and immunoprecipitation analysis of metabolically labelled cells utilising defined post-translational modifications to indicate the intracellular position of the protein(s) of interest. However, neither of these procedures permits real-time in vivo study of the intracellular localisation of specific proteins. For that to occur, the protein(s) under investigation would have to be labelled in such a way as to allow its visualisation in intact cells. The isolation of cDNA clones encoding the green fluorescent protein (GFP) of the jellyfish *Aquorea victoria* would appear to have provided a suitable label for such experiments. The 238 amino acid GFP is present as a monomer in solution and emits green light upon excitation at an appropriate wavelength. The usefulness of GFP as an in vivo reporter tag has been enhanced by specific mutations in the sequence of the wild-type protein which have been shown to sharpen the peak of fluorescence emission, enhance the fluorescence intensity of the fluorophore and increase the rate at which the protein folds to generate a flu-orescent protein (see Cubitt et al., 1995, for recent review). Tagging TGN38 with GFP would thus be predicted to generate an integral membrane protein whose intracellular distribution could be monitored in intact cells. The availability of cDNA clones encoding GFP with improved fluorescence and folding properties combined with the recent observation that GFP-tagged proteins can be expressed and fluoresce in the secretory pathway of transiently transfected eukaryotic cells (Kaether and Gerdes, 1995) encouraged us to generate cDNA constructs encoding TGN38 tagged at either the N- or C terminus with GFP.

We have shown previously that significantly elevated levels of expression of ratTGN38 can lead to its mis-localisation in heterologous cells (Reaves and Banting, 1994a), and others have found that GFP-tagged integral membrane proteins expressed at high levels in transiently transfected cells can lead to an apparent accumulation of the tagged proteins in ER membranes (H. Pelham, personal communication). We therefore chose to express the TGN38-GFP constructs under the control of an inducible promoter in stably transfected rat (NRK) cells. We now show that both N- and C-terminally tagged TGN38 constructs are expressed in stably transfected cells. The tagged proteins are fluorescent and are correctly localised. The stable transfectants thus provide a convenient tool for the study of TGN membrane dynamics as shown by response of the transfected cells to various drug treatments. We also show that elevated expression of ratTGN38 in rat (NRK) cells has no major morphological effect on the TGN. This is in contrast to previously published observations in monkey (COS) cells, where elevated expression of ratTGN38 led to fragmentation of the TGN (Reaves and Banting, 1994a) and has implications for the role which TGN38 and its orthologues might play in maintaining the architecture of the TGN.

MATERIALS AND METHODS

GFP-tagged constructs

To produce TGN38-GFP, the S65T mutant GFP cDNA (Heim et al., 1994) was amplified by PCR from the plasmid pGFP-CMX (generous gift from Dr J. Pines, CRC Wellcome Institute, Cambridge) using primers which introduce *Hin*dIII sites at either ends of GFP cDNA (primer 1: 5′-CCCCAAGCTTGGTGGAGATATGAGTAAAGGAGAAGAACTTTTC-3′; primer 2: 5′-CCCCAAGCTTCTATTATTTGTATAGTTCATC-3′; the positions of GFP cDNA initiating methionine and the 'stop' codon are underlined in primers 1 and 2, respectively). The oligonucleotides used throughout this work were synthesised on a DuPont Coder 300 DNA synthesiser by Dr L. Hall, BBSRC Molecular Recognition Centre, University of Bristol. The PCR amplification was performed using *Pfu* thermostable DNA polymerase (Stratagene) in the buffer supplied and according to the manufacturer's instructions. Reaction parameters were as follows: 94°C, 1 minute; 52°C, 2 minutes; 72°C, 2 minutes, for 2 cycles, then 94°C, 1 minute; 65°C, 2 minutes; 72°C, 2 minutes, for 18 cycles. The resulting product was gel purified, the 5′-ends were phosphorylated with T4 polynucleotide kinase (Pharmacia) and subcloned into the *Sma*I site of pUC18. The absence of any PCR mutation was confirmed by sequencing. The *Hin*dIII GFP fragment was then excised from this vector and cloned into the *Hin*dIII site (nucleotide 1,076) of TGN38-ΔpMEP4, thus removing the stop codon of TGN38 cDNA. ΔpMEP4 vector is a deletion version of the eukaryotic expression vector pMEP4 (Stratagene), lacking a non-essential 4.5 kb region (nucleotides 5,570-10,114) (E. P. Roquemore, unpublished data).

GFP-TGN38 was constructed following a similar strategy, but using a pair of PCR primers containing *Bam*HI sites at either ends of the GFP cDNA. The resulting PCR fragment was cloned into the *Bcl*I site (nucleotide 181) of TGN38-ΔpMEP4. The sequences of the primers are: primer 3, 5′-CATGGGATCCGGGTGGAGGTATGAGTAAAG-3′; primer 4, 5′-CGATGGATCCCCACCTTTGTATAGTTCATCCAT-3′; underlined are the first methionine of GFP and the position of the 'stop' codon of GFP which has been mutated to encode a glycine residue.

Subcloning of humTGN46 into ΔpMEP4 and transfection of NRK cells

The full length cDNA coding sequence of humTGN46 in pSPORT (a generous gift from Dr S. Ponnambalam) was sub-cloned into the *Kpn*I/*Bam*HI sites of the multiple cloning site of ΔpMEP4. This directional cloning resulted in recombinants which carry humTGN46 in the correct orientation with respect to the human MTIIa promoter in the vector. The humTGN46ΔpMEP4 construct was linearised with *Cla*I prior to transfection into NRK cells. Transfections and subsequent selection procedures were as described below.

Cell culture and transfections

Normal rat kidney (NRK) cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics as described previously (Reaves and Banting, 1992).

For transient transfections, NRK cells were plated at 30-50% density onto round coverslips and 3 μg of either GFP-TGN38 or TGN38-GFP plasmid were introduced using the Transfectam reagent (Promega), following the manufacturer's instructions. Cells were imaged 48 hours after transfection. Stable transfections were performed using the same method but starting with a 50% confluent T75 flask of NRK cells and 25 μg linearised plasmid. Two days after transfection hygromycin B (Boehringer) was added to a final concentration of 0.4 mg/ml. Resistant clones were clearly visible 15 days following transfection.

Drug treatments, imaging and immunofluorescence

Unless otherwise indicated, transfected cells were incubated in the presence of 5 μM $CdCl_2$ for 16 hours prior to analysis. Treatments with BFA (Sigma), nocodazole, okadaic acid (Cambridge Bioscience) and chloroquine (Sigma) and the 15°C temperature block of transiently or stably transfected cells were performed as previously reported (Reaves et al., 1993). Real-time fluorescence signals produced by the transfected cells were generated under illumination from a xenon lamp and visualised with a fluorescein filter set (Zeiss, set 9; 450-490 nm excitation; 520 nm long pass) and ×40 (1.4 NA) fluar objective on a

Zeiss Axiovert 100TV fluorescence microscope and imaged using a Coolview CCD Camera System (Photonic Science) and Biovision software on a Power Macintosh 8100/100. The average time for image collection, i.e. continuous exposure to excitatory light, was one minute; cells were kept in complete darkness during periods between image acquisition. This illumination regime did not appear to be deleterious to the cells since illuminated cells remained viable, i.e. they could be used for repeated studies on successive days. Cells grown on coverslips in DMEM medium supplemented with 10% FBS and antibiotics as described previously (Reaves and Banting, 1992) were rinsed several times in PBS prior to image acquisition and maintained in PBS throughout the period of image acquisition. The majority of images were acquired from cells incubated at room temperature. Images from cells which had been pre-incubated at 15°C were obtained from cells which had been rinsed and subsequently maintained in ice-cold PBS in order to prevent traffic of newly-synthesised material out of the ER. Cells were washed and incubated in Krebs-Ringer bicarbonate (KRB: 135 mM NaCl, 3.6 mM KCl, 10 mM NaHepes, pH 7.4, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5.5 mM glucose, equilibrated with $0_2/CO_2$ (95:5) for the duration of the BFA time-course experiments and were maintained at 37°C on a thermoregulated microscope stage. PBS and KRB were used in these studies since the auto-fluorescence detected from cells incubated in these media was minimal compared to that detected from cells maintained in DMEM or a variety of other pH indicator-free media.

