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Studies on endocytic mechanisms of the Menkes copper-translocating P-type ATPase (ATP7A; MNK)

Endocytosis of the Menkes protein

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Abstract

The human X-linked recessive copper deficiency disorder, Menkes disease, is caused by mutations in the *ATP7A (MNK)* gene, which encodes a transmembrane copper-transporting P-type ATPase (MNK). The MNK protein is localised to the Golgi apparatus and relocalises to the plasma membrane when copper levels are elevated. Previous studies have identified a C-terminal di-leucine endocytic motif (L1487L1488) in MNK, thought to direct it into the clathrin-mediated endocytic pathway. To determine whether MNK is internalised via clathrin-dependent endocytosis, this pathway was blocked in MNK-overexpressing HeLa cells by the transient expression of dominant negative dynamin and Eps15 mutants. MNK internalisation was not inhibited in such cells. MNK internalisation was inhibited in cells treated with hypertonic sucrose that not only blocked clathrin-mediated endocytosis but also fluid-phase endocytosis. These studies, together with earlier studies on the requirement for L1487L1488, suggest that MNK can utilise both clathrin-dependent and clathrin-independent endocytosis in HeLa cells.

Introduction

Menkes disease is a rare, usually fatal X-linked recessive disorder of copper metabolism. The primary defect is that of malabsorption of dietary copper, leading to symptoms of copper deficiency that include severe neurological problems, connective tissue defects and hypothermia. The reduced activity of several copper-dependent enzymes such as dopamine β-hydroxylase, lysyl oxidase and cytochrome c oxidase contributes to the pleiotropic symptoms (Danks, 1995). The defective gene in Menkes disease, (*ATP7A; MNK*) encodes an integral membrane copper-transporting P-type ATPase (Chelly *et al.*, 1993; Mercer *et al.*, 1993; Vulpe *et al.*, 1993). P-type ATPases translocate cations across membranes using energy derived from ATP hydrolysis. Copper is an essential trace element yet requires tight homeostatic control, as excess copper is toxic. Evidence that the MNK protein is involved in copper efflux has come from our studies on copper-resistant Chinese hamster ovary (CHO-K1) cells that overexpress the hamster MNK homologue and have an increased ability to efflux copper (Camakaris *et al.*, 1995). MNK localisation studies on these cell lines have revealed a novel system of transport protein trafficking whereby the ligand itself directly and specifically stimulates the trafficking of its own transporter (Petris *et al.*, 1996). MNK is predominantly localised to the *trans*-Golgi network (TGN) where it delivers copper to copper-dependent enzymes that are synthesised within the secretory pathway, such as tyrosinase (Petris *et al.*, 2000). Increasing the extracellular copper levels leads to a rapid redistribution of MNK to the cell surface thus enabling excess cop-

per to be removed. MNK recycles back to the TGN after the restoration of basal copper levels and this trafficking of MNK between the TGN and cell surface appears to occur both constitutively and in a copper-regulated fashion (Petris *et al.*, 1996; Petris and Mercer, 1999). An understanding of MNK trafficking in basal and elevated copper conditions is required to elucidate the role of MNK in the delivery of copper to copper-dependent enzymes and in the maintenance of copper homeostasis. The precise mechanisms of MNK copper-regulated trafficking are not yet known. This study focuses on the mechanisms of MNK endocytosis.

Several protein domains and signals involved in MNK trafficking and subcellular localisation have been discovered. A Golgi localisation signal was found in trans-membrane domain 3 (Francis *et al.*, 1998) and one CXXC metal binding site at the N′ terminus is necessary and sufficient for the copper-induced redistribution of MNK from the TGN (Goodyer *et al.*, 1999; Strausak *et al.*, 1999). A di-leucine internalisation motif at the C′ terminus when mutated, led to an accumulation of MNK at the cell surface in basal medium but did not affect copper efflux (Petris *et al.*, 1998; Francis *et al.*, 1999). It was concluded that the MNK protein constitutively cycles between the TGN and plasma membrane, and that its internalisation from the plasma membrane was likely to be clathrin-dependent (Petris and Mercer, 1999).

Di-leucine motifs bind to the clathrin adaptor complexes, AP-1 and AP-2 at the TGN and plasma membrane, respectively (Dietrich *et al.*, 1997). AP-1 and AP-2 couple clathrin cages to cytosolic domains of proteins containing these motifs. Thus, the di-leucine motif has been reported to be involved in the sorting of lysosomal proteins from the TGN via clathrin-coated vesicles (Chen *et al.*, 1993), basolateral sorting in polarised cells (El Nemer *et al.*, 1999; Poyatos *et al.*, 2000), and in clathrin-mediated endocytosis (Corvera *et al.*, 1994; Haft *et al.*, 1994). One method of blocking clathrin-mediated pathways is to treat the cells with hypertonic sucrose medium, causing a reversible loss of membrane-associated clathrin lattices and a subsequent accumulation of abnormal clathrin microcages. This results in a disappearance of clathrin-coated pits at the plasma membrane, clathrin-coated vesicles, and clathrin-coated buds at the TGN (Carpentier *et al.*, 1989; Heuser and Anderson, 1989; Hansen *et al.*, 1993).

More specific methods of blocking clathrin-mediated endocytosis are to utilise dominant negative dynamin and Eps15 mutants. The 100 kDa GTPase, dynamin, plays an essential role in the late stages of endocytic clathrin-coated vesicle formation (van der Bliek *et al.*, 1993; Schmid *et al.*, 1998). The overexpression of mutant dynamin-1 has been reported to block the endocytosis of transferrin in HeLa and COS7 cells (Herskovits *et al.*, 1993; van der Bliek *et al.*, 1993) and to inhibit GLUT4 internalisation in transfected CHO and primary rat adipose cells (Omata *et al.*, 1997; Al-Hasani *et al.*, 1998). To investigate the role of dynamin in MNK endocytosis, we studied the subcellular localisation of MNK in MNK-overexpressing HeLa cells that were transiently transfected with either wildtype or a GTPase defective (K44A) dominant negative mutant of dynamin-1. Recently, two new dominant negative isoforms have been shown to strongly inhibit the internalisation of the M_2 muscarinic acetylcholine receptor and the angiotensin AT_{1A} receptor – processes not inhibited by the expression of K44A dynamin. They are N272 dynamin, which lacks the complete GTP-binding domain, and K535M dynamin, which is not stimulatable by phosphatidylinositol 4, 5-bisphosphate (Werbonat et al., 2000).

