5060–5068 *Nucleic Acids Research, 2006, Vol. 34, No. 18* *Published online 20 September 2006*
doi:10.1093/nar/gkl659

Multiple pathways regulate intracellular shuttling of MoKA, a co-activator of transcription factor KLF7

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Received April 5, 2006; Revised June 15, 2006; Accepted August 28, 2006

ABSTRACT

MoKA is a novel F-box containing protein that interacts with and stimulates the activity of transcription factor KLF7, a regulator of neuronal differentiation. MoKA accumulates throughout the cell and predominantly in the cytosol, consistent with the presence of several putative nuclear localization and export signals (NLSs and NESs). The present study was designed to refine the identity and location of the sequences responsible for MoKA intracellular shuttling and transcriptional activity. Forced expression of fusion proteins in mammalian cells demonstrated that only one of three putative NLSs potentially recognized by karyopherin receptors is involved in nuclear localization of MoKA. By contrast, three distinct sequences were found to participate in mediating cytoplasmic accumulation. One of them is structurally and functionally related to the leucine-rich export signal that interacts with the exportin 1 (CRM1) receptor. The other two export signals instead display either a novel leucine-rich sequence or an undefined peptide motif, and both appear to act through CRM1-independent pathways. Finally, transcriptional analyses using the chimeric GAL4 system mapped the major activation domain of MoKA to a highly acidic sequence that resides between the NLS and NES clusters.

INTRODUCTION

Transcriptional regulation of gene expression plays a central role in embryonic development, physiological growth and in the cellular responses to environmental stimuli, injury or disease (1). Transcription factors are in turn regulated by several different mechanisms. Among them, intracellular compartmentalization of protein complexes and trafficking between the nucleus and cytoplasm are some of the means to modulate the activity of transcription factors. Some transcription factors remain latent in the cytoplasmic compartment until a specific signal triggers their access to the nucleus to activate gene expression; others are induced by specific inhibitors to exit from the nucleus; and others shuttle continuously between these cellular compartments under the influence of nuclear import and export signals (2,3). Protein transport across the nuclear envelope is usually an active process that relies on the presence of signal sequences recognized by specific receptors and adaptors that translocate cargo through the nuclear pore complex (4–6). Several types of signals have been identified to date that interact with different transport pathways. The nuclear localization signal (NLS[1]) of the SV40 T antigen is the prototype of the basic NLS that is enriched in lysines and arginines (7). Another basic import signal rich in lysines and arginines is the so-called bipartite NLS motif, which contains two positively charged clusters separated by a linker region of 10–12 amino acids. Both types of NLSs are recognized by specific receptors (karyopherins or importins) (5). There are also several examples of import signals that deviate from the aforementioned motifs. They include NLSs rich in arginines and glycines or in glycines only, and the unusually large M9-type NLS that is rich in glycines and aromatic residues (8–10). By contrast, the pathways of signal-mediated nuclear export are less defined. The best characterized nuclear export signal (NES) consist of a short hydrophobic, leucine-rich sequence that was originally identified in the human immunodeficiency virus type I (HIV-1), Rev protein (11) and cellular kinase PKI (12). Most of the proteins containing this leucine-rich NES have been shown to be exported from the nucleus by interaction with the importin-β-related export factor CRM1, also known as exportin 1 (13–15). Other receptors that mediate export of specific substrates include CAS, exportin t, exportin 4, importin 13, and calreticulin (16–20). However, there is still a large number of shuttling proteins that display unique NESs and whose cognate receptors are yet to be identified (21–25).

We have recently described the isolation of MoKA, a novel F-box containing protein that superstimulates the activity of transcription factor KLF7 (26). KLF7 is a member of the mammalian Kruppel-like family of zinc finger proteins, which has been reported to control expression of the genes coding for the cell cycle regulators $p21^{Waf/Cip}$ and $p27^{kip}$ and the neurotrophin receptor TrkA (26–29). Gene function

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studies in mice have demonstrated the critical role of KLF7 in neuronal morphogenesis and survival of sensory neurons (28,29). MoKA is a rather large protein with a unique primary structure that is ubiquitously expressed at low level in the developing and adult mice, and which interacts with KLF7 through the F-box motif. Transient overexpression of fusion products harboring large deletions of MoKA have correlated separate clusters of putative basic NLSs and putative leucine-rich NESs with shuttling of the protein between the nucleus and the cytoplasm of asynchronously cycling cells (26).

An important limitation of these early studies is that they were performed using large deletions of MoKA and thus, albeit informative, they did not elucidate the contribution of individual signals to protein shuttling. As part of our ongoing effort to characterize MoKA function, the present study was therefore designed to provide a systematic and more detailed analysis of the factors responsible for the intracellular distribution of MoKA. The results of these experiments demonstrate that while a single basic NLS is sufficient for nuclear import of MoKA, cytoplasmic accumulation of the protein is instead under the control of both CRM1-dependent and CMR1-independent pathways that act distinctly from each other. As part of this study, we also show that the transcriptional activity of MoKA is mostly accounted for by an asparagines-rich sequence that resides between the putative NES and NLS clusters. Collectively, these and previous experiments suggest that MoKA is a modular protein made of structurally and functionally discrete peptide motifs.

