Original Paper

Beta-Like Importins Mediate the Nuclear Translocation of MAPKs

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Abstract
Background/Aims: The rapid nuclear translocation of signaling proteins upon stimulation is important for the regulation of de-novo gene expression. However, the molecular mechanisms of this translocation is not well understood, although some studies suggest that much of this translocation may be mediated by beta-like importins (Imps). Here we undertook to study the stimulated nuclear shuttling of JNK and p38 MAPKs. ***Methods:*** For this purpose, we used coimmunoprecipitation, proximity ligation assay, gel filtration and immunostaining to examine the mechanism of nuclear translocation of these proteins. ***Results:*** We found that JNK and p38 MAPKs translocate into the nucleus in a Ran dependent, but NLS- or NTS-independent manner, unrelated to their catalytic activity. We show that this translocation involves three β-like Imps, 3, 7 and 9. Knockdown of these Imps inhibits the nuclear translocation of the MAPKs, and thereby, phosphorylation of their transcription factor targets. We further demonstrate that the translocation requires the stimulated formation of heterotrimers composed of Imp3/Imp7/MAPK or Imp3/Imp9/MAPK. JNK1/2 and p38α/β bind to either Imp7 or Imp9 upon stimulated post-translational modifications of the two Imps, while Imp3 joins the complex after its stimulation-induced phosphorylation. Once formed, these heterotrimers move to the nuclear envelope where Imp3 remains, while Imp7 or Imp9 escort the MAPKs into the nucleus. ***Conclusion:*** These results suggest that β-like Imps are central mediators of stimulated nuclear translocation of signaling proteins, providing a central level of regulation of the induction of cellular processes such as transcription upon stimulation.

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Introduction

Rapid and massive nuclear translocation of signaling proteins is an important step in the induction of transcription upon extracellular stimulation [1, 2]. Despite the importance of this process, its molecular mechanism has only been elucidated for a few signaling components. The well-understood cases include signaling proteins such as NFkB [3], which utilize the classical nuclear localization signal (NLS)-mediated binding with importin-α (Impα) and Impβ [4]. Another signaling protein that utilizes NLS for its nuclear translocation

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upon stimulation is ERK5 [5]. However, many other signaling proteins (e.g. ERK, MEK, RSK, PKC, PKA) translocate to the nucleus shortly after stimulation, using distinct NLS- and Impα/β- independent mechanisms. The rapid nature of the nuclear translocation, and molecular weight of the shuttling proteins (>40 kDa) make it unlikely that their translocation is mediated by a passive diffusion. Several other stimulus-dependent signaling mechanisms for such proteins that were proposed include Imp-like activity by the shuttled protein itself (e.g. β-catenin [6]), or the use of shuttling proteins other than Impα/β (e.g. Smad4 [7]). We have previously elucidated the molecular mechanism of ERK1/2 translocation, showing that it involves a CK2-mediated phosphorylation of two Ser residues within a nuclear translocation signal (NTS) of ERK1/2 [8-10]. This phosphorylation allows interaction with Imp7, which further facilitates ERK1/2 shuttling via nuclear pores. Aside from ERK1/2, NTS-like sequences were only seen in a few other NLS-deficient translocating protein [SMAD3, MEK1 [8], and Egr1 [11]], indicating that other proteins use a distinct mechanism for their stimulated translocation.

The involvement of Imp7 in the nuclear translocation of ERK1/2 drew our attention to the group of less studied β-like Imps (Imp 2-13, also known as Karyopherin-βs; refs [12, 13]). This group of proteins shares a 10-20% sequence similarity amongst themselves as well as with Impβ. As in case of Impβ, they all contain helical HEAT repeats, and bind weakly to phenylalanine-glycine-repeats in the nuclear pore complex (NPC), required for directed cargo translocation. Finally, these Imps are regulated by the small GTPase Ran, which determines the directionality of the transport [14]. Most of these Imps are ubiquitously expressed, and have been shown to catalyze nuclear shuttling of both housekeeping and regulatory proteins. Among the regulatory proteins, c-Jun uses Imps β, 2, 5, 7, 9, and 13 for its nuclear translocation in non-stimulated or stimulated cells [15], SMAD4 uses Imp7 and Imp8 [7], and HIF1, Imp4 and Imp7 [16]. The translocation of these proteins, as well as of ERK1/2, and MEK1/2 led us to hypothesize that these Imps play a general role in the stimulated nuclear translocation of signaling proteins. Although the mechanism of action of these proteins is not fully understood, they may operate by more than one way to regulate the localization of the stimulated or housekeeping proteins [12].

In a search for NLS-independent shuttling proteins, we have studied the MAPK family members JNK and p38 [17]. In mammals, there are three JNK isoforms (JNK1-3) and four p38 ones (p38α-δ) that regulate a large number of stimulated cellular processes, including primerily stress responses. Unlike ERK1/2 and ERK5 [18], the subcellular localization of JNKs and p38s has not been properly established thus far. Similarly to ERK1/2, JNK1/2 and p38α/β, interact with cytosolic anchoring proteins in resting cells [19, 20], and translocate to the nucleus upon stimulation. Indeed, such a process was demonstrated for both JNK1/2 [21, 22] and p38α/β [23-25] in some systems. However, in others it was suggested that JNK1/2 [26, 27] and p38α/β [28, 29] are localized in the nucleus of resting cells, which may be responsible for some constant activities such as genome stability for JNK [30] and ROS production for p38 [31]. For the latter, it was even shown that after stimulation, it is exported out of the nucleus [32]. These conflicting data may indicate that the localization of these MAPKs is cell-type specific, but this still requires clarification.

In this study, we show that similar to ERK1/2, JNK1/2 and p38α/β are localized primarily in the cytoplasm of resting cells, and translocate to the nucleus upon stimulation. We further found that despite the pronounced similarity amongst the MAPKs, none of the JNK or p38 isoforms contain an ERK-NTS. Moreover, mutations in the equivalent regions did not have any effect on JNK1/2 or p38α/β translocation, which occurs independent of their activation. We found that the translocation of these MAPKs is mediated by the formation of either Imp3/7/MAPK or Imp3/9/MAPK heterotrimers. This heterotrimerization occurs upon stimulation by phosphorylation of Imp3, as well as by the binding of JNK1/2 and p38α/β to either Imp7 or Imp9. These heterotrimers then escort the MAPKs to the nuclear envelope, where Imp3 remains, whereas Imp7 or Imp9 enter the nucleus with the MAPK in a Ran-dependent manner. Thus, the stimulated nuclear translocation of MAPKs is mediated

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by distinct β-like Imps, demonstrating their role in the regulation of transcription and cell fate.

Materials and Methods

Reagents

Tetradecanoyl phorbol acetate (TPA), anisomicin, Polyethylenimine (PEI) and 4′6-diamino-2-phenylindole (DAPI) were obtained from Sigma (Rehovot, Israel). A/G beads were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Albumin bovine serum (BSA) was purchased from MP niomedical (Solon, OH, USA). Si RNAs and Dharmafect were from Thermo Fisher Scientific (CO, USA). Proximity ligation assay kit was from Olink Bioscience (Uppsala, Sweden). NBT/BCIP developing substrates were purchased from Promega (WI, USA), Calf Intestinal Phosphatase (CIP) was from New England Biolabs (MA, USA).

Antibodies

Anti Imp2, Imp3, Imp5, Imp7 Abs were from Abnova (Taipei, Taiwan). Anti Imp9, Imp13, pMEF2a Abs were from Novus (Littleton, USA). Anti Imp4 and Imp12 Abs were from Abcam (Cambridge, England). Anti GFP Ab was from Roche Diagnostics GmbH (Mannheim, Germany). Anti Imp8, Imp10, Imp11, JNK1, JNK2, p38β, pcMyc, Tubulin, GST and pATF2 Abs were from Santa Cruz Biotechnology (CA, USA). Abs against doubly phosphorylated ERK1/2 (pTEY-ERK), general ERK (gERK), doubly phosphorylated JNK (pJNK), general JNK1/2 (gJNK), doubly phosphorylated p38 (pp38), general p38 and c-Myc and Impβ were obtained from Sigma Israel (Rehovot, Israel). Anti pC-Jun, JNK2 and p38α were from Cell Signaling Technology (Boston, MA, USA). All antibodies were specific and recognize just one band in Western blot under the conditions used. Secondary antibodies conjugated to HRP or AP as well as secondary light chain specific secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA, USA).

Cell Culture and transfection

HeLa and MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1% Pen/Strep and 10% fetal bovine serum (FBS). HB2 cells were cultured in the same medium with the addition of hydrocortisone (0.5 mg/ml), and insulin (10 μg/ml). MCF10A cells were cultured in DMEM / F-12 with 5% horse serum, EGF (200 ng/ml), hydrocortisone (0.5 mg/ml), cholera toxin (100 ng/ml), insulin (10 μg/ml), 2 mM L-glutamine, 1% pen/strep and 10% FBS. HeLa cells were transfected using polyethylenimine (Sigma, [33]). SiRNAs were transfected using Dharmafect (Thermo Fisher Scientific, CO, USA).

Immunofluorescence microscopy

Cells were fixed in 3% paraformaldehyde in PBS (20 min, 23°C), and incubated with 2% Albumin bovine serum (BSA) in PBS (15 min, 23°C), followed by permeabilization with Triton X-100 (0.1% in PBS, 5 min, 23°C). Cells were incubated with the primary Abs (60 min, 23°C), washed three times with PBS and incubated with rhodamine-conjugated secondary Ab (60 min, 23°C), and DAPI. Slides were visualized by either fluorescence microscope (Olympus BX51, x40 magnification), or spinning disk confocal microscope (Zeiss, Cell observer SD, x100 magnification). Background correction, and contrast adjustment of raw data were performed using Photoshop (Adobe, CA, USA).

