Features in the N and C Termini of the MAPK-interacting Kinase Mnk1 Mediate Its Nucleocytoplasmic Shuttling*

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Eukaryotic initiation factor eIF4E binds to the 5′-cap structure of the mRNA and also to the molecular scaffold protein eIF4G. eIF4E is a phosphoprotein, and the kinases that act on it have been identified as the MAPK-interacting kinases Mnk1 and Mnk2. Mnk1/2 also bind to the scaffold protein eIF4G. The N-terminal region of Mnk1 has previously been shown to bind to importin α, a component of the nuclear transport machinery, although Mnk1 itself is cytoplasmic. Here we identify a CRM1-type nuclear export motif in the C-terminal part of Mnk1. Substitution of hydrophobic residues in this motif results in Mnk1 becoming nuclear. This has allowed us to study the features of Mnk1 that are involved in its transport to the nucleus. This process requires part, but not all, of a polybasic region near the N terminus of Mnk1. Residues required for nuclear transport are also required for its interaction with importin α. This polybasic region also serves a second function in that it is required for the binding of Mnk1 to eIF4G, although the residues involved in this interaction are not identical to those involved in the binding of Mnk1 to importin α. Interaction of Mnk1 with eIF4G promotes the phosphorylation of eIF4E. Mutations that reduce the binding of Mnk1 to eIF4G in vivo and in vitro also decrease the ability of Mnk1 to enhance eIF4E phosphorylation in vivo, underlining the importance of the eIF4G-Mnk1 interaction in this process.

The translation initiation factor (eIF) 4E binds specifically to the 5′-cap structure that is present on all eukaryotic cytoplasmic mRNAs. Overexpression of this factor can lead to transformation of cells (2, 3), and elevated levels of eIF4E correlate with the malignancy of human cancers (4), implying that eIF4E plays a key role in cellular regulation. Not surprisingly, the activity of eIF4E is regulated by several mechanisms.

One major regulatory mechanism is through the binding of 4E-Binding proteins (4E-BPs) to eIF4E (5, 6). The binding of 4E-BP1, the best studied of the 4E-BPs, is controlled by a complex mechanism involving phosphorylation at multiple sites, in which phosphorylation on some residues is dependent on the prior phosphorylation of other residues. The 4E-BPs bind to the so-called dorsal site of eIF4E. After phosphorylation and release of the 4E-BPs, eIF4G can bind to this site in eIF4E, resulting in the formation of the eIF4F complex (consisting of eIF4E, eIF4G, and the RNA helicase eIF4A). Subsequent binding of several other components of the translational machinery, e.g. eIF4B, eIF3, and the initiator-tRNA carrying 40 S ribosomal subunit leads to formation of active initiation complexes (7). Binding of 4E-BP1 to eIF4E therefore blocks the engagement of eIF4E with eIF4G and the formation of initiation factor complexes.

A second mechanism for regulating eIF4E function is through its phosphorylation at Ser-209. Recent work has indicated that this modification reduces the affinity of eIF4E for capped RNA (8, 9). Within the eIF4F complex, eIF4E can be phosphorylated by the MAPK-interacting kinases (Mnks) Mnk1 or Mnk2 (10–12; reviewed in Refs. 13 and 14). These kinases are under the control of the classic MAPK and the stress-activated p38 MAPK α/β pathways. The Mnks bind to the C terminus of eIF4G (10, 11), which is thought to ensure that phosphorylation of eIF4E only occurs within initiation complexes. Binding of Mnk1 to eIF4G involves a sequence near the N terminus of Mnk1 (11), although this has not been defined further. Replacement of the wild type eIF4E with a non-phosphorylatable form has been shown to reduce cell size in Drosophila, and to result in smaller flies, indicating that phosphorylation of eIF4E plays an important role in cell growth (15).

Several groups have reported that eIF4E is found in the nucleus as well as in the cytoplasm (reviewed in Ref. 16). Its exact role within this compartment is not clear, but it seems to play a role in the transport of certain mRNAs from the nucleus to the cytoplasm. The best described example is the mRNA encoding cyclin D1 (17). Overexpression of eIF4E results in increased levels of cytoplasmic cyclin D1 mRNA levels and consequently higher protein levels. This is thought to be one of the main causes for transformation of cells that overexpress eIF4E. The effects of eIF4E on cyclin D1 mRNA transport can be counteracted by overexpression of the promyelocytic leukemia protein, which reduces the affinity of eIF4E for cap structures (18). This may play a role on the transformation-repressing functions of promyelocytic leukemia protein.

