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Characterization of nuclear localization signal in mouse ING1 homolog protein☆

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Abstract

We reported previously that mouse ING1 homolog (mINGh), localized in the nucleus, enhanced cell death in HC11 mouse mammary epithelial cells. Analysis of the mINGh amino acid sequences revealed the presence of potential nuclear localization signal (NLS) and plant homeodomain (PHD) finger DNA binding domain. In the present study, NLS site in mINGh was determined using different pieces of mutant mINGh proteins, which were fused to green fluorescent protein (GFP), and transfected into HC11 cells. NLS of mINGh was split into two parts consisting of amino acids KEKK and KKLK. Mutation in NLS sites of mINGh resulted in no enhancement of the cell death when over-expressed. These results indicated that mINGh contains NLS of bipartite type, which is essential for the regulation of cell death.

Keywords: mINGh; NLS; Mammary epithelial cell; Cell death

Nuclear proteins, imported into the nucleus via nuclear pore complexes (NPCs), have important roles in nuclear events such as transcription, mRNA processing, chromosomal organization, and replication. NPCs permit passive diffusion of small molecules, such as ions and proteins of up to 20–40 kDa, and active translocation of large proteins via selective process using cytosolic receptors of the karyopherin family [1–3]. This selective and active nuclear protein transport is mediated by a nuclear localization signal (NLS). It is worthwhile to note that NLS is required in even smaller proteins for efficient targeting [4].

NLSs can be classified into several classes. The first consists of a single cluster of basic amino acids such as SV40 large T antigen (KKKRK) [5]. This type contains no bulky, acidic, or hydrophobic residues in the core and flanking regions. The second class contains bipartite sequences that carry two clusters of positively charged amino acid residues. These clusters are separated by a spacer region, such as NLS of *Xenopus laevis* nucleoplasmin (**KK**PAATKKAGQA**KKKK**) [6]. The third class is exemplified by c-MYC NLS, in which only three of the nine in the cluster are basic residues (PAA **KRVK**LD), where the N-terminal proline and C-terminal acidic residues are thought to be important for their function [7–9]. The fourth class includes various other NLSs, such as ribosomal proteins and hnRNPs [10,11].

We recently isolated a mouse ING1 homolog (mINGh) protein using yeast two hybridization methods [12]. mINGh enhances cell death under serum-starved condition and is expressed in various tissues. In addition, several transcripts are generated by alternative splicing. mINGh protein contains plant homeodomain (PHD) form of zinc finger domain which is commonly found in chromatin-associated proteins and lysine-rich region regarded as NLS and localizes to the nucleus such as ING1 protein [13,14].

In this study, we constructed mutants of mINGh for the identification of NLS and its function in HC11 mammary epithelial cells.

☆ *Abbreviations:* mINGh, mouse ING1 homolog; NLS, nuclear localization signal; GFP, green fluorescent protein.

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Materials and methods

Plasmid constructions. Mutation constructs were cloned into pEGFP-N2 in frame with GFP (Clontech, USA). cDNAs encoding various amino acid fragments or mutations of mINGh were generated by the polymerase chain reaction (PCR). Specific primers used are as follows:

1. 5′-TTGCTAGCGCCACCATGGCTGCTGGGATGTAT-3′
2. 5′-TTCCCGGGTTTCTTCTTCCGTTCTTG-3′
3. 5′-TTCCCGGGTGTGCGCACAAGTTTTAA-3′
4. 5′-TTGCTAGCATGGTGCAGCTGGCCATGCAG-3′
5. 5′-TTGCTAGCATGGGTAGCGCCCGCAGC-3′
6. 5′-TTGCTAGCATGCGGACCCAAAAGGAGAAAAAAGCT-3′
7. 5′-TTGCTAGCATGCGGACCCAAAACAACAACAACGCT-3′
8. 5′-TTGGGCCCCGTGCGCACAAGTTTTAACTTCTTCTG-3′
9. 5′-TTGGGCCCCGTGCGCACAAGGTTGTTGTTGTTCTG-3′
10. 5′-CGGACCCAAAACAACAACAACGCTGCCAGA-3′
11. 5′-TCTGGCAGCGTTGTTGTTGTTTTGGGTCCG-3′
12. 5′-TGTGCGCACAAGGTTGTTGTTGTTCTGGGC-3′
13. 5′-GCCCAGAACAACAACAACCTTGTGCGCACA-3′
14. 5′-TTCCCGGGCTATTTCTTCTTCCGTTCTTG-3′

The primer pairs for each construct, which contained *Nhe*I and *Apa*I restriction sites (underlined in the primer sequences) to facilitate cloning, are described in Table 1.

