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# Epitope mapping of two isoforms of a trans Golgi network specific integral membrane protein TGN38/41

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TGN38/41 is an integral membrane protein predominantly located in the trans Golgi network (TGN) of rat (NRK) cells. We have used a cDNA expression system to map the epitopes recognised by a panel of antibodies raised to TGN38/41 as a preliminary step in the accurate identification of the region(s) of the molecule responsible for its correct intracellular location. These studies have confirmed the predicted topology of the molecule, and have identified a region in the cytoplasmic domain which is immunologically (and hence potentially functionally) conserved between species.

Trans Golgi network (TGN); NRK; Brefeldin A (BFA)

## 1. INTRODUCTION

The classical secretory pathway in higher eukaryotic cells involves the passage of newly synthesised proteins through a discrete set of intracellular compartments before sorting and targeting to the appropriate destination. An organelle which plays a major role in the latter stages of this process has been termed the trans-Golgi network (TGN) [1], and has been shown to be an organelle independent of the Golgi stack [2–4]. The TGN also plays a role in the endocytic pathway, since certain receptors recycle between it and the cell surface [5]. Recent experiments have demonstrated that the fungal metabolite brefeldin A (BFA) affects both the secretory and endocytic pathways of many higher eukaryotic cells, causing (i) the contents of the Golgi stacks to redistribute into the endoplasmic reticulum (ER), (ii) the TGN to collapse upon the microtubule organising centre (MTOC), and (iii) endosomes to fuse with the TGN [2,4,6–13]. Several studies on the effects of BFA upon the TGN have relied upon antibodies to the protein TGN38 [4,11,12]. TGN38 is an integral membrane protein predominantly localised to the TGN of normal rat kidney (NRK) cells [14]. Isolation of cDNA clones encoding TGN38 identified a single long open reading frame which would specify a protein with a predicted molecular weight of 38 kDa [14]. Computer-assisted structural prediction analysis suggested that TGN38 would have an extra-cytoplasmic N-terminal domain, a single trans-membrane domain, and a cytoplasmic 'tail' of 33 amino acids [14]. Expression of wild-type rat TGN38 or TGN38 lacking the cytoplasmic 'tail' demonstrated that either (i) overexpression of the wild type molecule or (ii) deletion of the 'tail' is sufficient to cause mis-localisation of the protein, leading to its appearance at the cell surface rather than in the TGN [14]. These data suggested (i) the existence of saturable machinery responsible for the correct localisation of TGN38 and (ii) the presence of a localisation signal in the cytoplasmic tail of TGN38. We have recently isolated cDNA clones encoding an isoform of TGN38 designated TGN41 [15]. The two isoforms are collectively referred to as TGN38/41. The only difference between cDNAs encoding TGN38 and those encoding TGN41 is an eight base pair change, including a five base pair insertion in TGN41, in the region of the cDNA encoding the cytoplasmic tail. This insertion leads to a shift of reading frame, an extension of the 'tail', and a change in sequence at the C-terminus of the protein. Antibodies specific to TGN41 demonstrated that it too is predominantly localised to the TGN. In the course of our studies of TGN38 and TGN41 we, and others, have raised a variety of antibodies to them. We have now used a cDNA expression system to map the epitopes recognised by these antibodies and have used antibodies affinity purified on epitope clones in topological and immunolocalisation studies. Antibodies to defined epitopes will be of significant use in identifying regions of TGN38 and TGN41 involved in determining their faithful intracellular localisation.

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## 2. MATERIALS AND METHODS

### 2.1. *Construction and screening of epitope libraries*

The adaptor cloning strategy used to construct the epitope libraries has been described in detail before [16,17]. The starting material for

library construction was a full-length cDNA clone encoding TGN41 [15] in the plasmid vector pBs (Stratagene). 5 μg of this plasmid DNA was incubated with 10 ng DNAse I (Boehringer) at 20°C. A 'stop' Eppendorf tube was prepared containing 118 μl TE (10 mM Tris, pH 8.0, 1 mM EDTA), 2 μl 500 mM EDTA and 100 μl buffered phenol. 10 μl aliquots of reaction mix were transferred to the 'stop' tube after 0.5, 1, 2, 3, 4 and 5 min, vortexed and centrifuged prior to transferring the upper aqueous phases to new tubes. 10 μl aliquots of each sample were then loaded on a 2% agarose gel containing ethidium bromide and subjected to electrophoresis. The 2 min digestion time was found to produce fragments in the size range 100–1,000 bp; this sample was used for further stages. The digested DNA was then subjected to chloroform/isoamylalcohol extraction and ethanol precipitated.