Eps15 (EGFR pathway substrate clone 15) is a newly identified component of plasma membrane clathrin-coated pits that is ubiquitously and constitutively bound to AP-2 (Benmerah *et al.*, 1995). Eps15 plays a role in coated pit assembly and more specifically in the docking of AP-2 onto the plasma membrane. Overexpressing dominant negative mutants of Eps15 inhibits the endocytosis of transferrin and causes a loss in the punctate distribution of AP-2, clathrin and dynamin at the plasma membrane, implying the absence of coated pits (Benmerah *et al.*, 1998; Benmerah *et al.*, 1999). Such Eps15 constructs were transiently transfected into MNK-overexpressing HeLa cells to observe the effect on MNK endocytosis.

In the present study we have investigated the effect of blocking coated pit-dependent endocytosis on the internalisation of MNK. Observations of predominant plasma membrane MNK labelling in cells overexpressing MNK with a mutated dileucine internalisation motif led us to believe that MNK would accumulate at the cell surface of cells expressing mutant dynamin or Eps15. However, we observed no significant change in the steady state distribution of MNK in these cells. Hypertonic sucrose treatment, that blocked fluid-phase as well as clathrin-dependent endocytosis, led to an accumulation of MNK at the cell

surface, indicating an endocytic pathway for MNK that is clathrin-independent.

Materials and methods

Isolation of a stably expressing MNK HeLa cell line

Plasmid DNA of wildtype *MNK* cDNA inserted into the mammalian expression vector pCMB77 (Petris *et al.*, 1998) was prepared using midi columns (Qiagen). This DNA was used to stably transfect HeLa cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The resulting cell line, HeLa-5/MNK, was maintained in DMEM medium supplemented with 10% fetal calf serum (Trace Scientific), 4 mM L-glutamine, 830 μM $NaHCO_3$, 20 mM Hepes, 4.5 g/l glucose and 315 μM added $CuCl_2$. On average, 70–80% of HeLa-5/MNK cells expressed transfected human *MNK* cDNA.

Transfection experiments

Transient transfection of HeLa-5/MNK cells was carried out using Lipofectamine 2000 according to manufacturer's instructions and plasmid DNA prepared using Qiagen midi columns. Cells were seeded on glass coverslips 24 hours prior to transfection at a cell density of 6×10^4 cells per well in a 24 well tray and were incubated in the DNA mixture for 24 hours prior to processing for immunofluorescence.

Plasmid constructs

Wild-type (WT) and K44A dynamin-1 in pCB1 mammalian expression vectors were a kind gift from Dr. Sandra Schmid (Scripps Research Institute, CA). N272 and K535M dynamin-1 in pCMV96-7 were kindly supplied by Dr. J.P. Albanesi (University of Texas Southwestern Medical Center, Dallas, Texas) and Dr. van Koppen (Essen, Germany). The control Eps15 isoform (D3Δ2), DIII mutant (Benmerah *et al.*, 1995) and EΔ95/295 mutant (Benmerah *et al.*, 1998), were cloned into the EGFP-C2 expression vector (CLONTECH Laboratories). L1487AL1488A MNK has been previously described (Petris and Mercer, 1999).

Antibodies

Anti-MNK polyclonal rabbit antibodies raised to the MNK N-terminal region (Camakaris *et al.*, 1995) were diluted 1:300 for use in the immunofluorescence studies. Anti-dynamin antibodies (Hudy-1) (Benmerah *et al.*, 1999) were kindly provided by S. Schmid.

Indirect immunofluorescence

Cells were seeded onto 13 mm round glass coverslips 48 hours prior to experimentation. Post-experimentation, coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde/PBS for 15 minutes. Cells were then permeabilised in 0.1% Triton X-100/PBS for 5 minutes, washed with PBS, and blocked overnight at 4 °C in 1% bovine serum albumin (BSA)/PBS. All antibody incubations were for 1 hour in 1% BSA/PBS. Polyclonal MNK antiserum was visualised using either affinity-purified FITC-conjugated sheep anti-rabbit IgG antibodies (Silenus), Alexa-488^{TM} or Alexa-594^{TM}-conjugated goat anti-rabbit IgG antibodies (Molecular Probes). Anti-dynamin antibodies were visualised with Alexa-594^{TM}-conjugated goat anti-mouse IgG antibodies (Molecular Probes). Coverslips were washed overnight in PBS at 4 °C then mounted using 100 mg/ml DABCO [1,4-diazabicyclo-(2.2.2) octane] (Sigma) in 90% glycerol. Confocal microscopy was done on an Olympus BX60 microscope using a 60× PlanApo lens with a 1.40 N/A. This microscope was connected to an Optiscan F900e laser scanning unit with a Krypton/Argon ion laser of excitation wavelengths 488 nm (10 mW/15 mW) and 568 nm (10 mW/15 mW). Typical fluorescein and rhodamine filter sets were used. When collecting x-y serial sections for maximum brightness images the standard step size was 0.351 μm. In all experiments, at least 25 MNK-overexpressing cells were analysed and images shown are representative of what was observed.

Endocytosis controls

Endocytosis of fluorescently labelled transferrin (Tf) was performed on subconfluent HeLa-5/MNK cells grown on coverslips one day after transfection. The cells were first incubated in OPTI-MEM® I (Gibco BRL Life Technologies) for 10 minutes at 37 °C to eliminate receptor-bound Tf and then incubated in OPTI-MEM® I containing either 100 μg/ml FITC-conjugated transferrin (Molecular Probes) or 10 μg/ml Alexa-594^{TM}-conjugated transferrin (Molecular Probes) for 10 minutes. Cells were washed for 2 minutes in ice-cold acidic buffer as described previously (Petris and Mercer, 1999) to remove transferrin

bound to the cell surface. The cells were then fixed and processed for immunofluorescence as described above. For fluid-phase endocytosis, cells were incubated in treatment medium + 0.05% BSA containing 1 mg/ml FITC-dextran (M_r 70,000; lysine fixable; Molecular Probes) for 30 minutes at 37 °C as outlined in the Fig. legends. The cells were then washed for 2 minutes in ice-cold acidic buffer, fixed and processed for indirect immunofluorescence as described above.