MATERIAL AND METHODS

Preparation of plasmid constructs

The construct expressing the full-length MoKA-Flag fusion protein has been already described (26). Internal deletion of individual NESs [MoKA(Δ116–143)-Flag, MoKA(Δ142–203)-Flag, MoKA(Δ202–318)-Flag, and MoKA(Δ317–472)-Flag] or point mutations of the full-length protein were generated by PCR amplification using wild-type MoKA-Flag or wild-type MoKA-GFP as a template. In the case of the internal deletions, sequences were amplified with primer pairs that included XbaI and BamHI restriction enzyme sites, and the resulting products were subcloned into BamHI site of the pCMV-Tag 1 vector (Invitrogen). NESs-containing fragments (116–142 MoKA-GFP, 143–202 MoKA-GFP, 203–318 MoKA-GFP and 319–472 MoKA-GFP) were similarly generated by PCR amplification using appropriate primer pairs that included SalI and BamHI restriction enzyme sites. The resulting amplified products were then subcloned into SalI/BamHI sites of pEGFP-C3 vector (Clontech). Construct expressing wild-type MoKA and MoKA deletion mutants fused to the DNA-binding domain of GAL4 [(1–147)-GAL4] were generated by PCR amplification using MoKA-GFP as a template and appropriate primer pairs including EcoRI and BamHI restriction enzyme sites and subcloned into the pBXG1 vector (26). Constructs expressing MoKA fusion products of peptides containing NLS1, NLS2 and NLS3 (473–819, 820–1006, and 1007–1193 MoKA) were PCR amplified using MoKA-Flag as a template and subcloned into KpnI/NotI sites of the pcDNA3-PK-myc vector (kindly provided by Dr G. Dreyfuss, University of Pennsylvania School of Medicine, Philadelphia). Nucleotide substitutions were introduced into the MoKA sequence using the QuiKChange XL site-directed mutagenesis kit (Stratagene) as instructed by the manufacturer. All constructs were verified by DNA sequencing, and immunoblots were employed to verify the size of MoKA deletion products as well as to monitor that the various recombinant proteins were expressed at comparable levels.

Cell culture, DNA transfection and cell microscopy

Mouse NIH3T3 fibroblasts and green monkey kidney COS7 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum and/or calf serum (Hyclone) at 37°C in a 5% CO_2-humidified atmosphere. About 24 h before transfection, 8×10^4 cells were seeded on 2 cm^2 microscope cover glass (Fisherbrand) and grown in 24-well plate (Nalgene Nunc International). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. When needed, cell cultures were treated with 5 ng/ml of leptomycin B (LMB) (Sigma) or equal volumes of methanol 70% for 3 h before analysis. GFP-positive cells were visualized by auto-fluorecence microscopy, whereas antibodies against Flag, (1–147)GAL4 or myc were employed for the other cells transfections (26,30) Sub-cellular distribution of the fusion products was analyzed by confocal scanning microscopy (Zeiss LSM 510). The relative intracellular distribution of over-expressed proteins in each experimental sample was calculated as a nuclear to cytoplasmic ratio by measuring the intensity of the signals in each cellular compartment with the aid of the Photoshop software (Adobe Systems Inc., San Jose, CA). Measurements were performed on $\geqslant$100 cells from three independent transfections each performed in triplicate. Luciferase assay was described elsewhere (26). Statistical significance of the results was evaluated by the Student's *t*-test assuming $P \leqslant 0.05$ as significant.

RESULTS

One basic signal mediates nuclear localization of MoKA

We have previously shown that internal deletion of the 115/472 segment or elimination of the first 472 residues of MoKA lead to protein localization exclusively in the nucleus (26). These results were correlated with the presence within the 473/1193 segment of three putative basic NLSs located at positions 648/654 (NLS1), 901/904 (NLS2) and 1140/1146 (NLS3) (Figure 1A). In order to assess the contribution of individual signals to nuclear accumulation, MoKA peptides containing NLS1 (amino acids 473–819), NLS2 (amino acids 820–1006) or NLS3 (amino acids 1007–1193) were fused to myc-tagged pyruvate kinase (PK), an enzyme with exclusive localization in the cytosol, and transiently expressed into COS7 and NIH 3T3 cells (31). Transfected cells were analyzed by indirect immuno-fluorescence using anti-myc antibodies. In order to best appreciate changes in the intracellular distribution of fusion peptides irrespective of small differences in plasmid expression level and cell transfection efficiency, the intensity of the myc signal was measured in each cellular compartment