It is essential for adaptor ligation that the cDNA fragments have blunt ends; these were produced as described previously [16,17]. Adapted fragments were separated on a 2% low melting point agarose gel containing ethidium bromide. Gel slices in the ranges 100–200 bp, 200–400 bp and 400–600 bp were excised under 369 nm light, diluted 10-fold in TE, made to 50 mM NaCl and incubated at 65°C for 5 min to melt the agarose. The tubes were cooled to 37°C and 10 μl of kinased, adapted pGEX-3X (see below) added to each tube together with 0.1 vol. 10× ligase buffer (0.2 M HEPES, pH 7.6, 0.1 M $MgCl_2$, 0.1 M DTT, 6 nM γATP) and 2 μl ligase (1 unit/μl, Boehringer). Tubes were subsequently incubated for 2 h, at 37°C. Ligation products were used to transform *E. coli* strain XL-1 blue (Stratagene) by electroporation using a Bio-Rad Gene Pulser.

Since several of the antisera to be epitope mapped had been raised against β-galactosidase fusion proteins partially encoded by pUEX [18], a different vector was chosen to harbour the epitope libraries. They were constructed in the *Bam*HI site of the bacterial expression vector pGEX-3X (Pharmacia) which allows expression of correctly inserted, in frame, cDNA fragments as glutathione-*S*-transferase fusion proteins. Adapted pGEX-3X DNA was prepared following the procedure previously described for pEX1 DNA [17]. Fusion protein production was induced by growing colonies for 3 h on nitrocellulose filters which had been impregnated with 1 mM isopropylthio-β-D-galactoside (IPTG) Sigma Chemical Company (Poole, Dorset, UK).

### 2.2. *Preparation of double-stranded cDNA template for sequencing*

Plasmid DNA from epitope clones was isolated using the Promega (Promega Corporation, Madison, USA) 'magic mini prep' method following the manufacturer's protocol.

### 2.3. *Double-stranded sequencing of epitope clones*

This was performed using the Sequenase Version 2.0 kit (United States Biochemicals) and [$^{35}$S]dATP following the manufacturer's protocol.

### 2.4. *Immunological techniques*

Immunofluorescence analysis and BFA treatment of cells [4], immunoblot analysis [19] and affinity purification of antisera [20] were performed as previously described.

### 2.5. *Antibodies and cell lines used*

All the antibodies used are rabbit polyclonal antisera. Antisera 1 and 4 have been described previously [14,15]. Antisera 2 and 3 were raised in rabbits (New Zealand White) to a TGN41-β-galactosidase fusion protein produced by a pUEX1 construct. Antiserum 2 was a generous gift from Dr. K. Howell, University of Colorado, USA; antiserum 3 was raised by A. Wilde in this laboratory. Antiserum 5 was raised following immunisation of rabbits (New Zealand White) with the peptide CKRSKVTRRPKASDYQRLNLKL conjugated to thyroglobulin via its amino terminal Cysteine residue and a sulfo-SMCC bifunctional cross-linker Sigma Chemical Company (Poole, Dorset, UK) using previously published procedures [21]. The peptide was synthesised within the SERC Molecular Recognition Centre, University of Bristol. NRK [22], RF-1 (primary human fibroblast cell line generously provided by Dr. C. Paraskeva, University of Bristol), MDCK [23], LLC-RK$_1$ [24], and COS [25] cells were cultured as previously described [4].