Hypertonic medium treatment

HeLa-5/MNK cells were treated with hypertonic medium using a method adapted from a previous study (Heuser and Anderson, 1989). All incubations were at 37 °C. The effect hypertonic medium had on constitutive MNK trafficking was observed by pre-incubating the cells in serum-free medium (SFM) for 10 minutes, then incubating them in 0.45 M sucrose/SFM for a total of 3 hours. The effect on MNK endocytosis after the removal of elevated Cu was seen by pre-incubating the cells in SFM for 10 minutes, SFM + 150 μM Cu for 2 hours, 0.45 M sucrose/SFM + 65 μM Cu for 30 minutes, then in basal 0.45 M sucrose/SFM for 60 minutes. The control was SFM without added sucrose. After the above treatments, cells were washed in PBS then fixed and processed for immunofluorescence.

Results

MNK subcellular location in HeLa-5/MNK cells

Previous studies utilising mutant dynamin and Eps15 were carried out in HeLa cells (Damke *et al.*, 1994; Benmerah *et al.*, 1998; Benmerah *et al.*, 1999), therefore the present study on MNK endocytosis was completed in MNK-overexpressing HeLa cells (HeLa-5/MNK). As predicted from our earlier studies utilising CHO cells (Petris *et al.*, 1996), indirect immunofluorescence using HeLa-5/MNK cells showed a predominant perinuclear localisation of MNK in basal medium and a reversible translocation to the plasma membrane when incubated in increased copper concentrations (data not shown).

Internalisation of MNK is not inhibited by the expression of K44A dominant negative dynamin-1

Di-leucine motifs have been implicated to direct proteins to be internalised into clathrin-coated pits. We

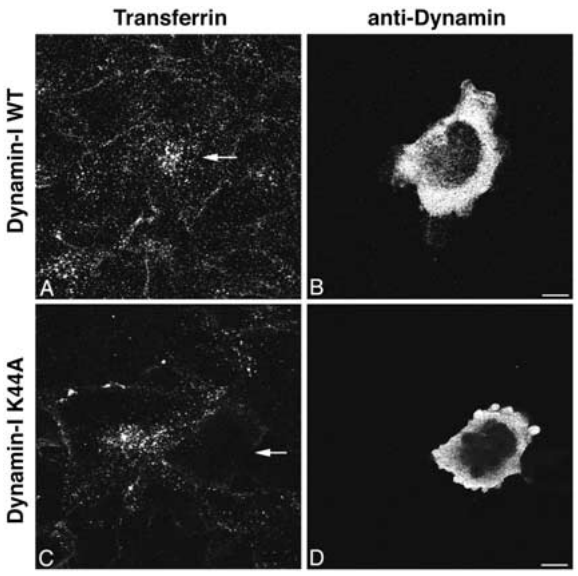

Fig. 1. Expression of K44A dynamin-1 inhibits clathrin-dependent endocytosis in HeLa-5/MNK cells. HeLa-5/MNK cells transiently transfected with wild-type (A, B) or K44A (C, D) dynamin-1 were incubated in the presence of FITC-conjugated transferrin for 10 minutes at 37 °C, then washed and fixed. Dynamin was visualised using monoclonal anti-dynamin antibodies (Hudy-1) followed by Alexa-594^{TM}-conjugated anti-mouse IgG. Arrows represent dynamin-transfected cells. Bars = 10 μm.

therefore hypothesised that MNK is internalised via clathrin-mediated endocytosis. In order to test this, we inhibited clathrin-dependent endocytosis in HeLa-5/MNK by transiently expressing both WT and a dominant negative GTPase deficient mutant form of dynamin-1, K44A (Figures 1, 2).

In control experiments, the expression of K44A dynamin-I significantly inhibited clathrin-mediated endocytosis in HeLa-5/MNK as seen by K44A dynamin-transfected cells exhibiting virtually no uptake of FITC-Tf (a bona fide marker of this kind of endocytosis) (Figure 1C). This contrasted with the endosomal labelling of FITC-Tf as seen in WT dynamin-expressing cells and neighbouring non-transfected cells (Figure 1A).

In order to test whether MNK is constitutively internalised via a dynamin-dependent route, HeLa-5/MNK cells grown in basal medium were fixed and processed for immunofluorescence 24 hours after being transiently transfected with either WT (Figure 2A, B) or K44A (Figure 2C,D) dynamin. No significant difference in the predominant perinuclear localisation of MNK between WT- and K44A-dynamin transfected cells could be seen. No difference was observed when the cells were processed for immunofluo-