For immunofluorescence analysis, cells were fixed and permeabilized with methanol or with paraformaldehyde and processed as described (Reaves et al., 1993). Anti-GFP (Clontech) and rabbit polyclonal anti-γ-adaptin (Dittié et al., 1996) (a generous gift from Dr S. Tooze, ICRF, London) primary antibodies were used at a final dilution of 1:250, the anti-humTGN46 rabbit polyclonal antiserum (GB1)(Ponnambalam et al., 1996) was used at a final dilution of 1:500, the anti-mannosidase II monoclonal antibody (Burke et al., 1982) was used as neat tissue culture supernatant, whilst anti-rabbit and anti-mouse FITC or TRITC labelled secondary antibodies (Amersham) were used at 1:500 final dilution.

For double labelling, coverslips of GFP-TGN38 cells were induced with 2.5 μM or 20 μM $CdCl_2$ plus Zn for 18 hours. Methanol fixed cells were blocked in 2% FBS and incubated in the presence of rabbit anti-γ-adaptin polyclonal antibody (1:250) or mannosidase II monoclonal antibody (as above) for 20 minutes, then the cells were washed 4× in PBS. A set of coverslips was incubated with TRITC-labelled secondary antibodies and mounted on slides. Another set of coverslips was washed and incubated with anti-GFP antibody (1:250), and then with FITC-labelled anti-rabbit antibody, prior to mounting on slides.

Quantitative immunoblot analysis

NRK cells (10^6) expressing GFP-TGN38 were incubated in culture medium containing 0, 5, 10 μM $CdCl_2$ or 20 μM $CdCl_2$ and 80 μM $ZnCl_2$ for 16 hours. Non-transfected NRK cells not exposed to cadmium were used as negative control. Whole cell lysates were prepared and an aliquot of each lysate (corresponding to 2×10^4 cells) was subjected to SDS-PAGE and transferred to nitrocellulose as previously described (Brake et al., 1990). The nitrocellulose membrane was incubated with a 1:100 dilution of anti-GFP polyclonal antibody (Clontech). Binding of primary antibody was detected by the ECL method (Amersham) according to the manufacturer's instructions. Chemiluminescence signals were quantified by densitometric scanning on a Chromoscan 3 densitometer (Joyce Loebl) according to published procedures (Belsham et al., 1980).

RESULTS

The construction of TGN38-GFP and GFP-TGN38 hybrids

RatTGN38 was tagged either at its extreme C terminus or near its mature N terminus with GFP (S65T/Q80R/I167T triple mutant) (see Fig. 1). The C-terminally tagged construct was generated by inserting cDNA sequence encoding GFP into the unique *Hin*dIII site immediately upstream of the 'stop' codon in ratTGN38. PCR, using appropriate oligonucleotide primers, was used to amplify the GFP sequence prior to this insertion. The oligonucleotide primers incorporated *Hin*dIII sites flanking the GFP coding sequence and were designed such that the final construct encoded a tri-Glycine linker between the C terminus of ratTGN38 (..NLKL) and the N terminus of GFP (MSKG..). The rationale behind the inclusion of the tri-glycine linker was that it might allow folding of GFP as a separate domain of the hybrid protein. The N-terminally tagged construct was generated by inserting cDNA sequence encoding GFP into the unique *Bcl*I site in ratTGN38. *Bcl*I generates an 'in-frame' cleavage of ratTGN38 between codons encoding amino acids 40 (Thr) and 41 (Asp) of the mature protein sequence. This region of the sequence is poorly conserved between ratTGN38 and its orthologues (Ponnambalam et al., 1996); we therefore argued that incorporation of GFP in this position would be unlikely to interfere with the function or trafficking of ratTGN38. The oligonucleotide primers used in this PCR amplification of GFP included sequences encoding 'PGGG' and 'GG' linkers at the N- and C-termini of the protein sequence. These sequences were included in the hope that they would increase the probability that the GFP region would be able to fold independently of the flanking TGN38 sequences. The N-terminally tagged construct (GFP-TGN38) encodes a protein with GFP expressed at the extra-cytoplasmic side of the membrane, whilst the C-terminally tagged construct (TGN38-GFP) encodes a protein with GFP expressed at the cytosolic side of the membrane (see Fig. 1).

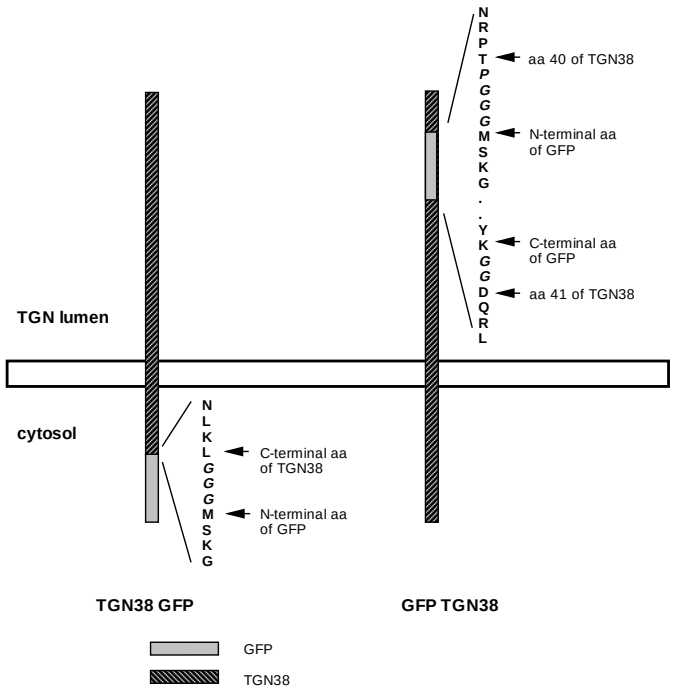


Fig. 1. Cartoon representations of TGN38-GFP and GFP-TGN38 constructs indicating positions of insertion of GFP sequences in ratTGN38. The amino acid sequence at TGN38-GFP and GFP-TGN38 junctions is presented, those amino acids not present in either TGN38 or GFP are indicated in italics.

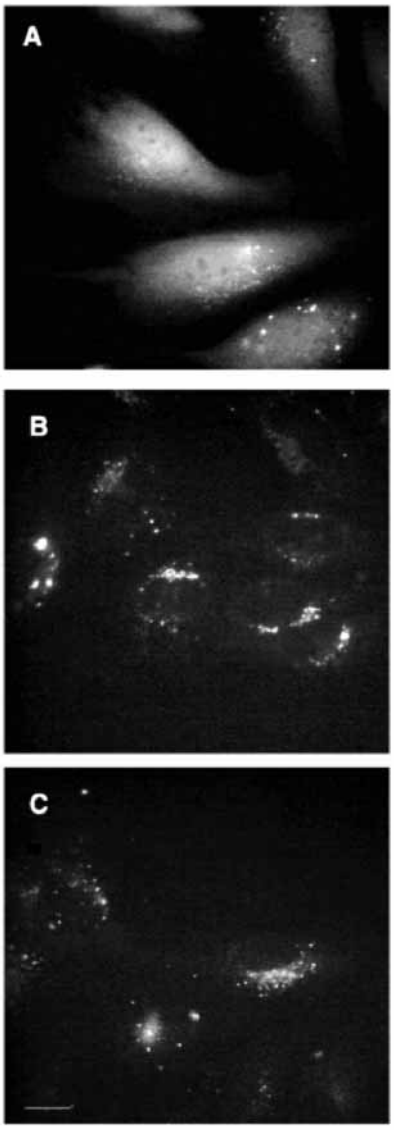


Fig. 2. Images of transiently transfected NRK cells expressing GFP alone (A), TGN38-GFP (B) or GFP-TGN38 (C). Bar, 10 μm.

The GFP-TGN38 and TGN38-GFP constructs were subcloned into the plasmid-based eukaryotic expression vector ΔpMEP4. Expression of appropriately inserted cDNAs is driven by the human metallothionein IIa (MtIIa) promoter in this vector. The MtIIa promoter is inducible by the addition of low levels of heavy metals to the medium bathing cultured cells. We have previously used this promoter for regulated expression of ratTGN38 in monkey (COS) and human (HeLa) cells (Reaves and Banting, 1994a).