## 3. RESULTS AND DISCUSSION

The epitope mapping strategy employed involves (i) subcloning random, overlapping fragments of appropriate cDNA into a plasmid expression vector to create an epitope library, (ii) expression of the proteins encoded by those random fragments as part of plasmid encoded fusion proteins, (iii) immunoscreening of the expressed protein fragments, (iv) isolation and sequencing of immuno-positive clones, (v) alignment of immuno-positive clones with the original cDNA sequence, (vi) identification of an epitope as the region of sequence shared by all clones immuno-positive with a specific antibody. The identification of epitopes can be further refined by alignment of the sequences of immuno-negative clones. This procedure has been described previously [16,17].

Three epitope libraries were constructed, each expressing a different size range of TGN41 cDNA fragments. The size ranges, 100–200 base pairs, 200–400 base pairs and 400–600 base pairs were used to encompass both linear and conformational epitopes. The three libraries were initially screened with a cocktail of five different antibodies, each of which recognises TGN38 and/or TGN41 (Table I). A total of 230 immuno-positive colonies were identified, picked onto 'master plates' and, in quintuplet, onto fresh nitrocellulose filters for rescreening with each individual antibody. The secondary screen identified 88 individual clones which were grouped according to their immunological reactivity,

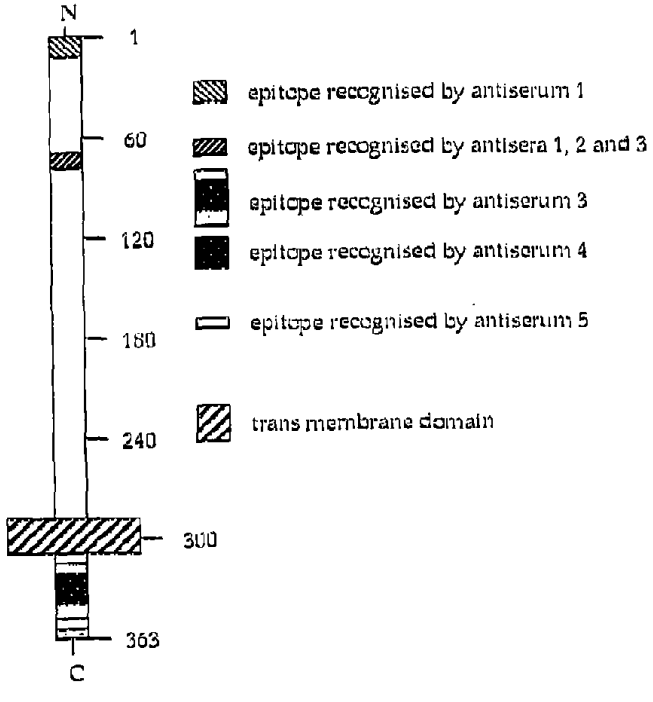


Fig. 1. Diagram of TGN41 illustrating positions of epitopes recognised by antisera 1–5. Numbers refer to amino acids in the mature protein sequence of TGN41, N = amino terminus, C = carboxy terminus.

