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Nuclear Localization Signal and p53 Binding Site in MAP/ERK Kinase Kinase 1 (MEKK1)

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Abstract

Previously, we showed that Mekk1 translocates to the nucleus, interacts with tumor suppressor protein p53 and co-represses PKD1 transcription via an atypical p53 binding site on the minimal PKD1 promoter (JBC 285:38818-38831, 2010). In this study, we report the mechanisms of Mekk1 nuclear transport and p53 binding. Using GFP-linked constitutively active-Mekk1 (CA-Mekk1) and a deletion strategy, we identified a nuclear localization signal (HRDVK) located at amino acid (aa) residues 1349–1353 in the C-terminal Mekk1 catalytic domain. Deletion of this sequence in CA-Mekk1 and full-length Mekk1 significantly reduced their nuclear translocation in both HEK293T and COS-1 cells. Using co-immunoprecipitation we identified an adjacent sequence (GANLID, aa 1354–1360) in Mekk1 responsible for p53 binding. Deletion of this sequence markedly reduced the interaction of Mekk1 with p53. Mekk1 does not appear to affect phosphorylation of Ser15, located in the Mdm2 interaction site, or other Ser residues in p53. However, Mekk1 mediates p53 protein stability in the presence of Mdm2 and reduces p53 ubiquitination, suggesting an interference with Mdm2-mediated degradation of p53 by the ubiquitin-proteasome pathway.

Keywords

p53; Mekk1; Nuclear localization signal; p53 binding sites

Introduction

The mammalian mitogen-activated protein kinase (MAPK) family of serine/threonine kinases is involved in a variety of cellular functions including proliferation, differentiation and apoptosis and includes at least three subgroups: ERKs (extracellular signal-regulated kinases), p38 MAPKs, and JNKs (the c-Jun N-terminal kinases, also known as stress-activated protein kinases or SAPKs) (Hagemann and Blank, 2001; Krishna and Narag,

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2008). Each MAPK is a member of one or more kinase cascades activated by phosphorylation in response to upstream signals. Thus a MAPK kinase kinase (MAPKKK or MAP3K) phosphorylates a MAP kinase kinase (MAPKK), which in turn phosphorylates a MAPK. Following activation, the MAPKs translocate to the nucleus to regulate the activity of transcription factors controlling a wide range of genes. The MAP3K which includes Raf and a group of MEK (MAP/ERK kinases) kinases (or Mekks), although they have the ability to activate ERK, appear to preferentially regulate the JNK pathway. One of the key MAP3K components of the stress-activated JNK pathway is Mekk1. The Mekk family consists of four members (Mekk1-4) based on similarity to the yeast MAP3Ks, Ste11p and Byr2p, in their catalytic domains. They all have a C-terminal catalytic domain and an N-terminal regulatory domain, but differ in size, sequence, intracellular distribution and kinase specificity. For example, Mekk1 is only 50% identical to Mekk2-4 in the kinase domain. Upon stimulation, Mekk1 phosphorylates Mkk4/Mkk7 (MAPKKs), which then phosphorylate and activate JNK (Healy et al, 2008; Das and Muniyappa, 2010; Schlesinger, 2002). Signaling initiated with the typically membrane-associated Mekk1 ends with activation of the transcription factor AP-1 (activator protein-1), which is a homo- or heterodimer of c-Jun with c-Fos or ATF2; or with other Mekk1-JNK responsive transcription factors including p53. The small GTPases, Ras, Rac, and cdc42, and their downstream effectors JNK and AP-1 have been linked to several diseases. Beside this MAPK cascade, an E3 ubiquitin ligase function has been recognized in Mekk1 that mediates degradation of ERK1/2 and c-Jun (Lu et al, 2002).

Since its discovery 30 years ago, the importance of p53 as an essential transcription factor for tumor suppression has become clear. The tumor suppressor p53 is widely known for its role in growth arrest, DNA repair, and apoptosis in response to cellular damage, and its inactivation by genetic mutation is one of the most frequent alterations in human cancers (Boehme and Blattner, 2009; Kruse and Gu, 2009; Vousden and Prives, 2009). The p53 protein prevents cellular transformation by regulating the cell cycle and promoting apoptosis. It elicits these functions via transcription-dependent and independent modes. In the transcription-dependent mode, p53 serves as a transcription factor by directly binding DNA at consensus sequences located in the regulatory regions of target genes, and it can serve as an activator or repressor depending on the target gene (Menendez et al, 2009). The p53 protein is unstable with a half-life ranging from 5–30 min; however, many cellular stresses such as hypoxia, DNA damage, oncogene activation, etc. can lead to its stabilization via a block of its degradation (Levine, 1997; Wu et al, 1997). In normal unstressed cells, the p53 protein is maintained at a low level owing to its continuous degradation, primarily mediated by Mdm2 (mouse double minute 2; human homolog is hdm2). Compelling genetic evidence has established the important physiological role of Mdm2 as a regulator of cellular p53: Mdm2 deficient mouse embryos die before E6.5, but are fully rescued if they are co-deficient of p53 (Jones et al, 1995; Montes et al, 1995). On the other hand, Mdm2 itself is a product of transcription induced by p53 (Wu et al, 1997; Picksley and lane, 1993). Thus, p53 and Mdm2 are linked through an autoregulatory loop. Besides Mdm2, numerous other proteins interact with p53, and these protein-protein interactions play an important role in regulation of p53 function at different levels (reviewed in Fernandez and Sot, 2010).

Recently, we reported a novel mechanism of transcriptional repression by Mekk1 in which its effect is mediated in the nucleus through interaction with the tumor suppressor protein p53 via an atypical p53 DNA binding motif in the PKD1 proximal promoter. Mekk1 kinase activity is not required for this repression, excluding the need for a Mekk1 mediated phosphorylation cascade. Furthermore, their interaction seemed to stabilize p53 protein in cells (Islam et al, 2010).

In the present study, we explore this interaction between Mekk1 and p53. We have identified a signal sequence in Mekk1 for its nuclear localization, and a sequence in Mekk1 responsible for p53 binding. Furthermore, a possible mechanism of p53 protein stabilization by Mekk1 is discussed.