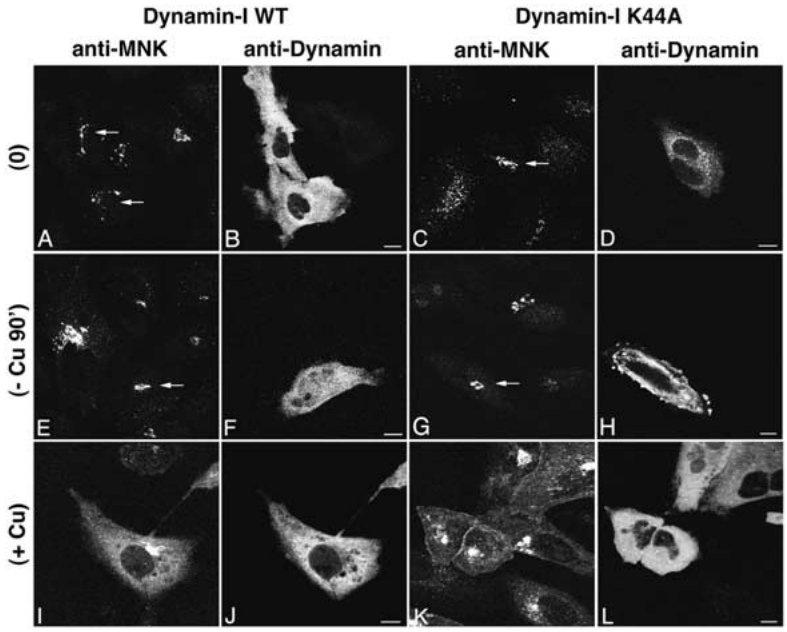

Fig. 2. Internalisation of MNK is not inhibited by the expression of K44A dynamin-1. HeLa-5/MNK cells were transiently transfected with either wild-type (A, B, E, F, I, J) or K44A (C, D, G, H, K, L) dynamin-1 and processed for immunofluorescence analysis after being grown in either basal medium (A–D), in basal medium for 90 minutes after a 2 hour pre-incubation in 630 μM Cu (E–H), or after a 2 hour incubation in 630 μM Cu (I–L). MNK was visualised using anti-MNK antiserum followed by FITC-conjugated anti-rabbit IgG (A, C, E, G, I, K). Dynamin-1 was visualised using monoclonal anti-dynamin antibodies (Hudy-1) and Alexa-594^{TM} -conjugated anti-mouse IgG (B, D, F, H, J, L). Arrows represent TGN labelling. Bars = 10 μm.

rescence 48 hours after transfection (data not shown). In light of the constitutive trafficking of MNK and the presence of the di-leucine internalisation motif, this result was unexpected. An accumulation of MNK was expected at the cell surface of cells in which clathrin-mediated endocytosis had been blocked.

To test the effect of mutant dynamin on MNK endocytosis after the removal of elevated copper, dynamin transfections were carried out on HeLa-5/MNK cells in normal conditions. The transfected cells were incubated for 2 hours in elevated copper and then returned to basal medium for 90 minutes. No inhibition of MNK endocytosis was observed based on there being no significant difference in MNK localisation in WT and K44A dynamin expressing cells (Figure 2E, F, G, H). The same results were achieved when Cu was removed for 30 and 60 minutes (data not shown). These data suggest MNK can be internalised via a dynamin-independent pathway.

As *in vitro* studies have implicated dynamin to be involved in the formation of both clathrin coated and non-clathrin coated vesicles from the TGN (Jones *et al.*, 1998), it was important to confirm that the apparent lack of inhibition of MNK endocytosis was not in fact due to an inhibition of MNK exocytosis from the TGN to the plasma membrane in K44A-expressing cells. Dynamin-I transfected cells were incubated in elevated copper for 2 hours then fixed and processed for immunofluorescence. Plasma membrane labelling of MNK was observed in both WT (Figure 2I, J) and K44A dynamin (Figure 2K, L) expressing cells. It was concluded that the expression of mutant dynamin did not interfere with MNK exocytosis in HeLa cells.

Internalisation of MNK is not inhibited by the expression of N272 nor K535M dominant negative dynamin-1

It has recently been shown that the internalisation of the M_2 muscarinic acetylcholine receptor and the angiotensin AT_{1A} receptor was inhibited by the N272 and K535M dominant negative dynamin-1 isoforms, but not K44A (Werbonat *et al.*, 2000). We investigated the effect of N272 and K353M dynamin on MNK internalisation. HeLa-5/MNK cells were transiently transfected with N272 and K353M dynamin-1, fixed 24 hours after transfection and immunolabelled for MNK and dynamin. Immunofluorescence microscopy showed no difference in MNK localisation between

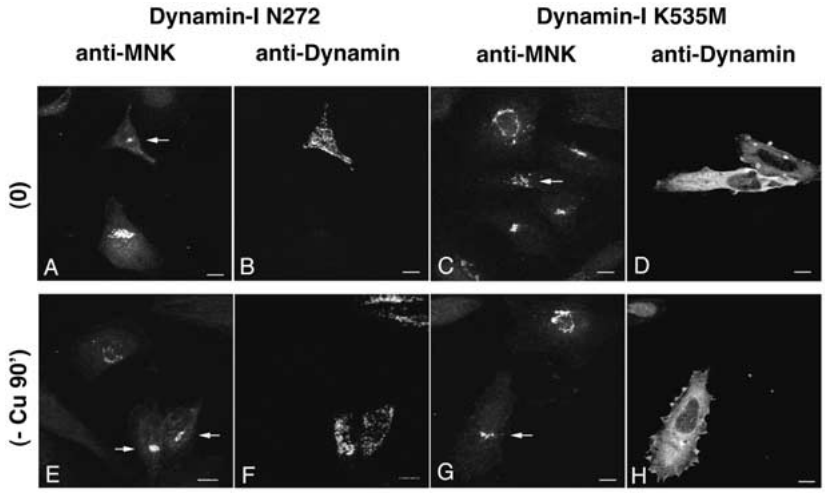

Fig. 3. Internalisation of MNK is not inhibited by the expression of N272 or K535M dynamin-1. HeLa-5/MNK cells were transiently transfected with either N272 (A, B, E, F) or K535M (C, D, G, H) dynamin-1 and processed for immunofluorescence analysis after being grown in either basal medium (A–D), or in basal medium for 90 minutes after a 2 hour pre-incubation in 630 μM Cu (E–H). MNK was visualised using anti-MNK antiserum followed by Alexa-488^{TM} -conjugated anti-rabbit IgG (A, C, E, G). Dynamin-1 was visualised using monoclonal anti-dynamin antibodies (Hudy-1) and Alexa-594^{TM} -conjugated anti-mouse IgG (B, D, F, H). Arrows represent TGN labelling. Bars = 10 μm.