The intracellular localization of Mnk1 has been shown to be sensitive to leptomycin B, a compound that blocks CRM1-mediated nuclear export (19). These findings imply that Mnk1 can enter the nucleus, and thus that it possesses motifs for nuclear localization (importin). This notion is consistent with the finding that Mnk1 binds to importin α in a yeast two-hybrid assay. This interaction, like its binding to eIF4G, appeared to involve

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§ The abbreviations used are: eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; GFP, green fluorescent protein; GST, glutathione S-transferase; HEK, human embryonic kidney; LMB, leptomycin B; MAPK, mitogen-activated protein kinase; Mnk, MAPK-interacting kinase; NES, nuclear export signal; NLS, nuclear localization signal; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin.
Localization Signals in Mnk1

EXPERIMENTAL PROCEDURES

Plasmids—pCSSMT-Mnk1 has been described previously (20). pEGFP-Mnk1 was made by cloning an HindIII-BamHI-digested PCR product, encoding the full-length Mnk1 into pEGFP-C1 (Clontech). Mutations in both pCSSMT-Mnk1 and pEGFP-Mnk1 that change residues Lys-14, Arg-15, Ser-380, Ser-383 to alanine, Leu-378 to serine, and triple mutations of KRR (residues 14–16) and KKR (residues 18–20) to alanine residues were created using the QuikChange® kit from Stratagene. The K18A and K19A mutations were introduced as PCR-generated HindIII-XmaI-digested fragments. PCR reactions were carried out using the high fidelity Expand HiFi® kit from Roche Applied Science. DNA sequencing was performed by the Sequencing Service (School of Life Sciences, University of Dundee, Scotland (available at www.dnaseq.co.uk)) using a DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers.

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells were grown in 10-cm plates in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Transient transfections were carried out by calcium phosphate precipitation of the DNA in BES-buffered saline as described earlier (21). For most purposes, 10 µg of DNA were used per 10-cm dish; however, for experiments where the phosphorylation of eIF4E was studied, it was important to achieve lower levels of expression of Mnk1, and 1.5 µg of Mnk1 vector plus 8.5 µg of carrier DNA (empty vector) was used.

Harvesting of Cells—After treatments, cells were washed once with phosphate-buffered saline and either harvested in 400 µl of harvesting buffer A (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 25 mM β-glycerophosphate, 0.2 mM EDTA, 10% glycerol, 1 mM Triton X-100, 1 mM diethiothreitol, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml antipain, and 1 µg/ml pepstatin) or (for studies on binding to eIF4G) in buffering of the DNA in BES-buffered saline as described earlier (21). For most purposes, 10 µg of DNA were used per 10-cm dish; however, for experiments where the phosphorylation of eIF4E was studied, it was important to achieve lower levels of expression of Mnk1, and 1.5 µg of Mnk1 vector plus 8.5 µg of carrier DNA (empty vector) was used.

Isoelectric Focusing—Endogenous eIF4E was purified from 200 µg of cell lysate protein by mGTP-Sepharose chromatography, resolved by isoelectric focusing, and detected by Western blotting as described previously (22).

Immunoprecipitation—For immunoprecipitations using anti-myc, 2 µl of antibody (9E10 from Sigma, catalog number M5546) was added to cell extracts (200 µg of protein) and incubated for 1 h at 4 °C. Subsequently, protein G-Sepharose beads (20 µl of packed beads per immuno-precipitation) of the appropriate harvesting buffer were added and allowed to bind the antibody for an additional hour at 4 °C. The beads were then washed three times with 500 µl of buffer and finally resuspended in SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE and Western blotting using appropriate antisera.