Methods

Plasmid constructs

The mammalian expression constructs for 1,493 amino acid containing full length Mekk1 and kinase-inactive Mekk1 (KI-M), in which the active lysine at 1235 has been replaced with a methionine (K1235M), were a generous gift of Dr. Widman (University of Lusanne, Switzerland), constitutively active Mekk1 (CA-M, 1174–1493 aa) was obtained from Stratagene (pFC-MEKK). This construct contains the C-terminal 293 amino acids of full-length Mekk1. p53 was a gift from Dr. Samir-El-Dahr (Tulane University Health Sciences Center) previously described. EGFP was obtained from Stratagene, and Mdm2 was from AddGene.

GFP fusion constructs and mutagenesis

A 970 bp CA-M comprising the coding sequence for C-terminal 293 amino acids (1174–1493 aa) of Mekk1 was PCR amplified using primer pairs GFP1 and 2 (Table 1). The forward primer GFP1 harbors an EcoRI site, while GFP2 contains a Bam HI site. The latter primer also mutates the stop codon in order to clone in frame upstream of GFP in the EGFP-N3 vector between EcoRI and BamHI. The sequential deletion constructs were created by PCR cloning using a forward primer harboring a designed EcoRI site in combination with the reverse primer GFP2 (Table 1). Following PCR, the fragments of varying sizes were restricted and cloned directionally into the EcoRI-BamH I site of the EGFP-N3 vector in frame with GFP protein. Some of these primers also contained an engineered in-frame start codon (ATG) downstream of the EcoRI restriction site.

All PCRs were performed in similar fashion: following a denaturation step at 95 °C for 3 min, 30 cycles of 94 °C for 30 sec, respective annealing temperatures (55–60 °C) for 45 sec and 72 °C for 60 sec, followed by a final extension of 7 min at 72 °C.

The deletion constructs (F, 1F or 2F) were created using primer pairs MKd1 and d2, MKdel21 and del22, or MKdel31 and del32 (Table 1) and the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. After verification by sequencing, the fragments were subcloned into the pEGFP-N3 vector in frame with GFP at the N-terminus. CA-M, Mekk1 or 1CA-M were created by swapping BsiWI and NheI fragments between F or 1F and CA-M or Mekk1.

Cell cultures and transfections

HEK293T, COS-1 and HCT p53^{-/-} cells (gift from Dr. Vogelstein, Johns Hopkins University medical Center) were obtained and were cultured as described (Islam et al, 2010): HEK293T and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotic (100 IU/ml penicillin, 100 µg/ml streptomycin). HCT116 p53^{-/-} cells were maintained in McCoy's media containing 10% FBS and antibiotic. All cells were grown at 37°C supplied with 5% CO₂. Following overnight culture in six-well plates, the cells were transfected with appropriate constructs using either the calcium phosphate method (HEK293T and HCT116 cells) or Lipofetamine (Gibco) (COS-1) according to the manufacture's instructions. The cells were harvested at 40 h and their protein concentration was determined using the BCA protein assay kit (Pierce).

Nuclear extract and immunoprecipitation

Nuclear extract was prepared from HEK293T or COS-1 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, 78833) following the manufacturer's instructions. Immunoprecipitation was carried out in the following way: to cells in six-well plates transfected with desired constructs was added 100 µl of CER buffer (Pierce) with protease inhibitor cocktail and the cells were stored at -20 °C for at least 1 h. After thawing the cells, 10 µl of 1× cell lysis buffer (Promega) was added, and the cells were scraped into 1.5 ml tubes. The extracts were then vortexed and microcentrifuged at full speed. The resulting supernatants were transferred to fresh tubes, and 170 µl of PBS and 20 µl of 5 M NaCl were added, and these were incubated with 5 µl of anti-p53 (SantaCruz, sc-6243) or anti-Mekk1 (SantaCruz, sc-252) antibodies on a rotating wheel overnight at 6°C. Then the antigen-bound primary antibodies were captured with Protein A/G plus-Agarose (SantaCruz, sc-2003) for 4 h, and were washed three times with PBS containing 0.5% Tx-100 and 0.5% NP40. The immunoprecipitates were analyzed by immunoblotting using either anti-Mekk1 or anti-p53 antibodies. AP-conjugated Clean-blot IP detection reagent (Thermo Scientific, 21233) was used as the secondary antibody.

SDS-PAGE and Western immunoblotting

The presence of various proteins in the samples was confirmed by 10 % SDS-PAGE, followed by electroblotting to PVDF membranes and detection using the respective primary antibodies: rabbit anti-p53 (SantaCruz, sc-6243, 1:1,500), rabbit anti-15-phosphoserine (SantaCruz, sc-101762, 1:2000), rabbit anti-Mekk1 (SantaCruz, sc-252, 1:1,500), mouse anti-β-tubulin (SantaCruz, sc-5286, 1:1,500), and rabbit anti-PARP-1 (SantaCruz, sc-74469, 1:1,500), mouse anti-β-actin (SantaCruz, sc-101762, 1:10,000), and mouse anti-Mdm2 (SantaCruz, sc-965 1:2000). Alkaline phosphate-conjugated secondary anti-rabbit (Sigma, A8025) or anti-mouse (Sigma, A7434) antibodies were used at 1:10,000 dilution. Following equilibration in chemiluminescence buffer (0.1 M diethanolamine, 1 mM MgCl₂, pH 9.5) for 5 min, the membranes were incubated with substrate CDP-Star (Amersham) for 5 min and exposed to film (RPI). For re-probing, the blots were stripped of primary and secondary antibodies using 20 mM Tris-HCl, pH 6.8 containing 1% SDS and 100 mM 2-mercaptoethanol at 55°C for 45 min on a rotating wheel (20 rpm), followed by extensive

washing with water and blocking with 5% non-fat dry milk for at least 2 h prior to incubating again with primary antibodies.

GST-fusion proteins and in-vitro p53-CA-M binding

CA-M or p53 in the pGEX vector was cultured for 5 h in the presence of IPTG added at log phase. Harvested cells were lysed by freezing and then sonication. The supernatant was allowed to bind GST-agarose overnight at 6 °C. The bound GST or its fusion proteins were eluted with reduced glutathione (10 mM). Purified GST or GST-p53 (0.5 µg) was allowed to bind a 96 well plate in 50 µl of 0.1 M bicarbonate buffer, pH 9.0 overnight at 6 °C.

