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Mechanisms Regulating the Nuclear Translocation of p38 MAP Kinase

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ABSTRACT

p38 mitogen-activated protein kinase (MAPK) is of fundamental importance in a cell's response to environmental stresses, cytokines and DNA damage. p38 resides in the cytoplasm of resting cells, and translocates into the nucleus upon activation, yet the exact mechanisms remain largely unclear. We show here that the phosphorylation-dependent nuclear translocation of p38 is a common phenomenon when cells are stimulated with various stresses. On the other hand, the nuclear export of p38 requires its dephosphorylation, and it is exported both in a MK2-dependent and a nuclear export signal (NES)-independent manner. Although different p38-regulated/activated protein kinase (PRAK) mutants all dictate the intracellular localization of p38, results from a *PRAK*-deficient cell line indicate that it plays no role in this process. Microtubule depolymerizing reagent nocodazole and dynein inhibitor EHNA both block the nuclear translocation of p38, demonstrating roles for microtubules and dynein in p38 transport. Taken together, stress-induced nuclear accumulation of p38 is a phosphorylation-dependent, microtubule- and dynein-associated process. J. Cell. Biochem. 110: 1420–1429, 2010.

KEY WORDS: P38 MAPK; STRESS; NUCLEAR TRANSLOCATION; NUCLEAR EXPORT

As a highly conserved subfamily of mitogen-activated protein kinases (MAPKs), p38 is an important mediator of the cellular responses to a wide variety of environmental stresses, such as ultraviolet (UV) radiation, oxidative stresses, heat or osmotic shock, bacterial lipopolysaccharide (LPS), and proinflammatory cytokines. p38 plays a critical role in many biological processes including cell growth, proliferation, differentiation, senescence, death, wound healing, inflammatory responses, and energy metabolism [Han et al., 1997; Bulavin et al., 2001; Kyriakis and Avruch, 2001]. p38 mediates stress signaling via simultaneous phosphorylation of the Thr180 and Tyr182 in the conserved TGY motif by its upstream MAP kinase kinase 3/6 (MKK3/6). Four distinct p38 subfamily members have been identified in mammalian cells so far, p38(α), p38β, p38γ, and p38δ [Kyriakis and Avruch, 2001]. p38 exerts its multiple functions mainly through various downstream target molecules including kinases such as MAPK-activated protein kinase 2/3 (MK2/3), MAPK-interacting kinase 1/2 (MNK1/2), p38-regulated/activated protein kinase (PRAK, also known as MK5), and mitogen- and stress-activated protein kinase 1/2 (MSK1/2). Nuclear transcription factors, such as activating transcription factor-2 (ATF-2), CHOP/GADD153, myocyte enhancer factor 2 (MEF2), Elk-1, and p53, are also important targets of p38 in gene expression regulation [Han et al., 1997; Bulavin et al., 2001; Kyriakis and Avruch, 2001].

Endogenous p38 is distributed both in the cytosol and nucleus in resting cells, and cytosolic p38 translocates into the nucleus upon activation to access its nuclear substrates. With the disappearance of the stimulation, inactivated p38 is exported to the cytosol to receive the next stimulation [Ferrigno et al., 1998; Kyriakis and Avruch, 2001; Brand et al., 2002; Lu et al., 2006; Zeidan et al., 2008; Wood et al., 2009]. Our previous study showed LPS stimulation promptly induced the nuclear translocation of p38 to mediate the up-regulation of tumor necrosis factor α (TNF-α) expression in monocytes, highlighting the importance of the location of p38 on its biological function [Jiang et al., 1999]. Thus, stimuli-induced intracellular redistribution is an important aspect for p38 to fulfill

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its cellular functions, through targeting to different substrates in different cell compartments.

Although nuclear translocation upon activation is observed in all MAPK family members, far less is known about the mechanism of this activation-dependent alteration in p38 localization, as compared with extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), two other MAPK subfamilies [Dickens et al., 1997; Khokhlatchev et al., 1998; Lenormand et al., 1998; Adachi et al., 1999; Blanco-Aparicio et al., 1999; Aplin et al., 2001; Matsubayashi et al., 2001; Aplin et al., 2002; Whitehurst et al., 2002; Charruyer et al., 2005; Chuderland et al., 2008]. The nuclear translocation of p38 depends on its phosphorylation but not its catalytic activity [Wood et al., 2009]. In budding yeast, a deletion of the importin-β homolog, NMD5, impaired nuclear accumulation of Hog1 (high osmolarity glycerol response), the p38 homolog, suggesting the nuclear translocation of p38 in yeast is a nuclear localization signal (NLS)-dependent active process [Ferrigno et al., 1998]. Since no typical NLS could be identified in all MAPKs, and no direct interactions have been detected between MAPKs and purified importins [Khokhlatchev et al., 1998; Adachi et al., 1999; Whitehurst et al., 2002; Charruyer et al., 2005; Chuderland et al., 2008], these enzymes may undergo nuclear translocation either through interaction with other proteins with a NLS as a carrier or by a specific sequence, which is designated as a nuclear translocation signal (NTS) in ERK. However, no such sequence has been identified in p38 yet.

There were several reports regarding the relationships of intracellular localizations between p38 and its phosphatases and downstream substrates, among them the NLS- and nuclear export signal (NES)-containing proteins MK2 and PRAK being the most extensively studied [Ben-levy et al., 1998; Seternes et al., 2002; New et al., 2003; White et al., 2007; Li et al., 2008; Gorog et al., 2009]. MK2 was regarded to be important in the nuclear export of p38, and the location of PRAK could be controlled by its docking interaction with p38. Other proteins have been reported to be involved in the determination of the intracellular localization of p38 including TAB-1 (TAK-1-binding protein) and MAPK phosphatases (MKPs) [Pratt et al., 2003; Lu et al., 2006]. However, the intracellular localization of p38 was suggested to be determined by different proteins in different studies, probably owing to the overexpressed mutants employed. Hence, although the intracellular localization of p38 may be a final result of interactions among multiple factors, it is still of necessity to determine the key regulator(s).

In the present study, we demonstrate that various stress stimuli can induce the phosphorylation-dependent nuclear translocation of p38. Although different PRAK and MK2 mutants all dictate the intracellular localization of p38, endogenous PRAK appears to play no role in this process, as indicated using a *PRAK*-deficient cell line. Moreover, the nuclear translocation of p38 is a microtubule- and dynein-dependent process. The nuclear export of p38 requires its dephosphorylation, and it is exported in a MK2-dependent and a NES-independent manner.