Fluorescence Microscopy—HEK293 cells were grown on coverslips, transfected, and grown for an additional 24 h. Coverslips were then washed with DAPI fixation solution (0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI), 20 mM Tris-HCl, pH 6.9, 0.1 mM MgCl₂, 0.1 mM EDTA in 90% methanol) and then incubated in the same solution for 15 min at made as a control (S380A) as indicated. D, mutation in the putative NES inhibits nuclear export. HEK293 cells were transfected with the indicated GFP-Mnk1 constructs and fixed after 24 h. GFP-tagged proteins were visualized by fluorescence microscopy (right panels). The panels on the left show the cells visualized by light contrast microscopy. The insets in the panels on the left show GFP-Mnk1 localization at a higher magnification. Similar data were obtained in five separate experiments.
Localization Signals in Mnk1

Assays for Binding of Mnk1 to Importin α—Hexahistidine-tagged importin α (His-importin α) was expressed in E. coli and purified on Talon® beads. Wild type Mhc-tagged Mnk1 or the indicated variants were expressed in transfected HEK 293 cells. The levels of the expressed Mnk1 proteins were determined by Western blotting with anti-Mhc. The extracts were subsequently incubated with His-importin α bound to beads. The part of the Coomassie Blue-stained gel with His-importin α is shown in the middle panel. After washing of the beads the amount of bound myc-Mnk1 was determined by Western blotting with antibodies directed against the myc-tag (lower panel). Similar data were obtained in four separate experiments. The relative amount of myc-Mnk1 bound was calculated as the signal for myc-Mnk1 (determined by densitometric analysis of the Western blot) divided by the signal for importin α (determined by Coomassie Blue staining of the gel), expressed as the percentage of the value for the wild type control. This is clearly an operational rather than an absolute ratio.

To study the binding of Mnk1 to eIF4G, 293 cells were transiently transfected with plasmids encoding myc-tagged wild type or mutant Mnk. After transfection, the cells were lysed with buffer C (1% [v/v] Triton X-100, 50 mM NaF, 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, 2 mM sodium orthovanadate, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mM PMSF). The lysates were then incubated (1 h, 4°C) with His-importin α bound to Talon® beads (Clontech). After washing the beads thrice with the same buffer, the amount of bound myc-Mnk1 was determined by Western blotting with anti-myc.

To study the possibility of competition between eIF4G and His-importin α for binding to Mnk1, GST-Mnk1 (expressed in E. coli (12)) was immobilized at a final concentration of 4 mg/ml on glutathione-Sepharose 4B beads. The beads were then incubated for 1 h in the presence of 5% (v/v) BSA. The amount of His-importin α was fixed, and the amount of His-eIF4G, was varied. The final concentration of NaCl in the buffer used in the assay was always adjusted to 50 mM.

To assess whether eIF4G interacted directly with importin α, it was expressed in E. coli as a GST fusion protein. The purified fusion protein was incubated with recombinant his-tagged importin α in the presence of 5% (v/v) BSA at 50 mM NaCl (as for the above experiments). A negative control was performed in which GST-eIF4Gc was omitted. The material bound to glutathione-Sepharose was analyzed by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

The C-terminal Part of Mnk1 Contains a Hydrophobic Nuclear Export Signal—Waskiewicz et al. (11) have reported that Mnk1 interacts with eIF4G and importin α in a yeast two-hybrid system. The interaction with eIF4G or importin α was abrogated by deletion of the N-terminal 23 amino acids, and it was suggested that the stretch of basic residues in the N

FIG. 3. Effects of mutations on the binding of Mnk1 to importin α. Hexahistidine-tagged (His) importin α was expressed in E. coli and purified on Talon® beads. Wild type Mhc-tagged Mnk1 or the indicated variants were expressed in transfected HEK 293 cells. The levels of the expressed Mnk1 proteins were determined by Western blotting with anti-Mhc. The extracts were subsequently incubated with His-importin α bound to beads. The part of the Coomassie Blue-stained gel with His-importin α is shown in the middle panel. After washing of the beads the amount of bound myc-Mnk1 was determined by Western blotting with antibodies directed against the myc-tag (lower panel). Similar data were obtained in four separate experiments. The relative amount of myc-Mnk1 bound was calculated as the signal for myc-Mnk1 (determined by densitometric analysis of the Western blot) divided by the signal for importin α (determined by Coomassie Blue staining of the gel), expressed as the percentage of the value for the wild type control. This is clearly an operational rather than an absolute ratio.

