Novel membrane traffic steps regulate the exocytosis of the Menkes disease ATPase

Christian Cobbold1, Sreenivasan Ponnambalam2, Michael J. Francis3 and Anthony P. Monaco1,*

1Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Headington, Oxford OX3 7BN, UK, 2School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK and 3Amersham Biosciences, Maynard Centre, Forest Farm, Whitchurch, Cardiff CF14 7YT, UK

Received June 27, 2002; Revised and Accepted August 19, 2002

The Menkes disease protein (ATP7A or MNK) is a P-type transmembrane ATPase that regulates translocation of cytosolic copper ions across intracellular membranes of compartments along the secretory pathway. In this study, we show that endogenous MNK in cultured cell lines is localized to the distal Golgi apparatus and translocates to the plasma membrane in response to exogenous copper ions. This transport event is not blocked by expression of a dominant-negative mutant protein kinase D, an enzyme implicated in regulating constitutive trafficking from the trans-Golgi network (TGN) to the plasma membrane, whereas constitutive transport of CD4 is inhibited. In contrast, protein kinase A inhibitors block copper-stimulated MNK delivery to the plasma membrane. Expression of constitutively active Rho GTPases such as Cdc42, Rac1 and RhoA reveals a requirement for Cdc42 in the trafficking of MNK to the cell surface. Furthermore, overexpression of WASp inhibits anterograde transport of MNK, further supporting regulation by the Cdc42 GTPase. These findings define a novel step in TGN-to-plasma membrane traffic required to export MNK to the cell surface.

INTRODUCTION

Menkes disease is a rare X-linked neurological disease that arises from a defect in copper transport characterized by progressive degeneration and death in early childhood (1). The ATP7A gene encodes a P-type ATPase (called MNK here for convenience) that functions as a copper transporter (2,3). Overexpression of the MNK gene product in cultured cell lines shows localization to the Golgi apparatus (4–7) and the functionally related ATP7B protein was thought to localize to a similar intracellular location (8,9). Recent evidence, however, suggests that ATP7B may reside in a late endosome compartment and not the trans-Golgi network (TGN) (10,11). Studies using transfected cells and copper-resistant cell lines show that overexpressed MNK protein cycles between the distal Golgi apparatus and plasma membrane in response to the levels of copper ions (5,6,12,13).

It has previously been shown that a specific transmembrane domain can mediate trans-Golgi localization and retention of MNK (14). In contrast, a naturally occurring minor splice isoform of MNK that lacks this transmembrane Golgi localization signal localizes to the endoplasmic reticulum (ER) (14). Importantly, patients with genetic mutations that result in expression of ER but not Golgi-resident MNK protein have a milder form of this disease called occipital horn syndrome (15).

One possibility is that the MNK ATPase would be transported to the plasma membrane along the constitutive secretory pathway in response to exogenous copper. However, the exact nature and identity of the transport intermediates that deliver MNK to the cell surface from the TGN and their membrane dynamics is largely unknown. Recently, however, an atypical protein kinase C member, protein kinase D [PKD, or protein kinase Cμ (PKCμ)] has been implicated in regulating the delivery of constitutively secreted proteins from the TGN to the plasma membrane (16–18). Overexpression of a dominant-negative PKD enzyme leads to TGN tubulation, and protein cargo specifically destined to the cell surface is found in these TGN-derived tubules (17).

Increasing evidence indicates that the actin cytoskeleton plays an important role in membrane trafficking. Different Rho GTPases could regulate vesicle fusion and fission events at the cell surface by transmitting signals to the actin cytoskeleton (19). The three most studied members of the Rho family, Cdc42, Rac1 and RhoA, have been shown to be involved in a variety of biological processes, including vesicular trafficking and endocytosis (20). Importantly, Cdc42 has been reported to inhibit the exit of basolateral proteins from the TGN whilst stimulating the exit of apical proteins (21). Many studies have focused on the identification of the molecular targets of Rho family members, and as such many putative effectors have been isolated. For example, downstream targets activated by Rac1, Cdc42 and
RhoA include POR1, WASp and ROKz, respectively, while PAK can be activated by both Rac1 and Cdc42 (22).