For the construction of pEGFP-mINGh-mutNLS and pcDNA-mINGh-mutNLS, three separate PCRs were performed and mINGh-mutNLS was generated by overlapping PCR [15] as follows: first PCR was performed with primers 1 and 11 or 10 and 2 and these two PCR products were combined; second PCR was performed with 1 and 12 or 13 and 2; after combining the two second PCR products, third PCR was performed with 1 and 2 or 1 and 14 and subcloned into pEGFP-N2 or pcDNA3 (Invitrogen, USA), respectively. All constructs were sequenced using ABI Prism 377 DNA Sequencer (Perkin–Elmer, USA).

Cell culture and transfection. HC11 cells were cultured in the growth medium containing RPMI1640 (GibcoBRL, USA), 10% heat-inactivated fetal bovine serum (GibcoBRL), 5 μg/ml insulin (Sigma, USA), 10 ng/ml EGF (Sigma), and 50 μg/ml gentamicin (Sigma) [16]. Transfection was carried out using Lipofectamine Plus Reagent (GibcoBRL) according to manufacturer's procedure. For stable transfection, cells were transfected with pcDNA-mINGh or pcDNA-mINGh-mutNLS and selected by growing in the medium containing G418 (400 μg/ml) for 3 weeks.

Microscopic observation. HC11 cells were seeded on glass coverslides, grown at 37 °C for 24 h, and transiently transfected with each construct. After fixation in 1% glutaraldehyde for 10 min, the cells were washed in phosphate-buffered saline (PBS) and visualized by confocal laser scanning microscopy.

Viability assay. Stable transfectants were seeded on 96-well plates at a density of 20,000 cells/well. On the following day, cells were incubated in the serum-free medium to induce apoptosis [17]. After 48 h, viable cells were estimated using Cell Proliferation Kit I (MTT) (Roche, USA) according to manufacturer's procedure. The proliferation levels are reported as the optical density (OD) readings; absorbance at 620 nm was subtracted from that at 540 nm for each well.

Table 1
Primer pairs for each construct

Name	Primer pair	Name	Primer pair
mINGh (1–248)	1 and 2	NN (131–166)	6 and 8
Δ1 (1–166)	1 and 3	NM	6 and 9
Δ2 (90–248)	4 and 2	MN	7 and 8
Δ3 (50–166)	5 and 3	MM	7 and 9
Δ4 (90–166)	4 and 3		

Results and discussion

Identification of deletion constucts for mINGh

Recently, we reported that mINGh contained PHD finger domain and putative NLS in the lysine-rich region (amino acids 127–162 of proteins). In addition, mINGh is predominantly localized in the nucleus of HC11 mammary epithelial cells [12]. Therefore, to determine the functional NLS of mINGh, we constructed several deletion mutants into pEGFP-N2 in frame with the cDNA encoding GFP and expressed the proteins in HC11 cells. A schematic representation of the deletion mutants is shown in Fig. 1A. All deletion mutants of mINGh except vector-only control were localized in the nucleus as in the intact form, which suggests that the NLS exists in the lysine-rich region of mINGh as expected (Fig. 1B).

mINGh contains a functional NLS

mINGh contained three putative NLSs in the lysine-rich region (amino acids ^{127}KGKK^{130}, ^{135}KEKK^{148}, and ^{159}KKLK^{162}). However, NN deletion construct of mIN Gh was predominantly localized in the nucleus of HC11 cells (Fig. 2). Furthermore, we obtained deletion forms of amino acids ^{129}KKS^{131} of the proteins in the mouse mammary gland by reverse transcription polymerase chain reaction (RT-PCR) and these forms also localized in the nucleus (data not shown). Therefore, we inferred that NLS of mINGh may be of a bipartite type.

To determine if the putative NLS sequences were functional, we constructed GFP fusion proteins possessing different mutations in the putative NLS (Fig. 2A). These constructs were transiently transfected into HC11 cells and the subcellular localization of these fusion proteins was monitored by confocal microscopy. As expected, amino acids 131–166, containing both NLSs, could target GFT protein to the nucleus. In contrary, mutation of the amino acids KEKK and/or KKLK could not induce nucleus localization of the full-length GFP fusion protein (Fig. 2B). These results clearly demonstrated that mINGh protein possesses functional NLS of bipartite type containing two regions separated by a space.