To study the binding of Mnk1 to eIF4G, 293 cells were transiently transfected with plasmids encoding myc-tagged wild type or mutant Mnk. After transfection, the cells were lysed with buffer C (1% [v/v] Triton X-100, 50 mM NaF, 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, 2 mM sodium orthovanadate, 14 mM [v/v] mercaptoethanol, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mM PMSF). The lysates were then incubated (1 h, 4°C) with His-importin α bound to Talon® beads (Clontech). After washing the beads thrice with the same buffer, the amount of bound myc-Mnk1 was determined by Western blotting with anti-myc.

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To assess whether eIF4G interacted directly with importin α, it was expressed in E. coli as a GST fusion protein. The purified fusion protein was incubated with recombinant his-tagged importin α in the presence of 5% (v/v) BSA at 50 mM NaCl (as for the above experiments). A negative control was performed in which GST-eIF4Gc was omitted. The material bound to glutathione-Sepharose was analyzed by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue.
FIG. 4. Mutations in Mnk1 that affect its binding to eIF4G also affect eIF4E phosphorylation in vivo. A, mutations in the basic region reduce eIF4G binding to Mnk1. HEK293 cells were transfected with the pCS3MT constructs to express the different myc-tagged Mnk1 variants, as indicated. After harvesting the cells, the myc-tagged Mnk1 proteins were immunoprecipitated in low salt buffer as described under “Experimental Procedures.” The amount of myc-Mnk1 and the presence of co-immunoprecipitated eIF4G were determined by SDS-PAGE and Western blotting with the appropriate antibodies. The top section shows the input eIF4G. The band below eIF4G in the middle section (marked with an asterisk) is a nonspecific cross-reacting band.

B, impaired phosphorylation of eIF4E in cells expressing Mnk1 variants with decreased eIF4G binding. Cells transfected with the same constructs as used in panel A were harvested, and eIF4E was purified by m7GTP-Sepharose chromatography. The phosphorylation state of eIF4E was determined by isoelectric focusing and Western blotting. The two forms of eIF4E are indicated on the right. Phosphorylation of eIF4E, given as a percentage of the total amount of eIF4E, was determined using the ImageJ software (available at rsb.info.nih.gov/ij/).

C, as described under “Experimental Procedures,” possible competition between eIF4Gc and importin α for binding to Mnk1 was studied by incubating recombinant GST-Mnk1 with a fixed amount of importin α and increasing amounts of eIF4Gc. The material bound to glutathione-Sepharose was analyzed by SDS-PAGE followed by staining of the gel with Coomassie Brilliant Blue. The last two lanes show 5% of...
terminus of Mnk1 (KKRRKKRR, residues 14–21 in the mouse sequence) might be involved in these interactions, because nuclear localization signals (NLS) often contain multiple basic residues (24). However, the interaction with importin α was not investigated further in that study, because immunofluorescence microscopy revealed that Mnk1 was localized in the cytoplasm, suggesting that Mnk1 might not actually be imported into the nucleus. However, McKendrick et al. (19) subsequently showed that the localization of Mnk1 is sensitive to leptomycin B (LMB), such that treatment of cells with this compound resulted in an increased proportion of the Mnk1 being found in the nuclear compartment. This finding implies that Mnk1 may shuttle between the nucleus and the cytoplasm.

We expressed a green fluorescent protein (GFP) fusion protein of Mnk1 by transient expression in transfected HEK 293 cells. Our data confirm the results of McKendrick et al. (19), i.e., that LMB treatment resulted in the accumulation of GFP-Mnk1 in the nucleus (Fig. 1A). To be sure that the cytoplasmic location seen in the absence of LMB is not due to the presence of the large GFP tag, the experiments were repeated with Mnk1 variants containing the much smaller myc tag. As shown in Fig. 1B, the myc-Mnk1 fusion protein is also primarily cytoplasmic in the absence of LMB and relocates completely to the nucleus when this compound is added. Thus, the sensitivity to LMB is likely an intrinsic property of Mnk1 itself rather than a feature conferred by the GFP tag. Indeed, other work has shown that the localization of GFP itself is not affected by LMB (25).

