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A Golgi localization signal identified in the Menkes recombinant protein

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Menkes disease arises from a genetic impairment in copper transport. The gene responsible for the phenotype has been identified as a copper transporting ATPase (*ATP7A*). Recently, the protein encoded by the *ATP7A* gene has been localized to the Golgi complex. In order to investigate the role of the Menkes disease protein in copper transport, recombinant constructs containing both the full-length open reading frame and an alternatively spliced form have been successfully expressed and localized in mammalian cells. Other studies of a patient with occipital horn syndrome, an allelic variant of Menkes disease, have demonstrated that only this alternatively spliced isoform and not the full-length form is expressed in this patient. The milder form of this patient's phenotype suggests that the alternatively spliced isoform has some functional role in copper transport. In the present study the full-length recombinant Menkes protein was shown by immunofluorescence to localize to the Golgi apparatus and the alternatively spliced form, lacking sequences for transmembrane domains 3 and 4 encoded by exon 10, was shown to localize to the endoplasmic reticulum. Using sequences from exon 10 fused to a non-Golgi reporter molecule, a 38 amino acid sequence containing transmembrane domain 3 of the Menkes protein was found to be sufficient for localization to the Golgi complex. Therefore, the protein sequence encoded by exon 10 may be responsible for this differential localization and both isoforms may be required for comprehensive transport of copper within the cell.

INTRODUCTION

Menkes disease is an X-linked fatal neurodegenerative disorder of copper transport, the incidence of which has been estimated to be between 1/250 000 and 1/298 000 live births (1), although in some populations it can be as high as 1/40 000 live births (2). The clinical symptoms include severe neurological degeneration, peculiar or kinky hair and focal cerebral and cerebellar degeneration (3,4). An allelic variant of Menkes disease, occipital horn syndrome (OHS) or X-cutis laxa, has also been described, the clinical symptoms of which are milder than those found in Menkes patients (5,6). Mutations giving rise to aberrant splicing of the Menkes gene have been shown to be responsible for the phenotype exhibited by OHS patients.

Menkes disease arises from a defect in the intestinal absorption of copper which leads to a phenotype of copper deficiency (7). The resulting symptoms arise because of a lack of both serum copper and essential copper-requiring enzymes, such as lysyl oxidase and cytochrome *c* oxidase (8–11).

The gene responsible for Menkes disease, *ATP7A*, was isolated in 1993 and encodes a protein with homology to the family of bacterial P-type ATPases (12–14). These are integral membrane proteins that use an aspartyl phosphate to transport cations across the membranes. Deletions, mutations and reduced mRNA synthesis are all evidence that *ATP7A* is responsible for Menkes disease. Studies have shown that the protein encoded by *ATP7A* can deliver copper into the secretory pathway of *Saccharomyces cerevisiae*, thereby confirming its role in copper transport (15). To simplify the terms used in this paper, we will refer to the *ATP7A* gene product as MNK.

More recently, the MNK protein has been localized to the *trans*-Golgi network (TGN) (16–18). Elegant studies with copper-resistant Chinese hamster ovary cells (CHO) suggest that the MNK protein cycles between the TGN and the plasma membrane (16), depending on the concentration of copper within the cell. TGN38 is another protein that has been shown to cycle between the TGN and the plasma membrane (19,20). A number of Golgi-resident proteins contain specific localization signals (21); it is thus feasible that MNK also contains similar signals.

cDNA clones and RT-PCR products that correspond to a frequently seen alternatively spliced variant have been isolated. This variant results in an in-frame deletion of transmembrane domains 3 and 4, arising from skipping of exon 10. This domain may contain a Golgi localization signal and two approaches were initiated to answer this. Firstly, epitope-tagged cDNA clones of the two isoforms were constructed and, secondly the transmembrane domain of the reporter molecule CD8 was replaced with

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sequences from exon 10. Detection of these recombinant proteins was then performed to establish subcellular localization and to test for a possible localization signal encoded by exon 10. The full-length form was shown by immunofluorescence to reside in the Golgi apparatus, whereas the alternatively spliced form was most consistent with a localization to the endoplasmic reticulum (ER). In addition, a 38 amino acid sequence containing transmembrane domain 3 of the Menkes protein may be responsible for Golgi retention of the Menkes protein.

RESULTS

Generation of recombinant constructs

Recombinant constructs expressing both the full-length form and the alternatively spliced form of the *MNK* gene were generated. The cDNA clones were isolated from a human fetal kidney library (12). A small portion of the 3′-end of the open reading frame (ORF) was not contained in these cDNAs and this fragment was obtained by RT-PCR on adult muscle first strand cDNA. The *MNK* recombinant constructs were completed using a partial digestion and ligation strategy in the vector pBluescript (Stratagene) (Materials and Methods and Fig. 1). In order to discriminate between the recombinant protein and the endogenous MNK protein, an epitope tag (MYC) recognized by the monoclonal antibody 9E10 was fused in-frame to the C-terminal end of the construct.