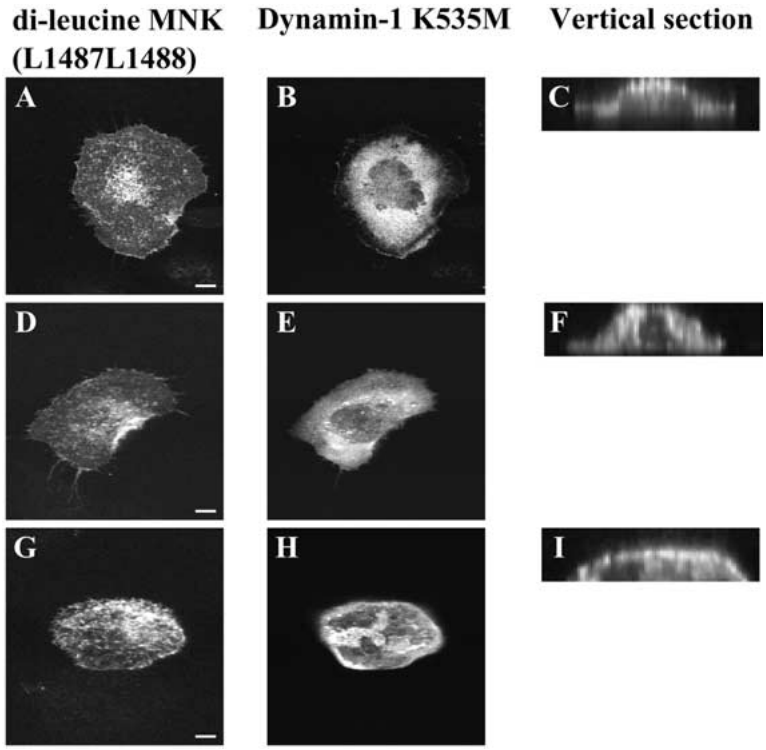

Fig. 4. Localisation of the MNK di- leucine mutant, L1487L1488, is not affected by expression of K535M dominant negative dynamin. Normal Hela cells transiently transfected with MNK dileucine mutant, L1487L1488 to A1487A1488, and the dominant negative mutant dynamin, K535M, were incubated in basal media (A), media supplemented with 630 μM $CuCl_2$ (D) and returned to basal media after pre-incubation in 630 μM $CuCl_2$ (G). Expression of K535M dynamin is shown in corresponding cells (B, E, H) and the XZ vertical sections of each cell (C, F, I) are shown to demonstrate labelling of the plasma membrane region. MNK was visualised using anti-MNK antiserum followed by Alexa-488^{TM} -conjugated anti-rabbit IgG. Dynamin-1 was visualised using monoclonal anti-dynamin antibodies (Hudy-1) and Alexa-594^{TM} -conjugated anti-mouse IgG. Bars = 10 μm.

transfected and untransfected neighbouring cells (Figure 3A–D). It was thus concluded N272 and K535M dynamin did not affect constitutive MNK internalisation. In order to test whether these dynamin isoforms inhibited MNK internalisation after the removal of excess copper, HeLa-5/MNK cells transiently transfected with N272 and K353M dynamin-1 were incubated for 2 hours in elevated copper and then returned to basal medium for 90 minutes. Immunofluorescence analysis again showed no significant difference in MNK localisation between transfected and untransfected cells (Figure 3E–H). All showed a predominant perinuclear localisation. The expression of N272 and K535M dynamin-1 strongly inhibited the uptake of Tf in HeLa-5/MNK cells (data not shown). Collectively, these data suggest that the constitutive and copper-induced internalisation of MNK is dynamin-independent.

Localisation of the MNK di- leucine mutant, L1487L1488, is not affected by expression of K535M dominant negative Dynamin

Previous studies in CHO-K1 have shown that the di-leucine mutant MNK, L1487AL1488A, MNK is predominantly trapped at the plasma membrane in both basal media and in elevated Cu media, and is required for MNK retrieval from the plasma membrane (Petris *et al.*, 1998; Francis *et al.*, 1999). To determine whether blocking clathrin mediated endocytosis has any effect on the plasma membrane localisation of di-leucine mutant MNK we transiently transfected both the L1487AL1488A MNK and K535M dynamin-1 into normal Hela cells. Cells were incubated either in normal basal media, media supplemented with 630 μM $CuCl_2$ for 120 minutes, or returned to basal media for 90 minutes after pre-incubation in 630 μM copper for 120 minutes. In all cases the di-leucine mutant MNK was predominantly localised to the plasma membrane region (Figure 4) and the implications of these findings are discussed in the discussion section.

Expression of mutant forms of Eps15 does not inhibit MNK internalisation

As Eps15 is constitutively bound to AP-2 at the plasma membrane, it can be used as a tool to specifically block coated pit-mediated endocytosis from the cell surface (Benmerah *et al.*, 1998; Benmerah *et al.*, 1999). The D3Δ2 control construct consists of the C′ domain of Eps15 (amino acids 529–897) lacking the AP-2 binding sites (amino acids 621–739); its

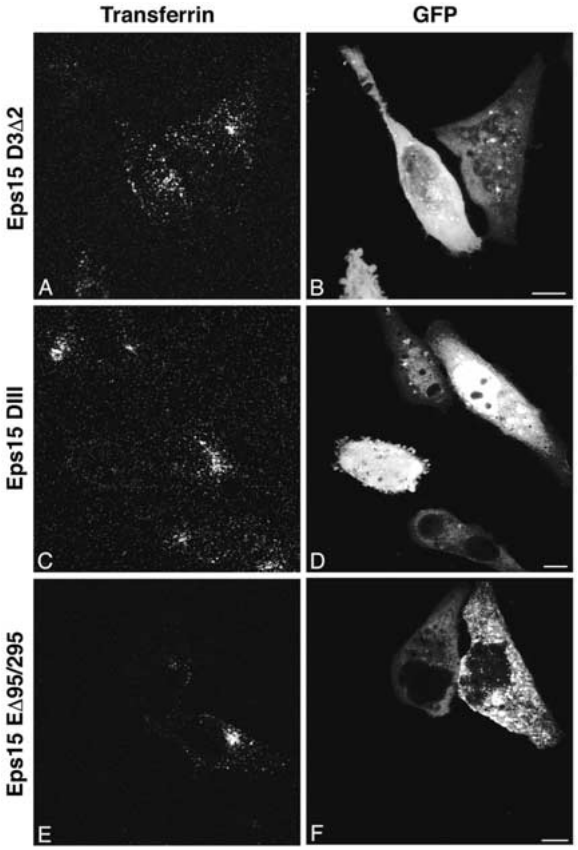

Fig. 5. Expression of mutant isoforms of Eps15 inhibits clathrin-mediated endocytosis in HeLa-5/MNK cells. HeLa-5/MNK cells were transiently transfected with either D3Δ2 (control) (A, B), DIII (C, D), or EΔ95/295 (E, F) GFP-Eps15 constructs then incubated in the presence of Alexa-594^{TM} -conjugated transferrin for 10 minutes at 37 °C and fixed. (A, C, E) Alexa-594^{TM} -conjugated transferrin. (B, D, F) GFP. Bars = 10 μm.