The effects of LMB on the localization of Mnk1 suggest that its export from the nucleus is mediated via CRM1. The consensus sequence for CRM1-mediated nuclear export signals is "D/ESFFDD" (where D represents a hydrophobic residue, and X is any amino acid). Inspection of the amino acid sequences of human or murine Mnk1 revealed a possible leucine-rich nuclear export signal (NES) is found in the C-terminal part of the protein (Fig. 1C). In murine Mnk1 the motif lies between residues 374 and 383 and has the sequence LAEGLCSMKL (where hydrophobic residues are shown in boldface).

To test whether this motif is involved in the export of Mnk1 from the nucleus, we mutated Leu-378 and Leu-383 individually to non-hydrophobic residues, i.e. serine or alanine, respectively. As a negative control, we also mutated a residue within the region of the motif but which does not form part of the hydrophobic consensus (Ser-380, to Ala). The resulting S380A mutant of Mnk1 was found almost exclusively in the cytoplasm (Fig. 1D, see insets for higher magnification images), as is the case for the wild type protein. In marked contrast, the variants L378S and L383A were both almost completely located in the nucleus, indicating that Leu-378 and Leu-383 play a key role in ensuring the cytoplasmic location of the wild type protein. This indicates that the series of hydrophobic residues highlighted in Fig. 1C is indeed likely to be a CRM1-binding nuclear export motif that mediates transfer of Mnk1 to the cytoplasm. Importantly, these data also strongly imply that Mnk1 does normally enter the nucleus (perhaps through its interaction with importin α), its overall cytoplasmic distribution being due to efficient re-export via CRM1, which is dependent upon the hydrophobic NES. Similar conclusions were recently drawn for the nucleocytoplasmic shuttling for several other translation factors (26).

Indeed, like Mnk1, a range of translation factors appears to be actively excluded from the nucleus.

**Nuclear Entry of Mnk1 Requires a Polybasic Region Near Its N Terminus**—Because the above data indicated that Mnk1 can enter the nucleus, and given the indications that the stretch of basic residues near the N terminus allowed interaction with the importin α (11), we next studied the role of this basic region in the nuclear import of Mnk1. To facilitate these studies, we made use of the L378S variant that, as described in Fig. 1, localizes to the nucleus due to inactivation of its NES. We anticipated that mutation of additional residues that are critical for nuclear import should result in the L378S variant becoming cytoplasmic. Alignment of the part of various Mnk sequences containing the basic stretch reveals several residues that are conserved among the Mnk1/2 proteins from mouse, human, and *Xenopus* (Fig. 2A).

Initially, we made two triple mutations in Mnk1 in which, respectively, the basic residues at positions 14–16 (KKR) or 18–20 (KKR) were changed to alanine (KKR/AAA and KKR/AAA variants, respectively; Fig. 2B). The resulting mutated proteins were expressed as fusion proteins with GFP in HEK293 cells. Immunofluorescence microscopy showed that the KRR/AAA variant of GFP-Mnk1[L378S] still localized to the nucleus. In contrast, the triple Ala mutation in the second part of the basic stretch completely abolished nuclear fluorescence (variant KKR/AAA; Fig. 2C). Apparently, the second part of this region is essential for nuclear import, whereas the first half of the basic region is dispensable for this process.

To pinpoint which residues within this polybasic region are critical for NES function, a set of single residue substitutions was created. The basic residues at positions 14, 15, 18, or 19 were individually replaced by alanines, and the effects of these changes on the localization of GFP-Mnk1[L378S] were studied. As shown in Fig. 2D, the behavior of the K14A and R15A variants was indistinguishable from that of GFP-Mnk1[L378S] itself: both were found almost entirely within the nucleus. This corroborates the above data, which showed that the first part of the polybasic region (in which these residues are situated) is dispensable for nuclear import of Mnk1. The K18A variant also showed a similar localization to the GFP-Mnk1[L378S] fusion, i.e. was nuclear. A high proportion of the K19A variant was also found in the nucleus, but substantially more of this variant was found in the cytoplasm than was the case for the other three mutants. These findings suggest that the most C-terminal part of the polybasic region is important for nuclear import, consistent with the findings for the triple mutants studied in Fig. 2C. The cytoplasmic localization of the KKR/AAA variant is presumably due to the alteration of Lys-19 and probably Arg-20 to alanine rather than the substitution of Lys-18.