MATERIALS AND METHODS

CONSTRUCTS AND ANTIBODIES

p38(WT), PRAK(WT), MK2(WT) were subcloned into pEGFP-C2 vector by PCR. EGFP-tagged p38(AF), p38(KM), PRAK(182A), PRAK(182D), PRAK(KM), PRAK(NLSm), PRAK(NESm), MK2(320A), MK2(320E), MK2(208A), MK2(208E), MK2(ΔNLS), and MK2(ΔNES) [Engel et al., 1998; New et al., 2003] were obtained by mutagenesis using MutanBEST mutagenesis kit (TaKaRa, Shiga, Japan). The primers used were synthesized by Invitrogen (Shanghai, China) and listed in Table I. All the constructs were finally confirmed by sequencing (Invitrogen, China). The antibodies used were as follows: anti-p38 antibody (Cell Signaling Technology, Danvers, MA, #9212), anti-p-p38 (Thr180/Tyr182) antibody (Cell Signaling Technology, #9211), anti-p-ATF-2 (Thr71) antibody (Cell Signaling Technology, #9221), anti-PRAK antibody (Stratagene, Cedar Creek, TX), anti-Lamin A/C antibody (Santa Cruz, Santa Cruz, CA), and anti-β-actin antibody (Cell Signaling Technology).

CELL CULTURE AND TRANSFECTION

Wild-type mouse embryonic fibroblasts (MEFs, C57B1/6 background), $PRAK^{+/+}$ and $PRAK^{-/-}$ MEFs, as well as NIH3T3 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL/Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Hyclone, Logan, UT) in the cell incubator (37°C, 5% CO_2). For transfection, 5×10^5 NIH3T3 cells were seeded into

TABLE I. Primer List for Constructs

Construct name	Forward primers (5′–3′)	Reverse primers (5′–3′)
EGFP-p38(WT)	tcaaagcttatgtcgcaggagaggcccacgt	taggatcctcaggactccatttcttcttggtca
EGFP-p38(AF)	gcaggcttcgtggctaccaggtggtacc	catctcatcatcagtgtgccgag
EGFP-p38(KM)	ttatgaagctgtcgagaccgt	ctgccacacgatgccccgtctt
EGFP-PRAK(WT)	tcaaagctttcggaggagagcgacatggac	taggatccttattgggattcgtgggacgtg
EGFP-PRAK(182A)	atggcaccccagttcacccctttattatg	caagtcaccttggtcaatcttg
EGFP-PRAK(182D)	atggacccccagttcacccctttattatg	caagtcaccttggtcaatcttg
EGFP-PRAK(KM)	atgattcttcttgatcgtccaaaagc	cagcgcaaaccgttcttgag
EGFP-PRAK(NLSm)	ttacttggcaccaagccaaaggacag	cagaatggggttgttcactgagt
EGFP-PRAK(NESm)	cactcagtgaacaaccccattctgcg	atcctggattctcatgttggcca
EGFP-MK2(WT)	tcaaagcttatgctgtcgggctctccgggcca	taggatccttagtgggcgagagccgcatcctcc
EGFP-MK2(320A)	gcaccactgcacaccagccgtgtcctgaa	ctgagggaccttcgtagattgcatga
EGFP-MK2(320E)	gaaccactgcacaccagccgtgtcctgaa	ctgagggaccttcgtagattgcatga
EGFP-MK2(208A)	gctccgtgttatacaccatacta	ggtcaaagagttgtgactggtgg
EGFP-MK2(208E)	gaaccgtgttatacaccatacta	ggtcaaagagttgtgactggtgg
EGFP-MK2(ΔNLS)	gctcgtgctgtggaggatgcggctct	tatcttgatctgctcatagtcaacac
EGFP-MK2(ΔNES)	cagatcaagataaagaagatagaaga	ctccttcaggacacggctggtgtgcagt

60 mm dishes. Twenty-four hours later, 3 μg DNA was mixed with 150 μl Opti-MEM (Invitrogen, CA), followed by further mixing with 15 μl Polyfect (Qiagen, Hilden, Germany). The mixture was incubated at room temperature for 15 min before adding to the cells. After 24 h incubation in the cell incubator, the cells were treated and observed using a DM-RA2 fluorescence microscope (Leica, Wetzlar, Germany).

REAGENTS

SB203580, PD98059, SP600125, paclitaxel, and cytochalasin D were purchased from Sigma-Aldrich (St. Louis, MO). Nocodazole, phalloidin, wortmannin, and leptomycin B were products of Calbiochem/Merck (San Diego, CA). For pretreatment, these reagents were added into the medium 30 min before stimulation, unless described specifically.

NUCLEAR AND CYTOPLASMIC EXTRACTION

Nuclear and cytoplasmic extraction was performed with NE-PER nuclear and cytoplasmic extraction reagents (Pierce/Thermo Scientific, Rockford, IL), following the guide of the manufacturer's protocol. MEFs (2×10^6) were used in the nuclear and cytoplasmic extraction, and about 100 and 200 μl of nuclear and cytoplasmic extracts were obtained, respectively. Twenty microliters of each sample was subjected to 12% SDS–PAGE. Lamin A/C was detected as positive control of the nuclear and cytoplasmic extracts.

IMMUNOBLOTTING

Cells were plated in 60 mm dishes and cultured to 80–90% confluence. The cells were washed with ice-cold PBS once, then lysed with 150 μl cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin) on ice for 20 min. Whole cell lysates were collected and centrifuged at 4°C, 12,000 rpm for 10 min, and 20 μg total proteins of the resultant supernatants were subjected to 12% SDS–PAGE. Western blot analyses were performed with primary antibodies and the corresponding horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Cell Signaling Technology), using a chemiluminescence detection system (Cell Signaling Technology) with the guide of the manufacturer's protocol. Images were obtained with Kodak IS2000R image station (Eastman Kodak Company, Rochester, NY).

IMMUNOCYTOCHEMISTRY

MEFs or NIH3T3 cells were washed with Tris-buffered saline (TBS) and fixed in 4% paraformaldehyde for 10 min. Cells were washed once with TBS, and then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After washing three times, the cells were quenched in 0.1% $NaBH_4$ in TBS for 5 min. The cells were blocked by incubation for 1 h in TBS containing 3% BSA, and then incubated with p38 or p-p38 specific antibodies at 4°C overnight. Cells were washed three times with TBS and incubated with Alexa 488 or Alexa 594-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR) for 45 min at room temperature, and then stained with 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) to visualize nuclei. Images were obtained with the A4, L5, or N3 filters of DM-RA2 fluorescence microscope (Leica) equipped with a Leica DC100 digital camera.