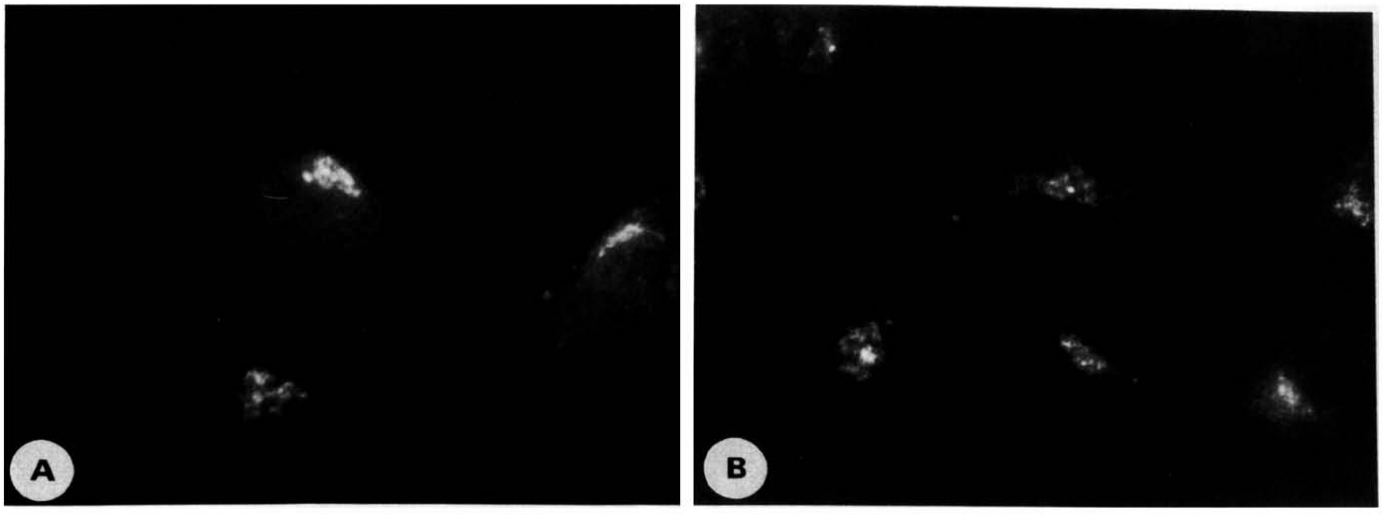


Fig. 2. Immunofluorescence analysis of methanol-fixed NRK cells using antiserum 1 (panel A) or antiserum 1 affinity purified on an epitope clone encoding amino acid residues 1–26 of the mature TGN41 protein (panel B).

i.e. those recognised by specific antisera were placed in different groups as were those recognised by different combinations of antisera. Many clones were positive with more than one antiserum, suggesting that some of the different antisera recognise the same or closely related epitopes.

A selection of clones from each group was then sequenced and the sequences aligned with the cDNA sequence of TGN41. Epitope boundaries were defined by identification of overlapping cDNA sequences from groups of clones immunopositive for each individual antibody. These data revealed that each antiserum recognises specific epitopes (Table I and Fig. 1). Antisera were affinity purified on clones expressing individual epitopes and used in immunofluorescence analysis of methanol-fixed NRK cells to confirm that antibodies recognising these epitopes still show the same pattern of staining as the initial polyclonal antiserum. All the affinity purified antisera gave a typical Golgi staining pattern (Fig. 2).

Table I

TGN41 epitopes

| Antiserum | Immunogen | Epitope(s) recognised amino acids of mature TGN41 |
|---|---|---|
| 1 | TGN38-β-galactosidase fusion protein | 1–13, 69–74 |
| 2 | TGN41-β-galactosidase fusion protein | 69–74 |
| 3 | TGN41-β-galactosidase fusion protein | 69–74, 316-355 |
| 4 | Synthetic peptide CKRSKVTRRPKASDYQRLNLKL | 320–338 |
| 5 | Synthetic peptide CKNLVLPADLFPNQEK | 359–363 |

The published antiserum to TGN38 (1 in Table I and Fig. 1) was raised against a TGN38-β-galactosidase fusion protein [14]. The sequences of several epitope clones recognised only by this antiserum express a region corresponding to amino acids −6 to +26 of TGN41. This defined the epitope recognised by this antiserum as lying between amino acid residues 1 and 26 of the mature protein. However the epitope was further refined by the antiserum's inability to recognise protein expressed by a clone which encodes a fragment of TGN41 beginning at amino acid 13 of the mature protein. Therefore the epitope within TGN38 and TGN41 recognised by this antibody lies within the first 13 amino acid residues of the mature protein. This antiserum is unique in recognising an epitope at the extreme amino terminus of the mature protein. However, it also detects a second epitope (centered around amino acids 69–74 of the mature protein) which is recognised by antisera 2 and 3 (Table I and Fig. 1). Antiserum 1, unlike antisera 2 and 3, is able to recognise TGN38/41 at the cell surface (Reaves, B., Horn, M. and Banting, G., submitted). This ability is presumably due to its unique ability to recognise the extreme amino-terminus of TGN38/41. The fact that antiserum 1 recognises an amino-terminal epitope, coupled with its ability to detect TGN38/41 at the cell surface, confirms the predicted topology of TGN38/41 [14] as a type I integral membrane protein.