**The More C-terminal Part of the Polybasic Motif of Mnk1 Is Required for Binding to Importin α**—A previous study had
shown that all or some of the first 23 amino acids of Mnk1 were required for its binding to importin α but had not further identified the exact binding site (11). The facts that importin α generally binds polybasic regions in its cargoes (27) and that mutation of three residues within this part of Mnk1 hinders nuclear transport (in the KKR/AAA mutant; Fig. 2C) suggested that this region might interact directly with importin α.

To test this idea, importin α was expressed as a hexahistidine fusion protein in E. coli, and purified on Talon® resin. This purified protein, still bound to this resin, was then incubated with wild type Myc-Mnk1 or with the various point mutants of Myc-tagged Mnk1 (all made in HEK293 cells). The His-importin α was then recovered by centrifugation, and the amount of associated myc-Mnk1 was assessed by SDS-PAGE followed by immunoblotting with anti-myc.

As shown in Fig. 3, wild type Mnk1 bound to His-importin α, as expected from earlier findings using the yeast two-hybrid system (11). The KRR/AAA variant of Mnk1 still bound to His-importin α, but the other triple mutant, KKR/AAA, showed only a very low level of association with His-importin α. This is completely consistent with the data of Fig. 2C, where the KRR/AAA variant was found to enter the nucleus but the KKR/AAA variant did not, and again suggests that the second part of the polybasic region is critical for nuclear import of Mnk1, but the first section is dispensable. (Given the slight variations in the levels of expression of the Mnk1 variants, it is important to consider the ratios of myc-Mnk1:His-importin α displayed below each lane in Fig. 3.)

Consistent with this, we found that single mutations that affect the first part of the motif (K14A and R15A) had little if any effect on the binding of Mnk1 to His-importin α, whereas the mutation K19A (in the second half of this feature) caused a marked reduction in His-importin α binding. In contrast, mutation of the immediate N-terminally neighboring residue, Lys-18, to alanine, had no discernible effect on binding to His-importin α (Fig. 3) consistent with its lack of effect on nuclear localization of GFP-Mnk1 L378S (Fig. 2D). A possible reason for this is that the K18A variant still retains three consecutive basic residues within the C-terminal part of the polybasic motif that may be important for importin α binding, whereas this is disrupted in the K19A mutant (see alignment in Fig. 2B).

Many nuclear localization sequences contain stretches of five consecutive basic residues (e.g. NLS in SV40 large T-antigen or lymphoid enhancer factor-1), although shorter stretches have also been shown to function in nuclear import in some cases (e.g. the NLS in myc or NF-κB p50, for a detailed study of NLS sequences see (27)). The K19A variant still contains a stretch of five consecutive basic residues (in the more N-terminal part of this polybasic region) but nonetheless binds poorly to His-importin α. This underlines the importance of the C-terminal section of this region in Mnk1 for nuclear import that was revealed by the finding that the KKR/AAA variant still enters the nucleus, whereas the KKR/AAA variant does not (Fig. 2C).

The observation that a substantial amount of the K19A mutant of Mnk1 L378S is found in the nucleus (Fig. 2D) seems to contradict the finding that the K19A mutation substantially decreases binding to His-importin α (Fig. 3). However, this mutation does not abolish binding to His-importin α. Although import of this mutant is clearly impaired, as indicated by the significant GFP signal seen in the cytoplasm, export is completely prevented by the Lys→Ser alteration in the NES, and thus, over time, the protein accumulates in the nucleus. The observation that the Mnk1 L378S[KKR/AAA] mutant is cytoplasmic suggests that its nuclear import is completely prevented by mutation of these three basic amino acid residues (Fig. 2D).