DNA Constructs and mutations

GFP-JNK1/2, p38α/β were cloned in pEGFP-C1 (Clontech, Mountain View, CA). JNK1/2 and p38α/β sequences were amplified from HeLa cells cDNA and flanked by EcoRI / BamHI for JNK2 and p38β and with XhoI / BamHI for JNK1 and p38α. Point mutations of JNK1/2 and p38α/β were performed by site-directed mutagenesis. GST-JNK1/2, p38α/β were cloned in pGEX-2T vector (GE healthcare, Buckinghamshire, UK) and flanked by SpeI/NotI restriction sites. Imps 3, 7 and 9 were cloned in pEGFP-C. Imps 7 and 9 were amplified from HeLa cells cDNA using specific primers flanked by BamHI/SalI for Imp7 and XhoI/SalI for Imp9, restriction sites. Imp3 was acquired from Forchheimer repository plasmid collection, and amplified using specific primers flanked by XhoI/EcoRI restriction sites. GST-Imp3, GST-Imp9 and His-Imp7 were a generous gift from Prof. Oded Livnah (Hebrew Univercity, Jeruslaem, Israel).

Coimmunoprecipitation (CoIP)

CoIP was done as previously descibed ([8]. Briefly, cell extracts were produced and incubated for 2 hr (4°C, with rotation) with A/G-agarose beads pre-linked with specific Abs (1 hr, 23°C). The bound A/G beads were washed, resuspended (1.5X sample buffer), boiled and subjected to Western blotting.

Proximity ligation assay (PLA)

Protein–protein interactions were detected with Duolink PLA Kit ([34]; Olink Bioscience), according to the manufacturer's protocol. Briefly, cells were grown, fixed and permeabilized as described. The samples were then incubated with primary Abs against two examined proteins (60 min, 23°C), washed (0.01 M Tris HCl pH 7.4, 0.15 M NaCl and 0.05% Tween 20), and then incubated with specific probes (60 min, 37°C), following by DAPI staining to visualize nuclei and washed (0.2 M Tris HCl pH 7.5, 0.15 M NaCl). The signal was visualized as distinct fluorescent spots by a fluorescence microscope (Olympus BX51, x40 magnification). Background correction, contrast adjustment and the quantification of the fluorescence signal were performed using Photoshop and ImageJ.

In vitro interaction assay

Cell extracts were produced as described and incubated for 2 hr (4°C) with A/G-agarose beads pre-linked with specific Abs (1 hr, 23°C). The bound A/G beads were sequentially washed with RadioImmune Precipitation Assay (RIPA) buffer, twice with 0.5M LiCl and twice with Buffer A (50 mM β-glycerophosphate pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1mM dithiothreitol, and 0.1 mM sodium vanadate). The bound A/G beads were then resuspended in Buffer A containing 0.01% BSA and aliquoted. GST tagged proteins were incubated with the beads 2 hr (4°C, with rotation) then washed and resuspended in 1.5 X sample buffer and boiled.

In vitro phosphatase assay

HeLa cells were grown to subconfluency, serum-starved (0.1% FBS for 16 hr) and then treated with stimuli or other drugs. Cell extracts were produced as described and incubated for 2 hr (4°C, with rotation) with A/G-agarose beads (Santa Cruz Biotechnology) pre-linked with specific Abs (1 hr, 23°C). The bound A/G beads were sequentially washed once with RIPA buffer, then twice with 0.5M LiCl and twice with Buffer A. The bound A/G bead sample, originated from the stimulated cells, was then incubated with Calf Intestinal Phosphatase (37°C, 1 hr, NEB, MA, USA) then washed. The samples were then resuspended in Buffer A containing 0.01% BSA and aliquoted-GST tagged proteins were incubated with beads (4°C, 2 hr), then washed and resuspended in sample buffer and boiled.

Subcellular Fractionation

Subcellular fractionation was performed essentially as described [8]. Briffly, Harvested cells were then resuspended in 200 μl of buffer H containing 0.1% Nonidet P-40. The lysates were mixed vigorously and centrifuged immediately as above to yield supernatants containing the cytosolic fraction. Nuclear proteins were extracted by resuspending the nuclear pellets in 200 μl of extraction buffer, waiting on ice for 5 min, brief sonication (2 x 5 sec, 40W, 4°C), vigorous mixing, and centrifugation. The protein concentration was determined by Coomassie protein assay reagent (Pierce). Both cytosolic and nuclear fractions were subjected to Western blotting.

Gel filtration

HeLa cells were serum starved (as above) and then were either stimulated with anisomycin (Anis, 0.5 μg/ml, 15 min) and TPA (250 nM, 15 min), or left untreated (NT). Cell extracts (25 mg of each treatment) were loaded on a 16/60 superdex 200 sizing column (flow rate 1 ml/min), and 1ml fractions were collected. The fractions were analyzed using Western blot with the appropriate Abs.

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Statistical Analysis

Data are expressed as mean ± S.E. Statistical evaluation was carried out using functional analysis and Student's t test (two-tailed) to test for differences between the control and experimental results. Values of $p < 0.05$ were considered statistically significant. In Fig. 1, 2, 3, MAPK staining that was either all over or primarily in the nucleus (more than 40% nuclear staining, no darker nuclear staining) was considered nuclear.

Results

JNK and p38 translocate into the nucleus using a NTS-independent mechanism

In our search for NLS-independent shuttling proteins, we have looked into the subcellular localization of JNK1/2 and p38α/β. Using immunostaining with specific antibodies (Abs) we found that, similar to ERK1/2, JNK1/2 and p38α/β are localized mainly in the cytoplasm of resting cells (Fig. 1A). Treating the cells with either stress or mitogenic stimulants (anisomycin and TPA, respectively), we observed a rapid and rubost nuclear translocation of all four MAPK isoforms, with only minor differences in their kinetics of translocation. This translocation of JNKs and p38s was confirmed using subcellular fractionation of anisomycin-treated HeLa cells (Fig. 1B). Interestingly, this translocation did not correlate with JNK1/2 and p38α/β activatory phosphorylation (Fig. 1C). Both JNK and p38 peak at 30 min after stimulation with anisomycin (fold activation avarages from three distinct experiments were 13.5± 1.5 fold for JNK and 9.6±2.2 fold for p38), while despite of the significant translocation, no activation was detected with TPA. These results indicate that the phosphorylation of JNK and p38 is not required for the translocation and that the mechanism of translocation of these four MAPKs is different from that of ERK1/2. Moreover, none of the JNK or p38 isoforms contained an NTS, and only two, JNK2 and p38β contained a phosphorylatable sequence (T-P-S) in the same kinase region (Fig. 2A). Nonetheless, the pronounced sequence and conformation similarities among JNK1/2 and p38α/β to ERK1/2 [17] prompted us to examine whether this region can act as an NTS in JNK1/2 or p38α/β as well. For this reason, we mutated the phosphorylatable ERK-NTS-aligned residues in JNK1/2 or p38α/β to either Ala or Glu residues in all four MAPKs. Unlike ERK1/2, overexpression of these mutants resulted in a similar distribution to that of WT proteins (Fig. 2B), indicating that JNK1/2 and p38α/β differ from ERK1/2 not only in the release from anchoring proteins [19, 20], but also in their NTS, and probably the whole Imp7-dependent mechanism of ERK translocation [8].

JNK1/2 and p38α/β interact with Imps 3, 7 and 9 upon stimulation

The lack of ERK1/2-like NTS, as well as a canonical or any atypical NLSs [35-37] prompted the search for their actual mechanism of translocation. To detect whether the process is energy or Ran-dependent, we knockdown Ran expression, and found that it is strongly involved in the stimulated nuclear translocation of JNK and p38 (Fig. 3). These results indicate that this translocation is not mediated by simple diffusion, but requires Ran-dependent transport mechanism. Since β-like Imps are Ran-dependent and have been implicated in the stimulated translocation of signaling proteins [12, 13], we hypothesised that one or more of these Imps are involved in this process. To examine it, we first used a CoIP screen to detect whether any of them could interact with JNK1/2 and p38α/β. Despite the expression of all these Imps in the examined cells, no significant Imp/MAPK interactions were detected in resting HeLa cells (Fig. 4A). However, IP of JNK1/2 and p38α/β in extracts of stimulated HeLa cells, revealed a varying degree of interaction of all four MAPKs with Imps 3, 7 and 9. Importantly, the screen and other IP experiments demonstrated only small differences in the association and timing of interactions between the components, suggesting a similar mode of regulation among them.