We have thus focused on the behaviour and trafficking of endogenous MNK protein in mammalian cells in response to copper ions and the factors that regulate this membrane trafficking event. To address these questions, we have analysed the trafficking of the endogenous MNK protein in non-overexpressing cells and show its localization to the distal secretory pathway. Comparison of MNK trafficking with a constitutively recycling TGN resident marker protein is used to monitor constitutive and copper-regulated protein transport events from the TGN to the plasma membrane. The requirement for soluble and membrane-associated cytosolic protein factors is tested using agents that disrupt or inhibit specific classes of proteins.

RESULTS

Copper-regulated subcellular localization of endogenous MNK protein

A rabbit polyclonal antibody to the C-terminal domain of the MNK protein detects a juxtanuclear staining pattern in HeLa cells (Fig. 1A). This pattern was compared with a key TGN resident and marker protein, TGN46 (Fig. 1B). The proteins displayed substantial co-distribution (Fig. 1C). To define MNK localization more closely, cells were incubated with the fungal metabolite brefeldin A (BFA) prior to immunofluorescence analysis. BFA induces the TGN to fuse with early endosomes, and with longer exposures condenses to a juxtanuclear structure known as the microtubule-organizing centre (23,24). Treatment of cells with BFA resulted in MNK contracting to a tight juxtanuclear location (Fig. 1D). The effect on the localization of TGN46 is shown in Figure 1E. Both proteins still displayed significant overlap (Fig. 1F). Recent results have shown that the related Wilson’s disease protein (ATP7B) may be localized to endosomes and not the TGN as has been described (8–11), and this may be the case for MNK. Proteins found within recycling endosomes, for example the transferrin receptor, are often observed within the TGN region of the cell. To further investigate the localization of MNK, the distribution of MNK was compared with that of the transferrin receptor. Extensive overlap of MNK with the transferrin receptor was observed in cells cultured in basal medium (Fig. 1G–I). Significantly, the localization of MNK and the transferrin receptor were subtly redistributed away from each other when cells were incubated with BFA (Fig. 1J–L). In addition, MNK did not co-localize with the early endosome marker EEA1 or the lysosome marker CD63 in the absence of BFA (data not shown). These data show strongly that MNK resides within the TGN, and not a post-TGN membrane compartment, and are consistent with previous reports suggesting localization of MNK at the TGN (4–7).

Western blotting using this antibody detects a 180 kDa polypeptide in HeLa cell lysates consistent with the calculated molecular mass of the mature MNK protein (data not shown). Stably transfected MNK overexpressing cells (13,25) or copper-resistant cell lines (6,12) show copper-dependent movement of this transmembrane protein to the cell surface. To test whether this is true for endogenously expressed MNK protein in non-overexpressing human HeLa cells, we treated cells with 200 μM copper (II) chloride for 2 hours prior to analysis by indirect immunofluorescence (Fig. 2A–C). Importantly, the MNK protein shows substantial redistribution to the plasma membrane (Fig. 2A). In contrast, TGN46 distribution was unaffected and showed a characteristic TGN/Golgi juxtanuclear staining pattern (Fig. 2B). Superimposition of both staining patterns after copper treatment reveals the majority of MNK now at the plasma membrane in the presence of exogenous copper ions; however, a small fraction of the MNK protein is still detected in a juxtanuclear compartment, which co-localizes with TGN46 (Fig. 2C).

The staining pattern for MNK protein in cells treated with exogenous copper ions suggested a net increase in MNK associated with the plasma membrane. The isolation of plasma membrane lawns is a well-established method of biochemically determining levels of proteins at the plasma membrane, including the TGN-to-plasma membrane trafficking of GLUT4 (26) and MNK (6). We used this method to analyse changes in MNK levels at the cell surface in response to copper. In basal medium, the levels of MNK associated with the plasma membrane lawns was low (Fig. 2D); however, on addition of copper for 2 hours, there was a striking increase in plasma membrane-associated MNK (Fig. 2E). Quantification reveals an ~5.5-fold increase in plasma membrane MNK levels in copper-treated cells compared with untreated controls (Fig. 2F).

To further analyze the kinetics of this copper-dependent redistribution of MNK to the plasma membrane, we treated cells with 200 μM copper chloride for different times up to 6 hours and measured the MNK levels at the plasma membrane (see Materials and Methods). Quantification of plasma membrane MNK levels show that significant amounts of plasma membrane MNK are first detected ~30 minutes after copper addition (Fig. 2F). Plasma membrane MNK levels then double between 0.5 and 2 hours after copper addition. MNK plasma membrane levels peak at a 6-fold increase 4–6 hours after copper addition (Fig. 2F).