RESULTS

DIFFERENT STRESSES-INDUCED NUCLEAR TRANSLOCATION OF P38

To explore the intracellular localization of p38 upon stress, MEFs were stimulated with arsenite, a classical cellular stress, and the intracellular localization of total p38 and phosphorylated p38 (p-p38) was observed. It was shown that in resting MEFs, p38 dispersed in the whole cell, however, after arsenite stimulation, most p38 displayed a nuclear localization. Arsenite-induced nuclear translocation of p38 was also observed in HeLa and SMMC-7721 cells (data not shown). For p-p38, the distribution and intensity of the fluorescence showed not only the nuclear localization of phosphorylated p38 upon arsenite stimulation, but also a significant activation of p38 (Fig. 1A). The analyses of nuclear and cytoplasmic extracts confirmed the results from immunostaining (Fig. 1B). With the stimulation of arsenite, p38 and its substrate, ATF-2, were both activated (Fig. 1C). Since p-p38 represents the activated p38 and it also confirms arsenite-induced nuclear translocation, we used the p-p38 antibody in the following studies on the mechanisms of its nuclear translocation.

We further wanted to know the effects of other types of stresses on the intracellular localization of p38. As shown in Figure 1D, UV radiation, anisomycin, LPS, hydrogen peroxide (H_2O_2), and sorbitol, all induced significant nuclear translocation of p38, suggesting stress-induced nuclear translocation of p38 is a common phenomenon.

The time course of arsenite-induced nuclear translocation of p38 was also studied. As shown in Figure 1E, transient arsenite treatment induced a rapid nuclear translocation of p38 within 15–30 min. The nuclear translocation peaked at 60 min, then decreased gradually, coming back to the resting level at 240 min. Since this time course is similar to the process of p38 activation (Fig. 1F), stress-induced nuclear translocation of p38 may be a phosphorylation-related process.

THE NUCLEAR TRANSLOCATION OF P38 IS A PHOSPHORYLATION-DEPENDENT PROCESS

To confirm the effect of the phosphorylation of p38 on its nuclear translocation, EGFP-tagged dominant negative mutant (TGY dual phosphorylation sites were changed to AGF) of p38, p38(AF), and kinase dead mutant (Lys53 in ATP binding site was changed to Met), p38(KM), were constructed. We chose NIH3T3 cells for transfection because of the low transfection efficiency of MEFs. As shown in Figure 2A, p38(AF), but not p38(KM), failed to translocate into the nucleus in response to arsenite treatment, suggesting the phosphorylation of p38, but not its kinase activity, plays a key role in determining its intracellular localization.

We further studied the role of p38's kinase activity on its nuclear translocation, with the widely used p38 specific inhibitor, SB203580, which blocks the kinase activity of p38, but not its phosphorylation. As shown in Figure 2B, SB203580 pretreatment had no effect on arsenite-induced nuclear translocation of p38, nor did ERK pathway inhibitor PD98059 or JNK inhibitor SP600125.

