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Conformational determinants of the intracellular localization of midkine

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Abstract

Midkine (MK) is a multifunctional growth factor and has been discovered to play important roles in carcinogenesis. MK has been reported to localize to the nucleus and nucleolus, however, the data are not consistent and the signals responsible for the localization are unknown. Here we reported that human MK exclusively localized to the nucleus and nucleolus in HepG2 cells by using GFP as a tracking molecule. In order to identify the motifs required for the nuclear localization and nucleolar accumulation, point- and deletion-mutations were introduced and the corresponding subcellular localizations were analyzed. Data revealed that (i) K79R81, K86K87, and the C-terminal tail of MK constitute the nuclear localization determinant of MK, and (ii) the C-terminal tail is the key element controlling MK nucleolar accumulation though the N-terminal tail, K79R81, and K86K87 also contribute to this process. Taken together, our results provide the first documentation about the determinants required for MK nuclear and nucleolar localization.

Keywords: Midkine; Green fluorescence protein; Nuclear localization; Nuclear localization signal; Nucleolar localization; Nuclear localization determinant

Midkine (MK) is a heparin-binding growth factor whose gene was first identified in embryonal carcinoma cells in early stages of retinoic acid-induced differentiation [1], and was found to be expressed at high levels in a variety of human carcinomas [2–6]. It has been reported that MK promotes the growth of Wilms' tumor cells and fibroblasts [7,8], the transformation of NIH3T3 cells [9], and the anti-apoptotic activity to Wilms' tumor cells treated with cisplatin [10]. MK also induces a strong angiogenic response in the rabbit corneal assay [11]. These biological activities implicate that MK plays an important role in carcinogenesis. However, the mechanisms of MK corresponding to these activities are only partly known. Several cell-surface receptors are discovered to bind to MK, including members of syndecan family [12–14], a proteoglycan receptor-type tyrosine kinase ζ (PTPζ) [15], a transmembrane protein low density lipoprotein (LDL) receptor-related protein (LRP) [16], and anaplastic lymphoma kinase (ALK) [17]. Recently, Shibata et al. showed that exogenous MK was endocytosed by cultured mouse L cells and then transported to the nucleus, and the nuclear translocation is necessary for the anti-apoptotic activity of MK [18], indicating that MK may act not only through receptor-induced signaling but also directly within cells. However, the data are inconsistent regarding the intracellular localization of MK. It has been reported that MK localizes in the nucleus [19–21], nucleolus [22], or cytosol [23,24].

The nucleus orchestrates the running of the cell and is now known to be a highly dynamic organelle containing

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dynamic compartments created by relatively immobile binding or assembly sites [25]. In order to function properly, the nucleus should rely on a constant flow of molecules between cytosol and nucleus. Many proteins can be translocated into the nucleus by the presence of an import signal such as nuclear localization signal or sequence (NLS) which can be specifically recognized by receptors on nuclear pore complex [26,27], or certain shuttling protein such as nucleolin [27,28]. After being transported into the nucleus, proteins can be docked at different subnuclear compartments. In a subnuclear structure such as nucleolus, proteins may accumulate in a steady-state compartment mediated by nucleolar localization sequence (NoLS) or domain which might interact with local high-affinity binding sites [25,29]. Thus far, the NLS and NoLS of MK remain unknown and the mechanism of MK intracellular localization is poorly understood. Here, we reported the intracellular localization of MK in HepG2 cells and the motifs responsible for the localization.

Materials and methods

Cell culture. HepG2 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) at 37 °C under 5% humidified CO_2 and passaged every 3 days by trypsinization.

Construction of the recombinant plasmids for transfection. The full-length human *MK* gene was obtained from the total RNA of HepG2 cells by reverse transcription polymerase chain reaction (RT-PCR) using MKFL5′ and MKFL3′ as the primers. All oligonucleotides used in our study were synthesized in Bioasia (Bioasia, Shanghai, China), and their sequences are shown in Table 1. After PCR amplification, the resulting fragment was digested with *Eco*RI and *Bam*HI, and inserted into the corresponding sites of pEGFP-N2 (enhanced green fluorescent protein N-terminal protein fusion vector, Clontech Laboratories, Palo Alto, CA), resulting in in-frame fusion to N-terminus of GFP. This plasmid was designated as pEGFP-MK.