However, on subsequent cloning of these inserts into the mammalian expression vector pCI-NEO (Promega) or pCEP4 (Invitrogen), recombinant clones were found to be deleted or rearranged. Because the clone was stable in pBluescript, it was assumed that the problem arose from either leaky expression in *Escherichia coli* and protein toxicity or instability generated by interaction of sequences between the expression vectors and cDNA sequence. Two alternative strategies were undertaken to overcome this problem. Firstly, intron 3 (~550 bp) from the *MNK* genomic sequence was ligated into the cDNA constructs. This would prevent protein translation beyond intron 3 of the MNK protein in *E.coli*. The protein would still be translated in mammalian cells because of the natural splicing mechanisms, which would eliminate the intronic sequence. However, this did not appear to prevent clone instability and suggested that if the rearrangements were the result of toxicity of the translated product, this product must be 5′ of intron 3, which contained the first copper binding domain. Secondly, the recombinant clones were grown at 30°C post-transformation and in culture and subsequently analysed for sequence integrity. Although many of the clones were found to be deleted and rearranged, clones were isolated which contained the correct sequence in both the full-length and alternatively spliced form under these revised conditions. The transfection studies reported in this paper were performed using the constructs MNKMYC, which contained the full-length ORF, and MNKMYC-E10, which lacked exon 10 but contained intron 3 positioned identically to that seen in the genomic sequence.

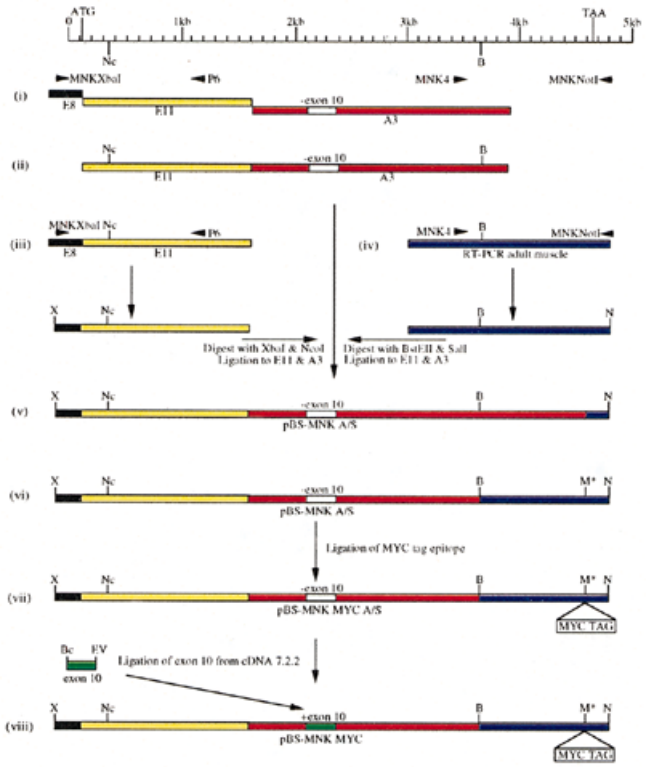

Figure 1. Construction of the recombinant constructs MNKMYC, containing the complete ORF, and MNKMYC-E10, the alternatively spliced form lacking exon 10. The complete ORF for the *MNK* gene is shown at the top of the diagram. (i) cDNAs E8, E11 and A3 are represented as black, yellow and red boxes respectively. cDNA A3 has an in-frame deletion of exon 10 and therefore represents the alternatively spliced form. The position of the *MNK* primers MNKXbaI, P6, MNK4 and MNKNotI are also shown. Both MNKMYC and MNKMYC-E10 were constructed using a combined strategy of cDNA ligation and PCR. (ii) cDNAs E11 and A3 were ligated together and cloned into pBS-Bluescript II KS. (iii) The PCR product of the ligation mix containing the cDNAs E8 and E11, using the primers MNKXbaI and MNKP6, was ligated into pGEM-T. (iv) This clone was then restriction digested with *Xba*I and *Nco*I and the resulting insert cloned into the vector containing cDNAs A3 and E11. (v) In order to generate the remaining 3′-sequence, RT-PCR was performed on first strand adult muscle using the primers MNK4 and MNKNotI. The resulting product was cloned into pGEM-T. *Sal*I and *Bst*EII were then used to liberate the insert prior to cloning into pBluescript containing E11, A3 and the 5′ PCR product (iv). (vi) The MYC epitope (EEQKLISEEDLLRKRRE) was inserted into the construct at an engineered *Mfe*I site at the C-terminus. (vii) Insertion of the tag was achieved by annealing together the primers MfeIMYCF and MfeIMYCR, which encoded the MYC epitope, followed by a stop codon and flanking *Mfe*I ends. (viii) Exon 10 was liberated from cDNA clone 7.2.2 using *Bcl*I and *Eco*RV and ligated into pCI-MNKMYC-E10 to give the full-length clone pCI-MNKMYC. Nc, *Nco*I; B, *Bst*EII; X, *Xba*I; N, *Not*I; Bc, *Bcl*I; EV, *Eco*RV; M*, engineered *Mfe*I site.