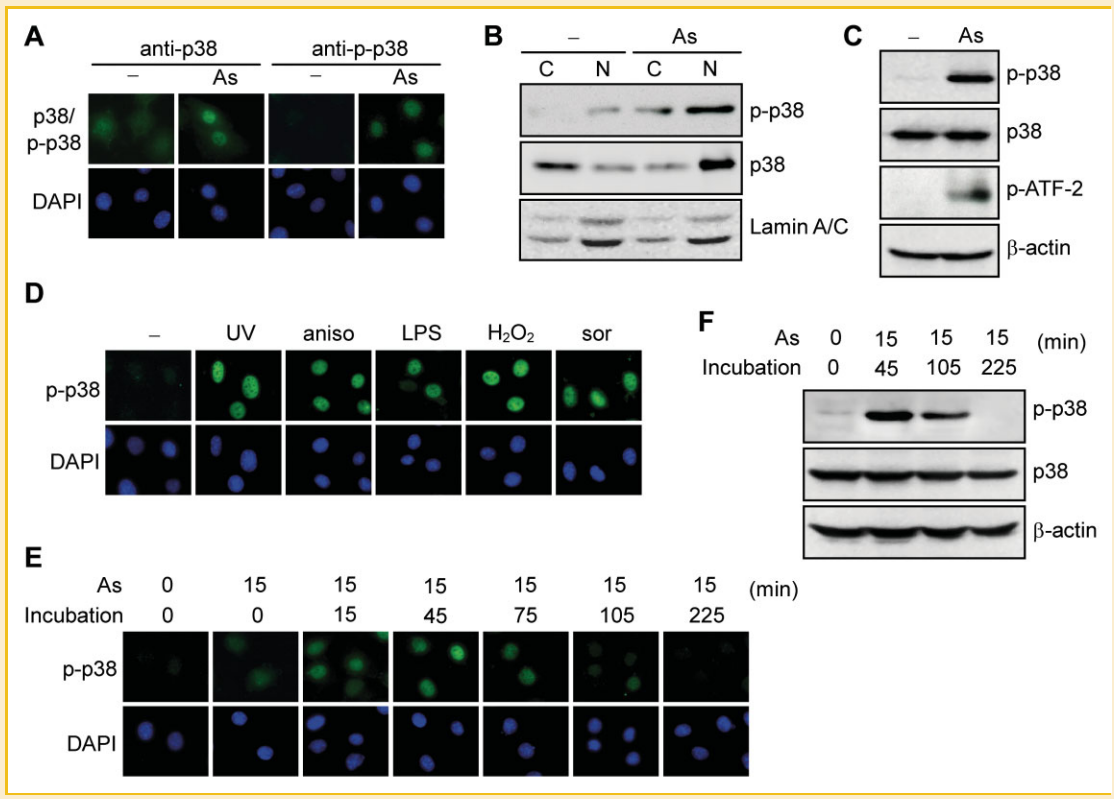

Fig. 1. Stress-induced nuclear translocation of p38. A: The nuclear translocation of total or phosphorylated p38 induced by arsenite. MEFs were treated with or without arsenite (As, 200 μM) for 1 h, and immunostaining was performed with p38 or p-p38 antibodies. B: Cell fractionation analyses of arsenite-induced nuclear translocation of p38. MEFs were treated with or without arsenite (As, 200 μM) for 1 h, followed by nuclear (N) and cytoplasmic (C) extraction. Western blot analyses were performed with p-p38, p38, and Lamin A/C antibodies. C: Arsenite-induced phosphorylation of p38 and ATF-2. MEFs were treated with or without arsenite (As, 200 μM) for 1 h, and Western blot analyses were performed with p-p38, p38, p-ATF-2, and β-actin antibodies. D: The nuclear translocation of phosphorylated p38 induced by different stresses. MEFs were treated with or without UV (30 J/m^2), anisomycin (aniso, 50 ng/ml), LPS (100 ng/ml), H_2O_2 (400 μM), or sorbitol (sor, 0.5 M) for 1 h, and immunostaining was performed with p-p38 antibody. E: The time course of nuclear translocation of p38 induced by transient arsenite treatment. MEFs were treated with or without arsenite (As, 200 μM) for 15 min, followed by incubation for the indicated time. Immunostaining was performed with p-p38 antibody. F: The phosphorylation of p38 induced by transient arsenite treatment. MEFs were treated with or without arsenite (As, 200 μM) for 15 min, followed by incubation of the indicated time, and Western blot analyses were performed with p-p38, p38, and β-actin antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

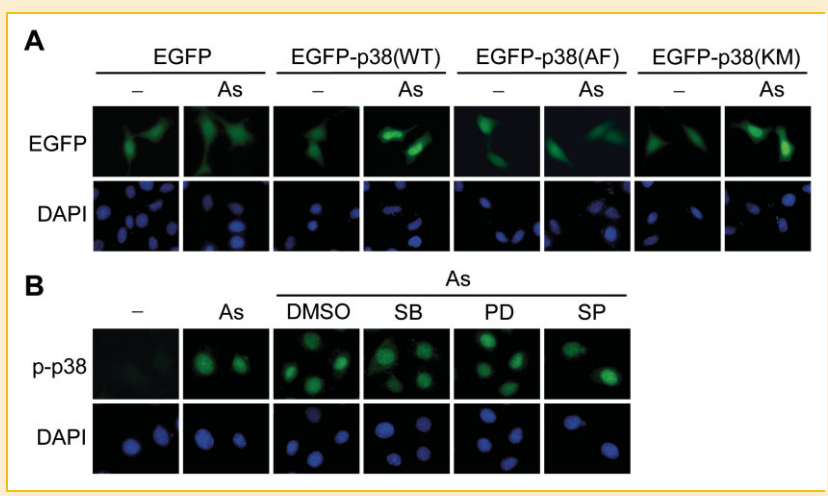

Fig. 2. The nuclear translocation of p38 is phosphorylation-dependent. A: The dominant negative mutant of p38, p38(AF), no longer undergoes nuclear translocation. NIH3T3 cells were transfected with EGFP vector, EGFP-p38(WT), EGFP-p38(AF), or EGFP-p38(KM). Twenty-four hours later, the cells were treated with or without arsenite (As, 200 μM) for 1 h. B, SB203580 pretreatment has no effect on the nuclear translocation of p38. MEFs were pretreated with or without SB203580 (SB, 20 μM), PD98059 (PD, 20 μM), SP600125 (SP, 20 μM), and stimulated with or without arsenite (As, 200 μM) for 1 h. Immunostaining was performed with p-p38 antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