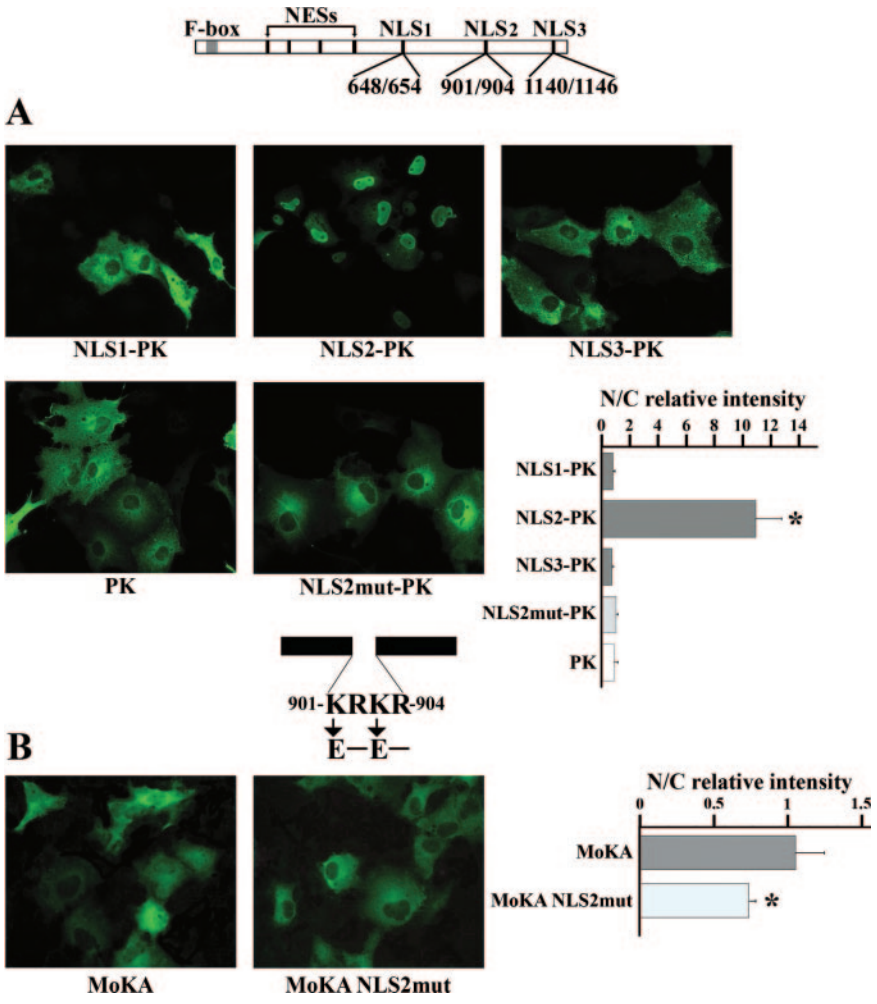

Figure 1. The basic NLS2 mediates nuclear import of MoKA. (**A**) At the top is a schematic representation of the MoKA protein structure showing the relative locations of the three putative basic NLSs identified by sequence homology. Below are illustrative confocal images of indirect immunofluorescence of COS7 cells transiently expressing NLSs1–3-containing peptides fused to PK-myc (NLS1-PK, NLS2-PK, and NLS3-PK) and with the NLS2-containing peptide carrying the K901E/K903E mutation fused to PK-myc (NLS2mut-PK). The bar graphs on the lower right summarize the individual N/C ratios of PK, NLS1-PK, NLS2-PK, NLS3-PK, and NLS2mut-PK fusion products. (**B**) Images of COS7 cells transiently expressing wild-type MoKA-GFP and a mutant version of it harboring the K901E/K903E mutations (MoKANLS2mut-GFP). Bar graphs on the right summarize the individual N/C ratios of MoKA-GFP and MoKANLS2mut-GFP fusion products.

and expressed as the nuclear to cytoplasmic (N/C) ratio for each experimental sample. Within the limitations of the experimental approach, these analyses gave at least an approximate idea of how mutations affected intracellular distribution of fusion products. Specifically, they revealed that only NLS2-PK accumulates in the nuclear compartment, thus excluding a major involvement of the sequences containing the putative signals NLS1 and NLS3 in this process (Figure 1A). Consistent with this result, transient expression of a PK-myc fusion protein in which the obligatory lysines of the hypothetical NLS2 had been replaced by glutamate residues (NLS2mut-PK) was found to localize exclusively in the cytoplasmic compartment (Figure 1A). A similar result was obtained when the same lysine residues were mutated in the full-length MoKA protein (Figure 1B). However the nuclear localization of the full-length mutant protein was not completely abolished conceivably implying that other factor(s) might participate in this process. This point notwithstanding, the mutagenesis experiments performed using both MoKA peptides and the full-length protein strongly implicated the basic NLS2 in the nuclear localization of MoKA.

Multiple signals mediate cytoplasmic accumulation of MoKA

The sequence believed to mediate MoKA localization into the cytoplasm corresponds to the amino-terminal third of the molecule and includes four putative NES-like motifs located at positions 132/141 (NES1), 194/201 (NES2), 307/316 (NES3) and 451–452/460 (NES4) (Figure 2A). Cell transfections were therefore performed to assess the intracellular localization of MoKA-Flag fusion protein containing deletions of each of the four putative NESs (Figure 2B). Indirect immunoflurescence using anti-Flag antibodies revealed that all but the NES1 deletion had a higher N/C than the wild-type product (Figure 2C). These loss-of-function experiments strongly suggested that at least three sequences (namely those harboring NESs2–4) are necessary for cytoplasmic