Protein localization of MNKMYC and MNKMYC-E10

Both forms, MNKMYC and MNKMYC-E10, were transfected into three different cell lines, COS-7 (SV40-transformed monkey fibroblasts), MRC5/V2 (SV40-transformed human fibroblasts) and HT1080 (naturally immortal human fibroblasts), using a cationic lipid transfection reagent, Superfect. Cells were left for periods of 24 and 48 h after transfection with Superfect prior to fixation with methanol. To confirm that the expressed proteins were of the expected size, lysates from the transfected cell lines were separated on a SDS–polyacrylamide gel and detected by western blotting using the 9E10 antibody and a secondary HRP-conjugated anti-mouse antibody (Fig. 2). A band of ~180 kDa is seen in both the alternatively spliced (lane 1) and the full-length tracks (lane 2), which is similar to the value expected

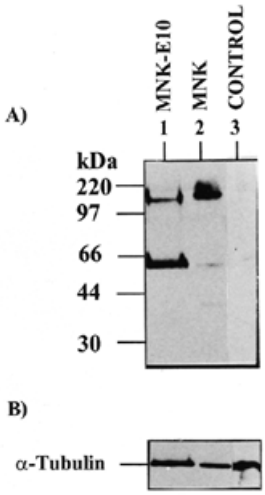

Figure 2. Western blot analysis of the protein products expressed by the full-length and alternatively spliced constructs. Whole cell extracts of untransfected and transfected MRC5/V2 cells were probed with either (**A**) mouse monoclonal antibody 9E10 or (**B**) anti-mouse α-tubulin followed by a goat anti-mouse HRP conjugate (Bio-Rad) and visualized using the ECL detection system (Amersham International). Lane 1, MNKMYC-E10; lane 2, MNKMYC; lane 3, MRC5/V2 untransfected control.

for the predicted size of the protein. The alternatively spliced product runs slightly faster than the full-length product, which is explained by the estimated 8 kDa difference between the two proteins. There is also a second band at ~66 kDa observed in both the full-length and alternatively spliced products. The proportion of the 66 to 180 kDa band is less in cells transfected with the full-length construct than that of the alternatively spliced form. An even fainter band representing a protein of ~46 kDa is seen only in MNKMYC (Fig. 2, lane 2). Although the lack of any signal in the control untransfected samples shows that all the signals observed in the transfected samples are specific for the Menkes protein, the nature of the smaller bands are difficult to interpret.

To detect the cellular localization of the two MYC-tagged MNK recombinant proteins, transiently transfected cells were fixed and visualized by indirect immunofluorescence using confocal laser microscopy. Localization patterns of expressed recombinant MNK isoforms were compared with the localization signals of a well-characterized human TGN resident, TGN46 (22,23). Figure 3 shows the results of this detection after transient expression (24 h) of the full-length ORF in MRC5/V2 cells. Figure 3A shows the staining pattern for TGN46 (red), which is visable as a juxtanuclear reticulum. The MNKMYC recombinant protein (green signal) gives a similar pattern, suggesting, as expected from previous studies with the endogenous protein, that it also resides in the Golgi apparatus (Fig. 3B). Superimposition of the two staining patterns (from Fig. 3A and B) reveals overlap between TGN46 and the MNKMYC proteins (Fig. 3C). However, transient transfection of the same cell lines with the construct lacking exon 10 shows a different cellular localization pattern (Fig. 4). The alternatively spliced MNK protein appears to be present in an extensive reticular pattern (Fig. 4B) characteristic of the endoplasmic reticulum (ER). Superimposition of the staining pattern for the ER marker calnexin (Fig. 4A) with MNKMYC-E10 reveals significant overlap (Fig. 4C). Nuclear staining is also observed with the anti-calnexin antibody due to a cross-reaction with the serum. Similar results were found in the COS7 and HT1080 cell lines when transfected with both the MNKMYC and MNKMYC-E10 constructs (data not shown).

Identification of a Golgi localization signal in TM3

To test the hypothesis that sequences in exon 10 may directly affect the localization of Menkes protein, hybrid molecules containing exon 10 sequences and those of the reporter molecule CD8 were analysed. This system has been used successfully in determining signals that localize TGN38 to the TGN (24). The wild-type CD8 protein contains a single transmembrane domain and localizes to the plasma membrane. Therefore, any resultant change in this localization observed in the hybrid molecules would be evidence that sequences in exon 10 contain organelle targeting sequences. The hybrid molecules were constructed as shown in Figure 5. A 38 amino acid sequence containing transmembrane domain 3 (TM3) from exon 10 and a sequence containing transmembrane domain 7 (TM7) or 8 (TM8), for control purposes, from the *ATP7A* gene were generated by PCR on cDNA using the primers TM3F, TM3R, TM7F, TM7R, TM8F and TM8R. Unfortunately, persistant problems with cloning of the TM4 PCR product prevented further analysis of this domain.