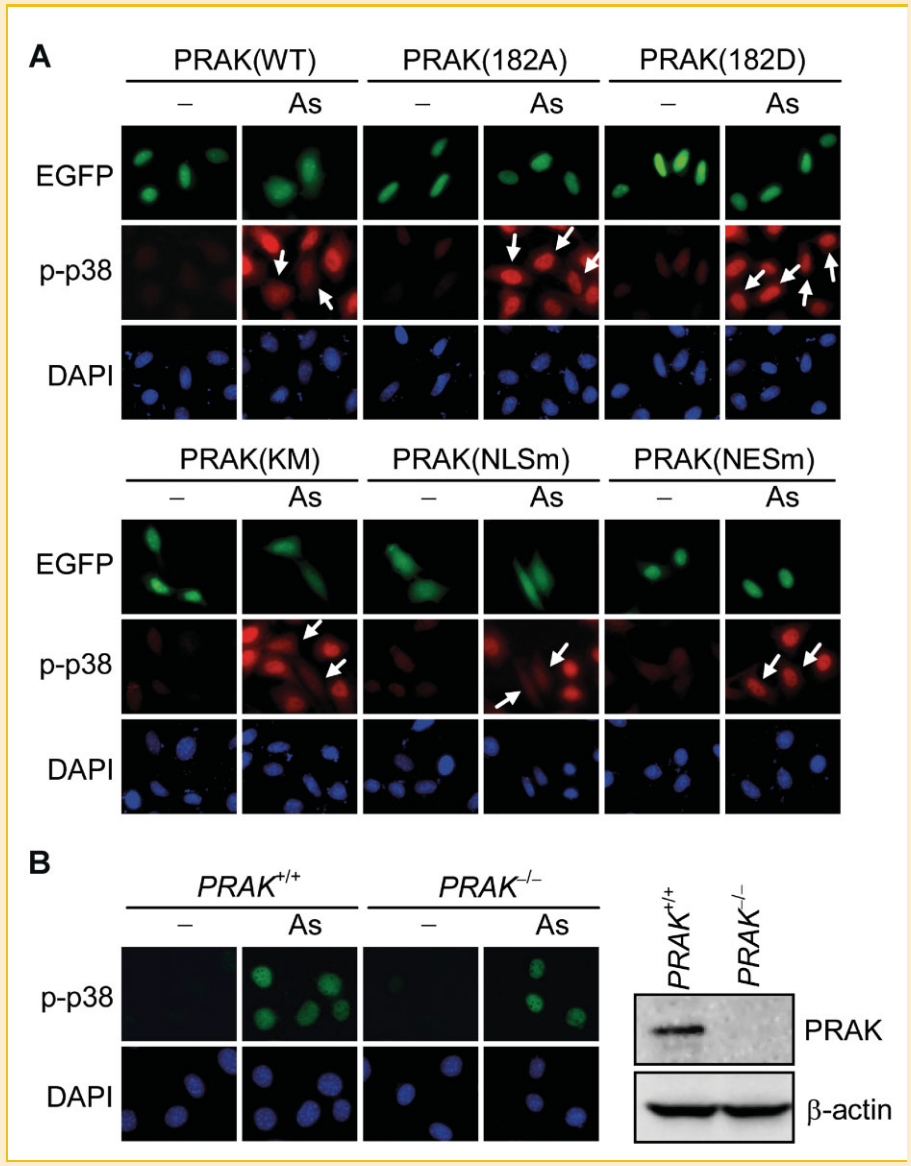

Fig. 3. Effects of PRAK on the nuclear translocation of p38. A: Effects of different PRAK mutants on the nuclear translocation of p38. NIH3T3 cells were transfected with EGFP-tagged PRAK(WT), PRAK(182A), PRAK(182D), PRAK(KM), PRAK(NLSm), or PRAK(NESm). Twenty-four hours later, the cells were stimulated with or without arsenite (As, 200 μM) for 1 h, then immunostaining was performed with p-p38 antibody. The white arrows indicate the cells expressing exogenous proteins. B: Effect of *PRAK* knockout on the nuclear translocation of p38. $PRAK^{+/+}$ or $PRAK^{-/-}$ cells were treated with or without arsenite (As, 200 μM) for 1 h, then immunostained with p-p38 antibody (left panel). The expression of PRAK in $PRAK^{+/+}$ or $PRAK^{-/-}$ cells was detected by immunoblotting with PRAK antibody (right panel). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

These results further confirmed the independence of the nuclear translocation of p38 to its kinase activity.

THE INTRACELLULAR LOCALIZATION OF P38 IS INDEPENDENT OF PRAK

Since there were reports about the role of p38 on the intracellular localization of its downstream kinase PRAK, we wanted to know if PRAK had the opposite effect and regulated the localization of p38. Different from p38, exogenous PRAK existed in the nucleus and translocated to the cytoplasm with arsenite stimulation, which was consistent with previous reports [Seternes et al., 2002; New et al., 2003]. Both the dominant negative mutant PRAK(182A) and the dominant positive mutant PRAK(182D) no longer underwent arsenite-induced nuclear export, while the kinase dead mutant PRAK(KM) maintained its stress-induced translocation. The NLS mutant PRAK(NLSm) localized in the whole cell, while the NES mutant PRAK(NESm) was located mainly in the nucleus, regardless of whether the cells were stimulated or not. Interestingly, the intracellular localization of p38 was strictly linked to the exogenously expressed wild-type or mutants of PRAK (Fig. 3A). However, the results obtained from *PRAK* knockout MEFs were totally different. In $PRAK^{-/-}$ cells, p38 still translocated into the nucleus with arsenite stimulation, showing no difference to that of $PRAK^{+/+}$ cells (Fig. 3B). This discrepancy may be because the results

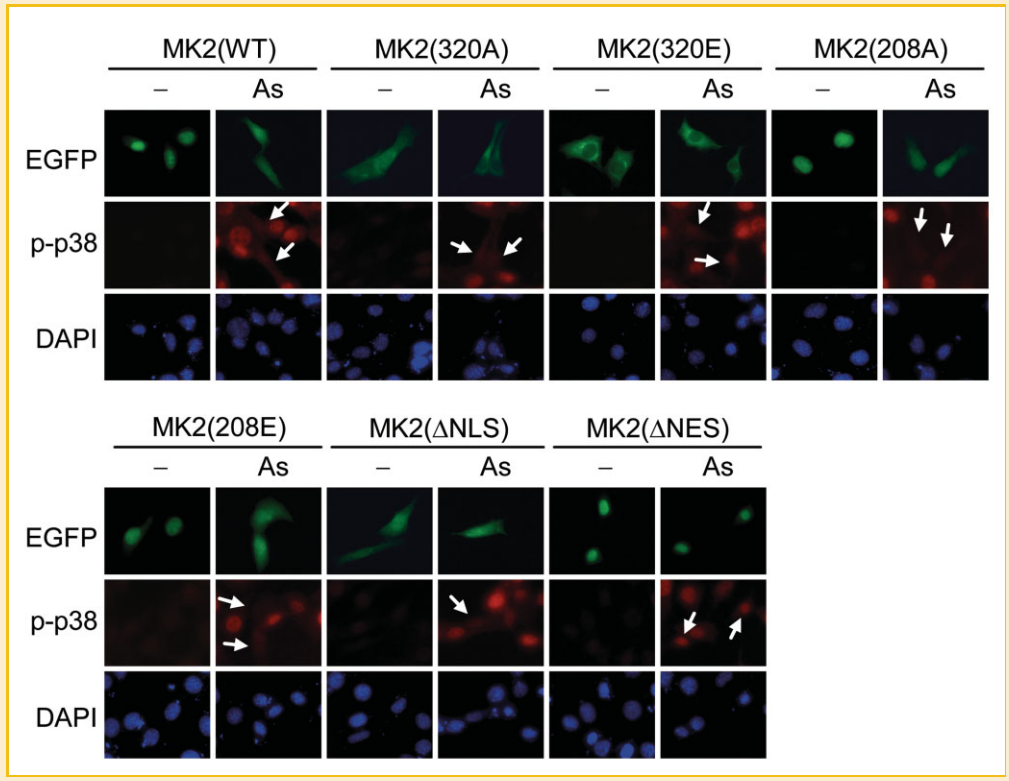

Fig. 4. Effects of MK2 on the nuclear translocation of p38. NIH3T3 cells were transfected with EGFP-tagged MK2(WT), MK2(320A), MK2(320E), MK2(208A), MK2(208E), MK2(ΔNLS), or MK2(ΔNES). Twenty-four hours later, the cells were stimulated with or without arsenite (As, 200 μM) for 1 h, then immunostaining was performed with p-p38 antibody. The white arrows indicate the cells expressing exogenous proteins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

from the exogenous, overexpressed mutants produce some artificial effects on p38 localization. Hence, under the physiological conditions, the intracellular localization of p38 should be independent of PRAK.

