Biochem. J. (1992) **283**, 313–316 Printed in Great Britain

RESEARCH COMMUNICATION

Identification, molecular characterization and immunolocalization of an isoform of the *trans*-Golgi-network (TGN)-specific integral membrane protein TGN38

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TGN38 is an integral membrane protein previously shown to be predominantly localized to the *trans*-Golgi network (TGN) of cells by virtue of a signal contained within its cytoplasmic 'tail' [Luzio, Brake, Banting, Howell, Braghetta & Stanley (1990) Biochem. J. **270**, 97–102]. We now (i) describe the isolation of cDNA clones encoding an isoform of TGN38, (ii) present the sequence of that isoform and (iii) describe the production and use of antibodies which specifically recognize the new isoform. This isoform, designated TGN41, is also predominantly localized to the TGN. The only sequence differences between the protein coding regions of cDNA clones encoding TGN38 and those encoding TGN41 occur within the region specifying the cytoplasmic tails of the two proteins. The TGN localization signal is shown to be within the sequence common to both proteins.

INTRODUCTION

The classical secretory pathway in higher eukaryotic cells involves the passage of newly secreted proteins through a discrete set of intracellular compartments before sorting and targeting to the appropriate membrane. The organelle from which proteins are delivered to the appropriate membrane has been termed the *trans*-Golgi network (TGN) (Griffiths & Simons, 1986) and has been shown to be an organelle independent of the Golgi stack (Doms *et al.*, 1989; Chege & Pfeffer, 1990; Reaves & Banting, 1992). The TGN also plays a role in the endocytic pathway, since certain receptors recycle between it and the cell surface (Stoorvogel *et al.*, 1989). Recent experiments have demonstrated that the fungal metabolite brefeldin A (BFA) affects both the secretory and endocytic pathways of 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 organizing centre (MTOC) and (iii) endosomes to fuse with the TGN (Misumi *et al.*, 1986; Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1990, 1991*a,b*; Donaldson *et al.*, 1990; Hunziker *et al.*, 1991; Orci *et al.*, 1991; Wood *et al.*, 1991; Reaves & Banting, 1992). Several studies on the effects of BFA upon the TGN have relied upon antibodies to the protein TGN38 (Lippincott-Schwartz *et al.*, 1991*b*; Hunziker *et al.*, 1991; Reaves & Banting, 1992). TGN38 is an integral membrane protein predominantly localized to the TGN of normal rat kidney (NRK) cells (Luzio *et al.*, 1990). Isolation of cDNA clones encoding TGN38 identified a single long open reading frame which would specify a protein with a predicted molecular mass of 38 kDa (Luzio *et al.*, 1990). Computer-assisted structural-prediction analysis suggested that TGN38 would have an extra-cytoplasmic *N*-terminal domain, a single transmembrane domain and a cytoplasmic 'tail' of 33 amino acids (Luzio *et al.*, 1990). 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 mislocalization of the protein, leading to its appearance at the cell surface rather than in the TGN (Luzio *et al.*, 1990). These data suggested (i) the existence of saturatable machinery responsible for the correct localization of TGN38 and (ii) the presence of a localization signal in the cytoplasmic tail of TGN38.

Given the growing interest in TGN38 as a marker for the TGN, we decided to study its cytoplasmic tail in more detail; in so doing we have identified an isoform with an 8 bp change, including a 5 bp insertion, in the region of the cDNA encoding the cytoplasmic tail. This insertion leads to a shift to reading frame, an extension of the tail and a change in sequence at the *C*-terminus of the protein. We present the sequence of this isoform of TGN38 and show, using antibodies specific to the new isoform, that it has an intracellular location similar to that of the original TGN38 molecule.

MATERIALS AND METHODS

Materials

Antibodies. The rabbit anti-(rat TGN38) polyclonal antiserum has been described previously (Luzio *et al.*, 1990). The rabbit anti-(rat TGN41) polyclonal antiserum was raised after immunization of rabbits (New Zealand White) with the hexadecapeptide CKNLVLPADLFPNQEK conjugated to thyroglobulin via its *N*-terminal cysteine residue and sulphosuccimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, a bifunctional cross-linker (Sigma Chemical Co., Poole, Dorset, U.K.) using previously published procedures (Green *et al.*, 1982). The peptide was synthesized within the Science and Engineering Research Council (SERC) Molecular Recognition Centre, University of Bristol. Rhodamine-labelled swine anti-rabbit IgG was from Dako (High Wycombe, Bucks., U.K.). Antibodies for use in immunofluorescence analysis were affinity-purified on bacterially

Abbreviations used: BFA, brefeldin A; ER, endoplasmic reticulum; MTOC, microtubule organizing centre; NRK, normal rat kidney; TGN, *trans*-Golgi network.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X64600.