The following site-directed MK variants were obtained by PCR using pEGFP-MK as the template. Mutations which altered the Lys at the position 111, 113, 115, and 116 to Ala were prepared using MKFL5′ as the sense primer and 4KtoA3′ as the antisense primer, and then the fragment *MKK111AK113AK115AK116A* was obtained. The DNA encoding N-terminal half part (MKNH, residues 1–59) and C-terminal half part (MKCH, residues 60–121) of MK was amplified using MKFL5′ and MKNH3′ as a pair of primers, and MKCH5′ and MKFL3′ as the other pair of primers. The DNA encoding C-terminal domain of MK (MKCD, residues 60–106) was amplified with MKCH5′ and ΔCT3′. To generate the C-terminal-tail-truncated variant MKΔCT (residues 1–106), and both N- and C-terminal-tail-truncated variant MKΔNCTs (residues 9–106), sense primer MKFL5′ and ΔNT5′, and antisense primer ΔCT3′ were used, respectively, in the two reactions.

The 15 amino acid C-terminal tail of MK was obtained by synthesizing two complementary DNAs CT plus and CT minus which correspond to 107-KTKAKAKAKKGKGKD-121 and annealing.

To replace the basic amino acid residues Lys79 and Arg81 with neutral residue Ala, the mega-primer PCR mutagenesis method [30] was used with minor modifications. Briefly, in the first round of PCR, MKFL3′ and the mutagenic primer K79AR81A5′ were used and the mega-primer containing mutant points was produced. The resulting fragment was then extracted using a gel extraction kit (Qiagen, Hilden, Germany) and eluted with 30 μl elution buffer. In the second round of PCR, 20 μl of purified mega-primer was incubated with the template pEGFP-MK in the absence of 5′ primer and the amplification conditions were: an initial denaturation at 94 °C for 5 min, 5 cycles reaction consisting of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C followed by a 35 min extension step at 72 °C. Five picomole of 5′ primer MKFL5′ was then added to the previous PCR tube and the reaction was carried on. After a 2 min initial denaturation step, the cycling conditions were: 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C for the first 5 cycles, and 1 min at 94 °C, 1 min at 56 °C, and 2 min at 72 °C for the subsequent 30 cycles followed by a final extension step at 72 °C for 10 min. The procedures to generate mutations changing the Lys86 and Lys87 to Ala were the same as described above but substituting the mutagenic primer K86AK87A5′ for K79AR81A5′ in the first round PCR. The procedure to obtain MK variant containing all four point-directed replacements was similar to the one for amplifying *MKK86AK87A* except using pEGFP-MKKRA (the plasmid containing *MKK79AR81A*) as the template. *MKCH* containing K79AR81A, K86AK87A or both, *MKCD* and *MKΔCT* containing both mutations were amplified by PCR in a manner similar to that described for amplifying *MKCH*, *MKCD*, and *MKΔCT*, except using constructed expression plasmids containing corresponding mutations as the templates.

All the fragments mentioned above were digested by *Eco*RI and *Bam*HI, and then inserted into vector pEGFP-N2.

The fragment *MKΔCT* containing K79AR81A and K86AK87A was cloned into a previously *Eco*RI + *Bam*HI digested vector pEGFP-C2 (enhanced green fluorescent protein C-terminal protein fusion

Table 1
DNA sequences of PCR primers used to generate GFP fusion proteins consisting of wild-type or mutant MK