Following that, the wells were washed twice with PBS and blocked in 5% BSA in PBS containing 0.5% Tx-100 (PBSTx) for 4 h at room temperature. Then, the wells were washed twice with PBSTx and once with 20 mM Tris-HCl, pH 7.5 and incubated with GST-CA-M (0.5 µg) in 50 µl of binding buffer (20 mM Tris-HCl, pH 7.5 containing 0.25 M NaCl, 0.5% Tx-100, 1 mM EDTA) for 3 h at room temperature with mild shaking. After that the wells were washed twice with PBSTx and incubated overnight with primary antibodies (anti-p53 or anti-Mekk1, 1:600 dilution) in blocking buffer. Following washing four times with PBSTx, the wells were incubated with secondary antibodies (1:1700 dilution) in PBSTx for 4 h. The wells were washed again four times with PBSTx and incubated 5 min at room temperature with 50 µl of 4-nitrophenylphosphate (4 mM) in 100 µl of 0.1 M bicarbonate buffer (pH 10.5). The absorbance of the color developed was read at 415 nm using a microplate reader (BioRad). In one set, the wells were treated with SDS buffer overnight at room temperature for western blotting.

Fluorescence microscopy

Approximately 1×10^5 cells/chamber from an overnight culture were transfected with various constructs for 40 h. The cells were fixed with 4% formaldehyde (30 min at 4°C), washed and permeabilized with 0.1% TX-100 in PBS. The GFP-fusion proteins were visualized at this point. For constructs without GFP, the fixed cells were blocked with 1% BSA prior to incubation with rabbit anti-Mekk1 (SantaCruz, sc-252, 1:300) overnight, followed by incubation with FITC conjugated anti-rabbit antibodies for 2 h. All cells were observed under a fluorescence microscope.

Results

Nuclear Localization Signal in Mekk1

Recently, we and others showed localization of Mekk1 (both overexpressed and endogenous) in both the nuclear and the cytosolic compartments. We further observed that Mekk1 physically associates with p53 in both compartments (Islam et al, 2010). Thus, we wondered whether Mekk1 translocates to the nucleus along with p53 following their association in the cytosolic compartment. To address this, we exploited mutant HCT116 cells deficient in p53 expression. As shown in the immunoblot analysis of the nuclear extracts (Fig. 1, NE), a significant portion of both the 36 kDa CA-Mekk1 (CA-M, upper) and the 196 kDa full-length Mekk1 (lower) localized into the nucleus when expressed ectopically in these cells (+). Full length (196 kDa) and its known major proteolytic fragment (90 kDa) were also seen in vector transfected cells (lower panel, right lanes). These

results suggested that association with p53 in the cytosol is not required for nuclear transport of Mekk1. Therefore, its nuclear translocation could depend on association with other nuclear-bound proteins or the presence of a nuclear localization signal (NLS) as found in many nuclear proteins.

Thus, we searched for the presence of an NLS in Mekk1 using PSORT. Two monopartite signals, consisting of a cluster of basic amino acids preceded by a helix-breaking residue, and a bipartite signal, consisting of two clusters of basic residues separated by 9–12 amino acids, were found in the N-terminal region of Mekk1 (Meares and Jopes, 2007). While one or more of these sequences could be responsible for the nuclear translocation of Mekk1, this would not explain the nuclear import of the C-terminal, constitutively active fragment, CA-M (1174–1493 aa). Thus, we hypothesized that a nuclear localization signal is present in the C-terminal 319 amino-acid sequence comprising CA-M. Upon inspection of the CA-M sequence, no site matching a known NLS motif was apparent. Therefore, to localize the NLS in CA-M we created a GFP fusion protein by linking CA-M in frame to the N-terminus of GFP (CA-M-GFP = CF) (Fig. 2A), from which sequentially truncated constructs were created by PCR cloning using a forward primer harboring a designed EcoRI site together with the reverse primer GFP2 (Table 1, Figs. 2A, B). Some of these primers also contained an engineered in frame start codon (ATG) downstream of the EcoRI site.

Serially truncated GFP fusion constructs were expressed in HEK293T cells, and the cells were fractionated into cytosolic and nuclear extracts and analyzed by immunoblot (Fig. 2C). To establish the fidelity of the cytosolic extract (CE) and nuclear extract (NE) we used the markers α -tubulin for cytosolic and/or PARP-1 for nuclear fractions (not shown). As shown in Fig. 2C, a significant fraction of the fusion constructs 17F, 16F and 15F translocated to the nucleus. The shorter constructs 13F and 5F did not, indicating that a sequence present in the 15F construct, but missing in the 13F construct, was required for nuclear transport, suggesting the presence of an NLS between 15F and 13F (Fig. 2B) of the CA-M sequence. Upon careful inspection, a basic, monopartite-like NLS sequence, HRDVK, within this region was found. To determine if this sequence has any role in nuclear transport, we created F by deleting HRDVK from the CF sequence (Fig. 2B). It is evident in Fig. 2D, that this deletion significantly reduced nuclear translocation of F compared to CF in HEK293T cells, even though more F was expressed (compare the ratios of CF and F in CE and NE of Fig. 2D), suggesting the possibility that this sequence (HRDVK) may serve as an NLS for Mekk1.

In order to eliminate the effect of GFP, if any, on the fusion constructs, and to determine whether this sequence is truly serving as an NLS in Mekk1, we produced CA-M and Mekk1 from CA-M and Mekk1, respectively, in which the sequence HRDVK was deleted (see Fig. 1B). Immunoblot analyses of the cytosolic and nuclear extracts of HEK293T cells transfected with these deleted and non-deleted constructs showed that while a significant portion of CA-M was nuclear localized, in contrast, CA-M was barely detected in the nucleus (Fig. 3A, left lanes). Both CA-M and CA-M were almost equally expressed eliminating the possibility that the difference was due to the level of expression. Similar results were also noted when tested in COS-1 cells (Fig. 3B, compare C vs. C in CE vs. NE), where it can be seen that there is a significant decrease of C in the nuclear extract

further confirming that the basic NLS-like sequence encoded in HRDVK was likely serving as a signal for nuclear localization of CA-M.

Nuclear localization of Mekk1 was significantly reduced compared to the full length Mekk1 (Fig. 3A, right lanes) in HEK293T cells, despite possessing several mono- and bi-partite basic type NLSs towards its N-terminus. Similar results were also obtained in COS cells (Fig. 3B, compare M vs. M in CE vs. NE). These results indicated that the sequence HRDVK present in both Mekk1 and CA-M is acting as the primary NLS for Mekk1, rather than the basic type-putative signals at the N-terminus. The GFP-tagged construct, F, was also decreased in the nuclear fraction (Fig. 3B).