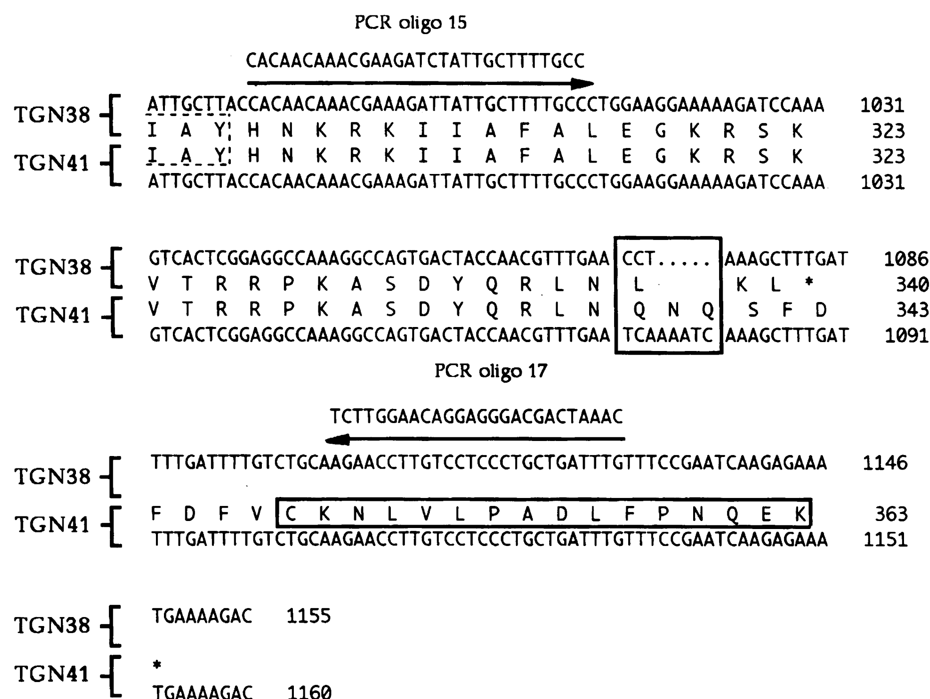

Fig. 1. DNA and protein sequences of TGN38 and TGN41 in the region corresponding to the cytoplasmic tails of the two molecules

The sequences of oligonucleotide primers used in PCR experiments are shown, and the direction in which they prime indicated by arrows above the relevant sequences. The region of DNA sequence which differs between TGN38 and TGN41 is boxed with solid lines, as is the TGN41 peptide sequence used for antibody production. The predicted *C*-terminus of the transmembrane regions of TGN38 and TGN41 is enclosed by broken lines. The asterisks (*) indicate stop codons.

expressed TGN38 and TGN41 fusion proteins as previously described (Brake *et al.*, 1990).

Oligonucleotides. All oligonucleotides were synthesized within the SERC Molecular Recognition Centre, University of Bristol, on a du Pont Coder 300 DNA synthesizer.

Methods

Cell culture, immunofluorescence microscopy (Reaves & Banting, 1992), library screening, hybridization assays and double-stranded cDNA sequencing (Stanley & Luzio, 1984; Brake *et al.*, 1990; Luzio *et al.*, 1990) were performed as previously described. Subcloning (Sambrook *et al.*, 1989) and PCR experiments (Kawasaki, 1990; Saiki, 1990) were performed using standard techniques. Fusion proteins were expressed from pUEX (Bressan & Stanley, 1987) constructs as previously described (Stanley & Luzio, 1984; Luzio *et al.*, 1990).

RESULTS AND DISCUSSION

PCR amplification of the cytoplasmic tail of TGN38

Immunoscreening a plasmid-based cDNA expression library with an antibody raised against rat liver Golgi integral membrane proteins led to the isolation of a group of eight cross-hybridizing clones. The cDNA from the clone with the longest insert was sequenced in its entirety and shown to encode the molecule now designated TGN38 (Luzio *et al.*, 1990). We subsequently sequenced the 5′ and 3′ ends of the remaining seven clones. All of the sequences obtained exactly match regions within the published sequence of TGN38.