In addition, we performed a proximity ligation assay (PLA), which is a CoIP-independent tool for protein-protein interaction studies (See Material and Methods). Similar to the CoIP results, no significant basal interactions between the examined Imps and MAPKs were
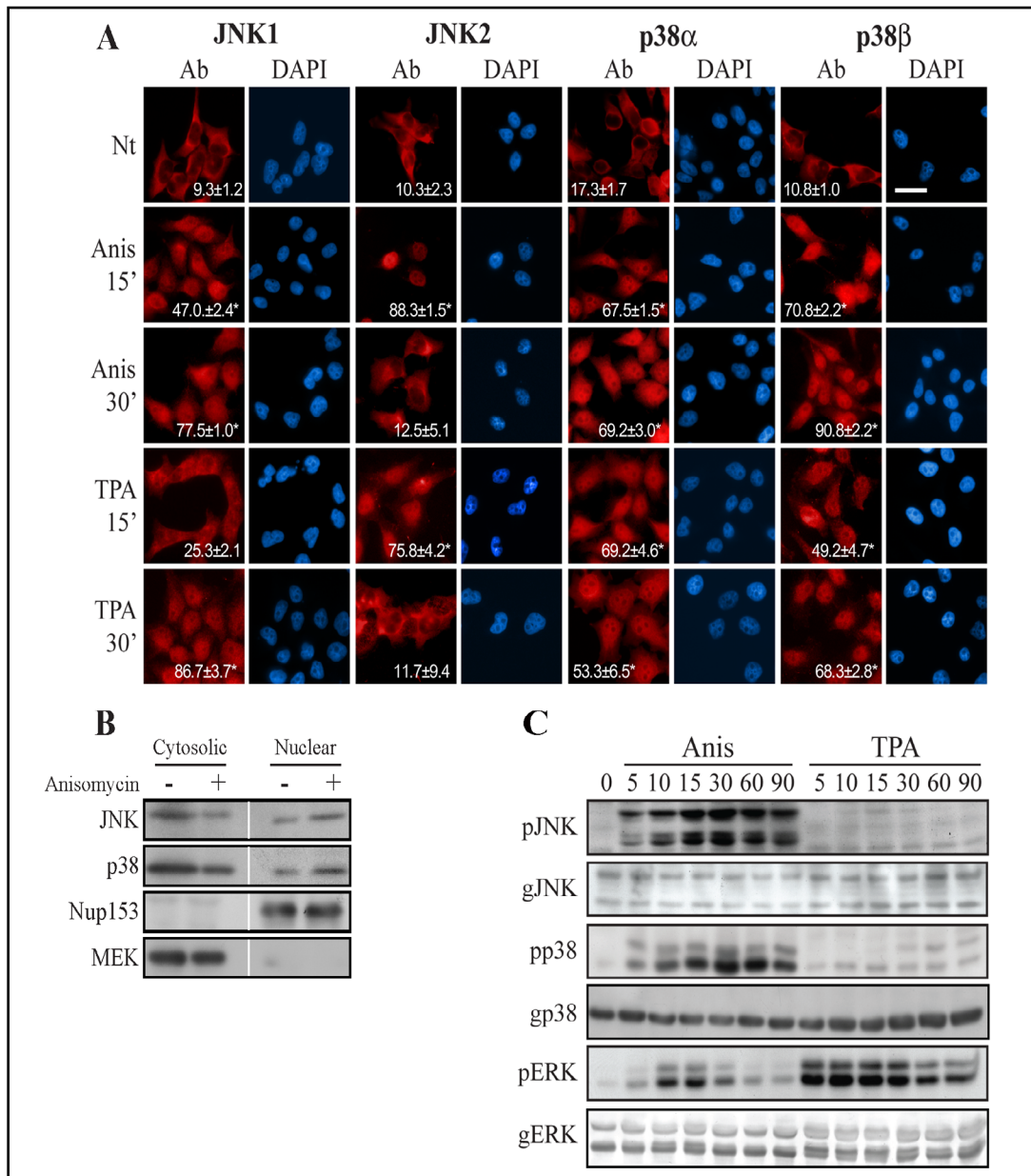
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Fig. 1. JNK1/2 and p38α/β translocate into the nucleus independently of their activatory phosphorylation. (A) Fluorescent microscopy demonstrate the stimulated nuclear translocation of JNK1/2 and p38α/β. HeLa cells were grown on slides to 70% confluence, serum starved (0.1% FBS, 16 hr), and then stimulated with anisomycin (Anis, 0.5 μg/ml) or TPA (250 nM) for the indicated times, or left untreated. Cells were fixed, and stained with anti JNK1, JNK2, p38α or p38β Abs as indicated, and DAPI to detect nuclei. Percent cells with >40% nuclear staining and standard error was determenined by counting six distinct fields of 20 cells each from two distinct experiments. * - P< 0.05 compaired to non-stimulated cells. The bar in the upper right panel is of 20 μM. (B) Subcellular fractionation confirms the nuclear translocation of JNK1/2 and p38 α/β. Serum-starved HeLa cells were either stimulated (Anis, 0.5 μg/ml, 15 min) or left untreated, and then harvested. Subcellular fractions of cytosolic and nuclear were produced as described under materials and methods, and the fractions were subjected to Western blot analysis with the indicated Abs. (C) The nuclear translocation of endogenous JNK1/2 and p38α/β is independent of their phosphorylation. HeLa cells were grown to 70% confluence, serum starved, and then stimulated for the indicated time points. Cell extracts were produced and subjected to Western blot analysis with the indicated Abs. The blots were developed with NBT/BCIP or ECL. The experiments were reproduced with very similar results three times.
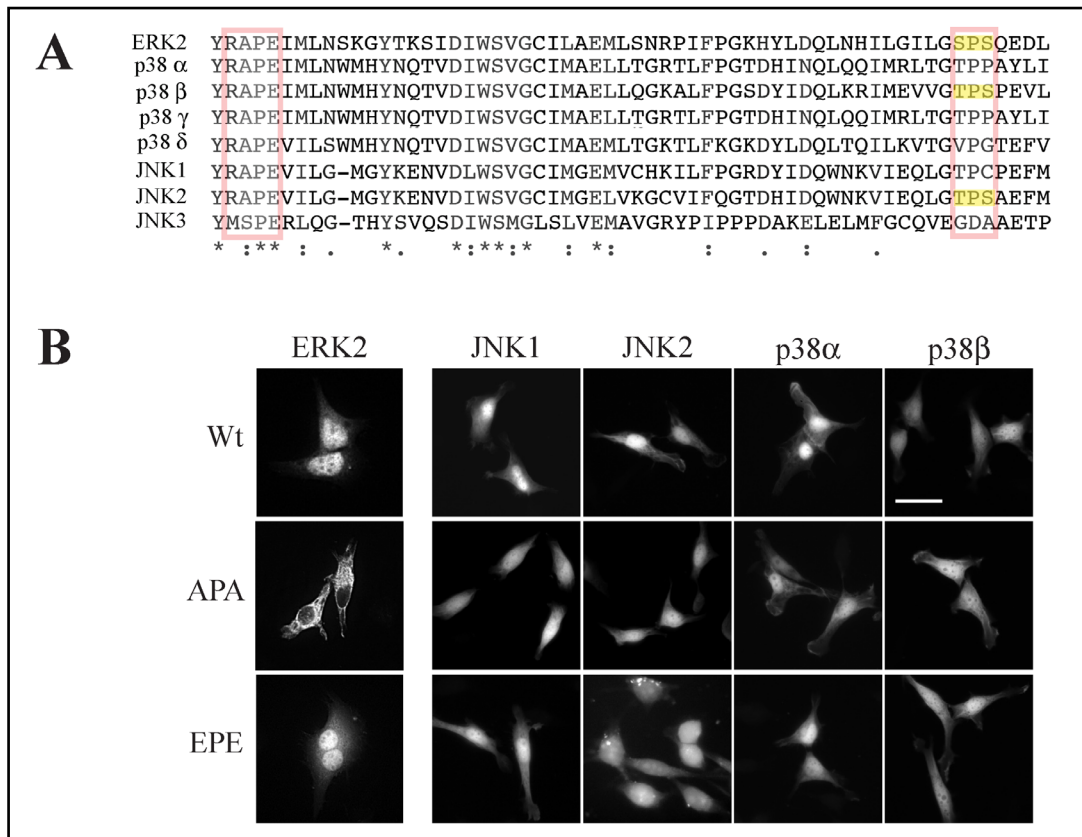
Fig. 2. JNK1/2 and p38α/β translocate into the nucleus using NTS-independent mechanism. (A) Sequence alignment of the kinase insert domain (KID) and the NTS in ERK2 compared with JNKs/p38s. The left side box mark the conserved kinase residues of the kinase subdomain VIII, that are adjacent to the KID. The right side box mark the region of ERK1/2's-NTS (SPS motif). The phosphorylatable residues in this region are marked in yellow. (B) Mutations in the NTS-homologous region do not affect the nuclear shuttling of JNK1/2 and p38α/β. HeLa cells were grown on slides to 70% confluence, transfected with JNK1-GFP (wt-JNK1), JNK2-GFP (wt-JNK2), p38α-GFP (wt-p38α) or p38β-GFP (wt-p38β) and their mutants (APA or EPE). Sixteen hr after transfection the cells were serum starved, fixed and visualized using a fluorescent microscope (Olympus BX51, x40 magnification). The results were reproducible and similar localization was observed in >50 cells for each condition. The bar in the upper right panel is of 20μM.