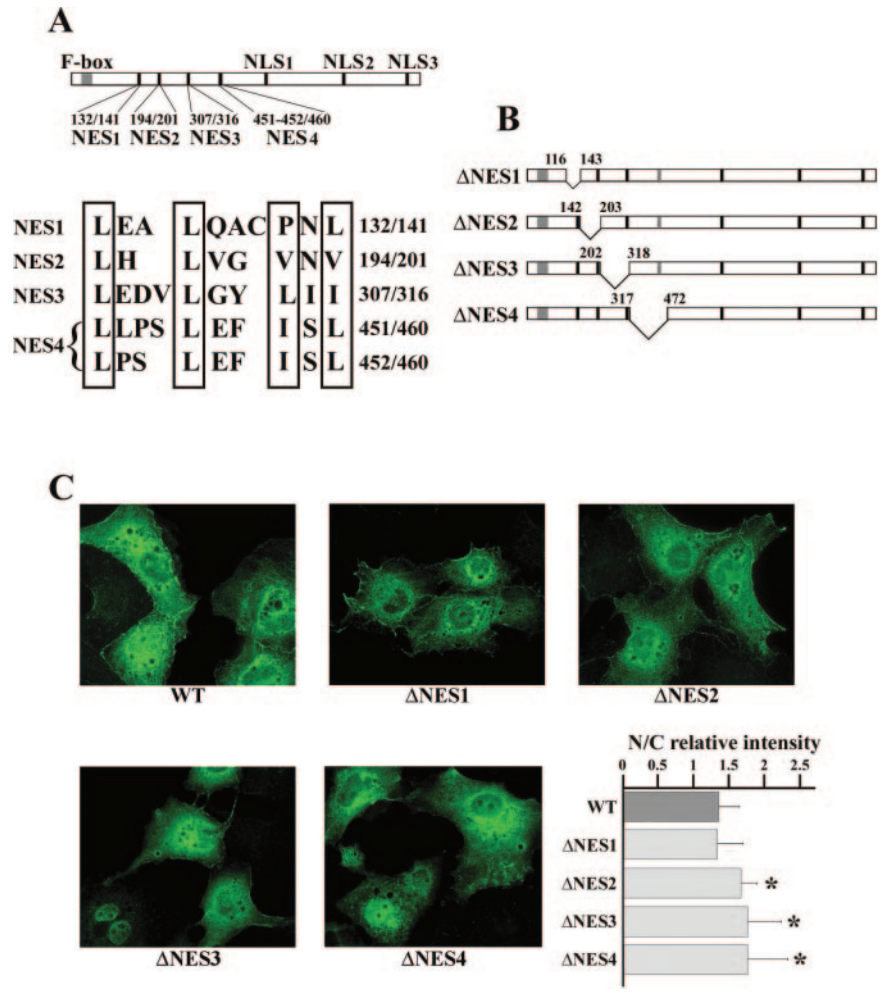

Figure 2. Three leucine-rich sequences mediate cytoplasmic localization of MoKA. (**A**) At the top is a schematic representation of MoKA protein structure showing the relative locations of the three putative leucine-rich NESs identified by sequence homology. Below is the amino acid alignment of the four NES-like motifs. (**B**) Schematic representation of the MoKA-Flag mutants without NES-like sequences (ΔNESs). (**C**) Illustrative confocal images showing indirect immunofluorescence of COS7 cells transiently expressing MoKA-Flag wild-type (WT) and MoKAΔNESs-Flag fusion proteins. The bar graphs on the right summarize the individual N/C ratios of full-length MoKA-Flag (WT) and of various MoKAΔNESs-Flag fusion proteins.

accumulation of MoKA. To investigate whether the peptides containing the putative NES2, NES3 or NES4 are also sufficient to allow cytoplasmic accumulation of MoKA, we examined the N/C ratio in cells transiently expressing proteins in which each of the four putative NESs was fused to the DNA-binding domain of GAL4 (32). The results of these transfection experiments confirmed the inability of NES1 to promote cytoplasmic accumulation of the GAL4 fusion product, in addition to suggesting that NES4 is the strongest signal since it completely counteracted the import signal of GAL4 (Figure 3). Qualitatively, if not quantitatively comparable results were obtained with MoKA-GFP fusion proteins (data not shown). We therefore concluded from these experiments that the peptides containing NESs2–4 are both necessary and sufficient to accumulate MoKA into the cytoplasmic compartment.

NES2 is a CRM1-binding site

The importin-β-related export factor CRM1 is the major receptor of shuttling proteins that harbor one or more leucine-rich NES (14). CRM1-mediated nuclear export can be effectively inhibited by LMB, which covalently binds a cysteine residue in the central domain of CRM1 (33,34). LMB was therefore used to assess whether or not CRM1 participates in the cytoplasmic localization of MoKA. Accordingly, COS7 and NIH3T3 cells expressing the wild-type MoKA-GFP fusion protein were treated with LMB or with the solvent (vehicle) for 3 h and then analyzed by confocal microscopy. Measurements of N/C ratios in each experimental sample revealed that LMB treatment reduces but does not eliminate cytoplasmic accumulation of MoKA-GFP (Figure 4A). Unfortunately, neither increasing the amount of the drug nor the time of treatment provided additional information, since both approaches resulted in substantial loss of MoKA-GFP positive cells (data not shown). This last point notwithstanding, the data suggested that one or more leucine-rich NESs interact with the CRM1 receptor.