NLS of mINGh is necessary for apoptotic effect

Since mINGh enhanced cell death under serum-starved condition, we examined whether the mutation of NLS affected cell death in mammary epithelial cells. NLS mutant form of mINGh was constructed into pEGFP-N2 or pcDNA3. HC11 cells were transfected with pcDNA-mINGh or pcDNA-mINGh-mutNLS and selected by growing in the medium containing G418 (400 μg/ml) for 3 weeks. Results revealed that pEGFP-

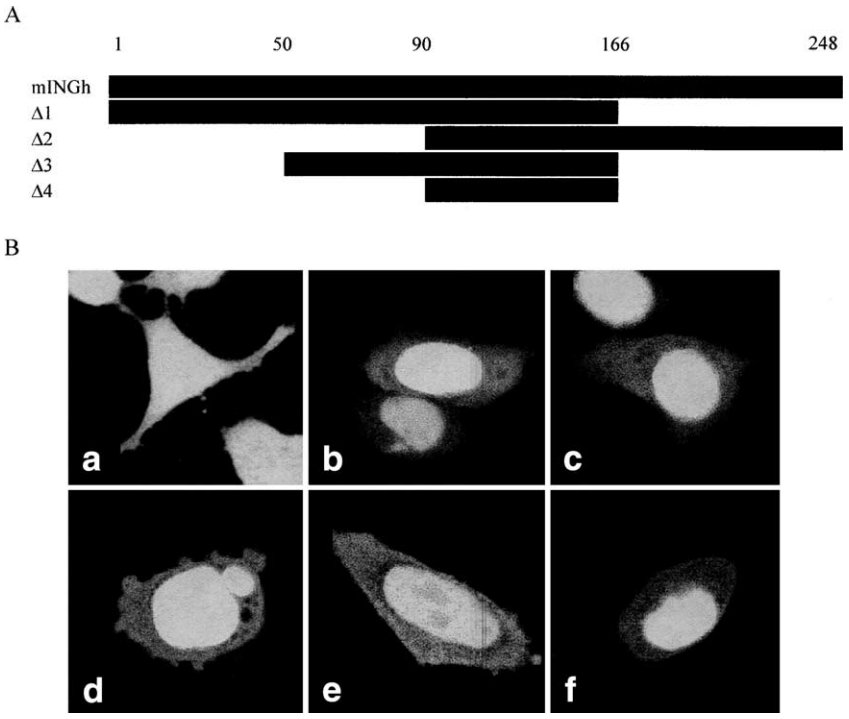

Fig. 1. Construction and subcellular localization of various deletion mutants. (A) Deletion mutants constructed into pEGFP-N2 in frame with GFP. Δ1, Δ2, Δ3, and Δ4 contained amino acids 1–166, 90–248, 50–166, and 90–166, respectively. (B) HC11 cells were transfected with vector only (a), pEGFP-mINGh (b), pEGFP-Δ1 (c), pEGFP-Δ2 (d), pEGFP-Δ3 (e), and pEGFP-Δ4 (f). After transfection for 48 h, cells were fixed with 1% glutaraldehyde and visualized through confocal microscopy.