Copper-dependent anterograde transport of MNK to the plasma membrane is not regulated by the constitutive route

It is possible that MNK is transported from the TGN to the cell surface via a specialized membrane traffic step, in comparison with a ubiquitous constitutive step in secretion. Therefore a key question is whether there is a link between the constitutive step in protein secretion from the TGN to the plasma membrane and the copper-dependent trafficking of MNK. It has recently been shown that PKD regulates constitutive secretion from the TGN to the plasma membrane (17,27). We transiently expressed the GST-tagged, wild-type PKD enzyme, and this protein is largely cytosolic (Fig. 3B). In the same cells, MNK shows a juxtanuclear localization (Fig. 3A: arrowheads) that again co-localizes with TGN46 (not shown). Expression of wild-type PKD enzyme (Fig. 3D) does not affect the copper-dependent translocation of MNK from the Golgi region to the plasma membrane (Fig. 3C: arrowhead). Liljedhal et al. (17) showed that the TGN-associated, dominant-negative PKD-K618N
protein inhibits the constitutive trafficking of proteins from the TGN. Transient expression of the PKD-K618N protein (Fig. 3F) shows substantial co-localization with MNK (Fig. 3E: arrowheads). Interestingly, this membrane-associated PKD mutant (Fig. 3H) does not affect copper-dependent transport of MNK from the Golgi to the plasma membrane (Fig. 3G: arrowheads), showing that this membrane traffic step from the TGN to the cell surface is independent of PKD regulation. A quantitative analysis of MNK plasma membrane levels in these cells is shown in Figure 3I.

It is possible that the levels of PKD-K618N expressed in these cells were not sufficient to block the constitutive

---

**Figure 1.** Comparison of the subcellular distribution of MNK with the TGN protein TGN46, and the recycling endosome marker, transferrin receptor. HeLa cells grown in basal medium were treated without (A–C and G–I) or with (D–F and J–L) brefeldin A for 1 h at 37°C and processed for indirect immunofluorescence and confocal microscopy. Bound rabbit anti-MNK (green; A, D, G and J), anti-sheep TGN46 (red; B and E), and anti-transferrin receptor (red; H and K) were detected using FITC-conjugated anti-rabbit, Texas Red-conjugated anti-sheep and Texas Red-conjugated anti-mouse secondary antibodies. (C) and (F), and (I) and (L), show superimpositions of MNK and TGN46, and MNK and transferrin receptor, staining patterns, respectively.
trafficking pathway. To confirm that constitutive exocytosis was inhibited, HeLa cells were transiently co-transfected with PKD-K618N and CD4, the latter being a constitutively secreted integral membrane protein that is transported to the cell surface (28). Transfected cells were incubated in the absence or presence of copper as described above, prior to visualization by fluorescence microscopy using antibodies to the respective proteins. In control untransfected cells not expressing PKD-K618N, CD4 is found at the cell surface in the absence or presence of copper (Fig. 4A and B). However, in PKD-K618N-expressing cells incubated in both the absence and the presence of copper (Fig. 4C–F), CD4 co-localizes with PKD in the Golgi apparatus. This is consistent with previous reports showing that transient PKD-K618N expression in HeLa cells prevents trafficking of CD4 from the TGN to the cell surface (17).

Copper-regulated anterograde transport of MNK requires protein kinase A activity

The above data suggested that copper-dependent trafficking of MNK to the cell surface is not regulated by PKD and is therefore not following the constitutive pathway. However, this