Since the results of transient expression studies had suggested that deletion of the cytoplasmic tail of TGN38 led to its mislocalization, we decided to ask whether the cytoplasmic tail itself was sufficient to specify localization of an integral membrane protein to the TGN. In order to address this question we chose to synthesize a cDNA construct which would encode a hybrid protein with the extra-cytoplasmic and transmembrane domains corresponding to a protein normally delivered to the plasma membrane coupled to the cytoplasmic tail of TGN38. The first step in this process was to use PCR to create a cDNA fragment, encoding the cytoplasmic tail of TGN38, with a restriction site at the 5′ end which would allow its subsequent ligation into the rest of the construct. Oligonucleotide primers 15 and 17 (Fig. 1) were used for this purpose. Primer 15 is at the 5′ end of the region encoding the cytoplasmic tail, and introduces a *Bgl*II restriction site into the sequence. Primer 17 is completely within the 3′ untranslated region of the published TGN38 sequence. As template for this PCR reaction we chose to use plasmid DNA from one of the immunoisolated clones described previously (Luzio *et al.*, 1990); however, we did not use DNA from the clone which was initially sequenced in its entirety. The resultant PCR product was subcloned into pBS$^+$ (Pharmacia) and sequenced in both directions. The sequence obtained was identical with the published sequence of TGN38 except for an 8 bp change that includes the insertion of 5 bp just upstream of the 5′ end of primer 17 (Fig. 1). This experiment was repeated and gave the same result. The region between primers 15 and 17 was then sequenced in all of the original immunoisolated clones; six of the eight encompassed this region, and of those six, four contained the published sequence and two the novel sequence. The complete coding region was sequenced from one of the two clones containing the novel tail sequence; no other modification to the published TGN38 sequence was observed. The 5 bp insertion changes the reading frame such that the three *C*-terminal amino acids of TGN38 are not present in the protein encoded by the novel sequence; instead, an extra 23 amino acids are encoded before a stop codon is reached (Fig. 1). The novel cDNA thus encodes a protein with a predicted molecular mass of 40989 Da; we therefore propose to call it 'TGN41'.

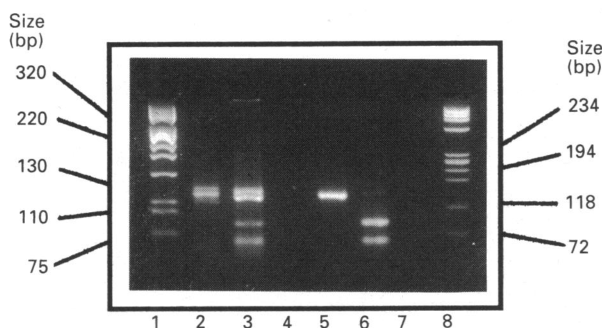

Fig. 2. Agarose-gel (6%) analysis of PCR products from rat epididymal mRNA using primers 15 and 17 (see Fig. 1)

Lanes 1 and 8, DNA size standards; lane 2, PCR products resulting from amplification of rat epididymal mRNA using primers 15 and 17; lane 3, products of *Hin*fI digestion of an aliquot of the material loaded in lane 2; lane 5, product of *Hin*fI digestion of the PCR product resulting from amplification, using primers 15 and 17, of plasmid DNA harbouring an insert encoding TGN38; lane 6, product of *Hin*fI digestion of the PCR product resulting from amplification, using primers 15 and 17, of plasmid DNA harbouring an insert encoding TGN41. Lanes 4 and 7 are 'empty'.

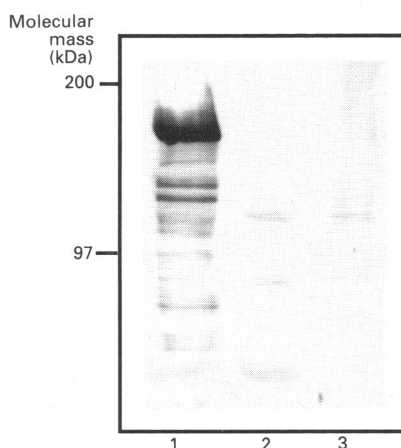

Fig. 3. Immunoblot, probed with antiserum raised against the TGN41 cytoplasmic tail peptide, of fusion proteins isolated from pUEX constructs encoding TGN41 (lane 1), TGN38 (lane 2) or rat asialoglycoprotein receptor (lane 3)