Expression of TGN38-GFP and GFP-TGN38 in transiently transfected NRK cells

Normal rat kidney (NRK) cells were grown on glass coverslips and transfected with either TGN38-GFP or GFP-TGN38 expression constructs. Control NRK cells were transfected with a construct designed to drive the expression of GFP alone. All cells were viewed down a Zeiss Axiovert 100TV fluorescence microscope 48 hours post-transfection. Diffuse cytosolic fluorescence was observed in those cells expressing GFP alone (Fig. 2A). In contrast, those cells expressing either TGN38-GFP or GFP-TGN38 generated a fluorescence signal in a juxta-nuclear position (Fig. 2B,C).

We have previously shown that, in NRK cells, the fungal metabolite brefeldin A (BFA) leads to a re-localisation of the TGN such that it co-localises with the microtubule organising centre (MTOC) (Reaves and Banting, 1992). In contrast, others have shown that BFA leads to a redistribution of the membrane and contents of the Golgi stack into the endoplasmic reticulum (ER)(Lippincott-Schwartz et al., 1991). Thus, although it is not possible to distinguish between the cisternae of the Golgi stack and the TGN of untreated cells at the light or fluorescence microscopy level, the different responses of these organelles to BFA allow them to be distinguished in BFA treated cells (Ladinsky and Howell, 1992; Reaves and Banting, 1992). Thus, if the juxta-nuclear pattern of fluorescence observed in NRK cells expressing either TGN38-GFP or GFP-TGN38 corresponded to a signal coming from the Golgi stack, the addition of BFA would be expected to lead to redistribution of the hybrid protein into the ER generating a more reticular pattern of fluorescence. However, if the juxta-nuclear pattern of fluorescence observed in NRK cells expressing either TGN38-GFP or GFP-TGN38 corresponded to a signal coming from the TGN then the addition of BFA would be expected to culminate in a focusing of the fluorescence signal over the MTOC. In fact, addition of BFA to these cells had no effect on the pattern of fluorescence (data not shown), implying that the hybrid proteins are in neither the TGN nor the Golgi stack. Thus, although the TGN38-GFP and GFP-TGN38 constructs both fluoresce in transiently transfected cells, neither protein is correctly localised. We reasoned that the mis-localisation of the hybrid proteins may well be due to aggregation of mis-folded proteins in a degradative compartment within the cell, and that the aggregation and mis-folding may have arisen by virtue of the elevated expression of the recombinant proteins in the transfected cells. GFP can dimerise under appropriate conditions (Cubitt et al., 1995): whilst this may not be a problem for soluble or secretory proteins tagged with GFP it may cause difficulties with similarly tagged, transiently expressed, membrane proteins. The high levels of membrane-associated GFP may provide a suitable local environment for dimerisation of the protein, an event which would lead to the dimerisation of the hybrid protein and may well affect its traffic through the secretory pathway. We argued that the generation of stably transfected cells expressing TGN38-GFP or GFP-TGN38 under the control of an inducible promoter would alleviate these potential problems and might allow the appropriate localisation of the hybrid proteins.

Expression of TGN38-GFP and GFP-TGN38 in stably transfected NRK cells

Stably transfected cells expressing TGN38-GFP or GFP-TGN38 under the control of the MtIIa promoter were generated and viewed as before following overnight induction of recombinant protein expression using 5 μg/ml $CdCl_2$, conditions previously shown to give expression and appropriate localisation of TGN38 in transfected cells (Reaves and Banting, 1992). Both constructs give a discrete juxta-nuclear pattern of fluorescence reminiscent of that seen in immunofluorescence analysis of NRK cells using antibodies to TGN38 (Fig. 3A,B). BFA was added to these cells in order to ascertain whether the fluor-

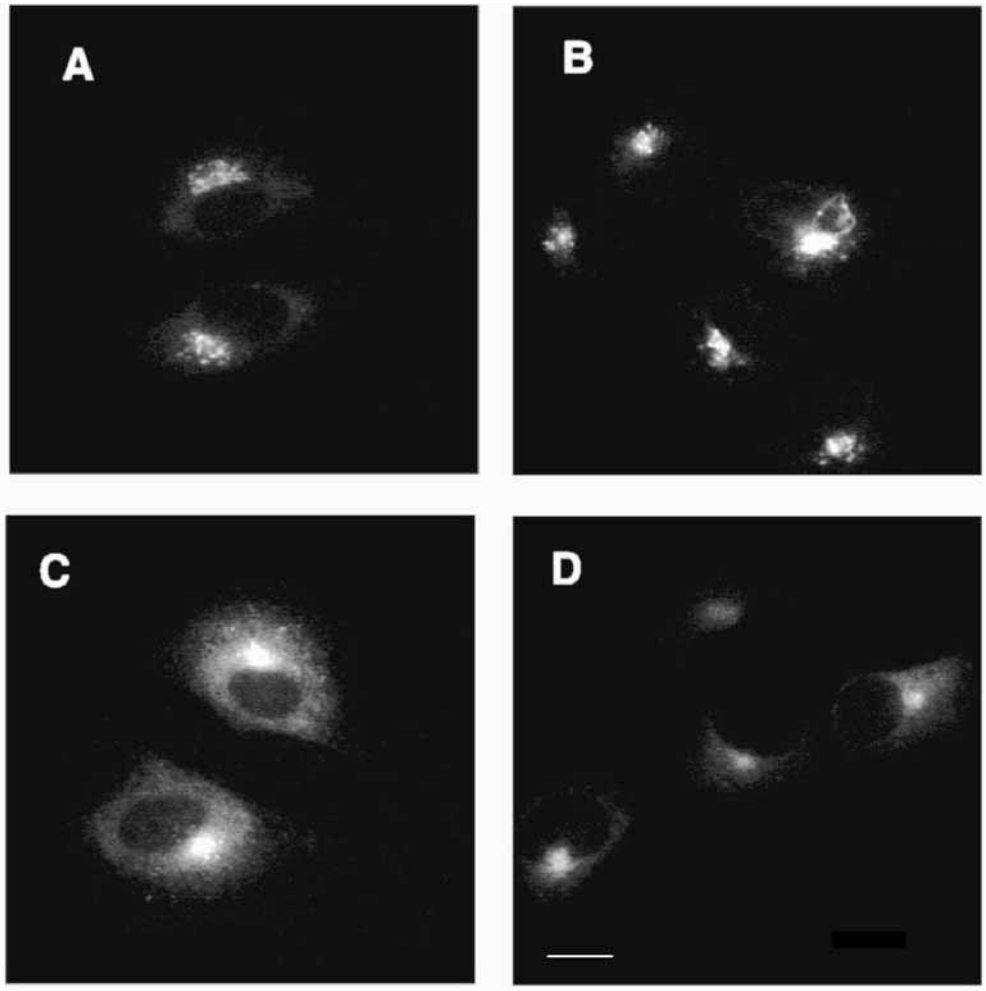


Fig. 3. Images of stably transfected NRK cells expressing GFP-TGN38 (A and C) or TGN38-GFP (B and D). Cells shown in C and D were incubated in the presence of 5 μg/ml BFA for one hour at 37°C prior to image capture. Bar, 10 μm.

escence signal came from the Golgi stack or TGN. BFA led to a collapse of the majority of the fluorescence signal in both cases (Fig. 3C,D). This focus of collapsed fluorescence co-localised with the MTOC (data not shown), thus establishing that both TGN38-GFP and GFP-TGN38 are predominantly localised to the TGN. Others have demonstrated that a proportion of TGN38 is in the *trans*-cisterna of the Golgi stack rather than the TGN (Rabouille et al., 1995), this material would be expected to redistribute into the ER and nuclear membranes upon treatment with BFA. The faint reticular and nuclear membrane fluorescent signal observed in the BFA treated cells (Fig. 3C,D) would correspond to this population of molecules.