Significant nuclear localization was observed by fluorescence/immunofluorescence microscopy in HEK293T cells transfected with Mekk1, CA-M, and CF (Fig. 3C). However, the corresponding deleted constructs (Mekk1, CA-M and F) in which the HRDVK sequence was deleted were predominantly localized in the cytosol and perinuclear region, further confirming the HRDVK sequence as the bonafide NLS for Mekk1.

We previously showed that CA-M (and Mekk1) interact with p53 in the nucleus and repress PKD1 (polycystic kidney disease gene 1) transcription via a p53 binding site in its proximal promoter region (Islam et al, 2010). Therefore, drastically reducing CA-M's nuclear import should impair its ability to repress the 0.2 kb promoter-luciferase reporter harboring the p53 site. To test this we examined whether the NLS-deleted mutant CA-M (C) has any effect on transcription of the PKD1 proximal promoter. The data presented in Fig. 4A show that

CA-M failed to repress the PKD1 0.2 kb proximal promoter as compared to that observed with CA-M (C). The expression levels of both CA-M and the somewhat shorter CA-M were comparable (Fig. 4B), thus suggesting that the difference in their repressive effects (Fig. 4A) is due to impaired nuclear import of CA-M.

p53-binding site in Mekk1

As mentioned above, Mekk1 and CA-M co-immunoprecipitate with p53 from both the cytosolic and nuclear fractions (Islam et al, 2010). Numerous proteins have been shown to interact with p53, directly or indirectly. Since co-immunoprecipitation from cell extracts could not rule out the possibility of indirect interaction via one or more of the many p53 binding proteins, and to demonstrate this interaction in vitro as well, we produced and purified both CA-M and p53 as fusion proteins linked to the C-terminal of GST (Glutathione-S-transferase). SDS-PAGE analysis in Fig. 5A showed that all three proteins, GST, CA-Mekk1-GST (CA-MG) and p53-GST (p53G), indicated by the asterisks, were relatively pure. ELISA-like assays, in which CA-MG was incubated with well-bound GST or p53G, and detected by anti-Mekk1 antibodies and alkaline phosphatase conjugated secondary antibodies, revealed significant binding of CA-MG to well bound p53G (Fig. 5B, far right bar). In contrast, there was almost no binding to GST protein only (middle bars). No significant ALP activities (conjugated to secondary antibodies) were observed in the wells (left and middle bars) incubated with either anti-Mekk1 or anti-p53 antibodies, thus eliminating the possibility of non-specific antibody binding to the wells. The results obtained by the ELISA-like assay in Fig. 5B were confirmed by western blot (shown in the inset in Fig. 5B) for the last three wells where a distinct CA-MG band can be seen in the last

lane. These findings further support a direct physical interaction between these two proteins, and suggest that binding was mediated via the C-terminal portion of Mekk1 (i.e., CA-M).

Peptide sequence comparisons between CA-M and known p53 binding proteins such as adenovirus E1B, SV40 T antigen and Mdm2, did not reveal any significant homology. To further characterize the interaction between CA-M and p53, we made use of the truncated CA-M-GFP (CF) constructs described in the previous section. HEK293T cells were transfected with CF, 15F, 13F, 5F and F were subjected to immunoprecipitation using anti-Mekk1 antibodies. As shown in Fig. S1, with evenly expressed constructs, p53 was coimmunoprecipitated with CF, 15F (1347–1493 aa) and even with F, but did not with 13F (1370–1493 aa) and the shorter 5F (1398–1493) (Fig. S1A), suggesting that the C-terminal amino acids between 1370–1493 are not involved in p53 binding, and that the binding domain is localized in the region containing amino acids 1347–1370.

As demonstrated above, the segment 1347–1370 also contains the NLS for Mekk1, however p53 was still co-immunoprecipitated with F (Fig. S1), thus eliminating the involvement of NLS sequence. To further narrow down the binding site, we created two additional deletion constructs in CF, 1F and 2F (Fig. 2B). Co-immunoprecipitation studies with these two deletion constructs in transfected HEK293T and COS-1 cells (Fig. 6A–B) using anti-p53 or anti-Mekk1 antibodies revealed that CF and 2F co-precipitated but 1F did not. The expression levels of p53 in all these cells were consistent, as were CF, 1F and 2F (inputs in Fig. 6A–B). These results together identified a short stretch of sequence, KGANLLID in CA-M possibly involved in p53 binding. Next, in order to eliminate fusion protein effects, we deleted the putative p53 binding sequence (KGANLLID) in CA-M to create 1CA-M and repeated the co-immunoprecipitation experiments using anti-p53 antibodies. As observed in Fig. 6C, compared to CA-M (C) there was significantly reduced co-precipitation with 1CA-M (1C), further supporting the role of this sequence in p53 binding.

Mekk1-mediated stabilization of p53

As observed earlier (Islam et al, 2010), ectopic expression of Mekk1, CA-M or KI-M (kinase inactive-Mekk1) in HEK293T cells increased p53 levels nearly two-fold: 1.8-fold in KI-M to 2.4 fold in CA-M. RT-PCR analysis of total RNA isolated from CA-M and Mekk1 transfected HEK293T cells ruled out the possibility of this increase resulting from increased p53 transcriptional activity (not shown).

In normal unstressed cells, p53 is under a complex, tight regulation. p53 protein is unstable and is maintained at a low level owing to its continuous degradation primarily mediated by Mdm2. Phosphorylation can also stabilize p53 by blocking its degradation.

To examine whether Mekk1 phosphorylates and stabilizes endogenous p53, lysates from HEK293T cells transfected with Mekk1, CA-M and KI-M were probed in western blots using anti-phospho-Ser15p53, as phosphorylation of Ser15 is known to have some effect on its stability. Only a slight increase in phosphorylation of p53 Ser15 in Mekk1 and CA-M transfected cells was observed compared to KI-M (Fig. S2A). This increase correlated well with the p53 protein increase, and the ratio of phospho-p53 over p53 essentially remained the same. Cellular and nuclear fractionation did not show much difference in

phosphorylation levels compared to vector transfected control cells (Fig. S2B). Several serine residues in p53 are phosphorylated, and since other serines might be phosphorylated, we reprobed the blots using phospho-Ser specific antibodies, which again revealed no difference (results not shown), confirming that the Mekk1-mediated stability of p53 is a phosphorylation independent event, as indicated by results with kinase inactive-Mekk1 (KI-M).