EFFECTS OF MK2 ON THE INTRACELLULAR LOCALIZATION OF P38

It has been confirmed that MK2, another important downstream kinase of p38, plays a key role in its nuclear export [Ben-levy et al., 1998; Gorog et al., 2009]. To elucidate if MK2 also effected stress-induced nuclear translocation of p38, the dominant negative mutants MK2(320A) or MK2(208A), the dominant positive mutants MK2(320E) or MK2(208E), as well as the mutants without NLS (MK2(ΔNLS)) or NES (MK2(ΔNES)) were expressed in NIH3T3 cells. As shown in Figure 4, wild-type MK2 was located in the nucleus and translocated to the cytosol with the stimulation of arsenite. For all the mutants, p38 exhibited strict co-localization with them, with or without arsenite stimulation. These results suggested that MK2 not only played a role in the nuclear export of p38, but also functioned as a p38 anchoring protein.

THE NUCLEAR TRANSLOCATION OF P38 IS MICROTUBULE- AND DYNEIN-DEPENDENT

To determine if the cytoskeleton is involved in the process of p38 nuclear translocation, the microtubule-depolymerizing reagent nocodazole, microtubule-stabilizing reagent paclitaxel, microfilament assembly-destroying reagent cytochalasin D and microfilament-stabilizing reagent phalloidin, were used for cell pretreatment. As shown in Figure 5A,B, only nocodazole blocked the nuclear translocation of p38. Moreover, nocodazole pretreatment blocked the phosphorylation of p38 and ATF-2 as well (Fig. 5C). These results demonstrated an important role for microtubules in both the activation and translocation of p38.

We further studied the effects of motor proteins on stress-induced nuclear translocation of p38. While myosin light chain kinase (MLCK) inhibitor wortmannin only showed a moderate blockade effect on the nuclear translocation of p38, dynein inhibitor EHNA pretreatment almost blocked this process entirely (Fig. 5A,B). Consistently, EHNA pretreatment also showed an inhibition effect on the phosphorylation of ATF-2 (Fig. 5C). These results suggested stress-induced nuclear translocation of p38 was an energy-consuming process, which mainly relied on the microtubule-based motor.

THE NUCLEAR EXPORT OF P38 DEPENDS ON ITS DEPHOSPHORYLATION

Since the nuclear translocation of p38 is related to its phosphorylation, we further investigated if the dephosphorylation of p38 was a prerequisite of the subsequent nuclear export. Different from transient stimulation with arsenite, p38 remained in the nucleus with extremely high activation after 4 h of continuous arsenite

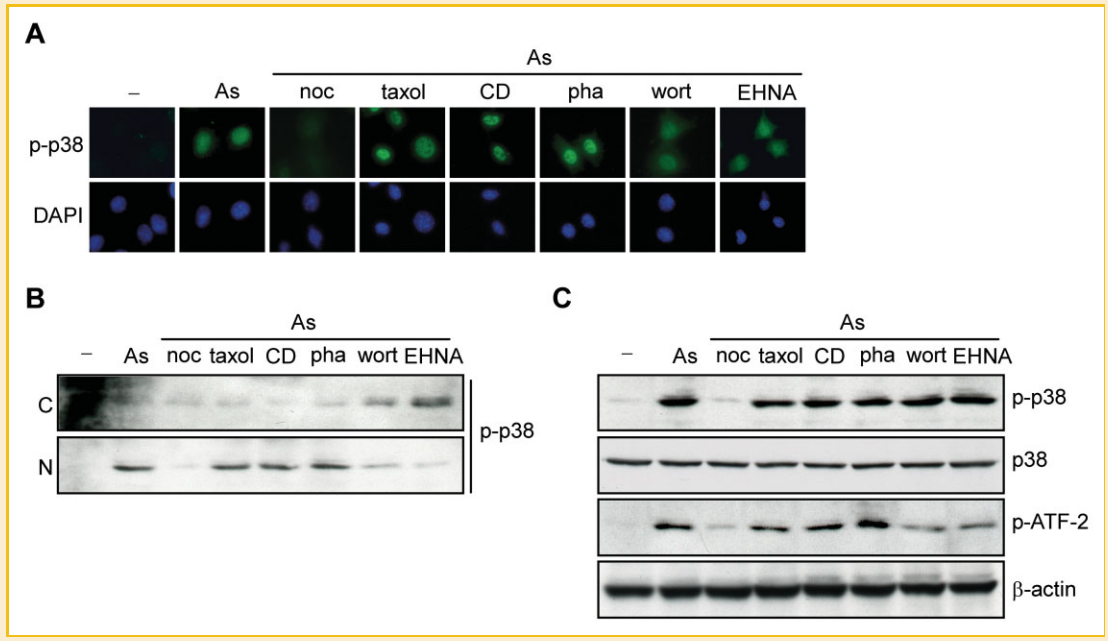

Fig. 5. Effects of the microtubule and dynein on the nuclear translocation of p38. A: MEFs were pretreated with or without nocodazole (noc, 20 μM), paclitaxel (taxol, 20 μM), cytochalasin D (CD, 200 nM), phalloidin (pha, 200 nM), wortmannin (wort, 200 nM), or EHNA (1 mM), and stimulated with or without arsenite (As, 200 μM) for 1 h, then immunostaining was performed with p-p38 antibody. B: MEFs were pretreated with or without chemicals described in A, and stimulated with or without arsenite (As, 200 μM) for 1 h, followed by nuclear (N) and cytoplasmic (C) extraction. Western blot analyses were performed with p-p38 antibody. C: MEFs were pretreated with or without chemicals described in A, and stimulated with or without arsenite (As, 200 μM) for 1 h, then Western blot analyses were performed with p-p38, p38, p-ATF-2, and β-actin antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

stimulation (Fig. 6A,B), demonstrating its nuclear export depends on its dephosphorylation.

Leptomycin B (LMB), a potent and specific nuclear export inhibitor, was employed to investigate if it had an effect on the nuclear export of p38. As shown in Figure 6C, LMB had no effect on the intracellular localization of p38 at all, suggesting p38 can be exported to the cytosol by a NES-independent manner. The same pretreatment with LMB blocked the