Isolation of further clones encoding TGN41

Further experiments were performed to confirm the existence of mRNA encoding TGN41. The rat liver cDNA library from which the original TGN38 clone was isolated was screened with an oligonucleotide probe corresponding to bases 35–55 of the published TGN38 sequence. In all, 39 independent clones were obtained after several rounds of screening and colony purification. Six of these were sequenced across the region between primers 15 and 17; two possessed the TGN41 sequence and four had the TGN38 sequence. Further support for the existence of mRNA encoding TGN41 came from PCR amplification, using primers 15 and 17, of mRNA isolated from rat epididymis. The TGN38 sequence should give a fragment of 150 bp and the TGN41 sequence a fragment of 155 bp. When the PCR products were analysed by agarose-gel electrophoresis, a diffuse band, corresponding to fragments in the range 150–155 bp, was observed (Fig. 2, lane 2). In control reactions, with no template added, no products were detected. The sequence alteration and 5 bp insertion found in TGN41 introduces a *Hin*fI restriction site not found in TGN38; we chose to digest the products of the mRNA PCR reaction with *Hin*fI before separation on an agarose gel. *Hin*fI digestion should cleave the TGN41 PCR product into two fragments of 90 bp and 65 bp, whereas the TGN38 PCR product should remain intact (150 bp). This is what we observed (Fig. 2, lane 3). Control *Hin*fI digests were performed on PCR products from the amplification, using primers 15 and 17, of plasmid DNAs harbouring inserts encoding TGN38 or TGN41. *Hin*fI failed to cleave the 150 bp TGN38 PCR product (Fig. 2, lane 5), but did cleave the TGN41 PCR product into two fragments of 90 bp and 65 bp (Fig. 2, lane 6). The 150 bp and 155 bp fragments obtained from PCR of rat epididymal mRNA were subsequently gel-purified, subcloned and sequenced. The 150 bp fragment corresponds to the TGN38 sequence and the 155 bp fragment to the TGN41 sequence.

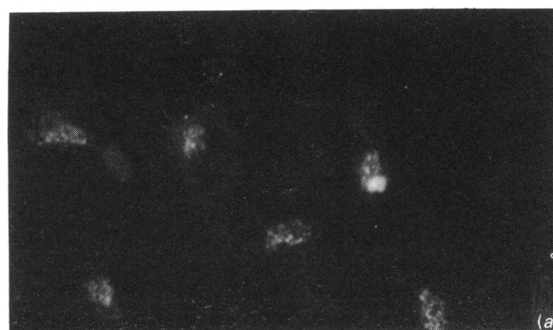

Fig. 4. Immunofluorescence staining of methanol-fixed NRK cells using a 1:1000 dilution of rhodamine-conjugated pig anti-rabbit second antibody to detect binding of (*a*) affinity-purified antibody to TGN41 tail peptide or (*b*) a 1:1000 dilution of antiserum raised against pUEX-encoded TGN38 fusion protein

Note: 1 cm ≡ 11.8 μm.

Antisera specific to TGN41: immunolocalization

Our PCR results implied the presence of an mRNA encoding TGN41; we therefore determined to investigate the existence of the corresponding protein. A polyclonal antiserum was raised in a rabbit to a synthetic hexadecapeptide coresponding to the unique *C*-terminal region of TGN41. This antiserum was used in immunoblot analysis of β-galactosidase fusion proteins encoded by pUEX constructs coding for either TGN38 or TGN41. It specifically recognizes the 160 kDa β-galactosidase–TGN41 fusion protein (Fig. 3). The antiserum was then affinity-purified on the β-galactosidase–TGN41 fusion protein and used in immunofluorescence analysis of methanol-fixed NRK cells, where it showed a perinuclear pattern of staining (Fig. 4*a*) reminiscent of that observed with antisera to TGN38 (Fig. 4*b*) (Luzio *et al.*, 1990; Reaves & Banting, 1992).

Conclusions

We have isolated, from various sources, cDNA clones encoding an isoform of the previously described TGN-specific integral membrane protein TGN38. We have raised antisera which specifically recognized the new isoform, and have immunolocalized it to a perinuclear region in NRK cells. The predicted molecular mass of the protein core of this molecule is approx. 41 kDa, hence the designation TGN41.

The original polyclonal antiserum to TGN38 (Luzio *et al.*, 1990) recognizes extracytoplamic epitopes on TGN38 (A. Wilde, unpublished work). This antiserum therefore detects both TGN38 and TGN41, since the extracytoplasmic domains of TGN38 and TGN41 share the same protein sequence. Thus, since immunoelectron microscopy using the original anti-TGN38 antiserum localized TGN38 to the TGN (Luzio *et al.*, 1990), TGN41 must also reside in the TGN.

It therefore appears that (i) the three *C*-terminal amino acids of the cytoplasmic tail of TGN38 are not required to prevent its appearance at the cell surface, and (ii) the addition of an extra 20 amino acids to the *C*-terminus of TGN38 (to create TGN41) has no gross effect on its intracellular location. The TGN localization signal must therefore be within the sequence common to both TGN38 and TGN41.

We are grateful to Dr. Graham Bloomberg (SERC Molecular Recognition Centre, University of Bristol) for peptide synthesis, Dr. Len Hall and Dr. David Emery (SERC Molecular Recognition Centre, University of Bristol) for oligonucleotide synthesis, Milena Girotti for rat epididymal RNA, and the Agriculture and Food Research Council and the Medical Research Council for financial support.

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Received 3 February 1992/13 February 1992; accepted 18 February 1992