detected (Fig. 4B). However, anisomycin or TPA stimulation resulted in a marked increase in the interaction of either JNK1/2 or p38α/β with Imps 3, 7 and 9. As would be expected from the shuttling role of the Imps, the interactions were found mostly in the cytoplasm and perinuclear regions. To verify these interactions, we repeated the CoIP experiments in MCF7 cells, and examined the interactions of overexpressed MAPKs with the Imps in HeLa cells. Both experiments confirmed that the interaction of MAPKs with of Imps 3, 7 and 9 is not specific only to endogenous HeLa proteins (Fig. 5). Moreover, we performed CoIP experiments using overexpressed MAPKs and found that in most cases they interact with Imp3, 7 and 9 as well. There were two reproducible exceptions to this type of interaction (JNK2 and Imp3 and p38β and Imp9 upon aniscomycin treatment). The reason for these changes is not clear at this stage, but might occur due to eronious localization or regulation of the overexpressed proteins. Together, our results may indicate that the stimulated interaction of Imps 3, 7 and 9 with the MAPKs first occurs in the cytoplasm, and the Imps are detached from the MAPKs either during the shuttling, or immediately after translocation.
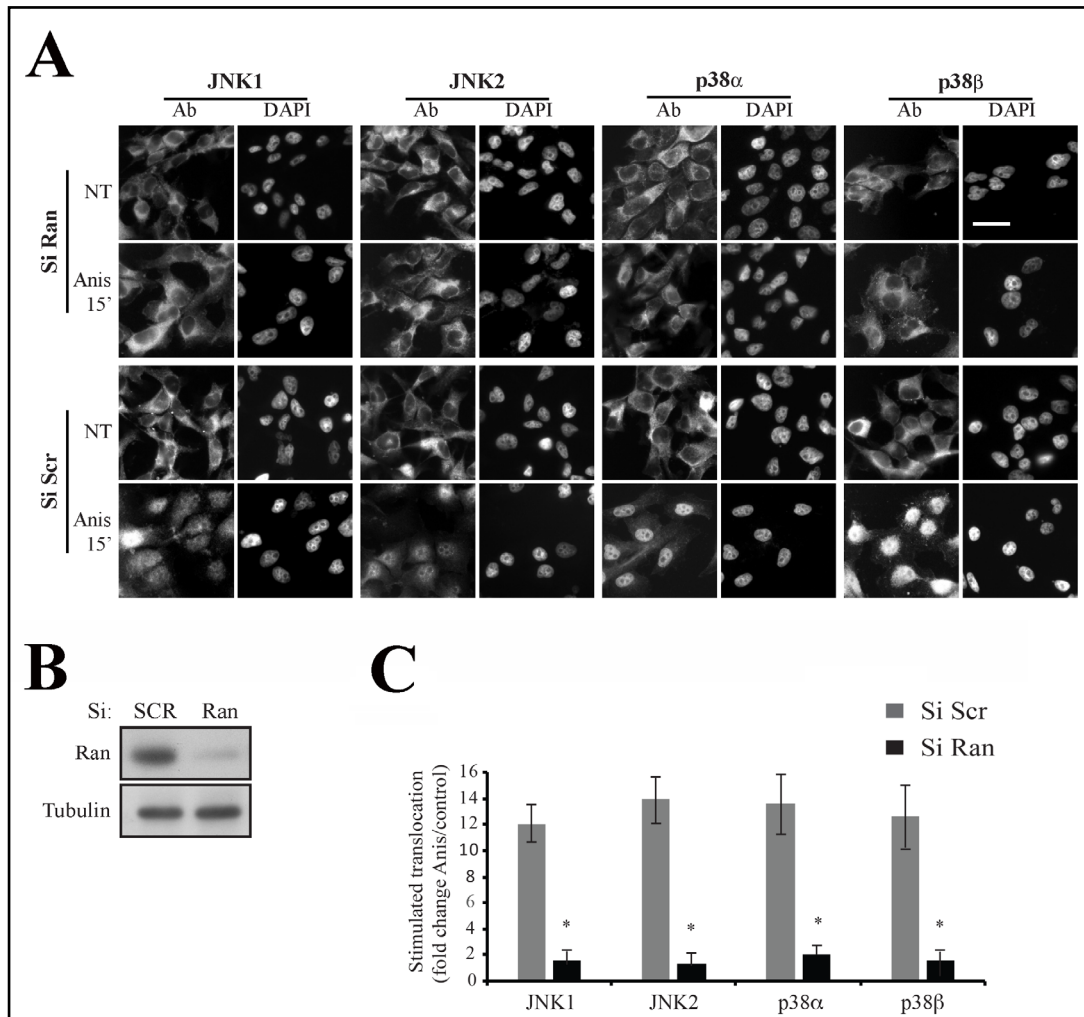
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Fig. 3. Ran is required for JNK1/2 and p38α/β nuclear translocation. HeLa cells were grown on cover slides to 30% confluence. SiRNA of Ran and a scrambled siRNA (SiScr; serves as a negative control) were then transfected to the cells using Dharmafect according to the manufacturer's instructions. Forty-eight hr after transfection, the cells were serum starved, and then either stimulated with anisomycin (anis; 0.5μg/ml, 15 min), or left untreated (NT) as indicated. The cells were then fixed, stained using the indicated anti MAPK Abs and visualized using a fluorescent microscope. The bar in the upper right panel is of 20 μM. B. SiRNA reduces the amount of Ran. To confirm the efficiency of the siRNA, cell were transfected with siRNAs as described, and were subjected to a Western blot analysis with the indicated Abs (lower panel). C. Quanntification. The results in A were quantified (total of 60 cells in 4 fields from two distinct experiment. The fold change in cells with nuclear staining (>40%) upon anisomycin stimulation was calculated in cells treated with anisomycin in both control Si Scr and Si Ran. * - P<0.01 between Anis treated and untreated cells.

Imps 3, 7 and 9 are required for JNK1/2 or p38α/β translocation into the nucleus

To further substantiate the role of Imps 3, 7, and 9 in the nuclear translocation of JNK1/2 or p38α/β, we used their siRNA to determine if the knockdown of these Imps affect the nuclear accumulation of MAPKs. A knockdown of Imp5, and scrambled siRNA of Imp3 served as negative controls. The siRNAs of the four Imps examined reduced the amount of the relevant Imps by more than 85% within 48 hr (Fig. 6). These knockdowns did not affect the cytoplasmic localization of endogenous JNK1/2 or p38α/β in resting cells (data not shown). However, the knockdown of Imps 3, 7, and 9, but not Imp5 or siRNA control, strongly modulated the stimulated nuclear shuttling of the examined MAPKs. The knockdown of Imp3 inhibited the translocation in ~80% of the cells, whereas the siRNA of Imp7 and Imp9
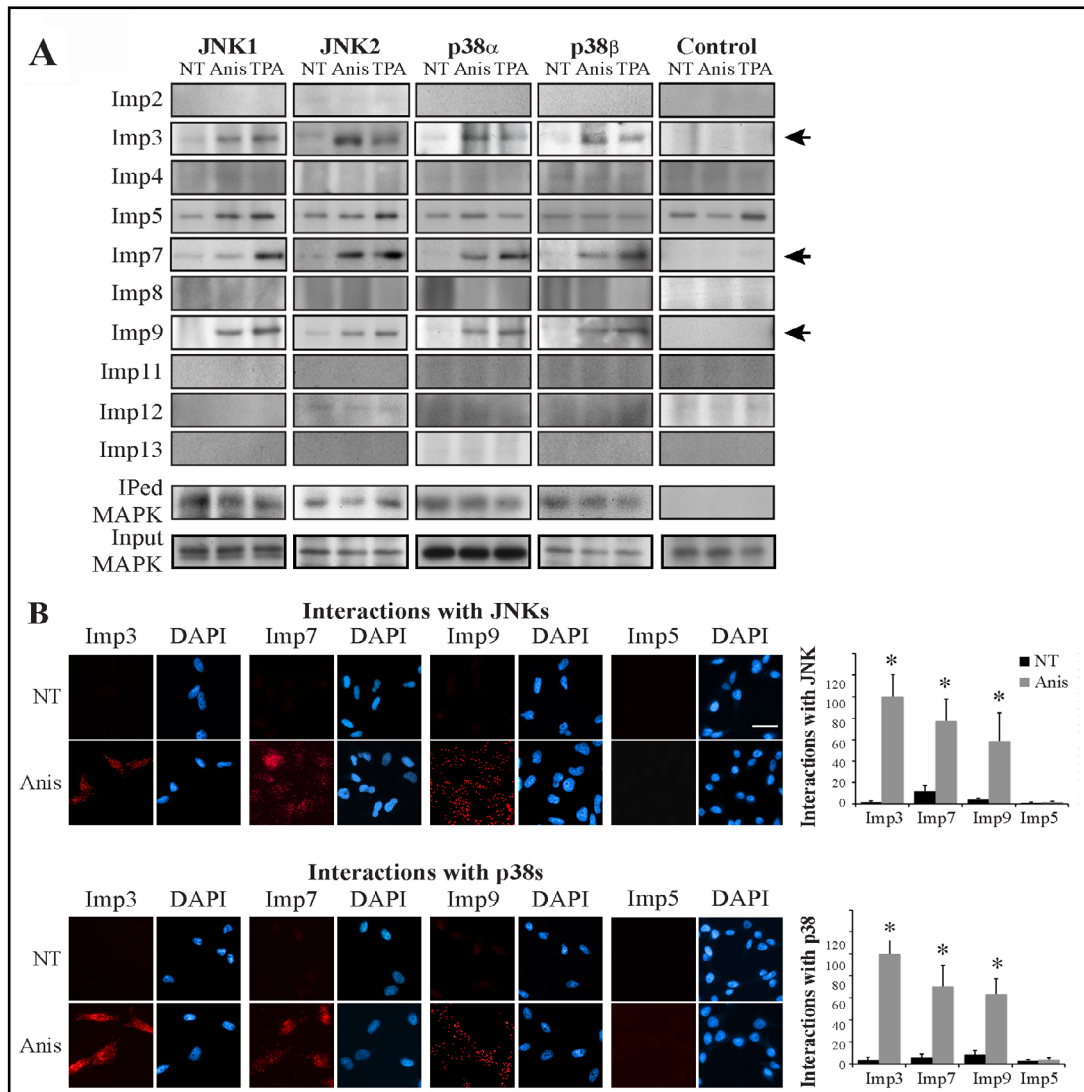
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Fig. 4. JNK1/2 and p38α/β interact with Imps 3, 7 and 9 in HeLa cells. (A) A CoIP screen to detect Imp/MAPK interactions. HeLa cells were grown to 70% confluence, serum starved (0.1% FBS, 16 hr), and then stimulated with anisomycin (Anis, 0.5 μg/ml, 15 min), TPA (250 nM, 15 min) or left untreated (NT). Cell extracts were subjected to CoIP with anti JNK1, JNK2, p38α, p38β Abs, or rabbit IgG as a negative control (Control). The interacting Imps and amount of IPed or input MAPKs (in extracts) were detected by Western blotting with the indicated Abs (bottom panel). Arrows indicate the interacting Imps. (B) Proximity ligation assay confirms interaction of JNKs/p38s with Imps 3, 7 and 9. HeLa cells were grown on slides to 70% confluence, serum starved and then either stimulated or left untreated (NT). Cells were fixed, and subjected to a PLA assay according to the manufacturer's instructions using the anti Imps together with either anti JNK (upper panels) or p38 (lower panels) Abs. The nuclei were detected using DAPI. Slides were visualized using a fluorescent microscope. The bar in the upper right panel is of 20 μM. Quantification of the intensity of the signal of 3 experiments (Bar graphs at right) was performed using ImageJ (* - p<0.0001).

prevented the translocation in only ~60%. Importantly, the combined knockdown of Imp7 and 9 significantly increased the effect of the individual ones to ~90% of the cells, indicating that these two Imps act additively in translocating stimulated MAPKs to the nucleus.