overexpression does not affect coated pit-dependent endocytosis (Benmerah *et al.*, 1999). The DIII mutant construct consists of the entire C′ domain (amino acids 529–897) and the EΔ95/295 mutant construct is an Eps15 deletion mutant lacking the second and third EH protein-protein interaction domains. Overexpression of either of these two latter mutant forms of Eps15 in HeLa cells leads to an inhibition of clathrin-dependent endocytosis as seen by a lack of uptake of Alexa^{594}-Tf in such cells compared to control D3Δ2-expressing cells or neighbouring non-transfected cells (Figure 5).

Transient expression of either DIII (Figure 6E–H) or EΔ95/295 (Figure 6I–L) Eps15 did not inhibit MNK internalisation in HeLa-5/MNK cells as compared to the D3Δ2 control (Figure 6A–D). Predominant perinuclear localisation was seen in all cells. This

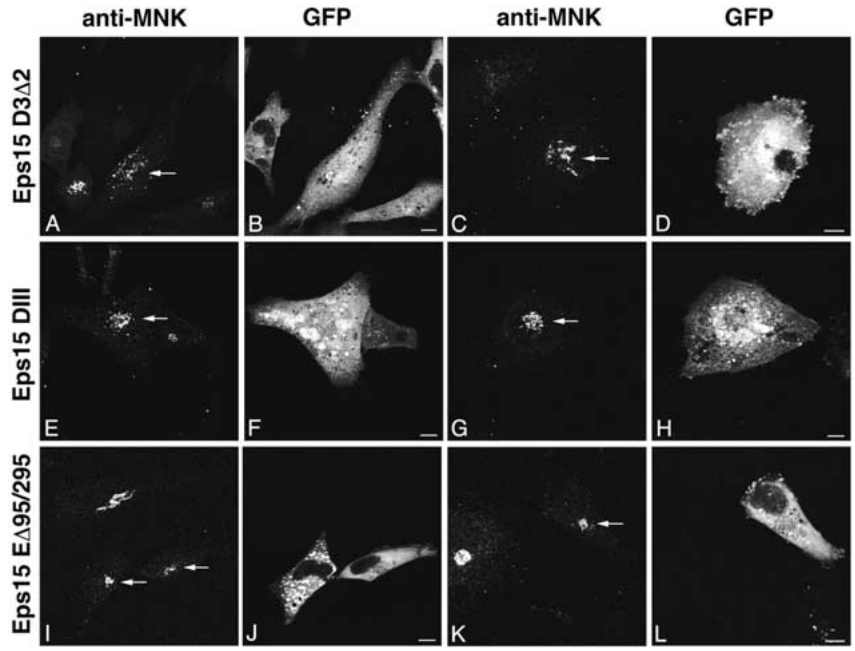

Fig. 6. Internalisation of MNK is not inhibited in cells expressing mutant isoforms of Eps15. HeLa-5/MNK cells were transiently transfected with either D3Δ2 (wild-type) (A–D), DIII (E–H), or EΔ95/295 (I–L) GFP-Eps15 constructs and incubated in either basal medium (A, B, E, F, I, J) or in basal medium for 90 minutes after a 90 minute pre-incubation in 630 μM $CuCl_2$ (C, D, G, H, K, L), then fixed and processed for immunofluorescence. MNK was labelled with polyclonal anti-MNK antiserum and Alexa-594^{TM} -conjugated anti-rabbit IgG (A, C, E, G, I, K). (B, D, F, J, L) GFP. Arrows represent TGN labelling. Bars = 10 μm.

was observed in cells incubated in basal medium and after the removal of elevated copper. The expression of either DIII or EΔ95/295 Eps15 did not inhibit MNK exocytosis from the TGN to the plasma membrane in the presence of elevated copper (data not shown). These data are consistent with the dynamin results and again do not support the hypothesis that MNK is internalised via a clathrin-dependent pathway.

Hypertonic sucrose treatment inhibits MNK internalisation

The third method employed in this study to block clathrin-mediated endocytosis was treatment with hypertonic sucrose that inhibits clathrin-dependent internalisation by causing a reversible loss of membrane-associated clathrin lattices. This method successfully inhibited clathrin-mediated endocytosis as seen by a complete lack of Alexa^{594}-Tf uptake in treated cells as compared to control cells (data not shown).

In order to determine whether hypertonic treatment also blocks fluid-phase endocytosis in HeLa cells, the effect on the net accumulation of FITC-dextran was observed. Rather than the endosomal FITC-dextran labelling seen in serum-free medium control cells (Figure 7B, C, H, I), hypertonic treatment caused a shift of labelling to the plasma membrane (Figure 7 E, F, K, L). The plasma membrane labelling of FITC-dextran can be seen in the vertical (z) optical sections of the hypertonic treated cells as compared to the SFM controls.

Hypertonic treatment inhibited MNK endocytosis in basal medium (Figure 7D) as well as after the removal of elevated copper (Figure 7J). In conclusion, MNK internalisation is not significantly inhibited when coated pit-dependent endocytosis is specifically blocked, but it is inhibited by hypertonic treatment under conditions where fluid-phase endocytosis is also blocked.