Time course of BFA response

Previous analyses of the time course of the effect of BFA upon cells have relied upon processing different groups of cells for immunofluorescence analysis at different time points following addition of the drug. The availability of stably transfected cells expressing GFP-tagged TGN38 means that such experiments can now be performed in real-time on the same group of live cells. Images from such a time-course are shown in Fig. 4. Within two minutes the fluorescent signal becomes slightly more diffuse, presumably corresponding to the tubulation of the TGN in response to BFA which has been reported previously (Reaves and Banting, 1992), before focusing in a juxta-nuclear position corresponding to the MTOC after 5-10 minutes. Thus, morphological changes to the TGN occurring as a result of incubation in the presence of BFA were observed at marginally earlier time points than previously described (Reaves and Banting, 1992), demonstrating one of the advantages of studying GFP-tagged membrane proteins in vivo as opposed to immunofluorescence analysis of fixed cells. The effect of BFA was fully reversible (data not shown).

Validity of transfectants

Confirmation that the GFP-TGN38 and TGN38-GFP constructs were being expressed in the different stably transfected cell lines was obtained in two ways. Firstly, whole cell lysate from each cell line was immunoprecipitated using a polyclonal antibody to TGN38 and the immunoprecipitated material subjected to immunoblot analysis using a polyclonal antibody to GFP. The anti-GFP antibody detected a protein of appropriate molecular mass in both samples and failed to detect anything in similar samples from non-transfected cells (data not shown). Secondly, PCR (using oligonucleotide primers designed to amplify sequences unique to each of the two constructs) demonstrated that there had been no cross-contamination between the two cell lines (Fig. 5).

Pharmacological and temperature effects on GFP tagged TGN38 expressed in stably transfected NRK cells

Drugs other than BFA have previously been shown to affect

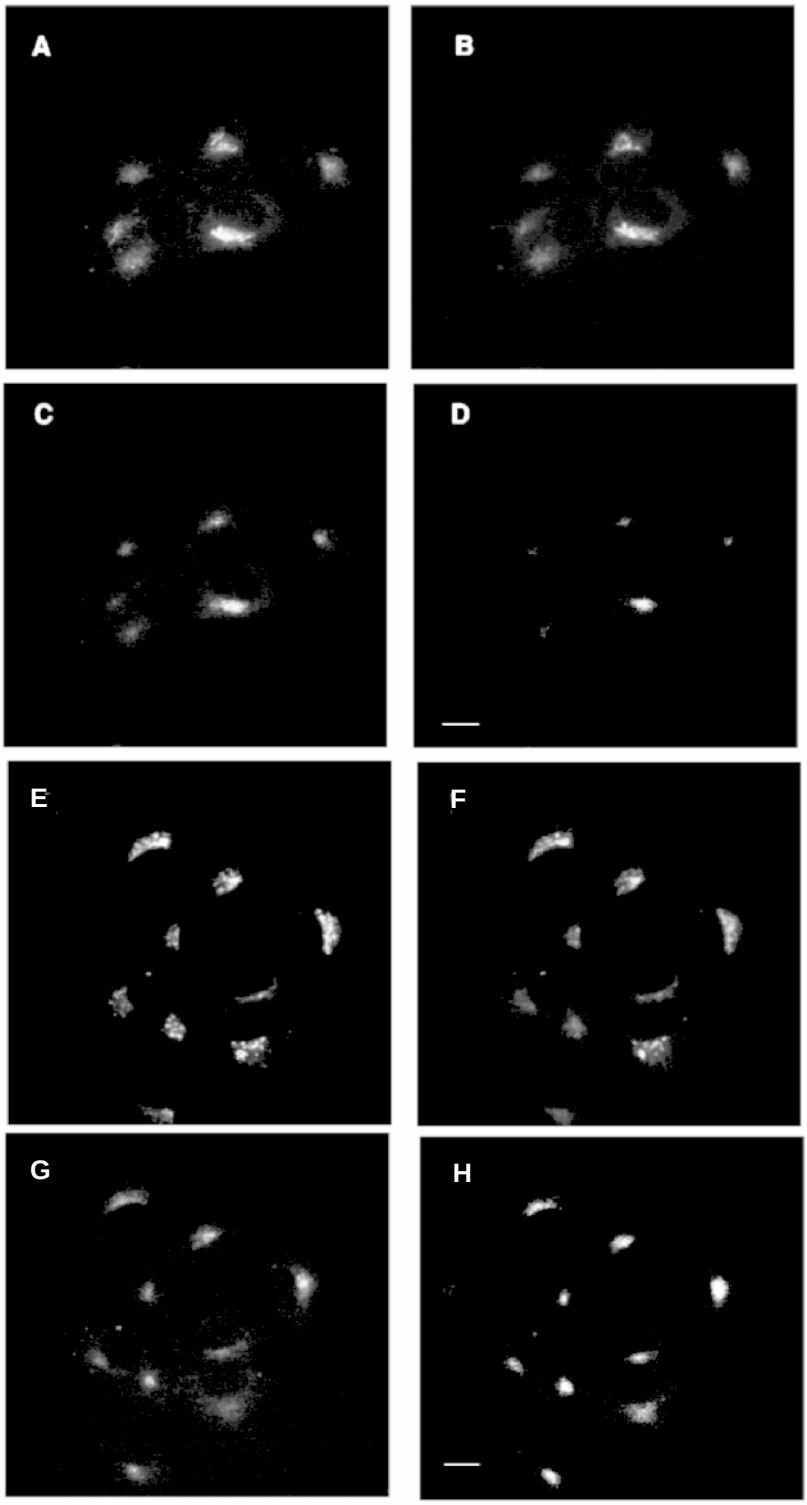
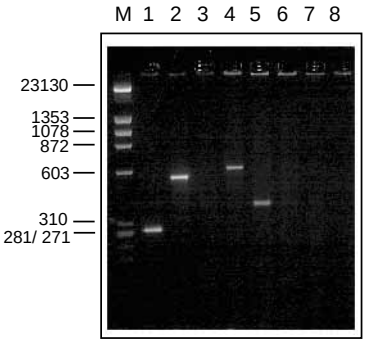


Fig. 4. (A-D) Time course of action of BFA. Stably transfected NRK cells expressing GFP-TGN38 were incubated in the presence of 5 μg/ml BFA at 37°C, and images captured at 0 (A), 2 (B), 5 (C) and 20 (D) minutes. Bar, 10 μm. (E-H) Stably transfected NRK cells expressing TGN38-GFP were incubated in the presence of 5 μg/ml BFA at 37°C, and images captured at 0 (E), 2 (F), 5 (G) and 20 (H) minutes. Bar, 10 μm.

the morphology of the TGN (Chapman and Munro, 1994; Horn and Banting, 1994; Reaves and Banting, 1992, 1994b). The microtubule depolymerising drug nocodazole leads to fragmentation of the TGN (Reaves and Banting, 1992); it has a similar effect in vivo as assayed using GFP-tagged TGN38 constructs (Fig. 6A). One of the effects of the weak base chloroquine is to block the formation of endocytic carrier vesicles on the endocytic pathway (Clague et al., 1994), this leads to an accumulation of the recycling population of TGN38 molecules in early endocytic structures (Reaves and Banting, 1994b). Chloroquine has similar effects on GFP-tagged TGN38 constructs in vivo (Fig. 6B), generating a pattern of fluorescence reminiscent of that observed in immunofluorescence analysis of cells incubated in the drug (Reaves and Banting, 1994b). The fragmentation of the TGN induced by incubation of cells in okadaic acid (Horn and Banting, 1994)



lane	primers	frg. length (bp)	cell line
1	a,b	290	TGN38-GFP
2	c,d	480	TGN38-GFP
3	e,f	-	TGN38-GFP
4	e,f	720	GFP-TGN38
5	g,h	380	GFP-TGN38
6	a,b	-	GFP-TGN38
7	a,b	-	NRK
8	e,f	-	NRK