To further study the effect of Imps 3, 7 and 9 knockdown on the translocation of JNK1/2 or p38α/β, we examined the phosphorylation of the nuclear JNK/p38 substrates c-Jun, ATF-2 and MEF2A [1]. Importantly, all three siRNAs significantly inhibited (>90%) the stimuli-
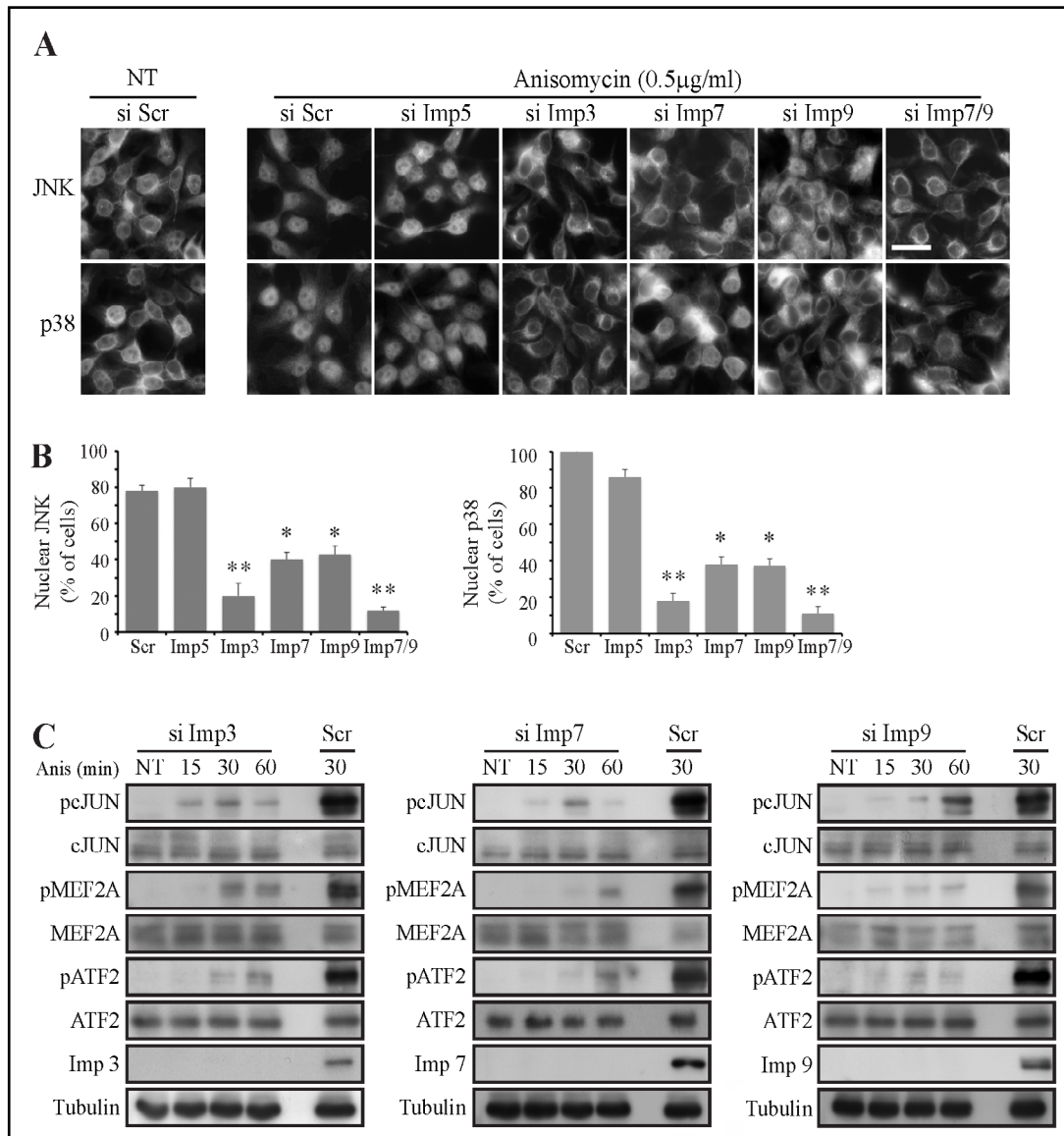
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Fig. 5. Endogenous and overexpressed JNKs and p38s CoIP with Imps 3, 7 and 9. (A) A CoIP screen to detect the Imps interacting with JNK1/2 and p38α/β in MCF7 cells. MCF7 cells were grown to 70% confluence, serums starved and then were stimulated with either anisomycin (Anis 0.5 μg/ml, 15 min), TPA (250 nM, 15 min) or left untreated (NT). Cells extracts were then subjected to CoIP with the indicated Abs. Imps, IPed MAPKs and inputs (in extracts) were detected by Western blot with the indicated Abs. (B) Interaction of overexpressed MAPKs with Imps 3,7 and 9 in HeLa cells. HeLa cells were transfected with either JNK2-GFP (wt-JNK2), p38β-GFP (wt-p38β) or GFP alone. Cells were serum starved, and then treated as described and subjected to CoIP with anti GFP Abs. The interacting Imps and the loading extracts were detected using Western blot analysis with the indicated Abs. Arrows indicate the interacting Imps. WCL is whole cell extract. (C) Loading control of overexpressed JNK2 and p38α. Extracts of HeLa cells overexpressing either JNK2-GFP or p38α-GFP or GFP alone were subjected to Western blot analysis with anti GFP Abs. The experiments were reproduced twice. The antibodies detected mainly the two bands (two start-sites) of the GFP-MAPKs and also few minor degradation products/impurities.

Fig. 6. Imps 3, 7 and 9 are required for JNK and p38 translocation and phosphorylation of downstream nuclear transcription factors. (A) siRNA of Imp3, 7, 9 but not of Imp5 inhibits the stimulated-nuclear translocation of JNKs and p38s. HeLa cells were grown on cover slides to 30% confluence. Si RNAs of the indicated Imps and siRNA control (scambled siRNA of Imp3; si Scr) were transfected to the cells. 48 hr after transfection, cells were serum starved, stimulated or left untreated (NT), fixed and stained using the indicated anti MAPK Abs. The bar in the upper right panel is of 20 μM. (B) Quantification of JNKs/p38s nuclear localization prior and upon stimulation. The nuclear localization of JNKs and p38s was calculated as the number of cells presenting nuclear staining (nuclear staining was considered for all cells in which > 30% of the stain was inside the nucleus) per the total cell number. The data shown represents mean ± S.E of three different experiments (* - p<0.0003, ** - p<0.00001). (C) SiRNA of Imp3, 7, 9 inhibits the induction of transcription regulated by JNKs/p38s. HeLa cells were transfected with the indicated siRNAs or control of scrambled siRNA of Imp3 (Scr), serum starved and then either stimulated with anisomycin (Anis 0.5 μg/ml) for the indicated times, or left untreated (NT). Cell extracts were subjected to a Western blot analysis with the indicated Abs. The experiments were reproduced twice.

induced phosphorylation of these transcription factors. Interestingly, the strong decrease in transcription factor phosphorylation did not correlate with the medium inhibtion of JNK/p38 translocation (~60%) upon Imp7 and Imp9 knockdown. This point was very reproducible accros experiments, and could be explained by the existence of a threshold of MAPK activity that is required for the full activation of the MAPK substrates. Such a threshold has been previously suggested for the action of MAPKs in several systems [38]. No influence of the siRNA of Imp3 and 9 on the ERK target C-Myc, ERK phosphorylation, or RSK were detected in any other proteins examined (data not shown), verifying the specificity of the effects. Thus, it appears that the three Imps are important for the translocation of both JNK1/2 and p38α/β, although the role of Imp3 may be more general.

In order to participate in the translocation process, it is possible that unlike Impα/β, Imps 3, 7 and 9 change their localization upon stimulation. Indeed, using fluorescent (Fig. 7A, B) and spinning disk confocal microscopies (Fig. 7C), we found that stimulation of both HeLa and HB2 cells causes a significant shift of Imps7 and Imp9 from the cytoplasm to the nucleus (Imps 7, 9). The localization of Imp3 upon stimulation was changed in a different manner, as some molecules shuttled to the nuclear rim (Fig. 7A), while another part remained in the cytoplasm (mainly in Hela cells). Impβ localization was not affected at all upon stimulation. The dynamic changes in Imps 3, 7 and 9 localization were confirmed by nuclear fractionation of stimulated HeLa cells as well (Fig. 7D). These results show for the first time that the β-like Imps are influenced by extracellular stimulation. It also further confirms that all three Imps are important for the nuclear translocation of JNK1/2 or p38α/β.