Figure 2. Copper-dependence of intracellular MNK distribution. (A–C) HeLa cells treated with 200 μM copper (II) chloride for 2 h at 37°C were processed for indirect immunofluorescence and confocal microscopy. Bound rabbit anti-MNK (green; A and C) and anti-sheep TGN46 (red; B and C) were detected using FITC conjugated anti-rabbit and Texas Red-conjugated anti-sheep secondary antibodies. (C) shows superimpositions of the MNK and TGN46 staining patterns. (D and E) Plasma membrane lawns isolated from HeLa cells incubated in the absence (D) or presence (E) of 200 μM copper (II) chloride for 2 h at 37°C were processed for immunofluorescence and confocal microscopy. Bound rabbit anti-MNK antibodies were detected using FITC-conjugated anti-rabbit secondary antibodies. (F) Histogram showing quantification of the relative levels of plasma membrane MNK in response to copper for increasing times. The SEM values in each experiment are indicated by the error bars.
transport event could be regulated by other protein kinases. To further analyse the role of protein kinases in MNK trafficking to the plasma membrane, HeLa cells were incubated with the protein kinase inhibitors staurosporine (abroad-spectrum protein kinase inhibitor), calphostin C [a protein kinase C (PKC) inhibitor], H-89 [a protein kinase A (PKA) inhibitor] and PKI(14-22) (a PKA inhibitor) prior to addition of copper chloride. In control cells (Fig. 5B) and cells incubated with calphostin C (Fig. 5D), MNK was transported to the plasma membrane. However, in cells pretreated with staurosporine (Fig. 5C), H-89 (Fig. 5E) or PKI(14-22) (Fig. 5), there was little detectable MNK staining at the plasma membrane, showing that PKA inhibitors block anterograde copper-regulated transport of MNK. The PKA activator forskolin had no effect on MNK distribution or trafficking (Fig. 5G). The quantitative effects of perturbing protein kinase activity are displayed in Figure 5G. These data shows that plasma membrane MNK levels are ~3-fold reduced from control levels (in the presence of copper) when cells are incubated in the presence of PKI(14-22), H-89 or staurosporine.

Copper-dependent anterograde transport of MNK to the plasma membrane is regulated by the Rho GTPase Cdc42

It has become increasingly clear that members of the Rho GTPase protein family play important roles in vesicular protein trafficking. To assess the role of Rho GTPases in the trafficking of MNK from the TGN to the cell surface, we expressed dominant-negative and constitutively active mutants of Cdc42, RhoA and Rac1 in HeLa cells. The dominant-negative mutants are known to inhibit the function of their endogenous counterparts (29), while the constitutively active mutants stimulate downstream effectors. The effect on MNK exocytosis of each construct was monitored by co-immunofluorescence microscopy using antibodies to MNK and Myc-tagged mutant Rho proteins.

We expressed the tagged, dominant-negative mutants of Cdc42 (Cdc42N17), Rac1 (Rac1N17) and RhoA (RhoAN19) and incubated cells in the presence of copper chloride for 2 hours (Fig. 6B, D and F, respectively). In the same cells, MNK shows a cell surface localization (Fig. 6A, C and E: arrowheads). Therefore, expression of these dominant-negative mutant proteins does not affect the copper-dependent translocation of MNK from the Golgi region to the plasma membrane. Similarly, expression of the constitutively active mutant protein of RhoA (RhoAV14) (Fig. 6L: arrowheads) did not significantly alter MNK trafficking induced by copper (Fig. 6K).

Figure 3. Copper-dependent MNK transport is not regulated by PKD. Transfected HeLa cells expressing wild-type PKD (A–D) or PKD-K618N (E–H) treated without (A, B, E and F), or with (C, D, G and H), 200 μM copper chloride for 2h at 37°C were processed for indirect immunofluorescence and confocal microscopy. Bound rabbit anti-MNK (red; A, C, E and G) and mouse anti-GST (green; B, D, F and H) were detected using Texas Red-conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antibodies. Arrows show MNK distribution at the TGN (A and E) and cell surface (C and G). (I) Histogram showing quantification of the relative levels of plasma membrane MNK in response to copper and expression of the indicated protein. The SEM values in each experiment are indicated by the error bars.
Kroschewski et al. (21) showed that the TGN-associated, Cdc42 constitutively active mutant protein Cdc42 V12 has differential effects on the trafficking of marker proteins from the TGN to basolateral and apical membranes. Expression of constitutively active Cdc42 protein (Fig. 6H; arrowheads) in cells expressing activated Cdc42, while presence of copper) in cells expressing activated Cdc42, while there is a 35% reduction in trafficking in activated Rac1-expressing cells. Overexpression of the mutant Rho proteins did not significantly alter the steady-state localization of MNK from the TGN (data not shown). Consistent with previous reports showing that Cdc42V12 expression can inhibit constitutive trafficking of proteins to the cell surface (21), CD4 co-localized with Cdc42V12 at the TGN in cells expressing CD4 and Cdc42V12 (data not shown).