Since Mekk1 physically associates with p53, it is possible that this interaction might be interfering with Mdm2 mediated degradation by the ubiquitin pathway. We tested this possibility using cells transfected with Mekk1 and CA-M in the presence or absence of Mdm2. As shown in Fig. 7, in contrast to significant degradation of p53 (Fig. 7A), all Mekk1 variants protected p53 from ectopically expressed Mdm2 (Fig. 7B). These results raised the possibility that Mekk1 and its derivatives interfere with the p53-Mdm2 interaction. This was further evident in the significantly reduced ubiquitination of p53 in Mekk1 and CA-M transfected cells, either in immunoblots of cell lysates (Fig. 7C) or with immunoprecipitated p53 (Fig. 7D).

Discussion

Recently, we showed that Mekk1 translocates to the nucleus, interacts with tumor suppressor protein p53 and together they co-repress PKD1 transcription via an atypical p53 binding site on the minimal PKD1 promoter (Islam et al., 2010). We further observed that Mekk1 stabilizes the p53 protein. In this study, we report the mechanisms for its nuclear transport and interaction with p53.

NLS in Mekk1

Full length Mekk1 is a 196 kDa protein that contains putative pleckstrin-homology domains (PHDs) at the extreme N-terminus and a kinase active site in the C-terminus (Fig. 1B). The PHDs are considered responsible for the localization of Mekk1 to specific regions of the cell membrane through an interaction with polyphosphoinositides. Recent observations by us and others showing membrane-localized Mekk1 (both exogenous and endogenous) and at least one of its proteolytic fragments in the nuclear compartment (Fig. 1A) was surprising. The constitutively active, C-terminal 293 aa containing Mekk1 (CA-M) was expected to be cytosolically localized. However, a portion of this CA-M was also observed in the nucleus (Islam et al, 2010). Moreover, co-immunoprecipitation with p53 in both compartments led us to ask the question: do Mekk1 or its fragments co-migrate to the nucleus with p53? To address this, we exploited HCT116 p53 null cells. These mutant cells do not express p53 protein (Kaese et al, 2004) In these cells, a significant fraction of ectopically expressed full-length Mekk1 and constitutively-active Mekk1 (CA-M) was found in the nucleus (Fig. 1). Endogenous Mekk1 was also seen in the nucleus of vector transfected cells. These results suggested that association with p53 in the cytosol is not required for the nuclear transport of Mekk1.

Cellular materials pass in and out of the nucleus through nuclear pores, which function as a semipermeable filter. Larger, soluble macromolecules, require a signal sequence known as the NLS, which is a stretch of amino acid sequence that mediates transport of proteins into

the nucleus. Positively charged residues are abundant in the NLS (Conti et al, 1998), although there are also glycine rich motifs with few basic residues (Bonifaci et al, 1997). The best known NLS motifs are monopartite and bipartite (reviewed in Boulikas, 1993). A typical monopartite motif consists of a cluster of basic amino acids preceded by a helix-breaking residue. The bipartite motif has two clusters of basic residues separated by 9–12 amino acids (Meares and Jopes, 2007). However, not all NLS sites comply with these simple rules (Hsieh et al, 1998; Irie et al, 2000) and many proteins that possess these sequences are cytosolic.

PSORT analysis of the Mekk1 protein sequence predicted several mono- and bipartite sequences near the N-terminal region. However, this region is absent in the N-terminally truncated CA-M, indicating if the nuclear import is NLS driven, it must be located within the 1174–1493 aa region of Mekk1. As there are examples of proteins smaller than 30 kDa that may diffuse into the nucleus without a functional NLS (Alber et al, 2007), CA-M was fused to GFP (~30 kDa). In addition, this also aided in SDS-PAGE analysis of shorter peptide fragments generated by deletion.

Results of immunoblot analyses of serially truncated fusion proteins in overexpressed cells, fractionated into cytosolic and nuclear extracts, suggested the presence of an NLS. Deletion of HRDVK, a basic, monopartite-like sequence present in this region, significantly reduced nuclear translocation of the F protein in contrast to the unmutated CF protein (Fig. 3B).

Further evidence that this basic sequence serves as a NLS and to eliminate any effect of GFP, was obtained by analysis of CA-M and Mekk1, generated by deletion of HRDVK in CA-M and Mekk1, respectively. Compared to the wild-type, CA-M was barely detected (Fig. 3A, left lanes) and Mekk1 was significantly reduced in the nucleus. These results discounted any role of the putative mono- and bipartite sequences identified towards the N-terminus. The residual nuclear presence of these deleted proteins without GFP could possibly suggest the presence of a minor NLS, which will require further investigation.

Additional support was obtained by fluorescence/ immunofluorescence microscopy studies (Fig. 3C). In contrast to significant nuclear localization of Mekk1, CA-M, and CF in transfected cells, the corresponding deleted constructs (Mekk1, CA-M and F) were predominantly cytosolic and perinuclear.

As noted above, NLS sequences are diverse and many proteins possessing them are in fact localized in the cytosol. Nuclear import of mRNA binding protein, A1 is mediated by a nonclassical NLS (Bonifaci et al, 1997; Pollard et al, 1996). Yeast protein, Np13p uses both an NLS that is comprised of a repetitive RGG (arginine-glycine-glycine) motif and another that is a nonrepetitive sequence (Yun and Fu, 2000). In addition, nuclear import of this protein is also affected by methylation of arginine in RGG and phosphorylation of a serine in the nonrepetitive sequence. The NLS of ADAR1 (RNA-specific adenosine deaminase) is localized in the C-terminus and consists of a bipartite basic amino acid motif plus the last 39 residues (Nie et al, 2004). Another interesting observation is that the *S. cerevisiae* La protein contains a signal in the C-terminal 113 amino acids that does not correspond to the NLS of human La protein (Rosenblum et al, 1998). The sequence identified in Mekk1 matches well

with the basic, monopartite like (BBXXB) NLS, the only difference being the inclusion of a histidine. Basic residues in known NLS sequences are mostly comprised of arginine with an occasional inclusion of lysine.