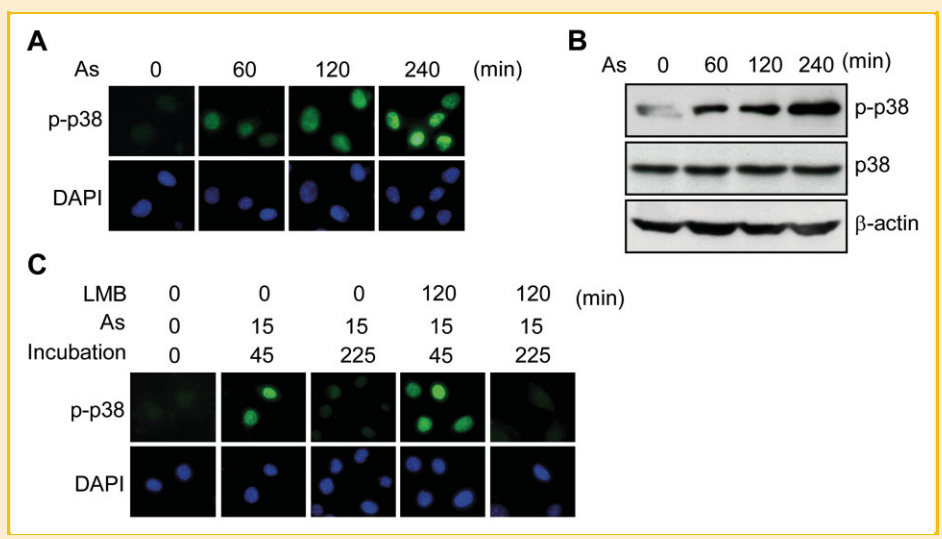

Fig. 6. The nuclear export of p38 is dephosphorylation-dependent. A: Time course of nuclear translocation of p38 induced by continuous arsenite treatment. MEFs were treated with or without arsenite (As, 200 μM) for the indicated time, and immunostaining was performed with p-p38 antibody. B: The phosphorylation of p38 induced by continuous arsenite treatment. MEFs were treated with arsenite (As, 200 μM) for indicated time, and Western blot analyses were performed with p-p38, p38, and β-actin antibodies. C: Effect of leptomycin B on the nuclear export of p38. MEFs were pretreated with or without leptomycin B (LMB, 200 nM) for 2 h, then treated with or without arsenite (As, 200 μM) for 15 min. After incubation for the indicated time, the cells were immunostained with p-p38 antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nuclear export of EGFP-tagged PRAK and MK2 (data not shown).

DISCUSSION

Compartmentalization of signaling molecules is an important regulatory mechanism that enables the cell to build up an elaborate network of signaling pathways, facilitating the efficiency and the fidelity of signal transduction [Teruel and Meyer, 2000; Cyer, 2001; van Baal et al., 2005]. The coupling of activation and nuclear translocation represents an important aspect of p38 signaling [Ferrigno et al., 1998; Zeidan et al., 2008; Wood et al., 2009]. Consistent with other reports [Ferrigno et al., 1998; Wood et al., 2009], we confirmed that the phosphorylation, but not the kinase activity of p38 determines its nuclear translocation, with dominant negative mutants and specific inhibitors.

To avoid the artificial effects of overexpression, we performed immunostaining to study the nuclear translocation of endogenous p38. We also used phospho-p38 antibody for this purpose since the phosphorylated p38 represents the activated form. An earlier report [Wood et al., 2009] revealed p38 accumulated in the nucleus in response to UV and X-ray radiation, but not to TNF-α, Fas ligand (FasL) and phorbol 12-myristate 13-acetate (PMA), and concluded only stimuli that induce DNA double-strand breaks (DSBs) induced nuclear translocation of p38. In our study, however, it was shown that not only UV radiation, but also other classical stresses, such as arsenite, anisomycin, LPS, H_2O_2, and sorbitol, all induced the nuclear translocation of p38, suggesting its nuclear accumulation is a common phenomenon upon stress-induced activation. Since there are many different substrates of p38 in the nucleus, it may translocate to the nucleus to activate different nuclear targets and execute different signaling events upon different stimulations. This is unlikely to be a phenomenon that only occurs with stimuli that induce DSBs.

The traffic of some proteins between the cytoplasm and nucleus is mediated by a chaperon. In these systems, the NLS or NES is not in the proteins being transported, but in the chaperon [Ben-levy et al., 1998; Gorog et al., 2009]. In addition to stabilizing p38, downstream MK2, which carries a NLS and NES, was proved to transport p38 from the nucleus to the cytosol [Ben-levy et al., 1998; Gorog et al., 2009]. After activation, p38 enters the nucleus, binds to and phosphorylates MK2, and blocks its NLS as well. As a result of phosphorylation of Thr317 (Thr320 in mouse), but not Thr205 (Thr208 in mouse) by p38, the NES of MK2 is exposed, leading to its translocation from nucleus to cytoplasm [Engel et al., 1998]. Hence, p38 is coexported with MK2 to the cytoplasm. As the crystal structure study demonstrated, p38 and MK2 bind ''head-to-head'' with extensive intermolecular interactions [White et al., 2007]. Studies by Gorog et al. [2009] confirmed the role of MK2 in the nuclear export of p38 with *MK2*$^{-/-}$ mice. In our study, we found that p38 was redistributed by different MK2 mutants with different intracellular localizations. However, although studies by Engel et al. [1998] showed the nuclear export of GFP-MK2 was blocked by LMB and suggested it a chromosome region maintenance 1 (CRM1/exportin 1)-dependent process, we found that LMB pretreatment had no effect on the localization of p38, suggesting the export of p38 is not only mediated by a NES-containing protein. Thus, although MK2 is important in the nuclear export of p38, other mechanisms may also exist. In *MK2*$^{-/-}$ mice, the observation that nuclear export of phosphorylated p38 was only reduced, but not blocked [Gorog et al., 2009], also supported this conclusion.

Different from MK2 and p38, PRAK shuttles between the cytosol and the nucleus [Seternes et al., 2002; New et al., 2003]. The nuclear import of PRAK depends on its NLS, and its nuclear export requires p38-mediated phosphorylation on Thr182 and depends on CRM1 [Seternes et al., 2002; New et al., 2003]. However, the conclusions of the relationship between the intracellular localizations of PRAK and p38 mostly came from GFP fusion proteins, not the endogenous proteins. Moreover, endogenous PRAK mainly resides in the cytosol and exogenous overexpressed PRAK is in the nucleus [New et al., 2003]. In this regard, caution should be exercised when conclusions of PRAK-related localizations are drawn. We first identified PRAK as a p38 substrate and found p38 played a role in the localization of PRAK [New et al., 1998; New et al., 2003], and in the present study we also discovered that different mutants of PRAK could inversely redistribute the intracellular location of p38. However, with a *PRAK* knockout cell line, we found there was no contribution from PRAK in the nuclear translocation of p38. In the artificial systems, we do not know which one is in charge and the results may mislead us. For example, from the overexpression results, it may be deduced that MK2 carries p38 to the cytosol, and meanwhile p38 causes the relocalization of PRAK to the cytoplasm, showing an obvious contradiction to the role of the NLS and NES of PRAK itself. Hence, maybe all conclusions drawn from the experiments with overexpressed mutants need to be reconsidered.