Imps 7/9-JNK/p38 complexes interacts with phosphorylated Imp3

To obtain a better insight into the MAPKs-Imps interactions, we looked into the *in vitro* interaction of GST-p38α/β and GST-JNK1/2, with purified Imps from extracts of non-stimulated or stimulated Imp-overexpressing HeLa cells. Although the MAPKs interacted with Imps 7 or 9 from stimulated cells as expected, no *in vitro* interaction was detected with Imp3 (Fig. 8A). Moreover, CoIP experiment revealed that Imp3 is not required for the interaction of JNK or p38 MAPKs with Imp7/9 (Fig. 8B). These results indicate that JNKs and p38s directly interact with either Imp7 or Imp9, but not Imp3, and these interactions do not require Imp3.

The interaction of the MAPKs with Imps 3, 7 and 9 in cells (Fig. 4 and 5) raised the question as to what is the role of each of the Imps in the process of nuclear translocation. The finding that: i) the siRNA of Imp3 had a higher effect than that of Imp7 and Imp9 (Fig. 6), ii) the siRNAs of Imp7 and 9 had an additive effect, iii) Imp3 did not directly interact with JNK1/2 or p38α/β *in vitro* (Fig. 8A), and iv) Imp3 is not required for the MAPKs interactions with Imp7 or Imp9 (Fig. 8B), led to the hypothesis that the MAPKs bind either to Imp7 or Imp9, within heterotrimers of Imp3/Imp7/MAPK or Imp3/Imp9/MAPK. In order to examine this hypothesis, and establish a complex formation between endogenous Imps 3, and 9, we monitored these possible interactions by PLA. No interaction between any of the examined Imps was detected in resting HeLa cells (Fig. 9A, B). However, this was dramatically changed upon stimulation, which induced interactions between Imp3 and either Imp7 or Imp9, but not between Imp7 and Imp9. These results were further confirmed by CoIP experiments of stimulated extracts from HeLa cells as well (Fig. 9C). Interestingly, *in vitro* interaction of recombinant Imp7 and Imp9 (purified from bacteria) with immunoprecipitated (IPed) Imp3 from non-stimulated or stimulated cells (Fig. 9D), demonstrated that Imp3 is modified upon stimulation to allow the interaction. Treatment of the IPed Imp3 with alkaline phosphatase (CIP) reduced the interaction, strongly indicating that the interaction with either Imp7 or imp9 requires the phosphorylation of Imp3. This figure demonstrates that, as expected, there was no interaction between Imp7 and Imp9. This lack of interaction was not affected by CIP as well.
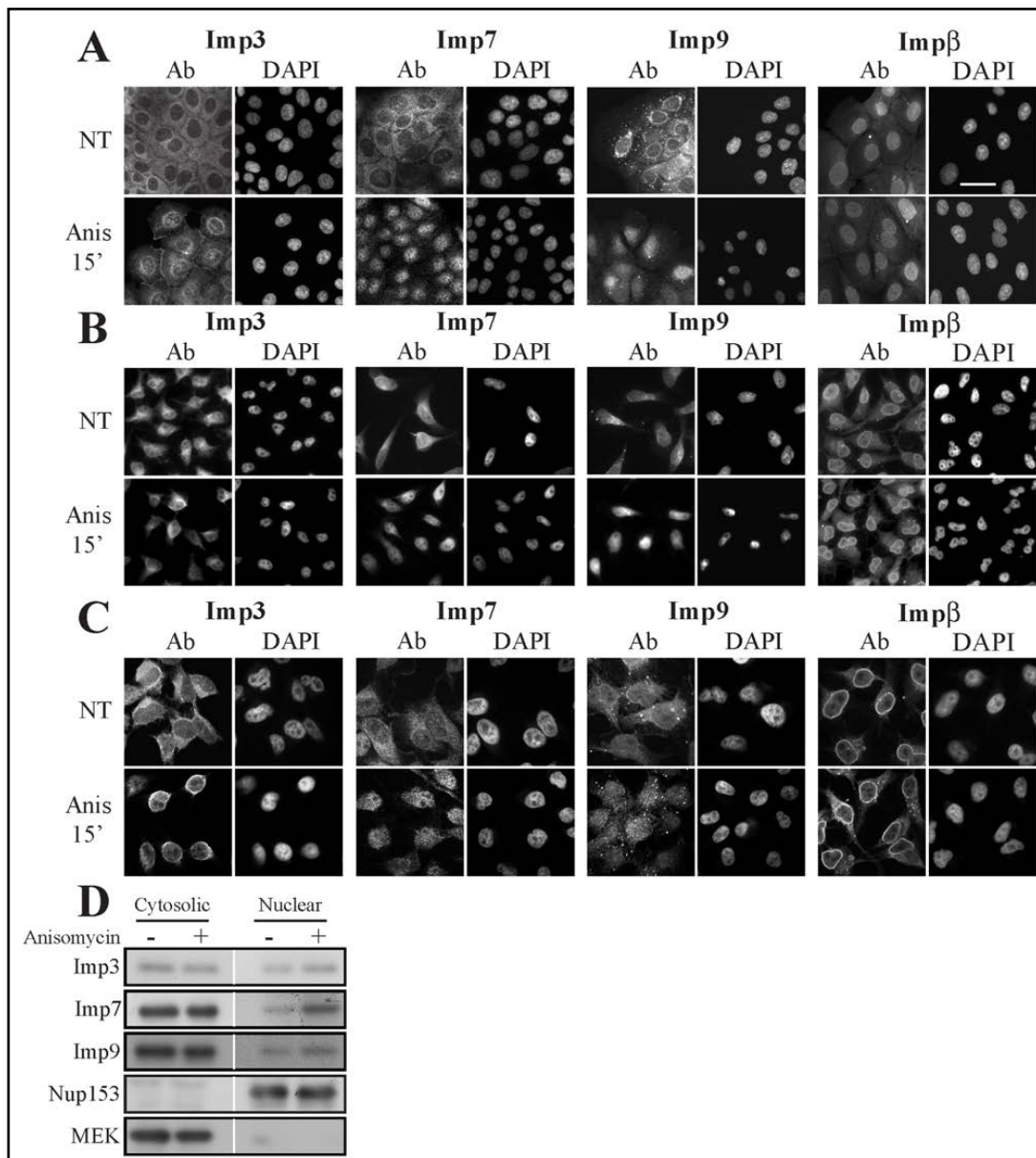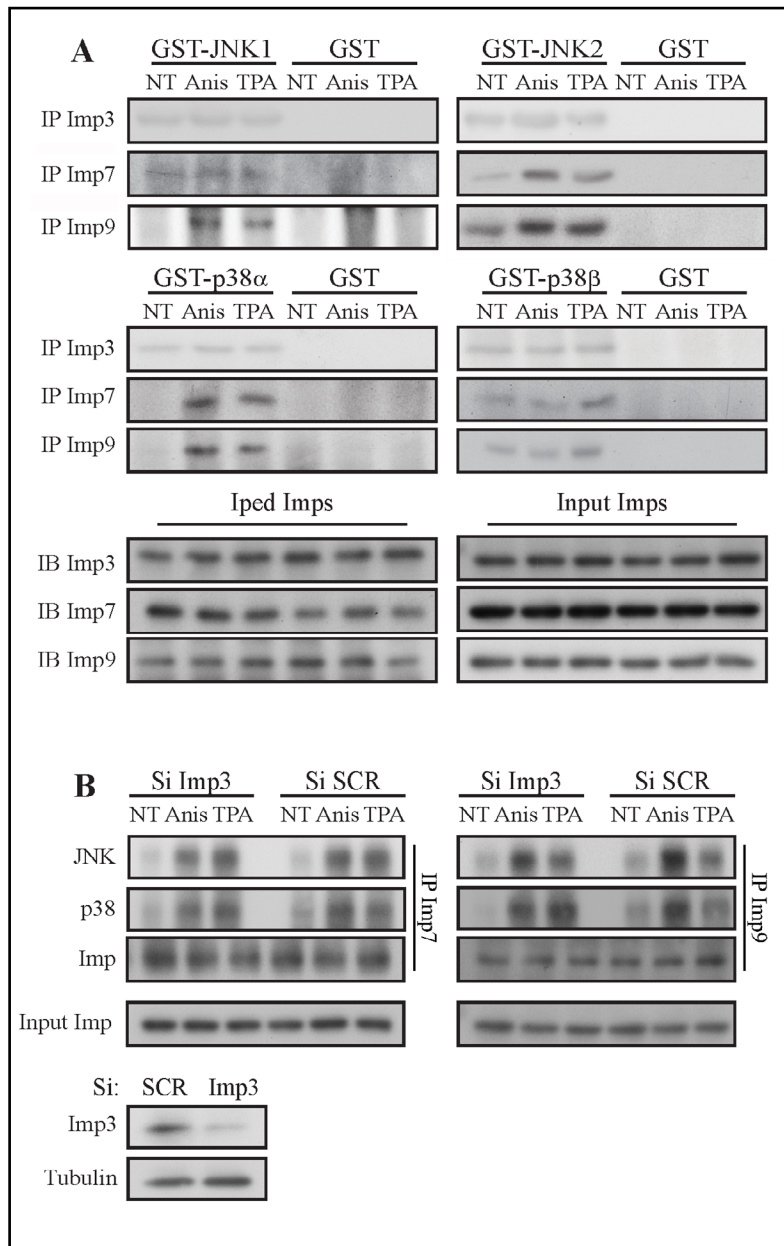
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Fig. 7. The subcellular localization of Imps3, 7 and 9 in resting and stimulated cells. Fluorescent microscopy detects the nuclear shuttling of Imp3/7/9. HB2 (A) or HeLa (B, C) cells were grown on slides to 70% confluence, serum starved, and then either stimulated with anisomycin (Anis 0.5 µg/ml 15 min) or left untreated (NT). Cells were fixed and stained using the indicated Abs. The nuclei were detected using DAPI and slides were visualized using either a fluorescent microscope (A, B) or Zeiss Spinning Disk confocal microscope (C). The bar in the upper right panel of A is of 20 µM. (D) Subcellular fractionation confirms the nuclear translocation of Imps 3,7 and 9. The same samples of Fig. 1D were used here. Serum-starved HeLa cells were either stimulated (Anis, 0.5 µg/ml, 15 min) or left untreated then harvested. Subcellular fractions of cytosolic and nuclear were produced as described, and subjected to Western blot analysis with the indicated Abs.