The NLS sequence of Mekk1 is 100% conserved in a wide range of species including human, mouse, cat, dolphin, rabbit, horse, xenopus and chicken. Comparison of human and mouse full length Mekk1 sequences shows 89% overall homology. Interestingly, most of the differences lie in the N-terminus near the mono/bipartite sequences.

p53-binding site in Mekk1

Mekk1 and CA-M interact with p53 and act as a co-repressor of p53 on PKD1 promoter and other promoters (Islam et al, 2010). Identification of their interacting sites may be useful for elucidating the mechanism of co-repression, especially as transcriptional repression by p53 is not well understood. The interaction shown by co-immunoprecipitation from cell extracts could be indirect via one of many p53 binding proteins. Thus, their direct, physical interaction was demonstrated using purified GST-fusion proteins in an ELISA-like assay, in which a significant CA-MG binding to well-bound p53G (Fig. 5B, far right bar) was observed. This physical interaction was also seen in western blot analysis. These results illustrated that a direct interaction with p53 is mediated via the C-terminal portion of Mekk1 (i.e., CA-M).

Significant sequence homology between CA-M and several known p53 binding proteins such as adenovirus E1B, SV40 T antigen and Mdm2, was not found. Therefore, we made use of the truncated CA-M-GFP constructs. HEK293T cells transfected with CF, 15F, 13F, and 5F were subjected to immunoprecipitation using anti-Mekk1 or anti-p53 antibodies. p53 was coimmunoprecipitated with CF and 15F, but not with 13F and the shorter 5F, suggesting that the binding site is localized in the region between 15F and 13F containing amino acids 1347–1370. Stronger binding of 15F compared to CF could possibly be due to residues N-terminus to 15F (e.g., 1174–1347) that may play some role in conformational stability or regulating this interaction.

The segment 1347–1370 also contains the NLS for Mekk1. F (which lacks the NLS) was able to coimmunoprecipitate with p53 (Fig. S1, Fig. 6), suggesting that the remainder of 1347–1370 sequence (Fig. 2B) contains the p53 binding site. Half of this sequence was deleted in 1F and the remaining half in 2F. These studies revealed that 2F co-immunoprecipitated with p53, but 1F did not. Thus, the sequence KGANLLID appeared to be involved in p53 binding. When this sequence was deleted in CA-M, 1CA-M (1C), significantly reduced co-IP was noticed (Fig. 6C), supporting its role in p53 binding. This sequence, including the 24 aa stretch surrounding the NLS, is 100% conserved in a wide range of species including human, mouse, cat, dolphin, rabbit, horse, xenopus and chicken, consistent with a evolutionarily conserved role.

A short sequence (PCSSAPSV; aa 967–975) was identified in the ~90 kDa fragment of Mekk1 (Fig. 1B) that interacts with c-Abl upon induction in U937 cells (Kharbanda et al, 2000). Mekk1, besides mediating kinase activity through its kinase domain, ubiquitinates c-

Jun through a SWIM domain and ERK1/2 through a PHD domain (Lu et al, 2002) using its E3 ubiquitin ligase activity.

Numerous proteins have been identified as p53 interacting proteins, including viral proteins such as HPV E6, SV40-Tag; transcription factors such as TBP, TFIID; protein kinases such as CK2, HIPK2, JNK1; histone modifiers such as HDAC1, p300/CBP; redox sensitive proteins such as HIF-1 α , Ref-1; replication repair proteins such as TFIID-XPB, RP-A; ubiquitination/deubiquitination proteins such as Mdm2, HAUSP; and other activator proteins such as 14-3-3 α and HMG-1 (reviewed in Fernandez and Sot, 2010). Much less is known about binding sites in these interacting proteins and only in a few are the sequences mapped. Perhaps the most extensively studied one is Mdm2, an E3 ubiquitin ligase that regulates p53 cellular levels through degradation, mediated by an evolutionarily conserved C-terminal RING finger domain, by transferring mono-ubiquitin to p53 C-terminal lysine residues (Fang et al, 2000). Mdm2 also inhibits transcriptional activity of p53 through its N-terminal transactivation domain (TAD). The crystal structure of the p53-Mdm2 complex localized aa residues 25–102 of Mdm2 consisting of two structurally similar portions interacting with the p53 TAD (Chen et al, 2000; Kussie et al, 1996). Within this region, N-terminal or internal deletions reduced p53 binding significantly. However, the first 150 amino acids of Mdm2 synthesized in vitro as a GST fusion protein did not bind p53 in vitro (Chen et al, 2000). The authors suggested that sequences in another region also contribute structural elements required for functional p53 binding. It is also possible that the conformational requirement was not achieved in this in vitro system. Similarly, as relatively stronger binding was observed with 15F than CF, other sequences besides this binding site in Mekk1 could contribute to or help regulate the stability of the p53-Mekk1 interaction. In the proposed binding model between the transcriptional coactivator p300 and p53, four domains of p300 (Taz1, Kiz, Taz2, IBiD) wrap around four TADs of tetrameric p53 (Teufel et al, 2007). In other binding mechanisms, the C-terminal DNA binding domain of positive cofactor 4 (PC4), a transcriptional coactivator (Rajagopalan et al, 2009); the C-terminal of BRCA2 (Rajagopalan et al, 2010); and a region spanning aa 179–190 of Rad51 (Friedler et al, 2005) were also shown to interact with p53.

Mekk1-mediated stabilization of p53

Recently, a study showed that Mekk1 regulates p53 levels by JNK-dependent phosphorylation (Fuchs et al, 1998). On the other hand, γ -irradiation, a poor activator of JNK, is thought to increase p53 levels through a JNK independent pathway (Maki and Howley, 1997). Forced expression of Mekk1, CA-M and KI-M (kinase inactive-Mekk1) in HEK293T cells increased p53 levels (Islam et al, 2010, Fig. S1A) nearly two-fold. The increase was slightly greater with kinase active vs kinase inactive Mekk1 suggesting both JNK-dependent and independent pathways may be involved.

p53 is under complex, tight regulation. In normal unstressed cells, the p53 protein is unstable and is maintained at a low level owing to its continuous degradation primarily mediated by Mdm2 and its co-operative protein Mdx (Mdm4) via the ubiquitin-proteasome pathway. However, many cellular stresses lead to its stabilization via a block in its degradation. Thus, disruption of the p53-Mdm2 complex by any means can be the pivotal

event prior to accumulation of p53 in the cell. The minimal Mdm2 binding site on the p53 protein has been mapped within aa residues 18–26 in the TAD (Shimizu et al, 2002; Yu et al, 2006). Checkpoint kinases (hCHK1, hCHK2) weaken p53-Mdm2 interaction through phosphorylation at several N-terminal serine residues (Chelab et al, 2000). Of the three potential residues (Ser15, Ser20 and Thr18) in the TAD binding domain, Thr18 phosphorylation weakens Mdm-2 binding in response to DNA damage (Lai et al, 2002). Ionizing radiation stabilizes p53 through a phosphorylation cascade which requires phosphorylation of Ser15 first, followed by Thr18 (Sakaguchi et al, 2000, reviewed in Fernandez and Sot, 2010).