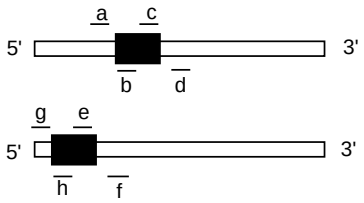


Fig. 5. PCR to confirm the identity of GFP constructs within stably transfected cell lines. Oligonucleotides which would allow the PCR amplification of specific GFP/TGN38 hybrid sequences were designed. The cartoon at the bottom of the figure represents cDNA encoding TGN38-GFP (upper cartoon) or GFP-TGN38 (lower cartoon); positions at which primers a-f anneal are indicated. Primer pairs a-b and c-d were designed to allow the PCR amplification of products from DNA encoding TGN38-GFP (lanes 1 and 2), but not from DNA encoding GFP-TGN38 (lane 6 for a-b pair). Similarly, primer pairs e-f and g-h were designed to allow the PCR amplification of products from DNA encoding GFP-TGN38 (lanes 4 and 5), but not from DNA encoding TGN38-GFP (lane 3 for e-f pair). No primer pairs permitted PCR amplification from non-transfected NRK cells (lanes 7 and 8 for a-b and e-f primer pairs). In order to perform these experiments, 10^7 cells were harvested by trypsinisation and resuspended in 200 μl sterile PBS; 5 μl of this suspension were boiled for 5 minutes prior to addition to a standard PCR mix (see Materials and Methods) using the primers indicated in the figure. PCR products were separated on a 2% NuSieve (FMC, USA) agarose gel and visualised under UV illumination following EtBr staining. The expected sizes of PCR products are as indicated. M, size standards (bp).

is also observed in vivo in cells expressing the GFP-tagged TGN38 constructs (data not shown).

In addition to their pharmacological sensitivity, membrane traffic pathways in mammalian cells have been shown to be temperature dependent (Matlin and Simons, 1983; Saraste and Kuismanen, 1984). Incubation of cells at 15°C has been shown to block transport from the ER to the Golgi stack (Saraste and Kuismanen, 1984); so, at this temperature, newly synthesised proteins accumulate in the ER. Thus cells expressing the GFP-tagged TGN38 constructs were incubated at 15°C prior to image acquisition in order to demonstrate that the TGN-localised GFP-tagged hybrid proteins were indeed reaching their destination following insertion into the ER membrane. Fig. 7 shows that, under these conditions, a strong GFP signal is observed in the nuclear and ER (reticular pattern) membranes. This signal corresponds to the material which has been synthesised during the temperature block and is in addition to the pre-existing, TGN-localised signal. Thus the GFP-tagged proteins are passing through the ER on their way to the TGN.

Elevated expression of GFP-tagged TGN38 in NRK cells

We have previously shown, using the MtIIa inducible expression system, that elevated expression of ratTGN38 in stably transfected COS cells leads to altered TGN morphology (Reaves and Banting, 1994a). In these experiments, COS cells which over-expressed ratTGN38 were fixed and subjected to dual label immunofluorescence analysis using a rabbit polyclonal antibody to TGN38 and a monoclonal antibody to γ-adaptin (γ-adaptin is a peripheral membrane protein specifically associated with the TGN; Ahle et al., 1988; Robinson and Kreis, 1992). We showed that in COS cells which express elevated levels of ratTGN38 there is a distension and fragmentation of the TGN with all those structures which are immunopositive for TGN38 being immunopositive for γ-adaptin (Reaves and Banting, 1994a). These data were interpreted as showing that over-expression of ratTGN38 in COS cells leads to fragmentation of the TGN. We chose to repeat these experiments using stably transfected NRK cells expressing TGN38-GFP or GFP-TGN38 transfectants in order to study the dynamics of the fragmentation process.

Cells expressing increasing amounts of GFP-tagged TGN38 show a concomitant increase in GFP fluorescence in vivo (Fig. 8). However, even at the highest level of fluorescence, there is no detectable change in the gross morphology of the TGN (Fig. 8D). Quantitative immunoblot analysis of whole cell lysate from stably transfected NRK cells expressing GFP-tagged TGN38 using antibodies to either TGN38 (to detect both the endogenous and recombinant protein) or GFP (to detect the recombinant protein alone) was performed in order to assess the level of expression of the recombinant protein in the transfected cells. The lysates were prepared from cells which had been incubated in increasing amounts of $CdCl_2$ in order to induce expression of the recombinant protein and corresponded to the conditions used to generate the fluorescence images presented in Fig. 8A-D. The level of expression of recombinant protein in stable transfectants incubated in the absence of $CdCl_2$ was found to be 7.5× that of the endogenous protein (data not shown); this increased a further 6× in cells incubated in the presence of 20 μM $CdCl_2$ and 80 μM $ZnCl_2$ (Fig. 8E). Thus, the cells presented in Fig. 8D express GFP-tagged TGN38 at a level corresponding to approximately 45× that of the endogenous protein in the same cells. This is a significantly greater increase in expression than the ~10× increase which we have previously shown to lead to fragmentation of the TGN when ratTGN38 is expressed in COS cells (Reaves

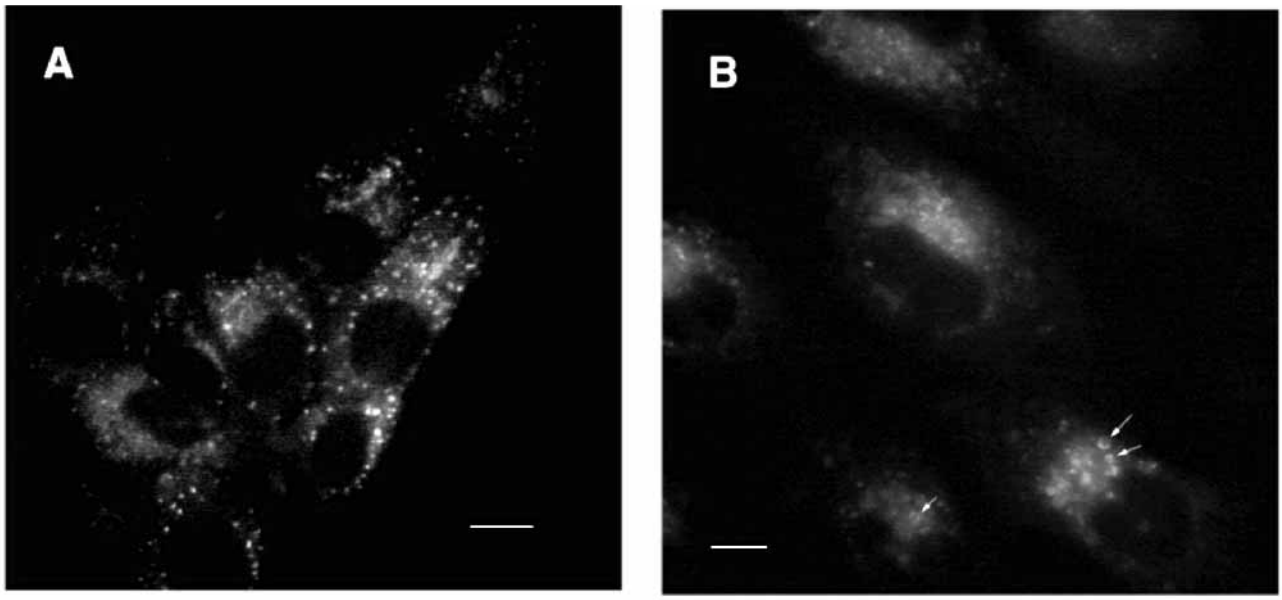


Fig. 6. Stably transfected NRK cells expressing GFP-TGN38 were incubated in the presence of nocodazole 20 μg/ml (A) or chloroquine 200 μM (B) for one hour prior to image capture. Arrows indicate 'doughnut-like' structures in chloroquine treated cells. Bar, 10 μm.

and Banting, 1994a). Thus, in contrast to the situation in monkey (COS) cells, elevated expression of ratTGN38 in rat (NRK) cells does not appear to disrupt the organisation of the TGN. We considered it possible, although unlikely, that the presence of the GFP tag (at either end of ratTGN38) was in some way preventing fragmentation of the TGN at elevated levels of expression of the recombinant protein and therefore chose to express GFP-tagged ratTGN38 in stably transfected COS cells. Stable transfectants were selected, transferred to coverslips and incubated in increasing concentrations of $CdCl_2$ prior to image acquisition. Elevated expression of GFP-tagged ratTGN38 in COS cells led to a fragmentation of the TGN (Fig. 9) as previously described for the elevated expression of ratTGN38 in COS cells (Reaves and Banting, 1994a).