Gel filtration analysis of MAPKs-Imps interactions

To further validate this mechanism, we used gel filtration to separate unbound proteins from higher MW complexes. Indeed, Imps 3, 7 and 9 from resting cells appeared at their expected monomeric MW (~90 kDa for Imp3 and ~120 kDa for Imp7or Imp9; Fig. 10A). Imp3 also appeared in a ~160 kDa peak, which may represent either a homo or hetero

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Fig. 8. Imp7 and Imp9 but not Imp3 directly interact with JNK/p38 MAPKs. (A) *In vitro* binding assay demonstrate a direct Imp7 or Imp9 interaction with JNK1/2 and p38α/β. Imps 3, 7 and 9 were IPed from either stimulated or untreated cells (NT) and extensively washed with RIPA buffer following LiCl (0.5 M) and buffer A. To examine association, 500 ng of each indicated GST-MAPK were incubated with the indicated IPed Imps (2 hr with rotation) following washing and resuspention with sample buffer. Interacting MAPKs were detected using Western blotting with the indicated Abs. (B) Imp3 is not required for Imp7 or Imp9 interactions with MAPKs. SiRNAs of Imp3 and scrambled si of Imp3 that served as control (Si SCR) were transfected to HeLa cells, as described. Cell extracts were then subjected to CoIP with the indicated Abs. The amount of CoIPed MAPKs, Imp and input Imps were detected by Western blotting with the indicated Abs. To confirm the efficiency of the siRNA, cells were transfected with the indicated siRNAs, and then subjected to a Western blot analysis with the indicated Abs (lower panels). The experiments were reproduced three times.

dimer/trimer of the protein. In addition, a small amount of all three non-stimulated Imps was detected in another, 220-400 kDa (designated as ~280 kDa) peak (Fig. 10A). This relatively wide ~280 kDa peak may correspond to either a dimer of Imps, a heterotrimer containing a dimer of Imps with additional 30-80 kDa proteins (e.g. MAPKs), or a complex of the Imps with any high MW proteins (100-200 kDa). Importantly, the relative amount of the Imps in the various peaks was dramatically changed in extracts from stimulated cells. Thus, the amount of Imps 3, 7 and 9 in the lower MW peaks significantly decreased, while that in the higher ones, correspondingly, increased. In parallel, JNK, and p38 shifted from a sharp peak at ~40 kDa, to a very wide peak after stimulation (Fig. 10C).
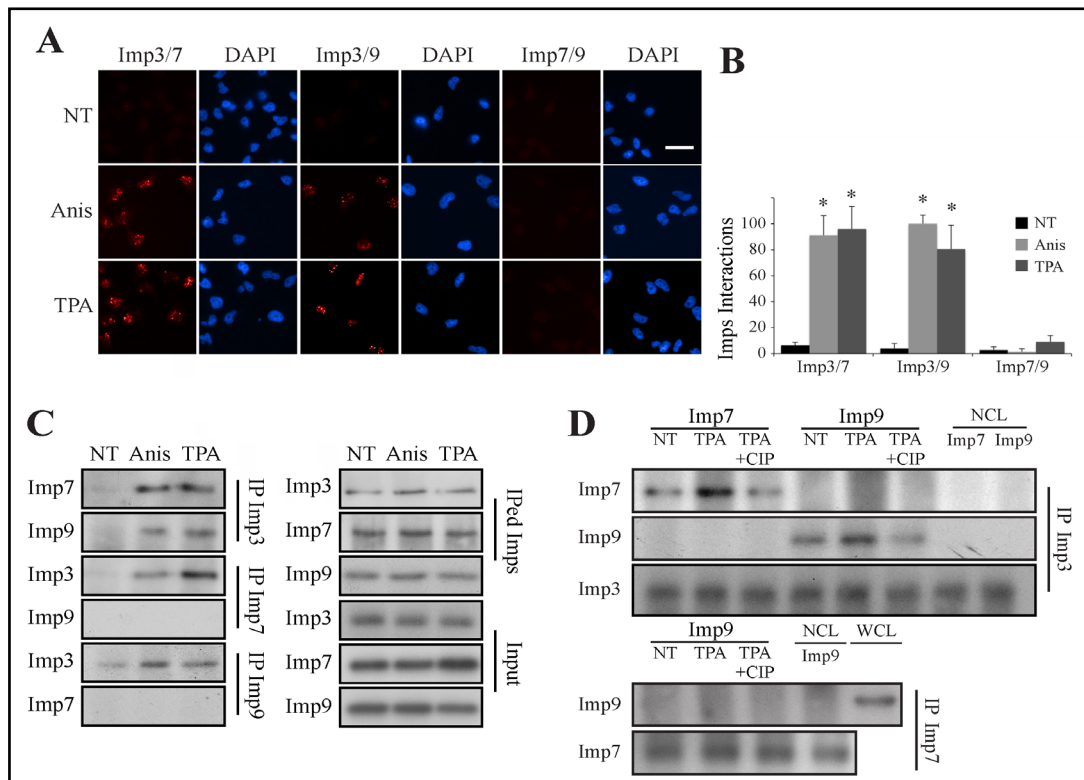
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Fig. 9. Imp3 interacts with Imp7/9-MAPK dimer in a phospho-dependent manner. (A) Proximity ligation assay demonstrates stimulus-dependent interaction of Imp3 with Imp7/9. HeLa cells were grown on slides to 70% confluence, serum starved and either stimulated with anisomycin (Anis, 0.5 μg/ml, 15 min) or left untreated (NT). The cells were then fixed and subjected to a PLA using the indicated Abs and visualized using a fluorescent microscope. The bar in the upper right panel is of 20 μM. (B) Quantification of the intensity of the signals was performed using ImageJ. The data shown represents mean ± S.E of three different experiments (* - p<0.0005). (C) CoIP assay confirms the interaction of Imp3/7 and Imp3/9. Serum-starved HeLa cells were stimulated with either anisomycin (Anis 0.5 μg/ml, 15 min), TPA (250 nM, 15 min) or left untreated (NT). Cells extracts were then subjected to CoIP with indicated Abs. Imps, IPed Imps and inputs were detected by Western blot with the indicated Abs. (D) The interaction of Imp3 with Imp7/9 is dependent on Imp3 phosphorylation. IPed Imp3 (upper panels) or Imp7 (lower panels) were purified from either stimulated or untreated cells, as described. Purified Imps were then incubated with calf intestinal phosphatase, (CIP, 1 hr, 37°C) or left untreated followed by washing. Recombinant Imps were incubated with the IPed Imps (2 hr, 37°C) and then washed. The interacting Imps were detected using Western blotting with the indicated Abs (NCL - no cell lysate, WCL - whole cell lysate). The experiments were reproduced twice.