In order to identify which of the candidate NESs is involved in CRM1-mediated nuclear export, COS 7 cells were transfected with expression vectors containing NES2, NES3 or NES4 fused to GFP and then treated with LMB. The effect

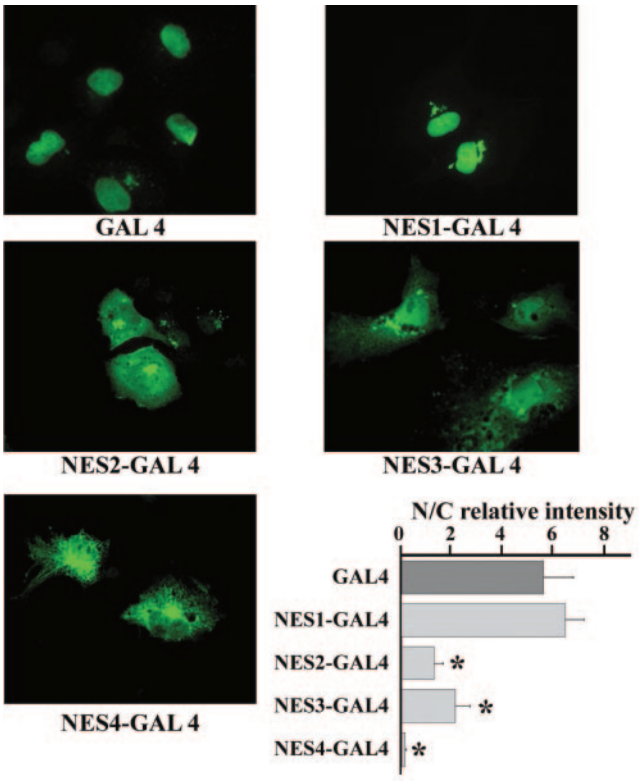

Figure 3. NESs2–4 counteract nuclear localization by the NLS of GAL4. Illustrative confocal images of indirect immunofluoresece of COS7 cells transiently expressing (1–147)GAL4 alone or fused to various NES peptides of MoKA. The bar graphs at the bottom summarize the individual N/C ratios of GAL4 and MoKA-NESs-GAL4 fusion products.

of the drug on the sub-cellular distribution of the fusion products was evaluated with respect to untreated cultures expressing GFP alone or fused to the MoKA peptides. Whereas LMB had no effect on the intracellular distribution of either NES3-GFP or NES4-GFP, the same treatment increased the N/C ratio of NES2-GFP to resemble that of GFP alone in untreated cells (Figure 4B). This finding strongly suggested that the leucine-rich NES2-containing peptide is the major, if not the sole, mediator of CRM1 activity during MoKA nuclear export.

To corroborate the above conclusion, we evaluated the consequences on nuclear export of amino acid substitutions within each of the three leucine-rich sequences of MoKA. Specifically, we mutated two highly conserved hydrophobic residues of the putative NES2, NES3 or NES4 and expressed the mutant and wild-type NES-GFP fusion products in COS7 cells. Consistent with the LMB experiments, only the amino acid substitutions in NES2 were found to increase the N/C ratio to resemble those of the wild-type NES2-GFP protein in LMB-treated cells and of GFP in untreated cells (Figures 4A and 5A). Collectively, these results demonstrated that cytoplasmic accumulation of MoKA is mediated in part by interaction between CMR1 and NES2.

MoKA contains two novel sequences mediating cytoplasmic accumulation

The results of the above experiments implied that putative NESs previously identified by sequence homology were not responsible for cytoplasmic accumulation of the 203/318 and 319/472 peptides identified here by functional assays. We therefore searched for more divergent leucine-rich NES-like sequences within the 203/318 and 319/472 peptides. Whereas three potential leucine-rich NES-like motifs were noted within the 203/318 peptide, none was found within the 319/472 peptide. The former elements include a putative NES-like sequence at positions 230/239 (LCISLRTFVM; NES3a) and two putative overlapping NES-like sequences at position 264/275 (LEHLEMVRVPFL and LEHLEMVRVPFL; NES3b and NES3c). The lack of other recognizable NES-like motifs in the 319/472 peptide prompted us to conclude that this highly hydrophobic segment of MoKA might conceivably be implicated in cytoplasmic accumulation of MoKA. The identity of this element, however, was not investigated further in the present study. Instead, our efforts were devoted to elucidate the role of NESs3a–c.

The highly conserved leucines of NESs3a–c were converted into alanines within the context of the (203/318)MoKA-GFP fusion product, and the N/C ratios of each of the resulting 203/318 mutant peptides [L230A/L234A (NES3a-mut), L264A/L267A (NES3b-mut), and L267A/L275A (NES3c-mut), L264A/275A (NES3b, c-mut1)] were independently measured in transfected COS7 cells (Figure 5B). With the exception of the mutant product harboring NES3a-mut, all the others were found to increase the relative N/C ratio of (203/318) MoKA-GFP (Figure 5B). This finding raised the possibility that the overlapping sequences of NES3b and NES3c may actually constitute a single sequence involved in cytoplasmic accumulation. To test this hypothesis, we designed another 203/318 mutant sequence in which alanine residues were introduced in place of the three leucines (L264A/L267A/L275A) in the sequence that overlaps NES3b and NES3c (NES3b, c-mut2) (Figure 5B). The triple amino acid substitution (NES3b, c-mut2) resulted in even higher N/C ratio than the other mutants (Figure 5B). Interestingly, NES3b, c-mut2 did not display the same intracellular distribution as GFP alone (Figure 5B). Collectively, these results indicated that the leucines at 264, 267 and 275 are necessary but not sufficient to mediate cytoplasmic accumulation of the (203/318) MoKA peptide. Consistent with this conclusion, amino acid substitutions of other hydrophobic residues in the 264/275 sequence did not affect subcellular distribution of (203/318) MoKA-GFP (data not shown).