A direct corollary of these observations is that elevated expression of the monkey orthologue of ratTGN38 in rat (NRK) cells would lead to fragmentation of the TGN in a manner analogous to the fragmentation of the TGN which occurs in monkey (COS) cells expressing elevated levels of ratTGN38. It was not possible to perform this experiment precisely since: (i) the only monkey species from which the ratTGN38 orthologue has been molecularly cloned is the macaque (Ponnambalam et al., 1996) whilst COS cells are derived from the African Green Monkey; and (ii) no antibodies have been shown to detect either the macaque or COS orthologue of ratTGN38. We therefore chose to express humTGN46 (the human orthologue of ratTGN38; for which a species specific polyclonal antibody is available; Ponnambalam et al., 1996) in NRK cells, arguing that expression of any primate orthologue of ratTGN38 in a rat cell would be analogous to expression of any other primate orthologue of ratTGN38 in the same background. A cDNA encoding full-length humTGN46 was therefore sub-cloned into ΔpMEP4 and the resulting construct used to transfect NRK cells (see Materials and Methods). Stable transfectants were selected, transferred to coverslips and incubated in increasing concentrations of $CdCl_2$ prior to processing for immunofluorescence analysis using a polyclonal antibody to humTGN46 and an FITC-conjugated secondary antibody (Fig. 10). The elevated expression of humTGN46 induced by $CdCl_2$ can be seen to lead to a fragmentation of the TGN, as previously described for the elevated expression of ratTGN38 in COS cells (Reaves and Banting, 1994a). In contrast, elevated expression of humTGN46 in human (HeLa) cells has no gross morphological effect on the TGN (S. Ponnambalam, personal communication).

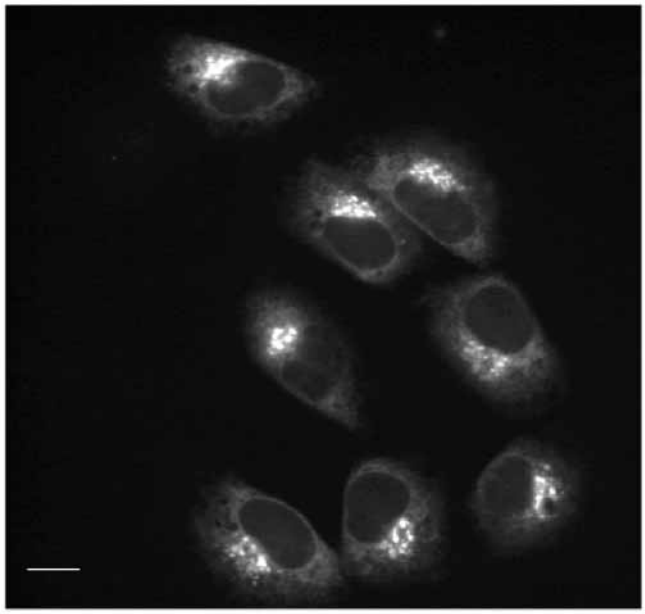
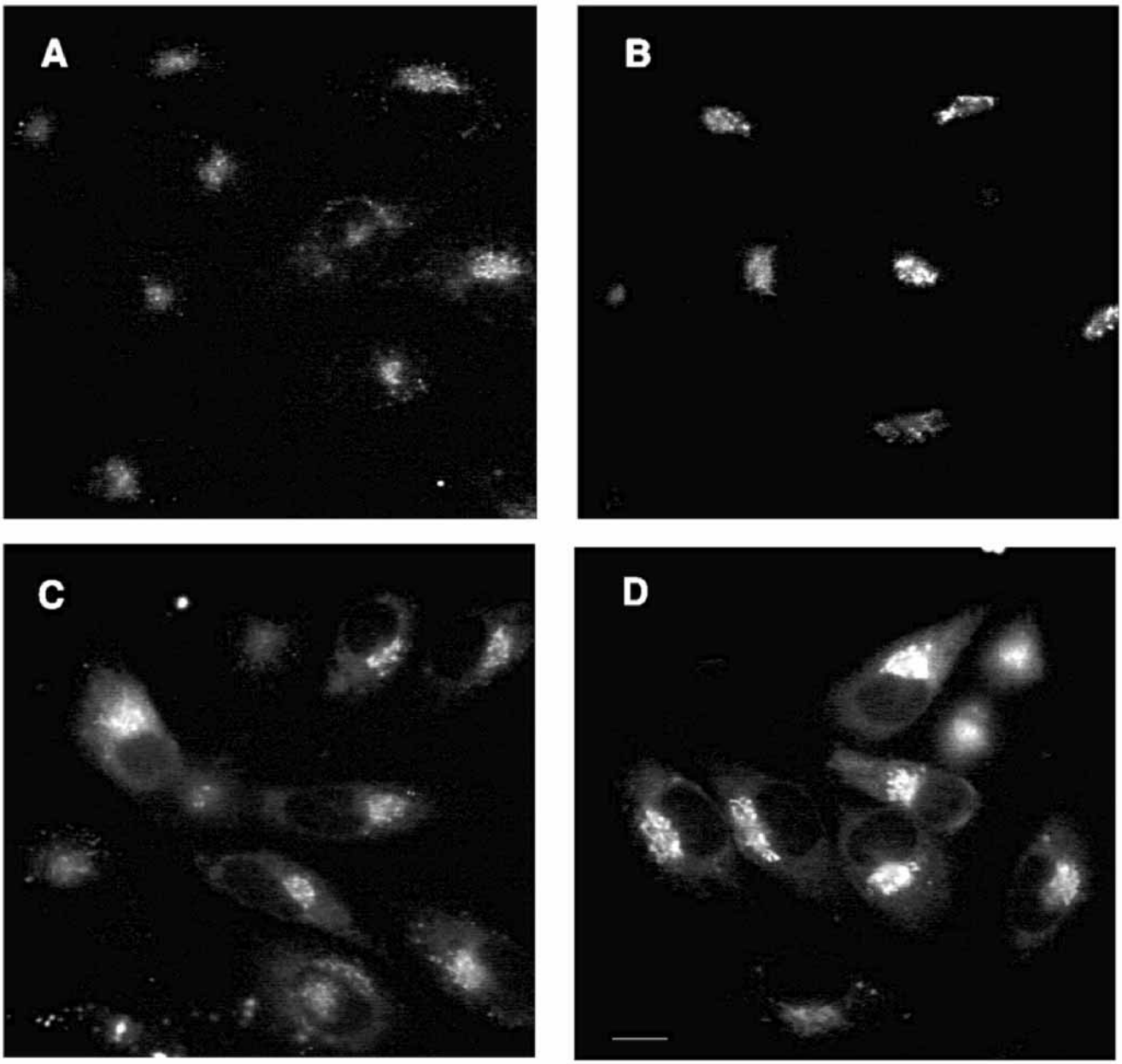


Fig. 7. Stably transfected NRK cells expressing GFP-TGN38 were incubated at 15°C for one hour prior to image capture. Bar, 10 μm.