An antiserum raised to a TGN41-β-galactosidase fusion protein (5 in Table I and Fig. 1) recognises epitopes in both the lumenal and the cytoplasmic domains of TGN38 and TGN41. The cytoplasmic domain epitope it recognises encompasses that recognised by antiserum 4. The epitope recognised by this anti-peptide antiserum could not be refined further than the sequence of the peptide used as the immunogen. However this antibody revealed a property that was unique among the other

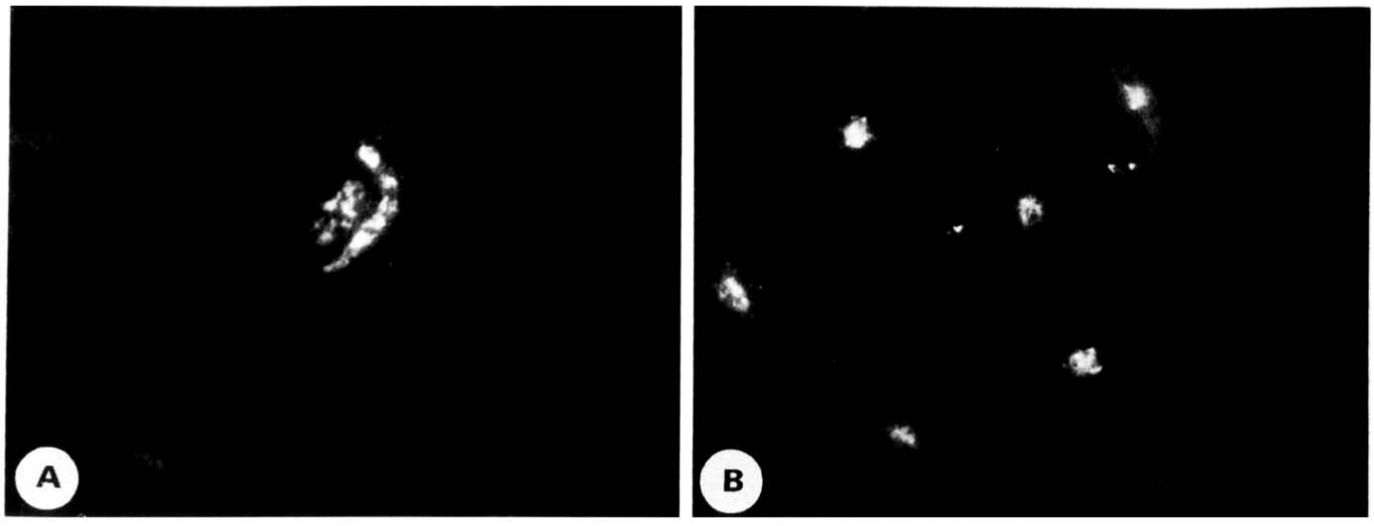


Fig. 3. Immunofluorescence analysis of methanol-fixed human (RF-1) (panel A) and simian (COS) (panel B) cells using antiserum 4 as the primary antibody.

antibodies raised to TGN38/41. It was found to have cross species reactivity (Fig. 3). When used in immunofluorescence studies on human fibroblast (RF-1), canine (MDCK), lupine (LLC-RK$_1$) and on simian (COS) cells a characteristic, juxtanuclear Golgi pattern of staining was observed. The conservation of this epitope may reflect a functional role for this region within the protein.

Antiserum 5 had previously been shown to be specific for TGN41 [15] and on epitope mapping was found to recognise a very well defined epitope. One clone, which expresses only the terminal 5 amino acid residues of TGN41, was weakly recognised by this antiserum. However, it proved impossible to affinity purify antibody on this epitope clone. This would suggest that the 5 carboxy terminal amino acid residues represent only the core of the epitope and that for affinity purification purposes either (i) the whole of the epitope is required or (ii) the sequence prior to this region is required for the correct folding of the epitope.

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