Primer name	Primer sequence[a]	Restriction site
MKFL5′	G**GAATTC**ATGAAAAAGAAAGATAAGGTGAAGAAGGGCGG	*Eco*RI
MKFL3′	CG**GGATCC**GGTCCTTTCCCTTCCCTTTCTTG	*Bam*HI
4KtoA3′	CG**GGATCC**GGTCCTTTCCCTTCCCTGCTGCGGCTGCGGCTGCTGCTTTGGT	*Bam*HI
ΔCT3′	CG**GGATCC**CGGGGGTGCAGGGCTTG	*Bam*HI
ΔNT5′	G**GAATTC**ATGGGCGGCCCGGGGAGC	*Eco*RI
MKCH5′	G**GAATTC**ATGGCCGACTGCAAGTACAAGTTTGAG	*Eco*RI
MKNH3′	CG**GGATCC**CTCCAAACTCCTTCTTCCAGTTGC	*Bam*HI
CT plus	G**GAATTC**ATGAAGACCAAAGCAAAGGCCAAAGCCAAGAAAGGGAAGGGAAAGGACCGGATCCCG	*Eco*RI
CT minus	CG**GGATCC**GGTCCTTTCCCTTCCCTTTCTTGGCTTTGGCCTTTGCTTTGGTCTTCATGAATTCC	*Bam*HI
K79AR81A5′	CAGGCACCGCAGTCGCTCAAGGCAC	
K86AK87A5′	GGCACCCTGGCAGCTGCGCGCTAC	

[a] Restriction sites are in bold type.

vector, Clontech Laboratories, Palo Alto, CA), resulting in in-frame fusion to C-terminus of GFP.

All constructs were checked for sequence integrity (Bioasia, Shanghai, China) before conducting transfections.

Transfection and intracellular localization analysis. Transient transfections were performed with Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were plated at a density of 1×10^5 cells per well on a 35 mm dish 24 h before transfections and transfected with 0.4 μg of each expression plasmid. After 16 h, live images were obtained with a Leica TCS SP spectral confocal microscope (Leica, Heidelberg, Germany) using a 40× 1 NA oil immersion objective.

Results and discussion

MK exclusively localized to the nucleus

Previous studies have reported the diverse subcellular localizations of mouse or human MK [18–21,23,24]. In order to decide the subcellular localization of human MK in HepG2 cells, we amplified MK wild-type cDNA and fused it to the 5′ end of GFP cDNA in-frame in pEGFP-N2. The subcellular localization of GFP (Fig. 1A, panel a) and fusion protein MK-GFP (Fig. 1A, panel b) was examined by using a laser confocal microscope. In the GFP control, fluorescence was detected diffusely over the entire cell body except in the nucleolus (Fig. 1B, panel a), whereas the fusion protein MK-GFP localized exclusively to the nucleus (Fig. 1B, panel b). Fig. 2 showed the time-lapse imaging of MK-GFP nuclear translocation. These results indicated that MK can actively drive GFP to the nucleus and therefore MK has a nuclear transporting ability.

Identification of nuclear localization determinant in MK

Most growth factors are less than 45 kDa in molecular weight, which means that they might diffuse freely between cytoplasm and nucleus [31]. However, many of them require certain NLSs which may be recognized by members of importin superfamily or nucleolin, and are actively transported into the nucleus by such signals [27]. According to our result that MK exclusively localized to the nucleus, we considered that a functional NLS might be present within this factor although the molecular weight of MK is only 13 kDa. We first examined the MK full sequence using on-line programs to predict whether MK contains a putative NLS, and the sequence 111KAKAKKG117 in the C-terminal tail was detected as a candidate in the program PredictNLS. Since basic amino residues are the characteristic of an NLS [27], we mutated all the Lys in this putative NLS to Ala to examine whether this 'NLS' is a true one (Fig. 3A, panel a).