Studies by Zeidan et al. [2008] revealed a leptin-induced nuclear translocation of p38, which depends on intact caveolae, RhoA activity and actin dynamics to mediate cardiomyocyte hypertrophy. However, in our study, we found microtubules but not the actin cytoskeleton were critical in this process, as cytochalasin D and phalloidin both had no effects on the nuclear translocation of p38 and its subsequent phosphorylation of ATF-2. Consistently, a dynein (a microtubule-associated motor [Schliwa and Woehlke, 2003]) inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), also blocked these processes, while myosin (a microfilament-associated motor [Schliwa and Woehlke, 2003]) light chain kinase (MLCK) inhibitor, wortmannin, only had a moderate effect. In fact, the association of MAPKs to microtubules has been known for many years [Reszka et al., 1995]. Our previous study has shown that the binding of MKK6 to p150 dynactin–dynein complex and subsequent transporting to microtubules is the prerequisite for its activation of p38 [Cheung et al., 2004], and we also observed the co-localization of p38 with different MKK6 mutants (data not shown). This can also explain why the microtubule-depolymerizing reagent nocodazole not only blocked the nuclear translocation of p38, but also its activation and subsequent phosphorylation of ATF-2. The microtubule-stabilizing reagent paclitaxel had no effect on either of these two processes, possibly because the structure of the microtubules was not affected. Thus, the nuclear translocation of p38 should be an energy-consuming process, mainly dependent on the microtubules

and dynein. The discrepancy between these results and those from Zeidan et al. may be due to the different stimuli and cell types used.

p38 was inactivated by MKPs through dephosphorylation [Owens and Keyse, 2007]. MKPs were also observed to have effects on the intracellular localization of p38, as catalytically inactive MKP-1 docks phospho-p38 in the cytoplasm [Pratt et al., 2003]. Nevertheless, the major role of MKPs on the intracellular localization of p38 may be owing to its dephosphorylation effect, by which p38-MK2 complex as an enzyme–substrate complex remains stable after MK2 phosphorylation [Kotlyarov et al., 2002]. This notion was supported by our observation that nuclear translocated p38 with a transient arsenite stimulation returned to the cytosol, while remained in the nucleus with a continuous stimulation.

Although ERK, JNK, and p38 are highly homologous proteins, it seems that they do not share similar mechanisms in their translocation, except the dependence on phosphorylation [Ferrigno et al., 1998; Khokhlatchev et al., 1998; Charruyer et al., 2005]. Using overexpression systems, many p38-related proteins, such as MKs and MKPs, were suggested to play roles in its intracellular localization. However, these may not be the genuine effectors regulating this process under physiological conditions. Moreover, the results from MAPKs and their homologous proteins in mammalian cells and yeast do not reflect each other, making these studies more complicated. For instance, the mechanisms by which ERK or JNK translocate to the nucleus are not identified in yeast yet. The homologous protein of p38 in yeast, Hog1, can translocate into the nucleus in an importin-independent manner, without interacting with an NLS-containing chaperon [Ferrigno et al., 1998]. In mammalian cells, this may not be the case, since the corresponding sequence to the NTS of ERK in p38 has not been identified. The most probable manner by which p38 translocates into the nucleus in mammalian cells seems to be via a NLS-containing chaperone.

Based on the previous knowledge of the nuclear translocation of p38 and our findings, we propose a model of the dynamics localization of p38 (Fig. 7). In resting cells, inactive p38 resides primarily in the cytosol. Stress-induced MKK3/6 activation results in phosphorylation of p38 located on microtubules. Phosphorylated p38 undergoes a change in conformation, leading to its interaction with a NLS-containing protein (or through another protein which interacts with this NLS-containing protein). With the help of this protein, as well as the assistance of dynein, p38 translocates into the nucleus along the microtubule. Apparently, this NLS-containing protein is neither MK2 nor PRAK. After phosphorylating MK2, nuclear localized p38 is dephosphorylated by MKPs. Inactivated p38 returns to the cytosol, partly through phosphorylated MK2 acting as a carrier, to receive the next stimulation. Hence, the next key task is to identify this NLS-containing protein, and potentially disclose a novel mechanism for the nuclear translocation of MAPK family members.

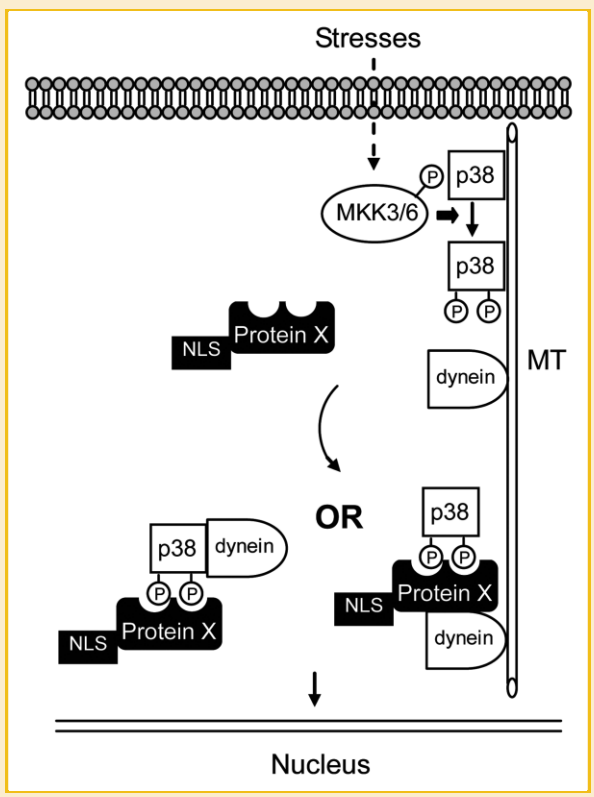

Fig. 7. A model for the nuclear translocation of p38. In resting cells, inactive p38 resides primarily in the cytosol. Upon stresses, activated MKK3/6 phosphorylates p38 on microtubules (MT). Phosphorylated p38 interacts with a NLS-containing protein (protein X). With the help of this protein X and dynein, p38 translocates into the nucleus along the microtubule.

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