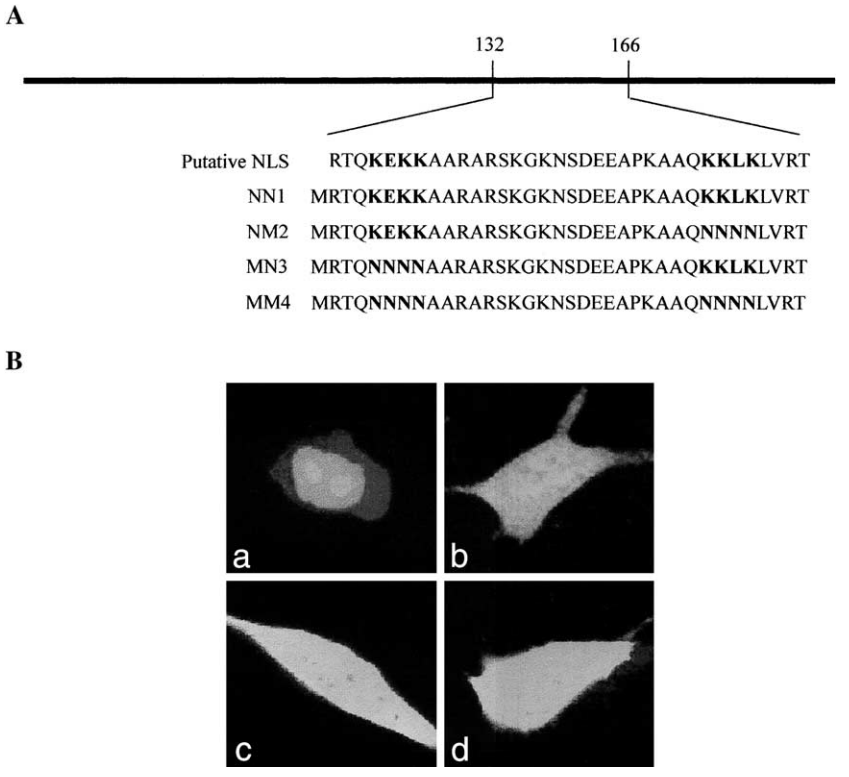

Fig. 2. Identification of functional NLS in mINGh. NLS mutants of mINGh (A) were generated and transfected into cells. Cells were visualized (B) by confocal microscopy. Panels a, b, c, and d represent NN1, NM2, MN3, and MM4, respectively.

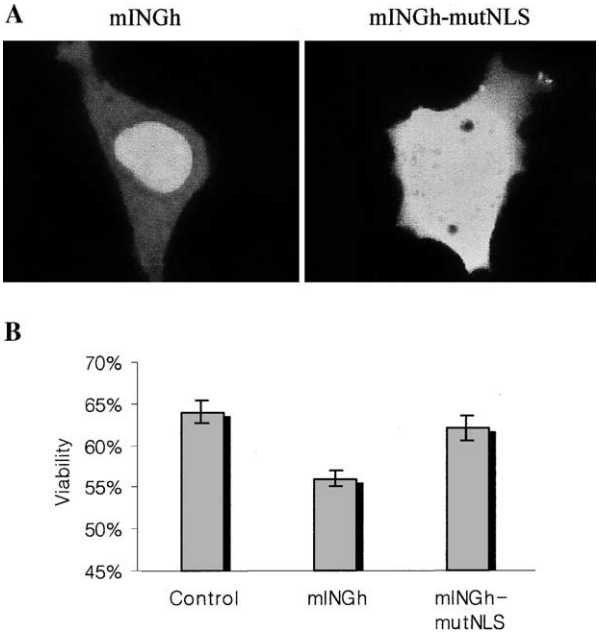

Fig. 3. Effect of NLS mutant form of mINGh in mammary epithelial cells. (A) HC11 cells were transfected with pEGFP-mINGh or pEGFP-mINGh-mutNLS and visualized using confocal microscopy. (B) Stable cells containing pcDNA-mINGh or pcDNA-mINGh-mutNLS were incubated under serum-starved condition for 48 h and viability was assessed using MTT assay. Values are means ± SD of at least three independent experiments.

mINGh-mutNLS was not able to induce the localization to the nucleus, while pEGFP-mINGh was (Fig. 3A). In addition, cells expressing mINGh enhanced cell death, whereas those transfected with pcDNA-mINGh-mutNLS showed no difference to the vector-only control (Fig. 3B). These results indicate that mINGh is required in the nucleus, by NLS in this case, to induce cell death in the mammary epithelial cells.

In summary, we identified functional NLS of mINGh by deletion and mutation analyses. It was found that mINGh has a bipartite type of NLS, which contains amino acids KEKK and KKLK in the lysine-rich region and is actively transported into the nucleus. The short form of mINGh and Δ1 mutant without PHD finger domain also localized into the nucleus and enhanced cell death [12]. However, once NLS region is mutated, the mutant form of mINGh was not able to enhance cell death in mammary epithelial cells. Furthermore, over-expression of ING1, an isolated tumor-suppressor gene, leads to the enhancement of apoptosis under serum-starved condition and cell death by ING1 is synergistic with the action of Myc [18,19]. Therefore, we suggest that enhanced cell death by mINGh is due to the interaction with certain proteins in the nucleus rather than by inducing activation of transcription factors.

Acknowledgment

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