WASp mediates the action of Cdc42V12 on actin polymerization

Well-known targets of Cdc42 include members of the Wiskott–Aldrich syndrome protein (WASp) family and PAK1-3. WASp proteins mediate the action of the Rho protein on the Arp2/3 complex to induce actin polymerization (30–32), while PAK can cause actin filament stabilization or decreased actomyosin contraction, depending on its target (22). In the next experiment, we wanted to determine whether WASp mimicked the inhibitory effect on exocytosis observed in cells transfected with activated Cdc42. Figure 7B (arrowhead) shows that overexpression of the WASp protein inhibits the copper-dependent transport of MNK from the Golgi to the plasma membrane (Fig. 7A; arrowhead). A quantitative analysis shows that MNK levels at the cell surface are reduced to 55% of control in cells expressing WASp. This suggests that MNK trafficking is linked to Cdc42, WASp and actin polymerization.

To confirm that WASp was having an effect on the actin cytoskeleton in our system, cells expressing WASp were incubated with copper and then the distribution of actin and WASp was compared. Consistent with previous reports (31), higher-expressing cells showed cluster formation of WASp-containing particles (Fig. 7D). Double labelling with anti-actin antibody revealed striking co-localization of polymerized actin with WASp (Fig. 7C; arrowheads), indicating a disruption of the endogenous actin cytoskeleton. In an attempt to provide a direct link and mechanism for the Cdc42 regulation of MNK trafficking, we looked at the effect of expressing constitutively active Cdc42 on the localization of actin. As was observed for WASp, overexpression of Cdc42V12 (Fig. 7F; arrowheads) caused an increase in the level of polymerized actin detected in cells (Fig. 7E: arrowheads). Together, these data suggest that constitutively active Cdc42 is activating WASp, which then disrupts the actin cytoskeleton.

DISCUSSION

The MNK ATPase is a key copper transporter necessary for normal mammalian development. This study shows that this transmembrane protein cycles from the TGN to the plasma membrane in a copper-dependent manner via a novel membrane traffic route. The MNK protein localizes to a distal Golgi subcompartment, which also contains TGN46, a key TGN resident in human cells. This is consistent with previous reports showing localization of MNK at the TGN (4–7). The plasma membrane enrichment for MNK in the presence of excess copper ions indicates that different trafficking pathways from the TGN operate to deliver MNK and TGN46 to the plasma membrane. Importantly, our work is the first to show...
that endogenous mammalian MNK in non-overexpressing cells can translocate from the TGN to the plasma membrane via a copper-dependent membrane traffic step.

Quantitative studies show that MNK levels at the plasma membrane are 6-fold enriched in the presence of exogenous copper ions. However, the kinetics of this event is relatively slow and significantly slower than the time taken for constitutively secreted proteins to move from the TGN to the plasma membrane. Expression of a dominant-negative TGN-to-plasma membrane regulator, PKD (16,17,27,33) does not block copper-induced MNK redistribution to the plasma membrane, while trafficking of CD4 is blocked. This indicates the existence of a unique and novel membrane traffic step that is not involved in the bulk flow of constitutively expressed proteins along the secretory pathway to the plasma membrane.

The actin cytoskeleton provides a superstructure to help define cell shape, and contributes in controlling a wide variety of cellular processes, including vesicular trafficking (34). It is now well established that Rho proteins play an important role in exocytic pathways, and that the Rho GTPases control the organization and dynamics of the actin cytoskeleton (20,34). To date, the most compelling evidence of a role for Rho GTPases in exocytosis from the TGN has come from studies on Cdc42 (21,35,36). Our results showed that, of the Rho GTPases studied, only constitutively active Cdc42, and to a lesser extent constitutively active Rac1, had any inhibitory effect on the copper-dependent trafficking of MNK to the cell surface. These findings have similarities to studies in neuroendocrine PC12 cells showing that the presence of constitutively activated Cdc42 diminished regulated exocytosis (35). Interestingly, Frantz et al. (35) also demonstrated that constitutively active Rac1 inhibits regulated exocytosis.

We also attempted to identify the downstream targets of constitutively Cdc42 and Rac1. The members of the WASp family are well-known targets of Cdc42 and mediate the action of the GTPase on actin polymerization. Our data argue for the involvement of WASp in the Cdc42-induced inhibition of MNK trafficking. Overexpression of WASp inhibited copper-regulated exocytosis of MNK to the cell surface. WASp activated by Cdc42 can act as a scaffold allowing recruitment of actin monomers and other required machinery (e.g. the Arp 2/3 complex) enhancing actin polymerization (22).