A distinct acidic sequence confers transcriptional activity to MoKA

We have previously shown that MoKA binds KLF7 through the F-box, and that this interaction results in enhanced stimulation of the $p21^{cip/waf}$ promoter by the transcription factor (26). The chimeric GAL4 system was therefore employed in the present study to identify the activation domain of MoKA. Towards this end, COS7 cells were transiently co-transfected with increasing amounts of the MoKA-GAL4 expression plasmid and the Luciferase reporter plasmid driven by multiple GAL4 binding sites (G5GAL4-Lux). These functional assays demonstrated that artificial recruitment of full-length MoKA to a basal promoter can also stimulate

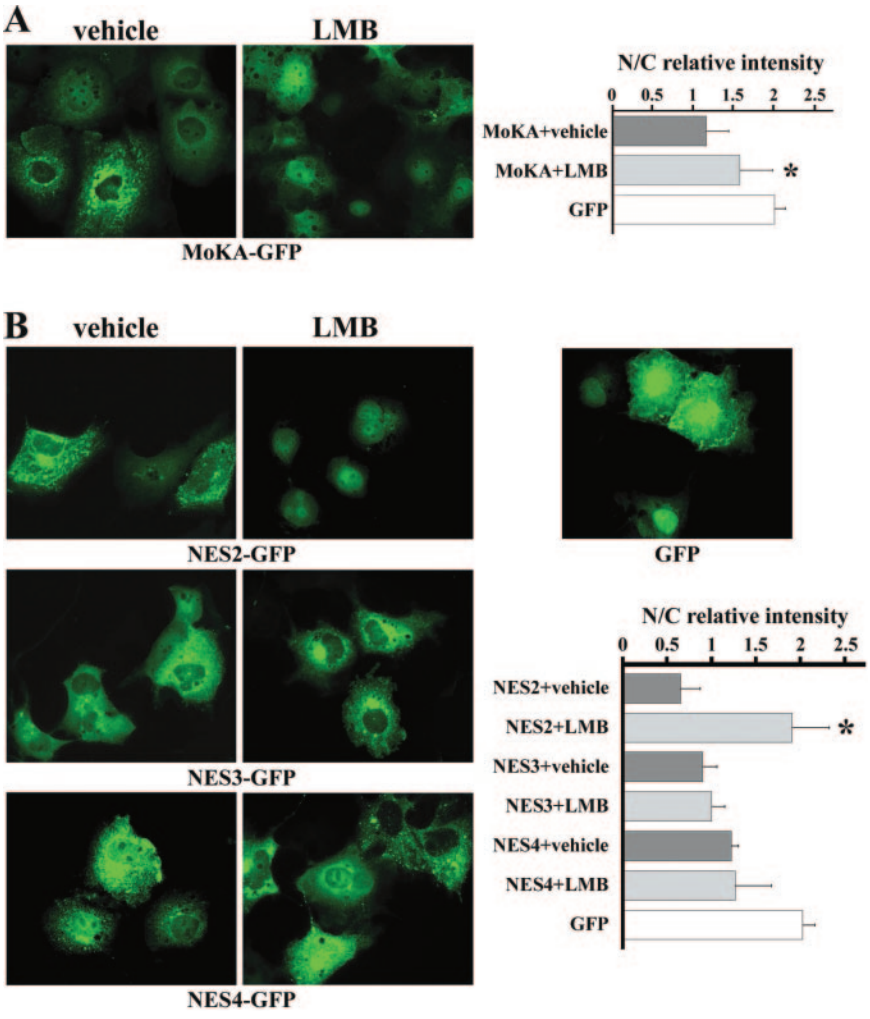

Figure 4. MoKA nuclear export is partially inhibited by LMB. (**A**) On the left are illustrative confocal images of COS7 cells transiently expressing MoKA-GFP full-length treated with LMB or vehicle. The bar graphs on the right summarize the individual N/C ratios of GFP and MoKA-GFP full-length in the absence (vehicle) or presence of LMB. (**B**) On the left are illustrative confocal images of COS7 cells transiently expressing NES-GFP products treated with LMB or vehicle. The bar graphs at the bottom summarize the individual N/C ratios of GFP and NES-GFP products in the absence (vehicle) or presence of LMB.

gene transcription in a dose-dependent manner (Figure 6). Next, cell co-transfections were repeated using different amino or carboxy-terminal deletions of MoKA fused to the DNA-binding domain of GAL4. The results narrowed down the transactivating domain of MoKA to an asparagine-rich sequence between residues 473 and 766 and thus, located between the NES and NLS clusters (Figure 6). Consistent with its prominent nuclear localization due to elimination of all putative NESs, (472–1193) MoKA-GAL4 stimulated reporter gene transcription more than full-length MoKA in cells transfected with comparable amounts of the respective plasmids (Figure 6).