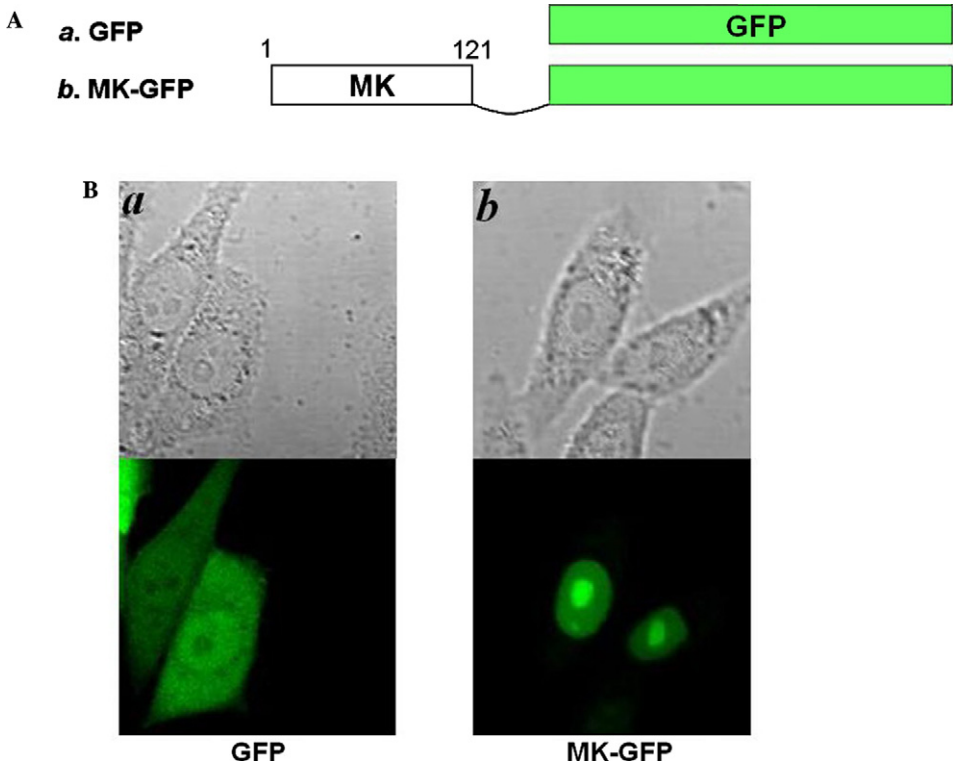

Fig. 1. MK-GFP localizes to the nucleus and nucleolus. (A) DNA construct of fusion protein. Full-length wild-type MK was cloned into the pEGFP-N2 (a) and yielded pEGFP-MK (b) encoding MK-GFP. White and green boxes indicate MK and GFP, respectively. (B) Confocal microscope images of HepG2 cells 16 h after transfection with pEGFP-N2 (a) and pEGFP-MK (b). Top panels are phase contrast images and bottom panels are fluorescence images showing the same fields to demonstrate representative fluorescent patterns.

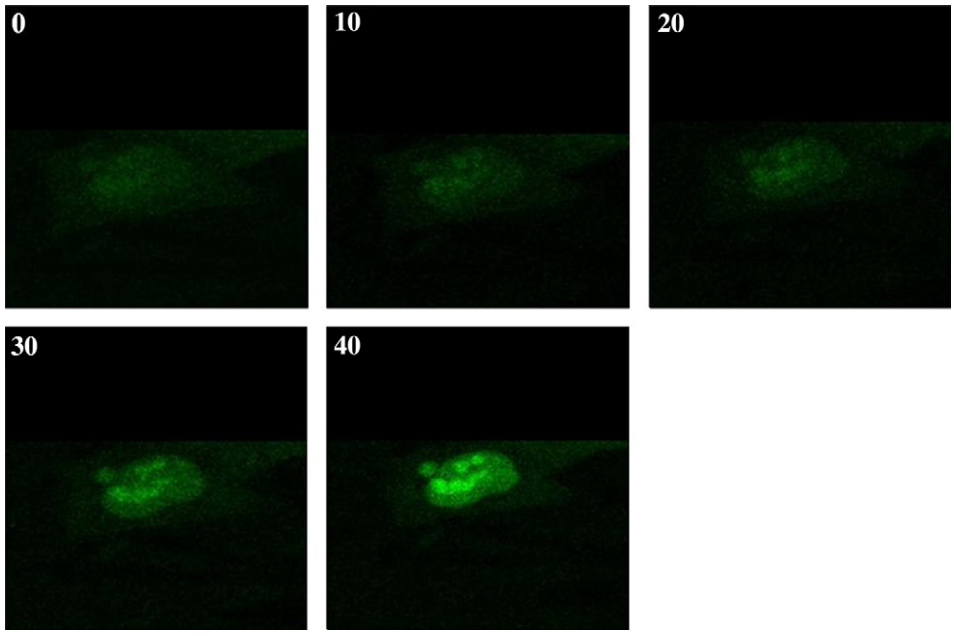

Fig. 2. Time-lapse imaging of the nuclear and nucleolar localization of MK-GFP fusion protein in HepG2 cells. The selected cell started to be monitored 8 h after transfection with pEGFP-MK and the images were taken at 10 min intervals. The number in each panel indicates minutes since initiation of observation.