As we have already established that MNK does not follow the constitutive pathway, how is the copper-dependent trafficking of MNK regulated? We can summarize our findings as shown in Figure 8. Newly synthesized MNK protein reaches the TGN, where it is retained. The MNK protein transports cytosolic copper across the TGN membranes in an ATP-dependent manner. Thus, newly synthesized copper-requiring proteins and enzymes [e.g. tyrosinase (37)] that arrive in the lumen of the TGN have sufficient copper ions to facilitate correct protein folding and enzymatic function. This could promote secretory protein egress out of the TGN and be part of a wider network of quality control mechanisms to facilitate degradation or retention of improperly folded or non-functional proteins, even within the distal part of the secretory pathway. The elevation of extracellular copper levels has multiple consequences. Copper ion levels within the cytoplasm increase, and this will have substantial cytotoxic effects by competitive inhibition in many divalent cation-regulated processes. To alleviate these toxic

Figure 5. Copper-dependent MNK transport is regulated by protein kinase A. HeLa cells treated without (A) or with (B–F) 200 μM copper (II) chloride for 2 h at 37°C in the absence (B), or presence of staurosporine (C), calphostin C (D), H-89 (E) or PKI(14-22) (F) were processed for indirect immunofluorescence and confocal microscopy. Bound rabbit anti-MNK (A–F) was detected using FITC-conjugated anti-rabbit secondary antibodies. (G) Histogram showing quantification of the relative levels of plasma membrane MNK in response to copper and the indicated chemical or expressed protein. The SEM values in each experiment are indicated by the error bars.
efforts, MNK protein is now needed at the plasma membrane to translocate copper ions from the cytosol to the extracellular milieu. MNK is relatively slowly transported from the TGN to the plasma membrane via a novel membrane traffic event that operates in a parallel, but independent, manner, in contrast to constitutive bulk secretion from the TGN. The relatively slow kinetics for MNK transport from the TGN to the plasma membrane may be due to a number of properties. One interpretation is that this step in membrane traffic is itself very slow and takes a relatively long time (a half-life of 30–60 minutes). However, it is difficult to envisage such slow movement of vesicles, since most of these processes are relatively rapid in vivo (38–41). Another possibility is that factors are recruited in a copper-dependent manner to facilitate vesicle biogenesis, fission and fusion. Alternatively, the packaging and export of MNK from the TGN into this specific transport step may be a copper-dependent event.

What are the factors that regulate this novel step in trafficking?

Our studies show that the Rho GTPase Cdc42, and its downstream target WASp, a protein involved in actin polymerization, regulates this trafficking step. However, previous studies have also implicated heterotrimeric G proteins (42–44), ARF1 (45) and Rab GTPases (46,47) in different TGN-to-plasma membrane traffic events. It is possible that some of these may also play a role. The PKA inhibitors H-89 and PKI(14-22) (48) both inhibited copper-dependent trafficking to the cell surface, suggesting a role for PKA. PKA is regulated through the second messenger cAMP in response to several extracellular signals, and in turn acts on a large variety of intracellular events, including different pathways and stages of membrane traffic (49,50). Interestingly, a role for PKA has been suggested for the budding of constitutive transport vesicles from the TGN (49). In this respect, it is interesting to note that the copper-regulated trafficking of MNK from the TGN to the cell surface is dependent on PKA activity and Cdc42, but not PKD, all of which are thought to be involved in constitutive protein trafficking. This is further evidence for the existence of a parallel, copper-regulated protein trafficking step in addition to the more common constitutive pathway. Future studies will determine the exact nature of the cytosolic and membrane-bound machinery needed to facilitate the copper-dependent transport of MNK.