The eIF4G Binding Site in Mnk1 Is Not Identical to the Importin α Binding Site—Earlier data have indicated that the N-terminal region of Mnk1 is involved in its interaction with eIF4G (11), although the residues within this region that bind eIF4G have not been identified. To test the role of the polybasic region in the binding of Mnk1 to eIF4G, Myc-tagged Mnk1, or the KRR/AAA or KKR/AAA substitutions, were expressed in HEK293 cells, and their association with eIF4G was analyzed by immunoprecipitating the Mnk1 with anti-myc and analyzing the immunoprecipitates by SDS-PAGE/Western blotting using anti-eIF4G or anti-Myc (as control for the expression of the Mnk1 proteins). Wild type Mnk1 clearly bound to eIF4G (Fig. 4A), whereas no eIF4G signal was seen for lysates from cells transfected with the empty vector. The KKR/AAA mutations in the C-terminal part of the polybasic region resulted in a drastic reduction in the ability of Mnk1 to bind to eIF4G (Fig. 4A). As described above, these substitutions also greatly decrease binding of Mnk1 to importin α and nuclear entry (Figs. 2C and 3). Thus, these residues in Mnk1 appear to be important for binding of both protein partners. In contrast, a marked difference was observed for the KRR/AAA substitutions, which decreased the binding of Myc-Mnk1 to eIF4G (Fig. 4A), even though they had no effect on binding to importin α or on nuclear entry (Figs. 2 and 3). This suggests that either both parts of the polybasic region are required for the binding of Mnk1 to eIF4G or that the presence of several basic residues in this region are required for stable binding to eIF4G.

In vivo, the phosphorylation of eIF4E by Mnk1 is greatly facilitated by the binding of both proteins to their common scaffold partner, eIF4G (10). Therefore, mutations in Mnk1 that affect its ability to bind to eIF4G should be manifested by alterations in the level of phosphorylation of eIF4E in cells that overexpress Mnk1. To study this, HEK293 cells were transfected with vectors encoding wild type Mnk1 or selected mutants, and 24 h later, lysates were prepared and analyzed by isoelectric focusing/immunoblotting to assess the level of phosphorylation of eIF4E.

The data in Fig. 4B clearly show that expression of wild type Myc-Mnk1 increases the phosphorylation of eIF4E to well above the basal level seen in cells transfected with the empty vector. Each of the triple mutations KKR/AAA and KKR/AAA greatly reduced the increment in eIF4E phosphorylation when compared with the wild type Mnk1. This is entirely consistent with the greatly reduced ability of these variant proteins to bind to eIF4G (Fig. 4A). The single mutations had much less effect, with only the K18A and K19A variants showing a reproducible decrease in eIF4E phosphorylation compared with the wild type Myc-Mnk1 (Fig. 4B, lower panel). This finding matches the relatively larger effects of these two mutations on binding to eIF4G observed in Fig. 4A, whereas the K14A and R15A variants bound eIF4G to similar extents as the wild type Mnk1. The latter two variants seem to promote similar levels of eIF4E phosphorylation to those seen with wild type Mnk1 when the slightly lower level of expression of the K14A variant is taken into account (Fig. 4B, upper panel). Mutation of K19 apparently decreases the binding of Mnk1 to His-importin α or eIF4G, whereas the K18A mutation affects only eIF4G binding.

eIF4G Does Not Compete with Importin α for Binding to Mnk1—The finding that the interaction of Mnk1 with importin α or eIF4G involves overlapping regions of the Mnk1 polypeptide suggested that these two proteins might compete with one another for binding to Mnk1. This could have important implications for the role of Mnk1 in controlling translation, on one hand, and its transfer into the nucleus, on the other.

To test whether competition between eIF4G and importin α
for binding to Mnk1 did indeed occur, recombinant GST-Mnk1 was incubated with a fixed amount of one partner (e.g. his tagged importin α), which was in excess over the amount of GST-Mnk1, while the amount of the other (e.g. his tagged eIF4Gc) was varied. The GST-Mnk1 and associated proteins were recovered on glutathione-Sepharose, and the bound proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining of the gel. The amounts of bound importin α and eIF4Gc, could be determined in this way and were quantitated using the LICOR Odyssey® imaging system. We took care to ensure that the total amount of the partners was sufficient to saturate the available Mnk1 and to perform the experiment in both possible ways (i.e. where the amount of either His-importin α or of eIF4Gc, fixed, while the quantity of the other protein was varied). The results show that, upon addition of increasing amounts of importin α, increased binding of importin α to Mnk1 was found, whereas binding of eIF4Gc was constant, or vice versa when the reverse experiment was performed (Fig. 4C).