DISCUSSION

MoKA was originally identified through a yeast two-hybrid screen of protein partners of KLF7, a transcription factor that was subsequently implicated in neurite outgrowth at several anatomical locations of the developing nervous system (26,28,29). These early experiments established that the F-box mediates binding of MoKA with KLF7, and that this interaction results in an increased stimulation of the $p21^{Waf/Cip}$ promoter by the transcription factor. They also indicated that MoKA displays a pattern of sub-cellular distribution characteristic of a shuttling protein with basal line localization predominantly in the cytoplasmic compartment. Additional analysis raised the possibility that multiple mechanisms might be involved in intracellular shuttling of this KLF7 co-activator. The evidence included the presence of potential signals for nuclear export in the amino-terminal third of the protein and for nuclear import in the carboxy-terminal half, and the potential for post-translational modifications of the intervening sequence. The present study extends the structural-functional characterization of MoKA by demonstrating that discrete sequences are involved in intracellular trafficking and transcriptional stimulation.

Elements implicated in MoKA sub-cellular localization were found to include one basic NLS, a variant NES that interacts with the export factor CRM1, a novel leucine-rich motif that acts in a CRM1-independent manner, and an ill-defined peptide sequence that is neither leucine-rich nor

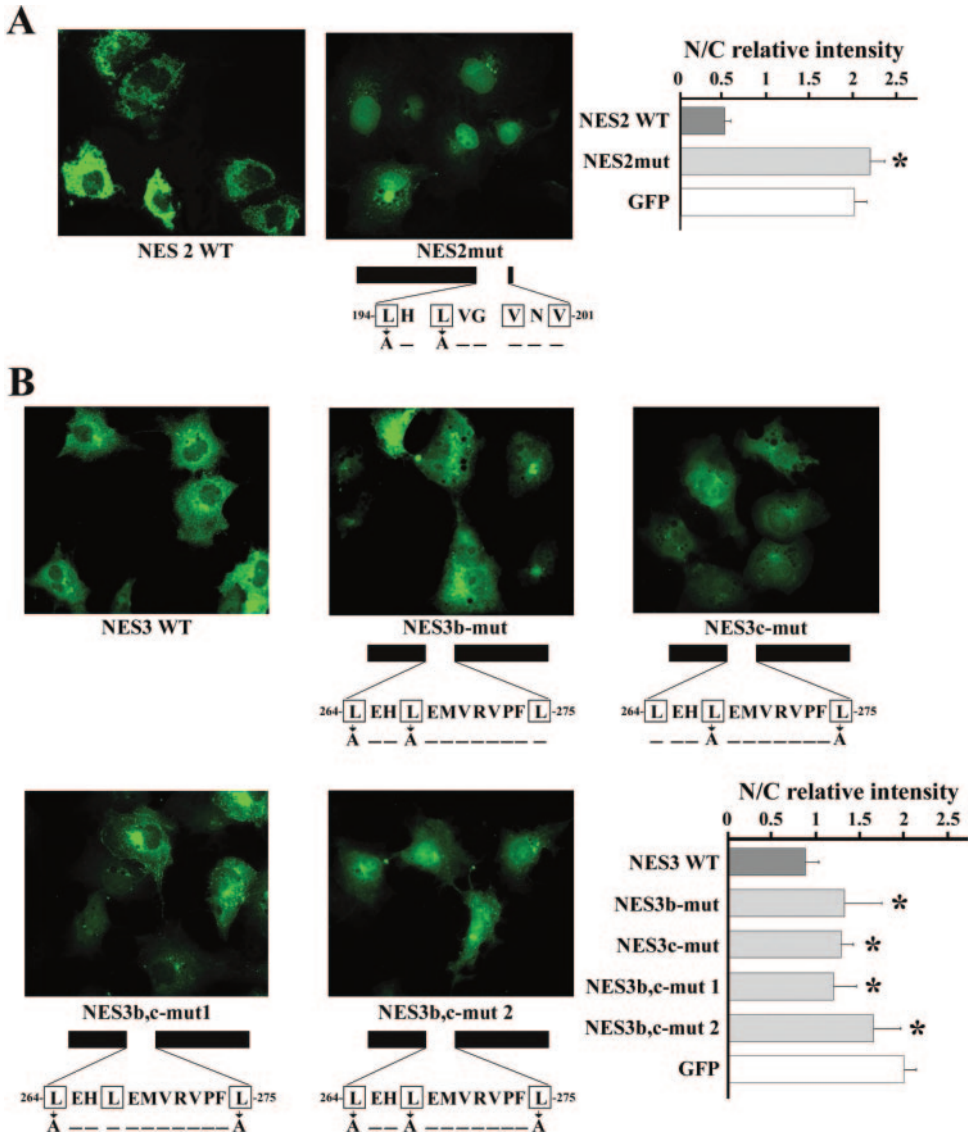

Figure 5. CRM1-dependent and independent pathways mediate cytoplasmic accumulation of MoKA. (**A**) On the left are illustrative confocal image showing COS7 cells transiently expressing wild-type NES2 or the L194A/L196A mutant (NES2-WT and NES2mut) fused to GFP. The bar graphs on the right summarize the individual N/C ratios of NES2-WT, NES2mut and GFP. (**B**) Illustrative images of COS7 cells transiently expressing the wild-type (203/318)MoKA peptide (NES3 WT) and NES3b-mut, NES3c-mut, NES3b,c-mut1, NES3b,c-mut2 mutants fused to GFP. The bar graphs at the bottom right summarize the individual N/C ratios of GFP, and of wild-type and mutants NES3 fusion products.

interacts with CRM1. Deletion experiments performed using the chimeric GAL4 system instead located the domain that is mostly responsible for the transcriptional potential of MoKA to a highly acidic segment residing between the putative NES and NLS clusters and downstream of the F-box. This 294 long region of MoKA contains a long stretch of asparagines and its known that 'acidic blobs' or negative noodles' are DNA activation domains (35–37).