The result showed that mutations changing these Lys to Ala hardly affected the subcellular localization pattern of MK (Fig. 3B, panel a), indicating that the 'NLS' predicted by the computer program is a pseudo one or not powerful enough to drive nuclear translocation of MK.

In an attempt to map the domain of MK responsible for the nuclear localization of the protein in HepG2 cells, we constructed two expression plasmids encoding GFP-tagged MKNH and MKCH (Fig. 3A, panels b and c), respectively. Subcellular localization analyses demonstrated that MKNH-GFP localized throughout the whole cell except to the nucleolus (Fig. 3B, panel b) while the pattern of MKCH-GFP (Fig. 3B, panel c) was identical to that of MK-GFP, indicating that the NLS may locate within the C-terminal half of MK.

As the C-terminal tail (107-KTKAKAKAKKGKG KD-121) is rich in basic amino acid residues, we first sought to characterize the role of this sequence in MK nuclear localization. This tail was deleted from MKCH to generate a plasmid encoding MKCD-GFP (Fig. 3A, panel e). The confocal microscope image showed the ratio of the intensity of GFP fluorescence in the nucleus and cytoplasm (the N/C ratio) of this variant to be markedly reduced (Fig. 3B, panel e) in comparison with that of wild-type MK-GFP or MKCH-GFP, demonstrating that the nuclear transporting ability of MKCD was remarkably diminished. To further test whether the C-terminal tail alone owns the nuclear transporting ability, we synthesized a DNA fragment corresponding to the tail and then ligated it with the GFP cDNA yielding MKCT-GFP (Fig. 3A, panel d). This tail actively drove GFP to the nucleus (Fig. 3B, panel d) though not as efficiently as wild-type MK or MKCH did. Moreover, totally deleting this tail did not lead to a uniformly diffused fluorescence (Figs. 3A and B, panel f) like the one of GFP control. Together these results indicated that the C-terminal tail of MK is the most important part of the NLS although it is not an entire one, and the one predicted by PredictNLS is not powerful enough to bring MK to the nucleus.

Our finding that the expression of MKCD-GFP in the nucleus was moderately stronger than in the cytosol offered us a clue that certain amino acid residues present within this domain also act in the process of MK nuclear localization. The previous NMR analyses have identified two heparin-binding sites located at the surface on one side of the C-terminal domain, which are basic amino acid clusters (Cluster I, K79R81K102; Cluster II, K86K87R89) [32], leading to our speculation that the residues in these two clusters may be involved in controlling the nuclear localization. To test this postulation, we systematically altered the duple residues K79R81 and K86K87 to Ala using site-directed mutagenesis. The nuclear localization of MKCH-GFP containing mutated sites K79AR81A (Fig. 4A, panel a) or K86AK87A (Fig. 4A, panel b) was partly inhibited (Fig. 4B, panels a and b) if taking the N/C ratio as an index. Compared with the two variants above, changing both the K79R81 and K86K87 to Ala decreased the N/C ratio of MKCH-GFP (Figs. 4A and B, panel c) at most. Furthermore, such a mutation made the distribution of MKCD-GFP to be identical to that of GFP (Figs. 4A and B,