Discussion

Previous studies on the cellular localisation of the MNK protein in CHO cells have demonstrated that MNK is principally located at the TGN under basal conditions and undergoes a reversible copper-responsive shift to the plasma membrane. Studies also suggest MNK constitutively recycles between the TGN and plasma membrane (Petris *et al.*, 1996; La

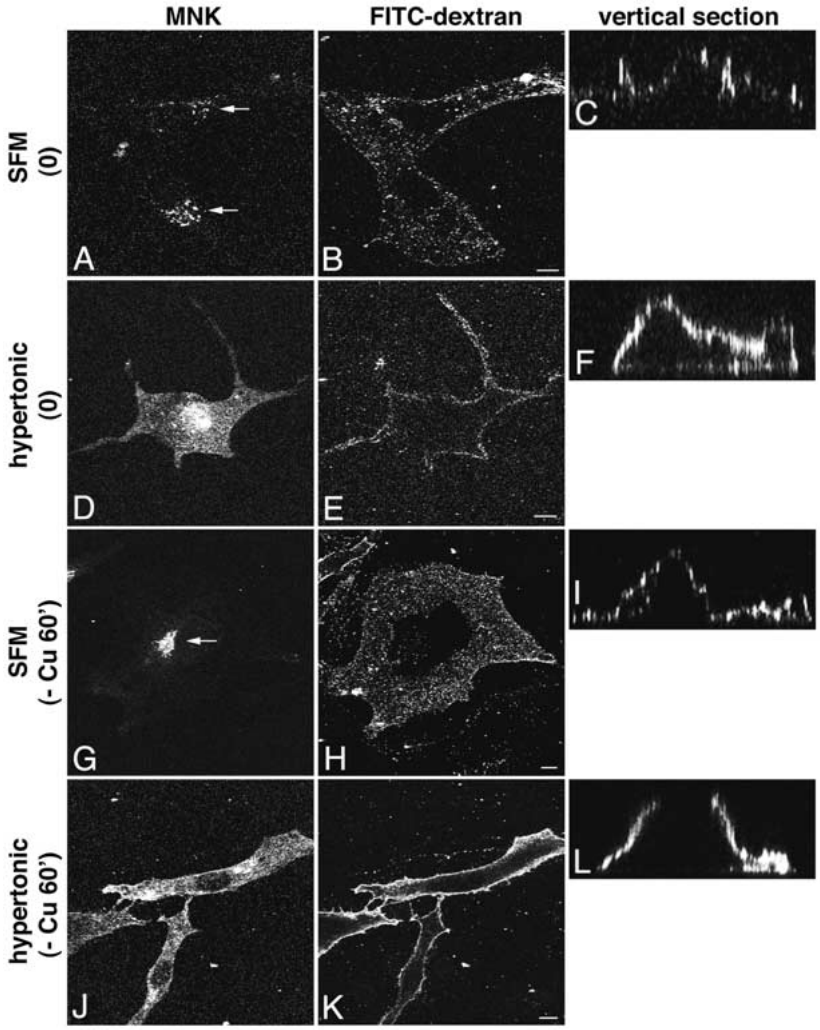

Fig. 7. Hypertonic medium inhibits fluid-phase endocytosis and causes an accumulation of MNK at the cell surface. All incubations were at 37 °C. HeLa-5/MNK cells were incubated in either serum-free medium (SFM) for 40 min (A–C); SFM for 10 min then 0.45 M sucrose/SFM for 3 h (D–F); SFM for 10 min, SFM + 158 μM Cu for 2 hours then returned to basal SFM for 60 min (G-I); SFM for 10 min, SFM + 158 μM Cu for 2 h, 0.45 M sucrose/SFM + 63 μM for 30 min then returned to basal 0.45 M sucrose/SFM for 60 min (J–L). All the above treatments included a 30 minute incubation in FITC-dextran during the last 30 minutes of treatment (B, C, E, F, H, I, K, L). MNK was visualised with polyclonal anti-MNK antiserum and Alexa-594^{TM} -conjugated anti-rabbit IgG (A, D, G, J). (C, F, I, L) vertical (z) optical sections of FITC-dextran. Arrows represent TGN labelling. Bars = 10 μm.

Fontaine *et al.*, 1998; Petris and Mercer, 1999). A di-leucine L1487L1488 motif in the C′ terminus of MNK has been shown to mediate MNK endocytosis from the plasma membrane (Francis *et al.*, 1998; Petris *et al.*, 1998; Petris and Mercer, 1999). As di-leucine motifs have been reported to be involved in clathrin-mediated endocytosis (Corvera et al., 1994; Haft *et al.*, 1994), we hypothesised that MNK would accumulate at the plasma membrane of cells in which clathrin-dependent endocytosis had been blocked.

The aim of this study was to determine if the primary endocytic route of the MNK protein was clathrin-dependent. To address this, we inhibited clathrin-mediated endocytosis in MNK-overexpressing HeLa cells and observed the effect this had on MNK localisation using confocal microscopy. In this study, we have demonstrated that the inhibition of clathrin-mediated endocytosis achieved by the over-expression of dominant negative dynamin-1 or mutant forms of Eps15 does not significantly alter the ability of the MNK protein to be internalised. The current

studies do not exclude the possibility of a small pool of MNK having its endocytosis inhibited under these conditions. Testing of this will depend on development of quantitative studies using stable transfectants and more sensitive methods of MNK detection.

The fact that the commonly used K44A dynamin did not inhibit MNK internalisation did not rule out the possibility that other mutant forms of dynamin could have an effect. The internalisation of the M_2 muscarinic acetylcholine receptor (M_2 mAChR) and angiotensin AT_{1A} receptor ($AT_{1A}R$) is not inhibited by the expression of K44A dynamin, which lacks only the first GTP-binding motif, and thus was traditionally thought to be dynamin-independent. It has been shown that the expression of a dynamin mutant lacking all three GTP-binding motifs (N272 dynamin) strongly inhibited the uptake of these receptors, as did expression of K535M dynamin which lacks phosphatidylinositol 4,5-bisphosphate-stimulated GTPase activity (Werbonat *et al.*, 2000). However, expression of a dominant-negative clathrin mutant or β-arrestin V53D, which inhibits clathrin-mediated internalisation, did not block the internalisation of M_2 mAChR (Werbonat *et al.*, 2000). The internalisation of M_2 mAChR is thus clathrin-independent but dynamin-dependent. To test this possibility for MNK endocytosis, the effect of N272 and K535M dynamin was studied. Neither dynamin isoform significantly inhibited MNK internalisation, as seen by indirect immunofluorescence. These data strongly suggest that MNK is internalised via a dynamin and Eps15-independent pathway.