Our cell transfection data clearly indicate that NLS2 is the only putative import signal capable of mediating nuclear accumulation of the otherwise cytoplasmic PK protein. This conclusion was further substantiated by the finding that mutations in the obligatory lysine residues of NLS2 re-localized the fusion protein into the cytoplasm. The results also suggest that this process is likely to be more complex since NLS2 was significantly less effective in mediating nuclear localization within the context of full-length MoKA. Although we have not provided supporting evidence, the composition of the peptide that mediates nuclear import (^{901}KRKR^{904}, NLS2) is at least consistent with the prediction that MoKA is likely to bind a karyopherin receptor (5). Several different mechanisms can direct cytoplasmic accumulation of a protein; they include NESs, interaction with inhibitory factors, interaction with anchoring proteins and conformational changes that modulate presentation of shuttling signals (38–45). Inhibition of the CRM1 pathway by LMB and site-directed mutagenesis of the putative NESs concurred in demonstrating that one of the mechanism of MoKA accumulation in the cytoplasmic compartment involves the classical CRM1 pathway and the leucine-rich NES located at position

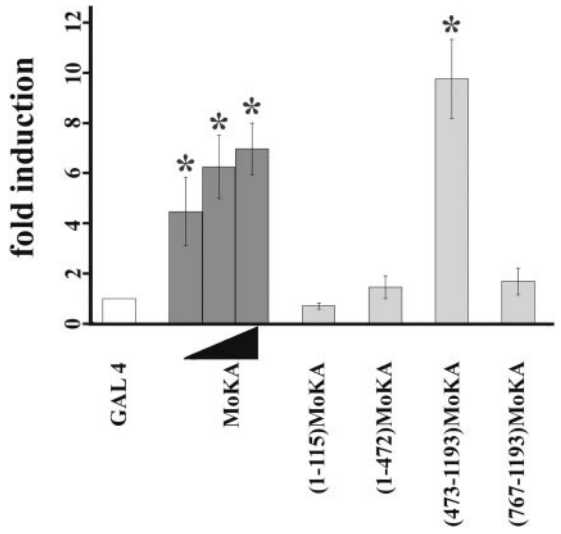

Figure 6. MoKA contains an activation domain. Luciferase activity of COS7 cells transiently co-transfected with MoKA-GAL4 (65, 200 and 650 ng) or equimolar amount of MoKA-GAL4 deletion mutants together with G5GAL4-Lux reporter plasmid.

194/201 (NES2). Indeed, our experiments strongly support the notion that the 203/318 and 319/472 peptides participate in this process as well through a CRM1-independent mechanism. These findings are in agreement with early evidence suggesting that the sequence context may be as equally important as the spacing between hydrophobic residues for the function of leucine-rich NESs (46).

Our study has also identified a novel leucine-rich motif, NES3b,c, which is made of two nearly overlapping NES-like sequence (LEHLEMVRVPFL and LEHLEMVRVPFL; LX_2LX_7L). Although mutagenesis experiments strongly suggested involvement of non-hydrophobic amino acids as well, they nevertheless demonstrated that leucines at 264, 267 and 275 are at least required to accumulate the 203/318 peptide into the cytoplasmic compartment. Two other proteins, RIP3 and HDAC 10, have been recently show to carry different leucine-rich stretches that functions as a CRM1-indipendent NES (47,48). Moreover, calreticulin has been shown to mediate the export of the protein kinase inhibitor in a NES-dependent, LMB-insensitive manner thus suggesting receptors other than CRM1 might recognize leucine-rich NESs (20). It is therefore conceivable to argue that the LX_2LX_7L motif of MoKA might be part of a protein-interaction domain binding to the nuclear pore complex, a nuclear exported protein, or a cytoplasmic anchoring protein. In contrast to the 203/318 fragment, mutagenesis of the leucine residues in the 319/472 peptide ruled out involvement of these amino acids in the cytoplasmic accumulation of MoKA. Unfortunately, lack of homology between the 319/472 peptide and any of the known export signals does not allow us to elaborate on the possible mechanism that may mediate cytoplasmic accumulation by this particular sequence of MoKA. We can only speculate that the highly hydrophobic nature of the 319–472 peptide might be a contributing factor in the base line distribution of MoKA predominantly in the cytoplasmic compartment (12,49). In conclusion, the present study has provided a fairly detailed characterization of the major structural-functional features of MoKA. This information will prove invaluable in guiding the interpretation of ongoing genetic experiments aimed at elucidating the function of MoKA during mammalian development and in the adult organism.

ACKNOWLEDGEMENTS

The authors thank K. Johnson for preparing the manuscript, Dr G. Dreyfuss for providing reagents and Dr Luca Carta for helpful suggestions. This work was supported by NIH grant AR38648, the St Giles Foundation and the James D. Farley family. Funding to pay the Open Access publication charges for this article was provided by National Institutes of Health.

Conflict of interest statement. None declared.

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