MATERIALS AND METHODS

Reagents and cell lines

All reagents and chemicals were from Sigma (Poole, UK) unless otherwise stated. The general protein kinase inhibitor, staurosporine (1 μM), PKA inhibitors PKI(14-22) (0.5 μM) and H-89 (20 μM), the PKC inhibitor calphostin C (0.1 μM), and the PKA activator forskolin (100 μM) were all purchased from Calbiochem (California). The protein kinase inhibitors were used at concentrations reported to specifically inhibit the indicated protein kinase (48,51–53). The Genejuice transfection reagent was obtained from Novagen (Madison, WI). HeLa cells were from ECACC (Porton Down, UK) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 2 mM glutamine supplemented with penicillin and streptomycin.
Immunofluorescence analysis, transfections and antibodies

For indirect immunofluorescence analysis, cells were grown to 40–80% confluence on glass coverslips, fixed in 4% (w/v) paraformaldehyde or methanol at −20°C and processed as previously described (54). Transient transfections were performed using Genejuice as per the manufacturer’s instructions, and cells were processed 24–48 h after transfection. The R70 rabbit antibody was raised against a peptide containing the sequence TSEPDKHSLL VGDFREDDDTAL in the MNK protein; this corresponds to the cytosolic portion of the extreme C terminus in the membrane protein. Antibody was purified from rabbit serum using a Protein G Sepharose column (Amersham Pharmacia Biotech) as per the manufacturer’s instructions. Mouse monoclonal antibody GST-2 to the GST
tag and mouse anti-actin antibody were from Sigma. Sheep polyclonal anti-TGN46 has been previously described (55,56). The monoclonal antibody against CD4 was purchased from Probes (Eugene, OR). Secondary antibodies used were FITC- or Texas Red-conjugated reagents from Molecular Probes (Eugene, OR). Cells were viewed using a Nikon Optiphot microscope and a 60× oil objective connected to a Bio-Rad 1024 confocal microscope. Images were collected using Bio-Rad Lasersharp software for viewing, analysis and quantification. To avoid bleed-through from the different channels, sequential capture of each image were performed on doubly labelled samples.

Isolation of plasma membrane lawns and quantification by immunofluorescence microscopy

The preparation of plasma membrane lawns is based upon that by Heuser et al. (57). Briefly, cells grown on coverslips were washed in PBS, and then coated with 0.2 mg/ml poly-L-lysine in PBS for 2 min at room temperature. Cells were rinsed three times in swelling buffer [0.7 mM magnesium acetate, 37 mM potassium acetate, 1.7 mM sodium acetate, 20 mM Hepes, 1 mM EGTA, pH 7.3, plus a protease inhibitor cocktail (Amer sham)] and then disrupted by placement under a 5 mm ultrasonic probe (Sonics Vibracell, UK) for 5 s at 40% power in 1 ml transport buffer (2 mM magnesium acetate, 110 mM potassium acetate, 5 mM sodium acetate, 20 mM Hepes, 1 mM EGTA, pH 7.3, plus protease inhibitor cocktail). Sonicated cells were immediately washed in PBS and fixed in −20°C methanol for 10 min prior to processing for immunofluorescence and confocal microscopy.

Fluorescence intensity was estimated as an average pixel intensity of digitized images from isolated plasma membrane lawns from membrane fragments of equivalent size from three different fields using laser scanning confocal microscope analysis software (Bio-Rad Laboratories). All images had identical scaling, and each experiment was repeated on at least four separate occasions. SEM values displayed on the histograms were calculated using Microsoft-Excel.

For transfection studies, fluorescence intensity of individual transfected cells was estimated as an average pixel intensity of digitized images measured from the plasma membrane regions using laser scanning confocal microscope analysis software (Bio-Rad Laboratories). The levels of plasma membrane MNK in each transfected cell was compared with at least three untransfected cells from the same field. At least 10 transfected cells were analysed from each experiment, and experiments were repeated a minimum of four times. SEM values displayed on the histograms were calculated using Microsoft-Excel.

Plasmids and cDNA constructs

The wild-type and mutant K618N PKD constructs previously described (17) were generously provided by Vivek Malhotra (University of California at San Diego). The clones for dominant-negative and constitutively active mutants of Cdc42, Rac1 and RhoA, and the wild-type WASp, were as described previously (35) and were kindly provided by Romano Regazzi (University of Lausanne, Switzerland). The pCMUV–CD4 construct has been described previously (58).

ACKNOWLEDGEMENTS

We thank Professor V. Malhotra (University of California at San Diego) for generously providing the wild-type and mutant K618N PKD constructs and for useful discussions. We are indebted to Professor R. Regazzi (University of Lausanne, Switzerland) for providing the plasmids encoding mutant WASp, Cdc42, RhoA and Rac1, and for his help and advice. This work was supported by funding from the Wellcome Trust. A.P.M is a Wellcome Principal Research Fellow.

REFERENCES