In order to ascertain that the higher MW peaks of the Imps and MAPKs are formed, at least partially, by interaction between MAPKs and dimers of Imps, we resorted to CoIP experiments. As expected, no association between the components was detected in the ~120 kDa peaks (Fig. 10B); this was also true for the higher MW peak (~160 kDa) of Imp3 (data not shown). On the other hand, Imps 3, 7 and 9 CoIPed both JNK and p38 proteins from the stimulated, 280 kDa peak. In addition, the Imp3 CoIPed both Imp7 and Imp9, while no interaction between Imp7 and Imp9 was detected. The lack of interaction between Imp7 and Imp9 was observed in higher MW fractions as well, clearly indicating that no Imp3/7/9 trimers are formed after stimulation. No reproducible differences in MAPKs/Imps binding affinity were detected under the distinct stimulations, indicating redundant activities of the dimers with MAPKs. These results further support the stimulated formation of heterotrimers of two Imps with one MAPK, and the role of these heterotrimers for proper translocation to the nucleus.
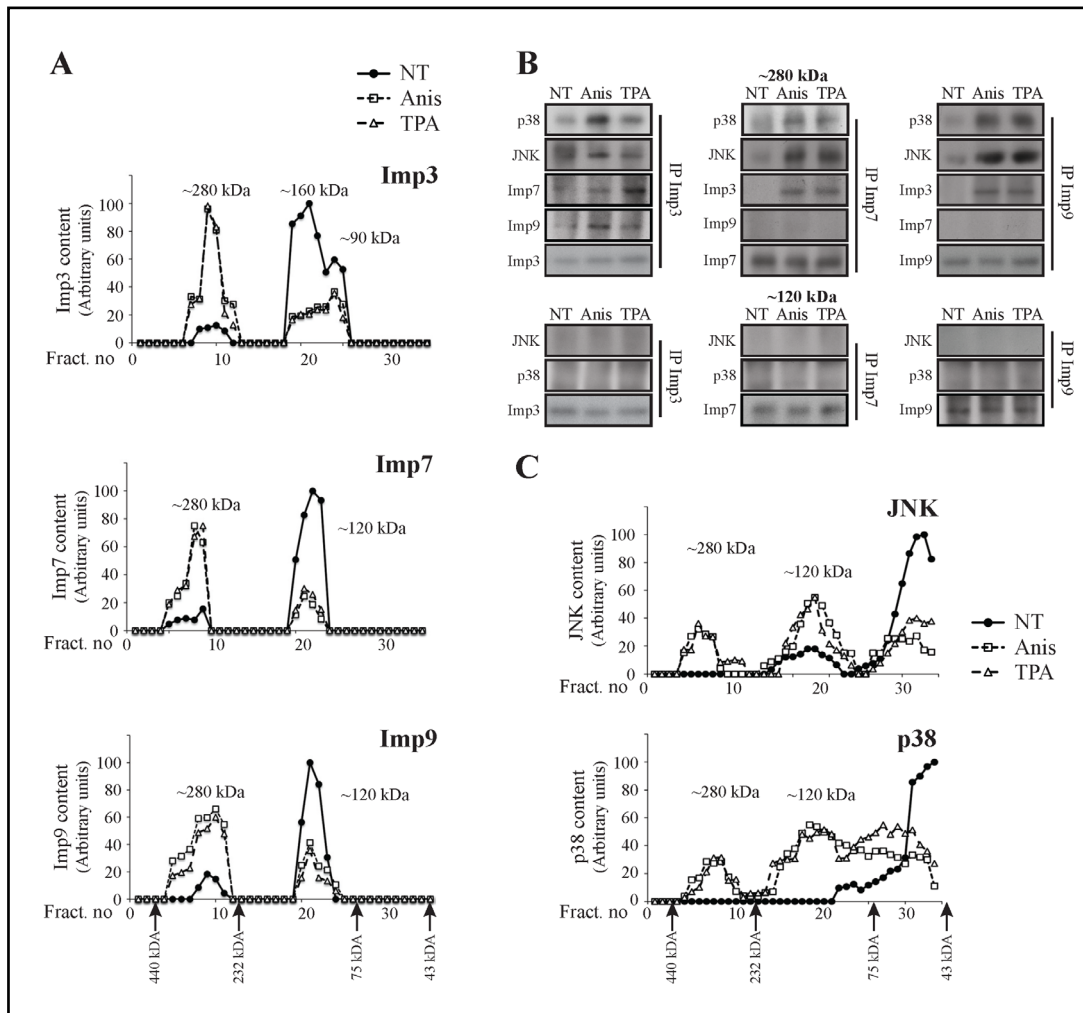
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Fig. 10. JNKs and p38s form complexes with dimers of either Imp3/7 or Imp3/9 after stimulation. (A) Gel filtration studies reveal MW shift of Imps 3, 7 and 9 upon stimulation. HeLa cells were serum starved and stimulated with anisomycin, TPA or left untreated (NT). Cell extracts (20 mg) were loaded on a 16/60 superdex 200 sizing column (1 ml/min), and 1ml fractions were collected. The fractions were then analyzed using Western blotting with the indicated Abs. (B) CoIP confirms association of JNKs and p38s with Imps dimers in the ~280 kDa but not the ~120 kDa peaks. Fractions representing the ~280 kDa and ~120 kDa peaks (fractions no. 9 and 22) from each of the Anis, TPA or non- treated stimulated columns, were subjected to CoIP with the indicated Imps Abs. The interacting proteins were detected using Western blotting with the indicated Abs. (C) JNK and p38 MAPKs form complexes upon stimulation. HeLa cells were serum starved and then stimulated with anisomycin, TPA or left untreated (NT). Cell extracts were loaded on a 16/60 superdex 200 sizing column (flow rate 1ml/min), and 1ml fractions were collected. The fractions were then analyzed, using Western blot with the anti JNK/p38 Abs, as indicated.

Discussion

Stimulation of cells by various extracellular agents induces changes in the localization of many signaling proteins into the nucleus (translocation), a step important for the regulation of transcription and induction of various cellular processes. Indeed, we have recently shown that prevention of the translocation of these MAPKs leads to a reduced proliferation of triple-negative breast cancer cells, reduced DSS-induced inflammation in mice, and reduced inflammation-related colon cancer in mice [25]. Despite the importance of the

stimulated translocation, the molecular mechanism that allows it, is not yet clear. Here, we studied the stimulated nuclear translocation of JNK1/2 and p38α/β, and found that all four kinases rapidly translocate to the nucleus upon stress and mitogenic stimuli. However, the molecular mechanism of these translocations seems to be distinct from that of ERK1/2 [8], or any other known translocation mechanisms [37, 39, 40]. Rather, the results presented here best fit a model in which upon stimulation, JNK1/2 and p38α/β are released from their cytoplasmic anchors by an activation-independent mechanism. This allows the formation of heterotrimers that include either active or inactive MAPKs, post-translationaly modified Imp7 or Imp9, and phosphorylated Imp3. The Imp3/Imp7 or Imp3/Imp9 dimers then escort the attached MAPKs to the nuclear envelope, where Imp3 remains, while Imp7/9 further penetrate into the nucleus together with the MAPKs (see scheme in [41]). Then, the complex is dissociated by GTP-Ran, which frees the MAPKs in the nucleus and allows the export of the Imps back to the cytosol.

MAPKs are just a small portion of the many proteins that translocate to the nucleus upon various extracellular stimulations. Thus, any stimuli induces a swift nuclear translocation of tens of millions of protein molecules, that are primarily required for the induction of transcription, and as a consequence, various cellular processes. This large scale and rapid translocation requires a handy and robust translocating machinary. Indeed, some of the proteins seem to use the canonical NLS-Impα/β for their translocation (e.g. NFkB [3], or ERK5 [5]). However, the limited number of Impα molecules, and the fact that most of them are preoccupied by housekeeping shuttling proteins [42], indicate that there are not sufficient free Impα molecules to carry out the rapid and massive translocation upon stimulation. In addition, the rapid nature of the process, as well as the size of most translocating proteins, do not allow a free diffusion or carrier-free penetration through NUPs interactions [39]. This suggests the action of stimulated, NLS- and diffusion-independent, translocation machineries. Indeed, our results do not show any interaction with either Impα or Impβ under the conditions used. However, a recent article has reported such interaction with Imps α2/β1 upon stress [43]. The reason for these discrepancy is not clear, but may occur due to distinct cell lines and stimulations used.

Our data here, as well as previous publications [12], strongly indicate that β-like Imps play key roles in NLS- and Impα-independent mechanisms. Despite the clear role of Imps 3, 7 and 9 in stimulation-dependent translocation shown here, previous studies demonstrated that these Imps induce nuclear shuttling of non-stimulated proteins as well. Therefore, it is likely that β-like Imps are able to mediate nuclear shuttling by more than one mechanism. An example for multiple mechanisms of action is demonstrated by Imp7, which induces shuttling of various proteins in both resting and stimulated cells. In resting cells, Imp7 appears to induce the nuclear shuttling of housekeeping (e.g. histones [44, 45], and ribosomal proteins [46]), as well as regulatory proteins (e.g. EZI [47], GR [48], and CDK5 activator [49]). Although Imp7 may act on its own, some reports demonstrated the requirement of its heterodimerization with Impβ [50, 51]. In all of the above cases, it was either shown, or speculated, that Imp7 binds canonical NLS or NLS-like sequences in their cargo. Interestingly, Imp7 also mediates the stimulated translocation of several signaling proteins (e.g. ERK1/2, MEK1/2, Smad3/4, EGR1) operating in a canonical NLS-independent manner by binding to specific sequences (e.g. NTS of ERK1/2) in its cargo [7, 8, 11, 52]. Here we show, yet another canonical NLS-independent mechanism for the stimuli-dependent nuclear translocation of JNK and p38 by Imp7. Thus, Imp7 is involved in multiple mechanisms and interactions with various cargo sequences to induce both stimulated and non-stimulated nuclear translocation of proteins. We also found that Imp7 undergoes a stimuli-induced modification to allow its binding to JNK and p38 MAPKs. Therefore, it is likely that the switch from one mechanism to another is mediated by post-translational modifications such as phosphorylation, ubiquitination or sulfation. The large variety of mechanisms by which Imp7 and the other β-like Imps operate may contribute to their specificity in regulating distinct processes upon different stimuli and conditions.

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Conclusion

In summary, we identified a novel mechanism of stimulated nuclear translocation of active/inactive JNK/p38 MAPKs, which is distinct from the stimulated translocation of ERK1/2. We show that this translocation involves binding of these MAPKs to either Imp7 or Imp9, which operate in conjugation with Imp3, in order to allow the nuclear translocation of the MAPKs. This is the first demonstration of dimerization of the β-like Imps with Imps other than Impβ itself. Unlike Impα/β, these heterotrimers then translocate to the perinuclear region, where they dissociate; Imp3 remains in the nuclear envelope, while the MAPKs, together with either Imp7 or Imp9, shuttle into the nucleus, in a Ran-dependent manner. This study clearly demonstrates that β-like Imps are central mediators of the stimulated nuclear translocation of JNK/p38 and the activation of transcription factors. Therefore, these components and the process of nuclear translocation are essential for the regulation of stimulated transcription and gene expression.

Disclosure Statement

The authors report no conflict of interest.

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