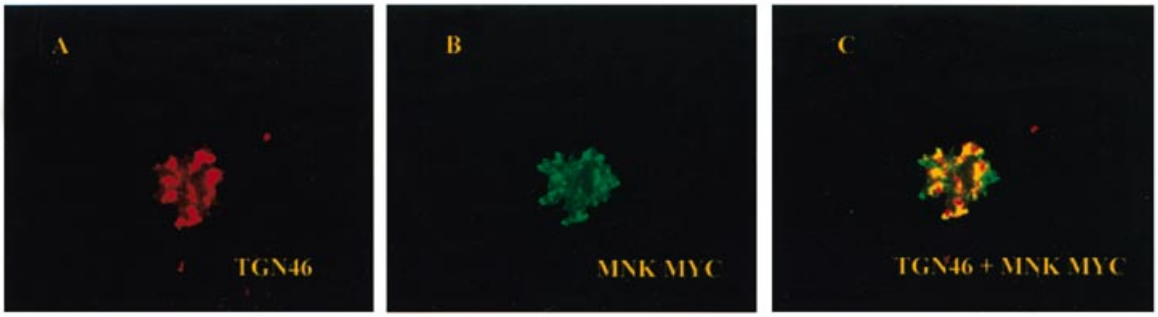

Figure 3. Immunofluorescence studies of the expressed protein from the MNKMYC construct transfected into MRC5/V2 cells. (**A**) The endogenous TGN46 protein was detected using rabbit anti-TGN46 antibody (P12) and anti-rabbit TRITC (tetramethylrhodamine isothyacyanate) secondary antibody (red signal). (**B**) The overexpressed recombinant protein from MNKMYC was detected using the anti-epitope tag (MYC) antibody 9E10 and the anti-mouse FITC (fluorescein isothiocyanate) secondary antibody (green signal). (**C**) The dual localization experiment shows the degree of overlap between the two proteins represented by the yellow signal.

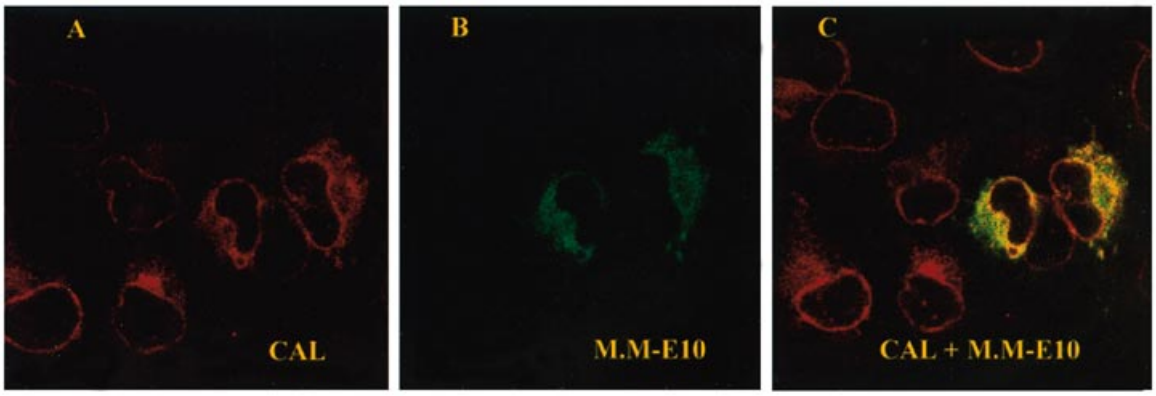

Figure 4. Immunofluorescence studies of the expressed protein from the alternatively spliced construct, MNKMYC-E10, in the MRC5/V2 cell line. (**A**) The endogenous calnexin protein (cal) was detected using the anti-calnexin protein antibody and anti-rabbit TRITC secondary antibody (red signal). (**B**) The overexpressed recombinant protein from MNKMYC-E10 (M.M-E10) was detected using the anti-epitope tag (MYC) antibody 9E10 and the anti-mouse FITC secondary antibody (green signal). (**C**) The dual localization experiment shows overlap between the two proteins represented by the yellow signal.