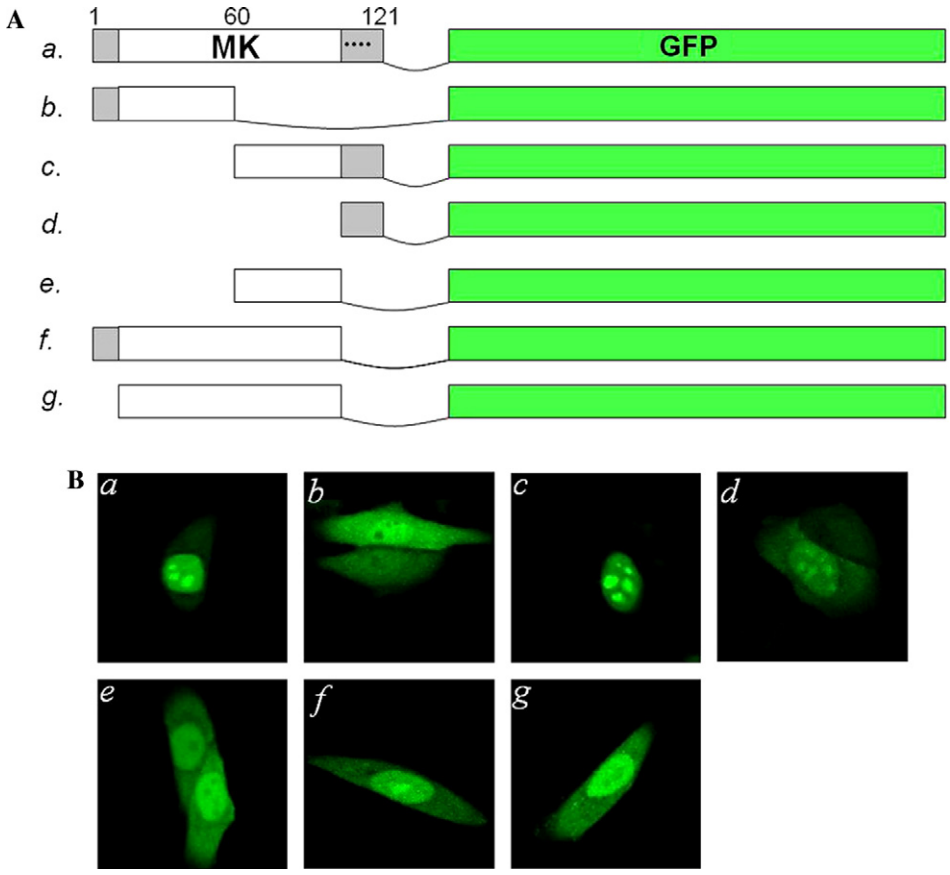

Fig. 3. Role of putative NLS predicted by computational program and each domain within MK in the nuclear and nucleolar localization. (A) DNA construct of each mutation. Full-length MK containing amino acid substitutions K111AK113AK115AK116A within the putative NLS was cloned into pEGFP-N2 and named pEGFP-MK4KA (a). The DNA encoding N-terminal half part (1–59), C-terminal half part (60–121), C-terminal tail (107–121) or C-terminal domain (60–106) of MK was cloned into the vector to form pEGFP-MKNH (b), pEGFP-MKCH (c), pEGFP-MKCT (d) or pEGFP-MKCD (e). C-terminal-tail-truncated (1–106) or both N- and C-terminal-tail-truncated MK (9–106) was introduced to yield pEGFP-MKΔCT (f) or pEGFP-MKΔNCTs (g). The gray boxes indicate the terminal tails within MK. The dots in the gray box indicate the amino acids mutated. (B) Confocal microscope images of HepG2 cells transfected with pEGFP-MK4KA (a), pEGFP-MKNH (b), pEGFP-MKCH (c), pEGFP-MKCT (d), pEGFP-MKCD (e), pEGFP-MKΔCT (f), or pEGFP-MKΔNCTs (g).

panel d). We therefore concluded that K79R81 and K86K87 are also key elements in the process of the nuclear localization of MK.

In order to further characterize the role of K79R81, K86K87, and C-terminal tail in directing full-length MK into the nucleus, we constructed two expression plasmids, which encode MKΔCT-GFP and GFP-MKΔCT containing both K79R81 and K86K87, respectively (Fig. 4A, panels f and g). The subcellular distribution patterns of the variants were (Fig. 4B, panels f and g) almost identical to that of GFP, whereas the N/C ratio of MK-GFP variants containing only either point-directed substitutions K79AR81AK86AK87A (Fig. 4A, panel e) or deletion of the C-terminal tail (Fig. 3A, panel f) was much higher (Figs. 4B, panel e; 3B, panel f), thereby confirming that these three elements, K79R81, K86K87, and the C-terminal tail, work together in controlling the nuclear localization of MK. However, the sequences do not belong to any canonical NLS. They form a conformational nuclear localization determinant instead of a linear NLS.