Electron microscopy (EM) studies of ultrathin cryosections have not detected significant MNK associated with caveolae (R. Parton, personal communication). Caveolae are indisputably involved in cholesterol transport and homeostasis (Fielding and Fielding, 1997), and in the clathrin-independent endocytosis of cholera toxin and some glycosylphosphatidyl inositol (GPI)-anchored proteins (Kurzchalia and Parton, 1999), but their role in constitutive endocytosis under basal conditions, however, has been questioned. Caveolae have been found to be highly immobile plasma membrane microdomains (Thomsen *et al.*, 2002), and hence are unlikely to participate in constitutive endocytic trafficking. Caveolae are therefore not likely to be the route of internalisation of MNK from the plasma membrane.

There is evidence of clathrin-independent pathways that do not involve caveolae. The concept of lipid rafts being responsible for clathrin- and caveolae-independent internalisation has recently been reviewed (Johannes and Lamaze, 2002; van der Goot and Gruenberg, 2002). Lipid rafts are dynamic, specialised microdomains of cholesterol and sphingolipids found in the plasma membrane (Simons and Ikonen, 1997). These domains are thought to be responsible for the internalisation of proteins such as the interleukin-2 (IL-2) receptor (Lamaze *et al.*, 2001) and GPI-anchored proteins (Pelkmans *et al.*, 2001), known to enter cells independently of clathrin and caveolae. Studies involving the Shiga toxin B-subunit have suggested that association with membrane microdomains is important for targeting molecules into the retrograde route from early endosomes to the TGN (Falguieres *et al.*, 2001). Collectively, these data suggest a possibility for MNK to be internalised via membrane microdomains or rafts. It is unclear at this stage whether membrane microdomains are exclusively clathrin- or dynamin- independent, but it is clear that there are numerous clathrin- and caveolin-independent entry routes due to different types of rafts and different endosomal compartments.

Blocking clathrin-dependent endocytosis using hypertonic sucrose medium has been well documented to inhibit clathrin-mediated endocytosis (Carpentier *et al.*, 1989; Heuser and Anderson, 1989; Hansen *et al.*, 1993) and dynamin dependent/clathrin independent endocytosis of M2MACLR (Roseberry and Hosey, 2000), but not to inhibit fluid-phase endocytosis (Oka *et al.*, 1989; Cupers *et al.*, 1994; Lukacs *et al.*, 1997). However, this treatment has been reported to inhibit the uptake of the fluid-phase endocytosis marker, horseradish peroxidase, in 3T3 L1 fibroblasts (Carpentier *et al.*, 1989). None of above studies were conducted in HeLa cells. We found in the current studies that hypertonic treatment blocked the uptake of the fluid-phase marker, FITC-dextran, in HeLa cells and therefore concluded that hypertonic treatment inhibited both clathrin-dependent and fluid-phase endocytosis in HeLa cells. Hypertonic treatment inhibited MNK internalisation in cells grown in basal medium and after the removal of elevated copper levels. As the endocytosis of MNK was affected by hypertonic treatment, as was fluid-phase endocytosis, we have concluded that MNK can be internalised via a clathrin-independent route which may be related to fluid-phase endocytosis.

How does one reconcile the apparent contradiction of the di-leucine results suggesting MNK is internalised via a clathrin-dependent route and the dynamin and Eps15 results suggesting MNK is internalised via

a clathrin-independent route? This can be explained if MNK were able to utilise more than one endocytic pathway. One is clathrin-dependent as dictated by the di-leucine internalisation motif, and the other is clathrin-independent.

Fluid-phase endocytosis has been shown not to be affected by expression of mutant dynamin (Herskovits *et al.*, 1993; Damke *et al.*, 1994; Damke *et al.*, 1995) or Eps15 (Benmerah *et al.*, 1999). Fluid-phase endocytosis has been shown to recover to wild-type levels within 30 minutes in cells expressing a temperature-sensitive dynamin. These cells rapidly and completely compensate for the loss of clathrin-mediated endocytosis by inducing an alternative endocytic pathway mutant (Lukacs *et al.*, 1997). If MNK were able to utilise both pathways and clathrin-independent endocytosis is induced (or at least unaffected) when clathrin-dependent endocytosis is blocked, it could explain the lack of visible inhibition of clathrin-dependent MNK internalisation in cells transiently transfected with K44A dynamin or mutant Eps15. It could simply be a 'shift in equilibrium' towards the MNK clathrin-independent pathway.

If MNK can still be internalised when clathrin-dependent endocytosis is blocked, as seen in cells expressing K44A dynamin or mutant Eps15, why does mutating the di-leucine motif lead to a significant accumulation of MNK at the cell surface? Why are these molecules not internalised via the clathrin-independent pathway? In order to test the hypothesis that there is up-regulation of a clathrin-independent pathway when clathrin and dynamin-dependent pathways are blocked, we investigated localisation of the L1487AL1488A mutant MNK in cells expressing dominant negative dynamin. If this hypothesis were true the expectation is that the dileucine mutant MNK would be internalised. The data presented in Figure 4 do not support this hypothesis.

These findings suggest that the C-terminal di-leucine motif could be required for both pathways of endocytosis of MNK, directly in the case of the clathrin-dependent pathway and either directly or indirectly (eg. by way of a di-leucine induced structural alteration) in the case of the clathrin-independent pathway. Studies observing the rates of MNK internalisation under various conditions should be informative as fluid-phase endocytosis is not as efficient as clathrin-dependent receptor-mediated endocytosis (Watts and Marsh, 1992; Lamaze and Schmid, 1995).

In conclusion, we have found that the MNK copper-transporting P-type ATPase can apparently utilise more than one endocytic pathway. Further studies are required to elucidate the relative proportions of the clathrin-dependent and clathrin-independent routes MNK can take.

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