The transmembrane domain sequences also included flanking amino acids which, in other proteins, have been shown to play an important role in Golgi localization (25,26). Nucleotides containing *Apa*I and *Sal*I restriction sites were added to the forward and reverse primers respectively. The transmembrane domain of wild-type CD8 (CD8WT) was removed by restriction digest with the enzymes *Apa*I and *Sal*I and replaced with either TM3 (CD8TM3), TM7 (CD8TM7) or TM8 (CD8TM8). The resultant constructs were transfected using identical conditions to the MNKMYC constructs and subsequently visualized using monoclonal antibodies specific to the CD8 protein. The results from transfection of these constructs can be seen in Figure 6. CD8WT is primarily localized to the plasma membrane and no co-localization with TGN46 was observed (Fig. 6A–C). However, CD8TM3 showed a staining pattern very similar to that seen with MNKMYC and TGN46 (Fig. 6D and E), suggesting that this CD8TM3 hybrid protein localizes to the Golgi apparatus. This is verified by the overlapping patterning observed with superimposition of TGN46 and CD8TM3 (Fig. 6F). In contrast, an extensive reticular pattern characteristic of the ER is observed with CD8TM7 (Fig. 6H) and no signal is observed in the Golgi complex. This is confirmed by the lack of overlap observed by superimposition of TGN46 (Fig. 6G) and CD8TM7 signals (Fig. 6I). It is possible that either TM7 contains an ER localization signal or perhaps, more likely, that CD8TM7 is a misfolded protein which is retained in the ER. Experiments using TM8 of the MNK protein (CD8TM8) showed localization to the plasma membrane similar to construct CD8WT (data not shown). These results suggest that Golgi localization is therefore mediated by specific sequences within CD8TM3.

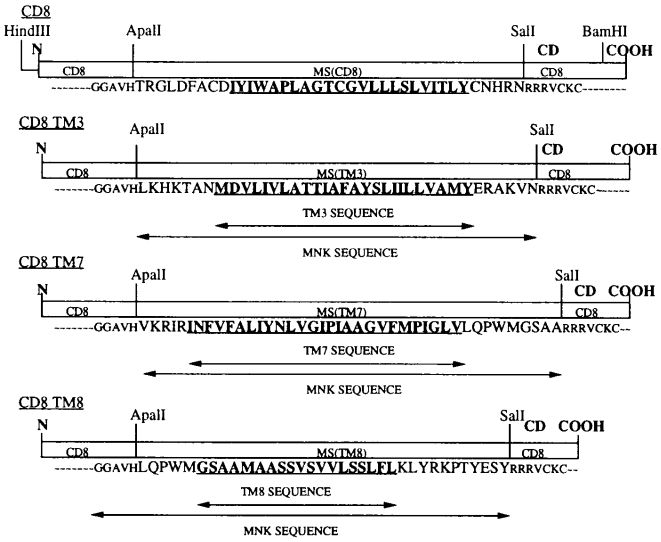

Figure 5. CD8 and the chimeric reporter constructs CD8TM3, CD8TM7 and CD8TM8. The membrane spanning domain of CD8 was excised using *Apa*I/*Sal*I restriction enzyme digestion and sequences containing the transmembrane domain and flanking sequences of TM3, TM7 and TM8 were ligated into CD8 to generate the constructs CD8TM3, CD8TM7 and CD8TM8 respectively. The Menkes amino acid sequence is contained within the *Apa*I and *Sal*I sites and the transmembrane domain is in bold. MS, membrane spanning domain; CD, cytoplasmic domain.

DISCUSSION

Efforts to make full-length cDNA constructs of the *MNK* gene have been hindered by stability problems. Difficulties when cloning other eukaryotic cDNAs in *E.coli* have also been observed and different strategies involving the mutagenesis of cryptic promoter sites and other 'toxic' sequences have been used successfully to overcome some of these problems (27). The stability of the *MNK* cDNA in pBluescript suggested that the instability observed when transferring the Menkes cDNA to a mammalian expression vector arose from a different problem from those described above. Either the instability of the clone was protein based or due to the interaction of nucleotide motifs within the *MNK* sequence and the expression vector. A strategy was successful where alteration of the transformation and culture conditions aided DNA sequence integrity and enabled recombinant clones representing the full-length (MNKMYC) and alternatively spliced variant (MNKMYC-E10) to be constructed.

The recombinant Menkes constructs have been successfully transfected and the proteins expressed in mammalian cell lines. Western blot and indirect immunofluorescence analysis indicate that the protein encoded by the full-length cDNA is a 180 kDa