The intrinsic fluorescence of GFP is retained after fixation

The fact that stably transfected NRK cells are capable of



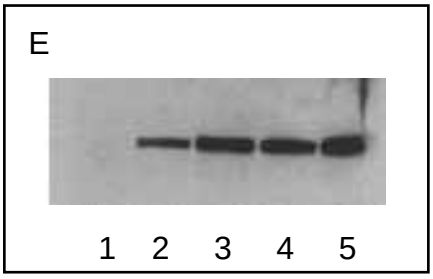


Fig. 8. Stably transfected NRK cells expressing GFP-TGN38 were incubated in the presence of increasing amounts of $CdCl_2$ (0 μM=A, 5 μM=B, 10 μM=C, or 20 μM $CdCl_2$ + 80 μM $ZnCl_2$=D) for 16 hours prior to image capture. (E) The results of immunoblot analysis (performed as described in Materials and Methods using a rabbit polyclonal antibody to GFP as primary antibody) of whole cell lysate prepared from non transfected NRK cells (lane 1) and stably transfected NRK cells incubated in the presence of increasing amounts of $CdCl_2$ (0 μM=2, 5 μM=3, 10 μM=4, or 20 μM $CdCl_2$ + 80 μM $ZnCl_2$=5) for 16 hours prior to lysate production. Bar, 10 μm.

expressing high levels of GFP-tagged ratTGN38 without deleterious effects to the morphology of the TGN is of benefit since it allows co-localisation experiments to be performed in fixed cells, comparing the intrinsic fluorescence pattern generated by GFP with that produced by conventional immunofluorescence labelling of a specific protein. Cells expressing elevated levels of GFP-tagged TGN38 were processed for dual label immunofluorescence analysis using antibodies to: (i) γ-adaptin (detected with a TRITC-conjugated secondary antibody)(Fig. 11A); and (ii) GFP (detected using a FITC-conjugated secondary antibody)(Fig. 11B). As expected, considerable co-localisation is observed. Similar, dual label

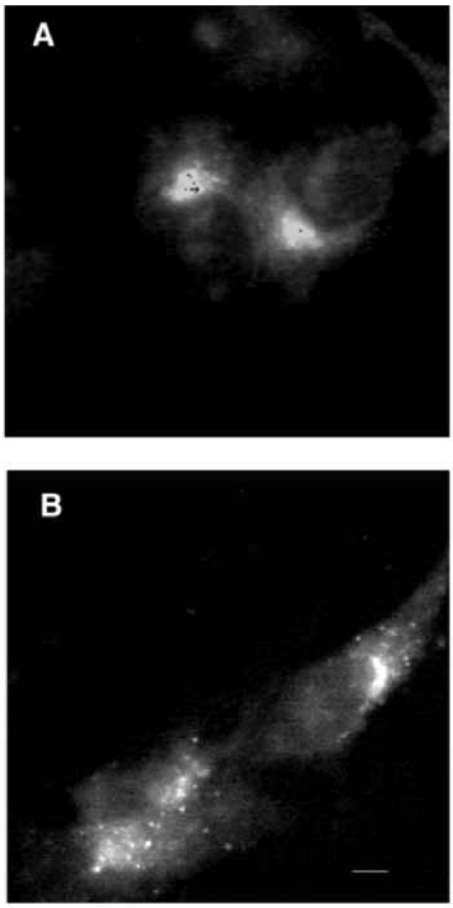


Fig. 9. Stably transfected COS cells expressing GFP-TGN38 were incubated in the presence of increasing amounts of $CdCl_2$ (2.5 μM=A, 20 μM=B) for 16 hours prior to image capture. Bar, 10 μm.

immunofluorescence analysis showed co-localisation (at this level of resolution) between GFP-tagged TGN38 and mannosidase II (an enzyme localised to the *cis*-medial cisternae of the Golgi stack (Baron and Garoff, 1990; Fig. 11C,D) as previously described for mannosidase II and endogenous TGN38 in NRK cells (Reaves and Banting, 1992). However, dual labelling is not required for co-localisation studies in cells expressing GFP-tagged TGN38. Cells expressing elevated levels of GFP-tagged TGN38 were processed for immunofluorescence analysis as before, using a monoclonal antibody to mannosidase II. Binding of the monoclonal antibody was detected with a TRITC-conjugated secondary antibody (Fig. 11E), whilst the intrinsic fluorescence of GFP indicated the position of GFP-tagged TGN38 (Fig. 11F) and, once again, demonstrated co-localisation (at this level of resolution) between GFP-tagged TGN38 and mannosidase II. Thus, the fluorescent signal from GFP survives both methanol and paraformaldehyde (Fig. 12) fixation, permitting facile co-localisation experiments to be performed.

DISCUSSION

We have presented data which show the viability of using GFP as an in vivo fluorescent tag for integral membrane proteins in mammalian cells. In rat (NRK) cells which were transiently transfected with expression constructs encoding either N- or C-terminally tagged ratTGN38 the recombinant protein generated a fluorescent signal but, for reasons which are still unclear, was incorrectly localised (Fig. 2). A possible explanation for mislocalisation of the hybrid proteins is that they may have become aggregated and ended up in a degradative compartment. In support of this explanation is the observation that GFP can dimerise under appropriate conditions (Cubitt et al., 1995).

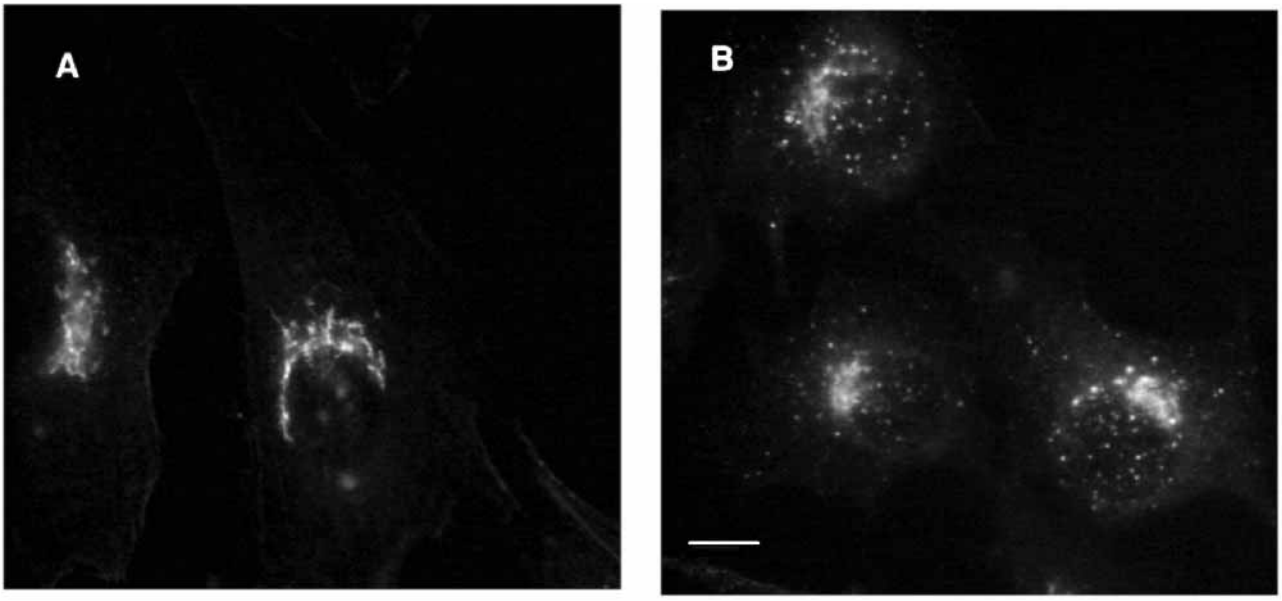


Fig. 10. Stably transfected NRK cells expressing humTGN46 were incubated in the presence of increasing amounts of $CdCl_2$ (2.5 μM=A, 20 μM=B) for 16 hours prior to methanol fixation and processing for immunofluorescence analysis using a rabbit polyclonal antibody to humTGN46 as primary antibody and a species specific TRITC-conjugated secondary antibody as described in Materials and Methods. Bar, 10 μm.