Since it has been reported that nucleolin binds to MK and leads to the nuclear translocation of MK [18,19], we postulate that the C-terminal tail, K79R81, and K86K87 may be the interacting sites within MK. In Shibata's reports, the MK-binding sites are mapped to acidic stretches in the N-terminal domain of nucleolin [18], which probably bind to the basic sequences located within MK, such as K79R81, K86K87 and the C-terminal tail; furthermore, the basic C-terminal tail located outside the domains forms a flexible structure and the two heparin-binding sites, Basic Cluster I (K79R81K102) and II (K86K87K89), are exposed on the same surface of MK [32]. Thereby it is sound to speculate that via the electrostatic interaction between negatively charged domains of nucleolin and positively charged atmosphere of Cluster I and II on the surface and the C-terminal tail, nucleolin binds to MK and then

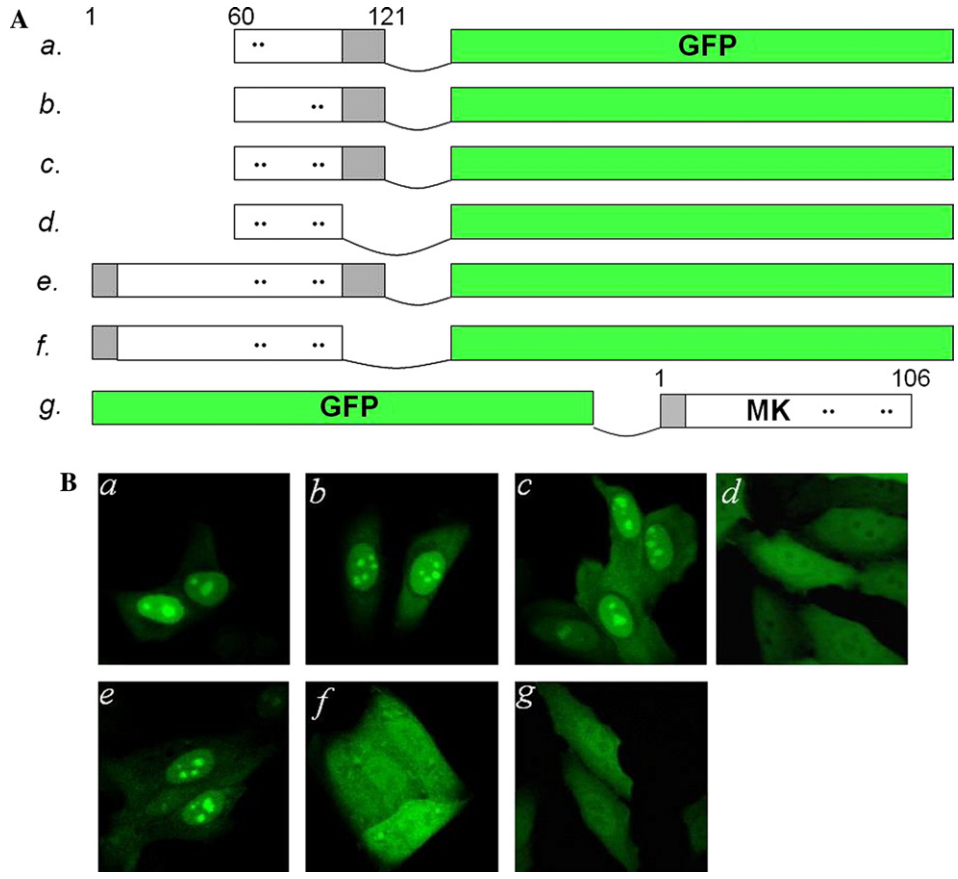

Fig. 4. The role of K79R81, K86K87, and the C-terminal tail in the nuclear and nucleolar localization of MK. (A) DNA constructs. N-terminal-half-truncated MK containing Lys/Arg-to-Ala changes at positions 79 and 81, 86 and 87, or all the four were named as pEGFP-MKCHKRA (a), pEGFP-MKCHKKA (b) or pEGFP-MKCH KRKKA (c). C-terminal domain, full-length MK or C-terminal-tail-truncated MK containing the four mutated points was used to generate pEGFP-MKCDKRKKA (d), pEGFP-MKKRKKA (e), pEGFP-MKΔCTKRKKA (f) or pEGFP-C2MKΔCTKRKKA (g). The dots in the white box are mutated points. (B) Confocal microscope images of HepG2 transfected with pEGFP-MKCHKRA (a), pEGFP-MKCH KKA (b), pEGFP-MKCHKRKKA (c), pEGFP-MKCDKRKKA (d), pEGFP-MKKRKKA (e), pEGFP-MKΔCTKRKKA (f), or pEGFP-C2MKΔCTKRKKA (g).