Thus, phosphorylation of Ser15 of endogenous p53 in HEK293T cells transfected with Mekk1, CA-M and KI-M was evaluated. Only a slight increase in phosphorylation of Ser15 in Mekk1 and CA-Mekk1 cells compared to KI-Mekk1 (Fig. S2A) was correlated with the p53 increase; the ratio of phospho-p53 over p53 protein essentially remained the same. Cytosolic and nuclear fractionation did not show much difference either, supporting the idea that the p53 increase is not phosphorylation dependent.

It is also possible that physical interaction between Mekk1-p53 might interfere with Mdm2-p53 and degradation by the ubiquitin pathway. All Mekk1 variants including KI-M protected p53 even in the presence of Mdm2, in contrast to significant degradation of p53 in Mdm2 expressing cells (Fig. 7A). Significantly reduced ubiquitination of p53 was seen in Mekk1 and CA-M transfected cells, either in immunoblots of cell lysates (Fig. 7C) or with immunoprecipitated p53 (Fig. 7D). These findings led us to speculate that p53 interacts with Mekk1 through its N-terminal domain, at or near the Mdm2 binding site. Since Mekk1 acts as a co-repressor of the p53 target gene PKD1, binding to the p53 N-terminal TAD to modulate its transcriptional activity seems logical. This is similar to the adenovirus E1B 55 kDa protein binding to the N-terminal region of p53 and inhibiting its transactivation (Yew and Berk, 1992).

Increased stability of p53 protein, reduced Mdm2-mediated degradation and reduced ubiquitination by forced expressed CA-M were also observed by Fuchs et al (1998). However, they did not observe any similar effect on p53 by kinase inactive Mekk1. The reason for these important differences is not clear, but could be due to difference in cell types and the kinase-inactive constructs used.

Mdm2 protein itself is also the focus of attention and is now also recognized as a principal target of signals that lead to p53 stabilization, prolonging p53 half-life from minutes to hours. Mdm2 undergoes multisite phosphorylation within the p53-binding domain and the central acidic domain required for degradation of p53 (Maya et al, 2001). A wide variety of kinases have been implicated in regulating Mdm2 phosphorylation and function including ATM, AKT, p38 MAPK, DNA-PK (DNA dependent protein kinase), CDK1 and CDK2 (cyclin A-dependent kinases) (Araki et al, 1999; Mayo et al, 1997; Zhang and Prives, 2001; Zhu et al, 2002). Deregulated oncogenes, such as Ras mutants, c-myc, or viral E1A stabilize and activate p53 by yet other mechanisms by interfering with Mdm2 E3 ubiquitin ligase activity (Honda and Yasuda, 1999). This study suggests yet another mechanism by virtue of

reducing ubiquitination, most likely by interfering with Mdm2 binding, by which Mekk1 stabilizes p53.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PKD	polycystic kidney disease
Mekk1	MAP/ERK kinase kinase 1
MAP3K	MAPkinase kinase kinase
CA-M	constitutively active Mekk1
KI-M	kinase-inactive Mekk1
CF	CA-M linked to GFP at the N-terminus; -F, truncated CA-M linked to GFP
CA-MG	CA-M linked to GST at the C-terminus
p53G	p53 linked to GST at the C-terminus.

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Figure 1.

(A) Nuclear localization of Mekk1 is not p53 dependent. HCT116 p53^{-/-} cells transfected with constitutively active-Mekk1, CA-M (+, upper), full length Mekk1 (+, lower) or empty vector (-) were fractionated into cytosolic (CE) and nuclear (NE) fractions. Following SDS-PAGE, the blot was probed with anti-Mekk1 antibodies. CA-M (36 kDa), full length Mekk1 (196 kDa) and its major fragment (90 kDa) and an unknown nuclear protein (*) are shown. The membranes, after stripping, were reprobed with anti-tubulin as a cytosolic marker. (B) Schematic representation of Mekk1 and constitutively active Mekk1 (CA-M). Also shown their deleted (white bars) variants in nuclear localization signal (Mekk1 and CA-M) or p53 binding site (1CA-M and 2CA-M). The kinase domain (KD) is located in the C-terminal region, and seven cysteines and one histidine containing the pleckstrin homology domain (PHD, 433–481) in the large noncatalytic or regulatory region. The start of the major 90 kDa proteolytic fragment is aa855.

Figure 2.

Nuclear localization signal in constitutively active Mekk1 (CA-M). (A) CA-M-GFP fusion protein (CF) and approximate primer positions shown with arrows for PCR cloning of various truncated constructs: forward primers 5F, 13F, 15F, 16F and 17F, and reverse primer GFP2. (B) Amino acid sequence of murine Mekk1 between amino acids 1342 and 1372, and the start sites for the 15F (I-1347) and 13F (D-1370) fusion proteins are shown by the arrows. The underlined amino acid residues were deleted in the respective deletion constructs shown by ΔF , $\Delta 1F$ or $\Delta 2F$. (C) HEK293T cells transfected with various GFP fusion constructs indicated in (A) were fractionated into cytosolic (CE) and nuclear (NE) fractions. Following SDS-PAGE, the membrane was probed with anti-Mekk1 antibodies and reprobbed with anti-tubulin antibodies as a cytosolic marker. (D) HEK293T cells transfected with CF, 15F, 13F and ΔF (HRDVK sequence deletion construct) were fractionated into

cytosolic (CE) and nuclear (NE) fractions. Following SDS-PAGE, the membrane was probed with anti-Mekk1 and reprobed with anti-tubulin antibodies.

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Figure 3.