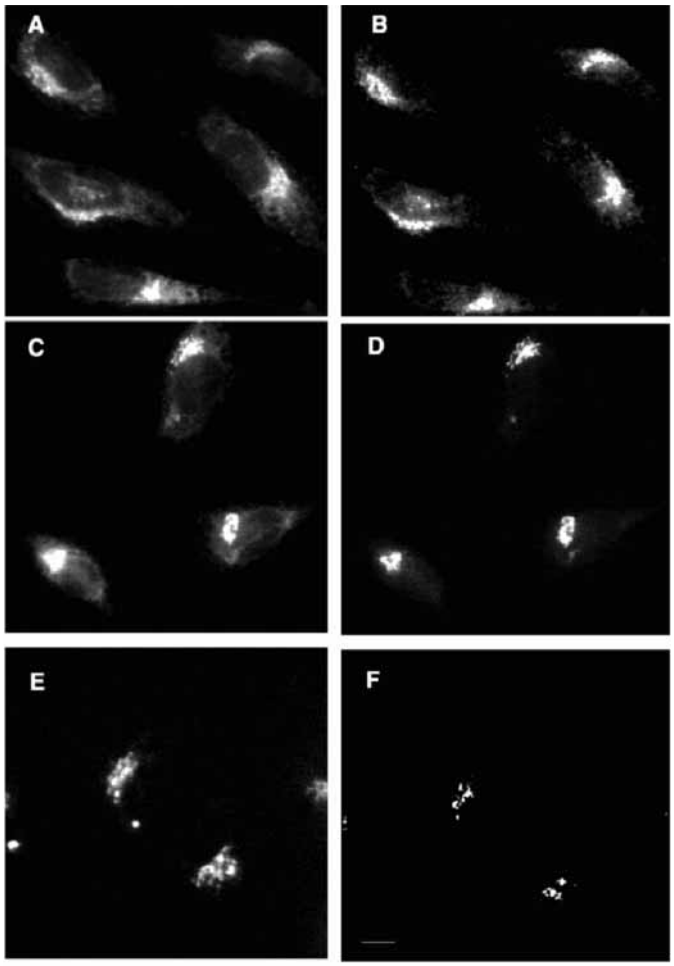


Fig. 11. Stably transfected NRK cells expressing GFP-TGN38 were processed for immunofluorescence analysis following methanol fixation (as described in Materials and Methods) using a polyclonal antibody vs γ-adaptin (A), a polyclonal antibody vs GFP (B and D), a monoclonal antibody vs mannosidase II (C and E), or the intrinsic fluorescence from GFP-tagged TGN38 (F). Binding of primary antibody was detected with a species specific, TRITC-conjugated (A,C,E) or FITC-conjugated (B and D) secondary antibody. Bar, 10 μm.

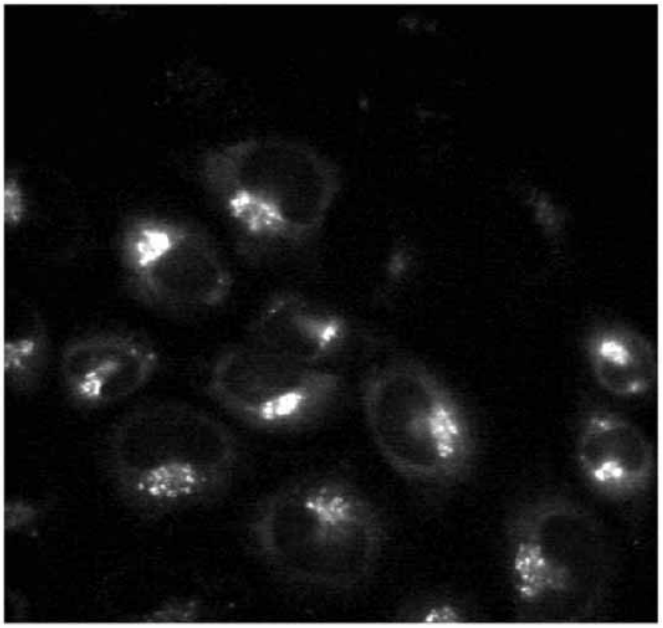


Fig. 12. Stably transfected NRK cells expressing GFP-TGN38 were processed for fluorescence analysis following paraformaldehyde fixation (as described in Materials and Methods) and images acquired using the intrinsic fluorescence from GFP-tagged TGN38. Bar, 10 μm.

Whilst this may not be a problem for soluble or secretory proteins tagged with GFP, it may cause difficulties with similarly tagged, transiently expressed, membrane proteins. Transient transfection generally leads to high level expression in the transfected cells; the high levels of membrane-associated GFP may provide a suitable local environment for dimerisation of the protein, an event which would lead to the dimerisation of the hybrid protein and may well affect its traffic through the secretory pathway, possibly leading to aggregation. Thus, the aggregation and mis-localisation of GFP-tagged ratTGN38 may have arisen by virtue of the elevated expression of the recombinant proteins in the transiently transfected cells. We argued that the generation of stably transfected cells expressing TGN38-GFP or GFP-TGN38 under the control of an inducible promoter would alleviate these potential problems and might allow the appropriate localisation of the hybrid proteins. It did. In stably transfected NRK cells both TGN38-GFP and GFP-TGN38 are expressed, fluoresce in vivo, and are appropriately localised. The latter shown by their response to BFA treatment (Fig. 3). Thus, GFP fluoresces when attached to either side of the TGN membrane. Its attachment to either the N- or C terminus of ratTGN38 does not significantly affect the intracellular localisation of that protein; indeed it provides a suitable label for the in vivo monitoring of TGN membrane dynamics. This is borne out by a study of the time course of the BFA induced collapse of the TGN upon the MTOC (Fig. 4); effects of BFA are discernible after two minutes, and are essentially complete after only five minutes. This is notably faster than previously observed in immunofluorescence analysis of BFA-treated cells (Reaves and Banting, 1992) and demonstrates one of the advantages of an in vivo reporter as opposed to post-fixation analysis.

We had anticipated that, as was the case with elevated expression of ratTGN38 in stably transfected monkey (COS) cells, elevated expression of GFP tagged ratTGN38 in rat (NRK) cells would lead to fragmentation of the TGN (Reaves and Banting, 1994a); a process which we could monitor in real time by virtue of the intrinsic GFP fluorescence of the hybrid protein. However, even at levels of expression significantly higher than those which had previously led to the fragmentation of the TGN in transfected COS cells, no fragmentation of the TGN occurred in NRK cells expressing GFP-tagged ratTGN38. Control experiments demonstrated that the GFP tag itself was not preventing fragmentation of the TGN (Fig. 9), whilst elevated expression of humTGN46 (the human orthologue of ratTGN38) in rat (NRK) cells did lead to fragmentation of the TGN (Fig. 10). A probable explanation for this observation can be found in recently published data concerning the sequences of TGN38 orthologues (Ponnambalam et al., 1996). It is of note that whilst those sequences responsible for the appropriate localisation (i.e.

the transmembrane and cytosolic domain) of TGN38 are highly conserved between the rodent and primate TGN38 orthologues, the corresponding lumenal domains differ markedly in amino acid sequence (Ponnambalam et al., 1996). In addition, it has been speculated that parallel and/or anti-parallel interactions may occur between these lumenal domains and, in so doing, play a role in the regulation of the architecture of the TGN (Ponnambalam et al., 1996). Thus, expression of ratTGN38 in monkey (COS) cells is the equivalent of expressing an appropriately localised, heavily mutated form of the monkey orthologue in those cells and would be predicted to interfere with interactions between lumenal domains of the endogenous protein (leading to fragmentation of the TGN at elevated levels of expression of the rat protein). No such disruption would be induced by elevated expression of ratTGN38 in a homologous system (i.e. expression of recombinant ratTGN38 in NRK cells) and no fragmentation of the TGN would occur. This is precisely what we have observed. These observations therefore support the hypothesis that part of the role played by TGN38 is in maintenance of the morphology of the TGN.

We also show that the GFP-tagged constructs retain their intrinsic fluorescence after methanol (Fig. 11) or PFA (Fig. 12) fixation, thus facilitating dual label immunofluorescence analysis. In addition, it is of note that after either methanol or paraformaldehyde fixation, there is lower background from the intrinsic fluorescence of GFP than there is from comparable immunofluorescent labelling.

The stable expression of GFP-tagged ratTGN38 in rat (NRK) cells has generated an invaluable tool for the in vivo study of membrane traffic dynamics and organelle morphology within the secretory pathway. The data we present clearly demonstrate that GFP can be expressed in stably transfected mammalian cells when attached to an integral membrane protein, that GFP is fluorescent on both the cytosolic and lumenal sides of intracellular membranes up to and including the TGN, that an inducible expression system allows regulated elevation of the intensity of the fluorescent signal obtained from GFP, and that GFP remains fluorescent after methanol or PFA fixation.

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Note added in proof

The use of GFP as a tool for the study of membrane dynamics was published elsewhere whilst this paper was under review: see **Cole, N. B., Smith, C. L., Sciaky, N., Terasaki, M., Edidin, M. and Lippincott-Schwartz, J.** (1996). Diffusional mobility of Golgi proteins in membranes of living cells. *Science* **273**, 797-801.

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