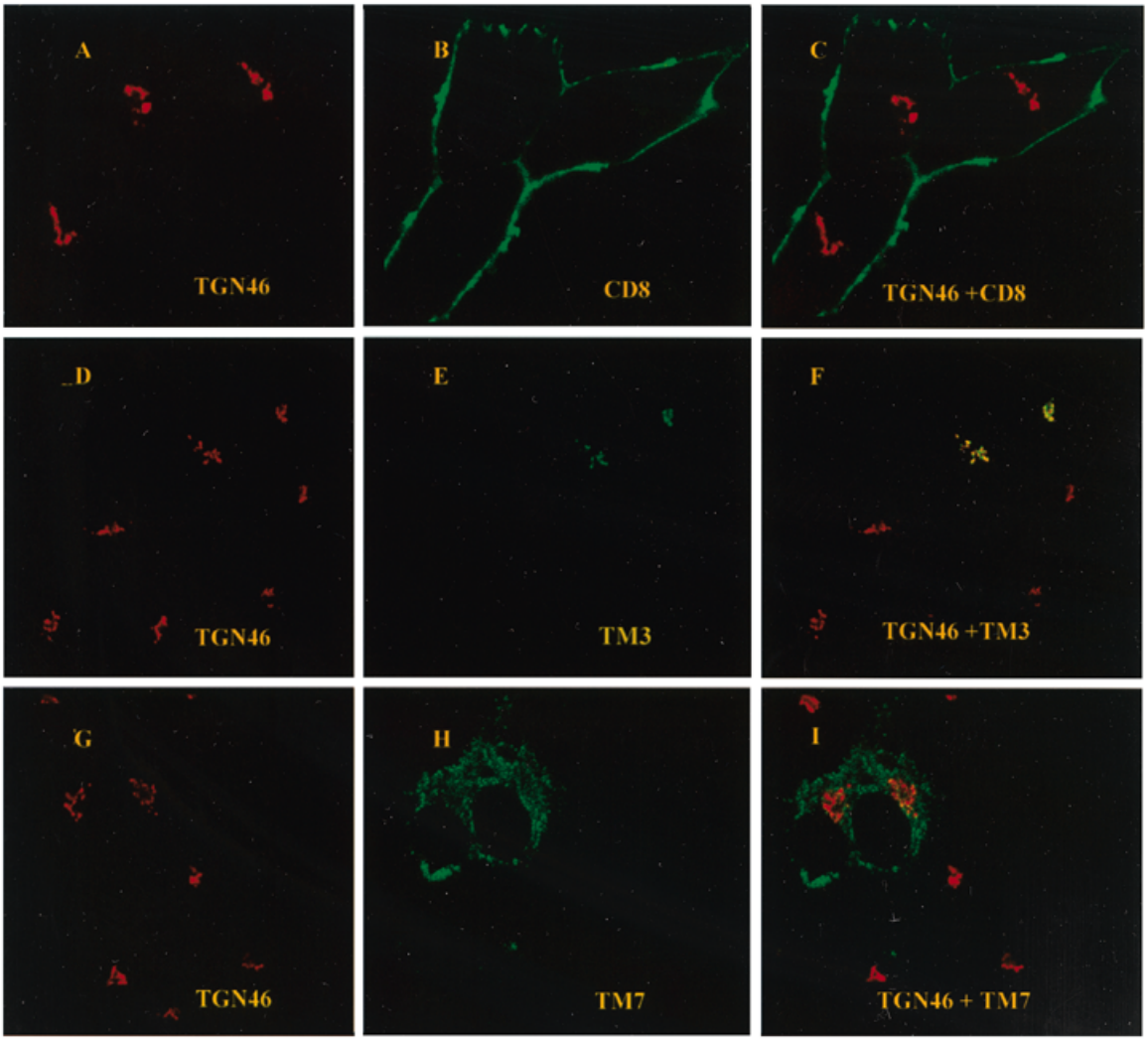

Figure 6. Immunofluorescence studies of CD8WT, CD8TM3 and CD8TM7 in a MRC5/V2 cell line. (**A**, **D** and **G**) TGN46 protein was detected using the TGN46 antibody (P12) and anti-rabbit TRITC secondary antibody (red signal). (**B**, **E** and **H**) The CD8 protein was detected using the anti-CD8 monoclonal antibody (OKT8) and the anti-mouse FITC secondary antibody (green signal). (**C**, **F** and **I**) Superimposition of these images is shown. Transfections were as follows: (A–C) CD8WT; (D–F) CD8TM3; (G–I) CD8TM7.

protein which localizes to the Golgi apparatus. Thus the full-length MNK recombinant protein localizes to a compartment that is morphologically similar to that of the endogenous protein (16–18). The localization of the MNK protein shows overlap with TGN46, a protein known to be resident in the TGN. Analysis of the alternatively spliced *MNK* gene product revealed a completely different intracellular localization. The protein no longer resides in the Golgi apparatus, but is detected in an extensive reticulum characteristic of the mammalian ER. This alternative splice has the effect of removing TM3 and TM4, when based on the current proposed protein model (13). Interestingly, all of the exon–intron boundaries of the *MNK* gene conform to the GT/AG rule except for the 5′ splice site of intron 9, flanking exon 10 (28,29).

The data suggest that a 38 amino acid sequence containing TM3 is sufficient for localization of a recombinant protein to the Golgi apparatus, which is consistent with other studies on the role of transmembrane domains in Golgi retention of other Golgi residents (21). The exact retention mechanism is unclear, although it has been suggested that the membrane spanning domains of Golgi-resident proteins interact and prevent protein incorporation into transport vesicles (30). Both glycosidases and glycosyltransferases that localize to the Golgi complex have been studied extensively and, although they all have a transmembrane domain that anchors them to the Golgi, a common Golgi localization signal is not revealed because of the lack of sequence homology between these domains (31). If MNK is a type II protein, as are almost all other Golgi proteins, then the orientation of TM3 in MNK would be the opposite to that of CD8, a type I protein. Therefore, in agreement with studies with the sialyltransferase transmembrane domain, the orientation of the domain is not critical for localization of the protein to the Golgi (32). Further studies involving site-directed mutagenesis of sequences in and flanking TM3 will aid the understanding of mechanisms of Golgi retention.