Nuclear localization signal in Mekk1. The sequence HRDVK was deleted in full length or constitutively active Mekk1 (CA-M) to create Mekk1 (M) or CA-M (C), respectively. HEK293T (A) or COS-1 (B) cells transfected with the Mekk1 or CA-M constructs as well as their respective wild-type constructs were fractionated into cytosolic (CE) and nuclear (NE) fractions. Following SDS-PAGE, the membranes were probed with anti-Mekk1 and reprobbed with anti-tubulin as a cytosolic marker. (C) HEK293T cells seeded in chamber slides were transfected with GFP, CF (CA-M-GFP) or its HRDVK sequence

deleted variant (F), CA-M, Mekk1 or their HRDVK sequence deleted (CA-M and Mekk1) variants. The cells were washed, fixed with 4% paraformaldehyde and washed. For constructs without GFP, the fixed cells were blocked with 1% BSA prior to incubation with rabbit anti-Mekk1, followed by FITC conjugated anti-rabbit antibodies. DAPI was used for nuclear stain. All cells were observed under a fluorescence microscope. scale bars equal 1 μ m.

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Figure 4.

CA-M represses the 0.2 kb PKD1 promoter, but not the NLS deleted CA-M. (A) The cells transfected with 0.3 μ g of the 0.2 kb PKD1 promoter in pGL3 vector together with 0.2 μ g of control vector (V), CA-M (C), or CA-M (Δ C) were subjected to luciferase reporter assay at 40 h following transfection. The normalized RLU value obtained in cells co-transfected with the promoter and control vector was set at 1.0. Each bar is the mean $\pm$ SD of a representative experiment (* $p < 0.01$). (B) shows the expression level of CA-M and CA-M (Δ C) in the transfected HEK293T cell lysates analyzed by immunoblotting using anti-Mekk1 antibodies. Actin is used as a loading control.

Figure 5. CA-M and p53 interact *in vitro*. (A) Both p53 and CA-M were purified as GST fusion proteins from *E. coli* cells: p53-GST (p53G), CA-M-GST (CA-MG). (B) GST or p53G was bound to the well indicated at the bottom, and then purified CA-MG was allowed to interact with well-bound GST or p53G at room temperature. The interacting CA-MG was detected using anti-Mekk1 antibodies and secondary antibodies conjugated with alkaline phosphatase. Quantification was performed using p-nitrophenylphosphate as a substrate and the product, p-nitrophenolate ion formed was measured at 405 nm. The measured

absorbance value for GST incubated with anti-Mekk1 was set to 1.0. Each bar is the mean of a representative experiment (**, $p < 0.005$) of a triplicate. Inset, immunoblot of the samples extracted from the last three wells with SDS buffer.

Figure 6. p53 binding site in CA-M. HEK293T (A) or COS-1 (B) cells transfected with either CA-M-GFP fusion protein (CF), or its deleted derivatives (Δ 1F or Δ 2F) were lysed (see Methods) and subjected to immunoprecipitation (IP) using anti-p53 or anti-Mek1 antibodies. The IPs and inputs (1:20 of total lysates) were analyzed by immunoblots (IB) using either anti-p53 or anti-Mek1 antibodies and alkaline phosphatase conjugated clean-blot IP detection reagent was used. (C) Inputs (1:20 of total lysates) and IPs from CA-M (C) and Δ 1CA-M

(1C) transfected HEK293T cells were analyzed by immunoblots using anti-Mekk1 or anti-p53 antibodies.

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Figure 7. Mek1 mediated p53 stability persists in the presence of overexpressed Mdm2. (A) Endogenous p53 levels in vector (V) or Mdm2 transfected HEK293T cells. Transfection of Mdm2 results in decreased p53. (B) HEK293T cells transfected with control vector (V), CA-M, Mek1, or KI-M together with Mdm2 were analyzed by immunoblotting using anti-p53. The membrane was re probed with anti-Mdm2 antibodies, and anti-Actin used as a loading control. Increased level of p53 persisted in cells transfected with Mek1 constructs despite the presence of ectopic Mdm2. (C) CA-M and Mek1 reduced total ubiquitination. Control vector (V), CA-M or Mek1 transfected HEK293T cells were immunoblotted using anti-Ub antibodies. Actin was used as a loading control. (D) Ubiquitination levels of p53 in cells transfected with Mek1 constructs. HEK293T cells transfected with control vector (V), CA-M, Mek1, KI-M or Mdm2 were lysed and immunoprecipitated with anti-p53 and immunoblotted with anti-Ub antibodies.

Table 1

Sequences of the primers used and their purposes.

Primers	Construct	Sequence	Purpose
GFP1	CF	5'-caagcttc <u>gaattc</u> tgccatggcgatgtcag-3'	Forward primer containing an EcoRI site (bold, underlined) for cloning of CA-M into EGFP-N3
GFP2		5'-ggaggcgat <u>ggaacca</u> aattaagtaccacgt g-3'	Reverse primer containing a Bam HI site (bold, underlined) for all PCR cloning into EGFP-N3 in frame with GFP.
MK5	5F	5'-gacaatt <u>gaattc</u> atggcgctg -3'	Forward primers that harbor an engineered EcoRI site (bold, underlined) for PCR cloning at EcoRI–Bam HI site of EGFP-N3. In some an engineered in-frame start codon, atg (underlined).
MK17	17F	5'-gcaactacaac <u>gaattc</u> catt gag-3'	
MK16	16F	5'-aatacgga <u>gaattc</u> atggagtcagtcg-3'	
MK13	13F	5'-gaggctgag <u>gaattc</u> caatgtttgga gctgctgcc-3'	
MK15	15F	5'-ccacgag <u>gaattc</u> agatgattcacag-3'	
MKd1	F	5'-cacgagaaccagatcatt/gccaacctgctcattgac-3'	Deletion of NLS site in CA-M / indicates the deletion site.
MKd2		5'-gacaatgagcaggttggc/aatgatctggttcctg-3'	
MKdel21	1F	5'-gatcattcacagagacgtc/agcaccggtcagaggctgag-3'	Deletion of binding site in CA-M / indicates the deletion site.
MKdel22		5'-ctcagcctctgaccgtgct/gacgtctctgtgaatgac-3'	
MKdel31	2F	5'-ctgctcattgacagacc/ttggagctgctccagg-3'	Deletion of binding site in CA-M / indicates the deletion site.
MKdel32		5'-cctggcagcagctccaaa/ggtgctgtcaatgagcag-3'	