initiates the nuclear import of MK. Linear NLSs are also reported to bind to the N-terminal domain of nucleolin [28]. Likewise, FGF-2, another low-molecular-weight growth factor rich in basic residues, is thought to be translocated into the nucleus and nucleolus primarily through binding to nucleolin [27].

Nucleolar accumulation of MK

Nucleolar accumulation of endogenous MK was found in the nucleolus of PHA-activated peripheral blood lymphocytes (PBL) [22]. In our study, we showed that endogenous GFP-tagged wild-type MK (Fig. 1A, panel b) accumulated in the nucleolus in HepG2 cells (Fig. 1B, panel b), while GFP control did not (Figs. 1A and B, panel a). Fig. 2 showed the time-lapse imaging of the nucleolar accumulation of MK and revealed that MK localized to the nucleolus since the earlier phase of the fusion protein expression. In one study concerning the nuclear targeting by MK, although MK was discovered to be translocated into the nucleus, it was excluded from the nucleolus according to the illustration [18]. The reason for the discrepancy is unclear. One possibility is that the subnuclear distribution of MK in various cell types is different.

So far, the motif that controls the nucleolar localization of MK has not been identified. Previous studies have reported that the sequences of some factors responsible for the nuclear and nucleolar localization overlap [33,34]. Thus, we hypothesized that the motif directing the nucleolar localization of MK may overlap with the one controlling the nuclear localization. To test the hypothesis, we examined the nucleolar localization of a series of point-directed and deletion variants. Our result that the C-terminal tail of MK (Fig. 3A, panel d) drove GFP into the nucleolus suggested that this stretch is the most important element responsible for the nucleolar accumulation (Fig. 3B, panel d). However, deletion of this stretch did not abolish MK nucleolar localization (Figs. 3A and B, panel f), which indicated other nucleolar

localization sequences present within the C-terminal-tail-truncated MK. Deletion of the N-terminal tail (Fig. 3A, panel g), or mutation of K79R81 and K86K87 to Ala (Fig. 4A, panels f and g) in MKΔCT essentially blocked the nucleolar accumulation of the fusion protein (Figs. 3B, panel g; 4B, panels f and g), indicating that these sequences also contribute to the nucleolar localization of MK. Recently, some researchers have proposed that sequences necessary and sufficient for proper localization into the nucleolus are more likely to act as retention signals rather than as classical targeting or transport signals and the basic amino acid residues within the proteins imported establish electrostatic interactions with acidic molecules residing in the nucleolus [25,35,36]. Thus, it leads to the speculation that the nucleolar accumulation of MK is an interaction with rRNA, rDNA or some possible proteins and requires some effective basic residues to bind to the targets. According to our investigation, the C-terminal tail, K79R81, K86K87, and N-terminal tail may together compose the structural determinant containing the effective basic residues which interacts with the targets in the nucleolus.

Several growth factors have been found to accumulate into the nucleolus but the relevance of this accumulation to the function remains unclear [37]. As MK promotes cell proliferation [7,8], and one role of the nucleolus is to control rRNA gene transcription, pre-rRNA processing, and nascent ribosome subunit assembly which could be the downstream elements of controlling cell proliferation, it is possible that the nucleolar accumulation of MK is relevant to its mitogenic activity.

In conclusion, we identified the conformational determinants for MK nuclear and nucleolar localization. We showed that K79R81, K86K87, and the C-terminal tail of MK together constitute the nuclear localization determinant for the first time. In nucleolar localization, our study revealed the C-terminal tail plays the most important role, and besides, the N-terminal tail, K79R81 and K86K87 also contribute to the process.

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