Differential cellular localization, resulting from alternative splicing, has been observed in other proteins, supporting the view

that alternative splicing has a role in both the localization and the function of some proteins (33–35). Exon 8 containing TM3 and TM4 of the Wilson disease gene (*ATP7B*) has also been shown to be alternatively spliced (36) and these differentially spliced products may provide functional isoforms of both the Menkes and Wilson genes. However, it is formally possible that the alternatively spliced isoform of the MNK protein has no functional role and that ER localization is due to misfolding of the alternatively spliced protein. In normal cells, no ER staining pattern, indicative of the alternatively spliced MNK isoform being present, has been reported in normal cells. This may result from low levels of the alternatively spliced mRNA transcript observed by other groups (29). A gene product lacking exon 10 sequences may have a minimal or different function in copper homeostasis. This is supported by studies of a patient with clinical symptoms of OHS who had an in-frame deletion of exon 10 and no exon 10-containing transcripts (37). The MNK protein localization in this patient resembles that of the alternatively spliced form in the present study. The milder phenotype exhibited by this patient, when compared with individuals with Menkes disease, suggests that the alternatively spliced form can function in copper transport, although TGN localization of the full-length form is essential for comprehensive copper transport to occur. It may be important for some copper-requiring enzymes to receive copper at an earlier stage in the secretory pathway, such as in the ER, and, in this way, the alternatively spliced form might play a functional role.

The constructs described in this paper are invaluable tools with which to identify the domains important in intracellular localization and thus allow a more in-depth study of the processes involved in the cellular trafficking of copper. If the MNK protein does transport copper to the plasma membrane, as a detoxifying mechanism, under conditions of increased copper in human cells, the use of these constructs will aid the understanding of how this is achieved.

MATERIALS AND METHODS

Construction of full-length and alternatively spliced products

In order to make constructs containing the full-length ORF and the alternatively spliced version of the *MNK* gene, cDNAs E11, E8 and A3 (26) and an RT-PCR product from adult muscle containing the 3′-end were used in a partial digest ligation strategy and cloned into pBluescript II KS (Stratagene). cDNA A3 lacked exon 10, representing the alternatively spliced form. In order to make the full-length form, exon 10 from cDNA 7.2.2 (a gift from C. Vulpe) was excised with *Bcl*I and *Eco*RV and ligated into E8 and A3. RT-PCR was performed on first strand adult muscle (kindly donated by L. Campbell and D. Blake) using the primers MNK4 (5′-AAC AGG AGC TGG ACA CTG AAA CCT-3′) and MNKNotI (5′-GTT GCG GCC GCA GCA CTC TCC ATG GCC TTT TA-3′) and used as described in Figure 1. An epitope tag (MYC) was incorporated into the C-terminus of the cDNA by engineering an *Mfe*I site at the C-terminus of the RT-PCR product. The *Mfe*I site was created by site-directed mutagenesis using the Transformer Site Directed Mutagenesis kit (Clontech) and the epitope tag was made by ligating the primers MfeIMYCF and MfeIMYCR together, which contained a stop codon and flanking *Mfe*I ends (MfeIMYCF, 5′-AAT TGA GGA GCA AAA GCT CAT TTC TGA AGA GGA CTT GTT GCG GAA ACG ACG AGA ATG A-3′; MfeIMYCR, 5′-AAT TTC ATT CTC GTC GTT TCC GCA ACA AGT CCT CTT CAG AAA TGA GCT TTT GCT CCT C-3′). The primers were phosphorylated using T4 polynucleotide kinase prior to annealing and ligation into the *Mfe*I sites. The MNK4/NotI fragment containing the in-frame insertion of the epitope tag was then used to replace the MNK4/NotI fragment in the alternatively spliced and full-length constructs. The full-length and alternatively spliced inserts could be excised using *Not*I digestion. These were then subcloned into the expression vector pCI-Neo (Promega) or pCEP4 (Invitrogen) and transformed into XL-1 Blue competent cells (Stratagene) and left to grow at 30°C for 48–60 h. Recombinant clones were then isolated by direct PCR of colonies using gene- and vector-specific primers to confirm orientation. DNA was prepared from positive clones (Qiagen). However, because of poor yields, a modified version of the Qiagen DNA maxiprep protocol was used. A single colony was used to inoculate 5 ml LB medium/ampicillin and left for 16–20 h at 30°C in a shaker. An aliquot of 1 ml of this culture was then used to inoculate 1500 ml LB medium which was left shaking at 30°C for 16–20 h. The lysis and neutralization buffers were increased 2-fold and the supernatant was purified using two maxiprep columns. The DNA was cleaned using 3- to 5-fold the recommended wash buffer. The DNA yield was ~150–200 µg/1.5 l culture medium. The resultant clones were verified by automated sequencing. Other primers used in the construction included MNKXbaI (5′-TAG TCT AGA GAA ACC AGG AAT GTA ATG AGG A-3′) and MNKP6 (5′-GCT GTT GAA TCA TTG G-3′)