One concern was that eIF4Gc might bind importin α directly, a feature that would have invalidated this approach. To test this, we expressed eIF4Gc, as a fusion protein with GST and tested its ability to bind to hexahistidine tagged importin α in pull downs on glutathione-Sepharose. As shown in Fig. 4D, no such direct interaction was observed, ruling this out as a possible explanation for the apparent lack of competition between eIF4G and importin α for binding to Mnk1.

In extensive in vitro experiments of this type, we never observed such competition. For further accuracy, the data were carefully quantitated: analysis of a typical experiment is shown in Fig. 4E. This suggests that the precise features within Mnk1 that bind these two partners are distinct, as also indicated by our other data using mutants of Mnk1 (see above). Because these experiments were performed with purified recombinant components, the data also confirm that Mnk1 and importin α interact directly, rather than indirectly through, e.g. a third common binding partner.

Concluding Remarks—The data presented here identify features in Mnk1 that are required for the trafficking of Mnk1 between the cytoplasm and the nucleus. Its N terminus contains a polybasic region that binds importin α and allows Mnk1 to enter the nucleus (Fig. 4F). Its C terminus contains a hydrophobic region that allows its CRM1-mediated export from the nucleus, so that, overall, Mnk1 is primarily a cytoplasmic protein. It is possible that under certain conditions the proportion of Mnk1 in the nucleus might be enhanced, e.g. if the function of its NES is impaired. This could happen, perhaps, as a consequence of phosphorylation of the conserved Ser-Pro phosphorylation site immediately C-terminal to this feature (Ser-384 in human Mnk1), which may be phosphorylated by MAPK or p38 MAPK (12). However, so far, we have not found any condition that alters the subcellular localization of Mnk1 in HEK293 cells. Conditions tested included ones that are known to activate MAPK and/or p38 MAPKs and which are known to enhance the phosphorylation and activation of Mnk1. Thus, under all the conditions we have tested in HEK293 cells, Mnk1 was cytoplasmic. In an earlier study, Waskiewicz et al. (11) showed that treatment of HEK293 cells with 12-O-tetradecanoylphorbol-13-acetate (which markedly activates Mnk1 (28)) failed to cause Mnk1 to enter the nucleus, although it did cause its localization to become perinuclear. The physiological significance of the presence in Mnk1 of a nuclear localization signal is thus so far unclear. The best characterized substrate of Mnk1, eIF4E, is found in the nucleus in a number of cell types (reviewed in Refs. 16). However, we consistently find that eIF4E is almost entirely cytoplasmic in HEK293 cells, whereas a much higher proportion of the eIF4E is nuclear in other cell types that we have tested (29). It is possible that HEK293 cells lack a component that is required for the efficient nuclear retention of eIF4E and Mnk1, which exists in some other cell types. This is not the promyelocytic leukemia protein, a nuclear binding partner for eIF4E (18), because this protein is present in the nuclei of HEK293 cells. Further work will be required to address this issue.

Earlier data had suggested that the N terminus of Mnk1 interacted with eIF4G as well as with importin α (11). Here we show that both interactions require the N-terminal polybasic region of Mnk1 and that this feature is required for its recruitment to initiation complexes to allow it efficiently to phosphorylate eIF4E in vivo. The requirements for binding to eIF4G and importin α are, however, distinct. Simultaneous alteration of three basic residues at either the N- or C-terminal end of the polybasic region abolishes the interaction with eIF4G, but only the C-terminal cluster appears essential for binding to importin α. Within that cluster, mutation of Lys-18 to alanine reduces binding to eIF4G but not to importin α. Binding to both partners seems to involve specific residues rather than just any of the basic residues in this region as exemplified by the effect of the K19A point mutation, but not the others, on importin α binding, and by the effects of the mutation of Lys-18 and Lys-19 but not Lys-14 or Arg-15 on binding to eIF4G.

These data provide further information on the functional motifs present in Mnk1 and that mediate its interactions with other proteins. In addition to the MAPK interaction motif located in the far C terminus, the C-terminal region also contains a hydrophobic NES, which presumably functions by binding CRM1 (Fig. 4E). The N-terminal part of the protein contains a polybasic region that mediates binding to the scaffold protein eIF4G, which recruits Mnk1 to functional initiation complexes containing its known substrate, eIF4E, and also binds importin α, and mediates the transfer of Mnk1 into the nucleus.

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