Construction of CD8 chimeric reporter molecules

CD8 hybrid molecules were constructed using a three way ligation strategy. This involved: (i) a *Hin*dIII–*Apa*I fragment containing the lumenal domain of the CD8 molecule; (ii) an *Apa*I–*Sal*I fragment containing either TM3 or TM7 of the *ATP7A* gene and (iii) a *Hin*dIII–*Bam*HI fragment containing the pCMUIV vector. The transmembrane domain sequences were generated by PCR using the primers flanking the transmembrane domain sequence of interest, TM3F (5′-GTG CAC CTG AAG CAT AAG ACA GCA-3′), TM3R (5′-GTC GAC GGT TCA CTT TGG CTC TCT CAT-3′), TM7F (5′-GTG CAC GTC AAG AGG ATT CGG ATA AAT TTT G-3′), TM7R (5′-GTC GAC GTG CTG CAG ATC CCA TCC AGG GCT G-3′), TM8F (5′-GTG CAC TTG CAG CCC TGG ATG GGA TCT GCA GCA-3′) and TM8R (5′-GTC GAC GAT AAC TCT CGT AAG TTG GTT TCC TG-3′). Each forward primer had an *Apa*I site added to the 5′-end and each reverse primer had a *Sal*I site added to the 5′-end. The PCR products were subcloned into pGEM-T and verified by automated sequencing. TM3 and TM7 were excised by restriction digestion with the enzymes *Apa*I and *Sal*I and ligated to the *Hin*dIII–*Apa*I fragment containing the lumenal domain of the CD8 molecule and the *Hin*dIII–*Bam*HI fragment containing the pCMUIV vector. Transformations were carried out with XL-1 Blue competent cells and recombinant clones analysed using construct-specific primer pairs. The resultant clones, CD8TM3 and CD8TM7, were verified by automated sequencing.

Immunofluorescence studies

COS-7, MRC5/V2 and HT1080 cell lines were grown on coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin/streptomycin and 2 mM glutamine to 40–60% confluency prior to transfection with

the MNK constructs using 30 µl Superfect (Qiagen). Cells were then left to express for 14–24 h prior to fixing in methanol at –20°C for 5 min and then stored at 4°C in phosphate-buffered saline (PBS). Immunochemical detection took place within 48 h of fixation. Coverslips were incubated in 0.2% bovine serum albumin in PBS for 10 min at room temperature and rinsed with PBS, and all antibody dilutions were made in PBS. The primary antibodies used were mouse 9E10 monoclonal antibody (Invitrogen) (1 mg/ml) diluted to 1/1000, rabbit polyclonal antibodies to TGN46 diluted to 1/1500, mouse monoclonal antibody (OKT8) to CD8 diluted to 1/100 and rabbit polyclonal antibodies to calnexin diluted to 1/100 (a gift from Hans Dieter Soling, Göttingen, Germany). Secondary antibodies were goat anti-rabbit conjugated to TRITC (1/500) (Sigma) and sheep anti-mouse conjugated to FITC (1/500) (Sigma). Antibody incubations were for 1 h at room temperature followed by a rinse in PBS. After the secondary layer incubation coverslips were rinsed, drained and mounted in Vectorshield antifade (Vector Laboratories) and viewed under a Nikon optiphot with a 60× oil objective. Images were captured using a Bio-Rad MRC 1024 confocal laser microscope and viewed with Lasersharp software (Bio-Rad). To avoid bleed through from the 488 (FITC) to 568 nm (TRITC) channel, sequential capture of signals from each wavelength was performed.

Western blot analysis

Cells were transfected and whole cell extracts of transfected and untransfected MRC5/V2 cells were obtained by scraping the cells into 2 ml phosphate-buffered saline (PBSA) and the mammalian protease inhibitor cocktail Complete™ (PBSAC; Boehringer Mannheim). The cell and PBSAC mix was centrifuged at 13 000 *g* for 5 min and the resultant pellet was resuspended in 50 µl PBSAC. An aliquot of 30 µl Laemmli buffer (Bio-Rad) was added to 15 µl whole cell extract and heated to 95°C for 5 min. The extracts were fractionated on a pre-made 4–15% SDS–polyacrylamide gel (Bio-Rad). Proteins were transferred to a nitrocellulose membrane (Hybond; Amersham International plc) by electroelution and the filter probed with the 9E10 antibody (1/1000) or anti-α-tubulin (1/3000) (Zymed Laboratories) and goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad). The protein was visualized using the ECL detection system according to the manufacturer's instructions (Amersham).

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