[Cell Cycle 7:2, 216-221; 15 January 2008]; ©2008 Landes Bioscience

Report

Basal c-Jun N-terminal kinases promote mitotic progression through histone H3 phosphorylation

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Abbreviations: NIMA, never in mitosis A; SAPK, stress-activated protein kinase; pH3, histone H3 phosphorylated at serine 10; MAPKs, mitogen-activated protein kinases; JNK, c-Jun N-terminal Kinase; DN-JNK1 or 2, dominant-negative JNK1 or 2

Key words: basal JNKs, SP600125, mitosis, histone H3, IMR90

Phosphorylation of histone H3 at serine 10 (S10) is essential for the onset of mitosis. Here, we show that basal c-Jun N-terminal kinases (JNKs) are required for mitotic histone H3-S10 phosphorylation in human primary fibroblast IMR90 cells. Inhibition of JNKs by specific pharmacologic inhibitors, expression of dominant-negative JNK1 and 2 mutants, or RNAi of JNK1 and 2 prevented phosphorylation of histone H3 at S10 in vivo. The JNK-specific inhibitor SP600125 blocked mitotic entry, as shown by its ability to prevent CDK1 dephosphorylation and cyclin A degradation. Basal JNK phosphorylation increased at G_2/M phase, although total JNK protein levels remained unchanged. In addition, basal JNKs were localized in nuclei and centrosomes during this time, suggesting that the nuclear localization of JNKs during G_2/M is tightly coupled with histone H3 phosphorylation. Basal JNKs were able to phosphorylate histone H3 in vitro and co-precipitation of histone H3 and JNKs was only detected at G_2/M. Taken together, these data strongly suggest that basal JNKs play a key role in controlling histone H3 phosphorylation for mitotic entry at G_2/M phase.

Introduction

Histone H3 associated with condensed chromosomes has long been recognized to become globally phosphorylated at serine 10 (S10) during mitosis. This phosphorylation of histone H3 has traditionally been regarded as a marker of mitosis.[1,2] Histone H3-S10 phosphorylation is involved in initiating, but not maintaining, mammalian chromosome condensation.[1] Recent reports revealed that never in mitosis A (NIMA) kinase and aurora kinase regulate the phosphorylation of histone H3 at S10 to trigger mitosis (reviewed in refs. 3 and 4).

In contrast to the global histone H3 phosphorylation associated with mitosis, a more selective pattern of histone H3-S10 phosphorylation is known to occur in response to various mitogens and stresses, and these stress-induced signaling pathways have been well documented.[5] The transient phosphorylation of histone H3 at S10 by mitogenic activation is closely related to the induction of immediate-early response genes, including the proto-oncogenes *c-fos* and *c-jun*.[6,7] Several kinases have been shown to be involved in histone H3-S10 phosphorylation elicited by mitogens or various extracellular stresses. They include the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERKs),[7,8] p38 kinases,[8,9] stress-activated protein kinase 1 and 2 (MSK1 and MSK2),[10] and ribosomal S6 kinase 2 (RSK2).[11]

C-Jun N-terminal kinases (JNKs) are members of MAPK family, well-known to be activated by diverse stresses. JNKs activated by extracellular stresses or inflammatory signals have been shown to phosphorylate histone H3 at S10, triggering transcriptional activation.[7,12] JNKs have other functions apart from those related to cellular stress responses. For example, they have been shown to regulate mitosis and the proliferation of unstressed cells. In embryonic fibroblasts, inactivation of MKK7, the upstream activating kinase of JNK, induces G_2/M cell cycle arrest and premature senescence, suggesting that MKK7-JNK signaling is directly responsible for the control of proliferation and cellular senescence (reviewed in refs. 13 and 14). Inhibition of JNKs using SP600125 or JNK1 and 2 antisense also leads to mitotic cell cycle arrest in several cancer cell lines.[15,16] However, the mechanisms by which JNKs modulate mitotic progression and cellular proliferation are not well understood. In this study, we demonstrate that the basal JNK activation at G_2/M transition mediates mitotic histone H3 phosphorylation at S10 to promote mitosis in primary fibroblast IMR90 cells.

Results

Selective inhibition of basal JNKs by SP600125 blocks cell proliferation and induces G_2/M cell cycle arrest. To approach the function of JNKs in the regulation of cell cycle progression, we first tested whether the selective inhibition of JNKs affects cell growth and proliferation. We chose to perform these tests in IMR90 human primary fibroblast cells, since transformed cell lines with oncogenic and/or tumor suppressor mutations may contain altered JNK signaling pathways. The results with IMR90 were also confirmed

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Submitted: 08/01/07; Revised: 10/11/07; Accepted: 10/11/07

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/article/5155

with mouse fibroblast NIH3T3 cells. Preliminary experiments with IMR90 and NIH3T3 cells revealed that a 72-hour incubation in 20 μM of the selective JNK inhibitor SP600125 resulted in approximately 50% viability as measured by MTT assays (data not shown). Based on these results, we used this concentration and treatment time of SP600125 for the cell growth and proliferation experiments. No increase in cell number was observed in IMR90 cells treated with SP600125, while untreated control cells continuously proliferated (Fig. 1A), demonstrating basal activity of JNKs is necessary for cell proliferation. This result was confirmed by examining cell proliferation in the presence of two other selective JNK inhibitors, JNK inhibitor I and JNK inhibitor III. IMR90 cells treated with each inhibitor also showed decreased cell proliferation (Fig. 1A). The function of basal JNKs for cell proliferation was further verified in cells where the expression of JNK1 and 2 was knocked downed by RNAi. Cell proliferation was reduced in IMR90 cells transfected with a combination of JNK1 and JNK2 RNAi duplex oligonucleotides and showed decreased JNK/SAPK expression, while cells transfected with control duplex proliferated continuously (Fig. 1B). These observations strongly support that the basal activity of JNKs is essential for cell proliferation.

To determine the stage of the cell cycle at which SP600125 may act to block cell proliferation, the DNA content of SP600125-treated IMR90 cells was analyzed by flow cytometry. Since IMR90 cells complete one cycle in roughly 24 hrs, analyses were performed at 24, 48 and 72 hr after SP600125 treatment. In control cultures, more than 80% of the cells contained 2N (G_1/S) DNA content. However, the majority (~80%) of SP600125-treated cells had 4N (G_2/M) DNA content at all time points examined (Fig. 1C). These results suggest that the inhibition of basal JNK activity may block proliferation by causing sustained cell cycle arrest at G_2/M.

To determine the precise stage of G_2/M arrest in SP600125-treated cells, we examined Cdc2 activation and cyclin A degradation in these cells. In mammalian cells, Cdc2 is activated at the beginning of mitosis via dephosphorylation at Y15.[18] Cyclin A becomes degraded in early mitosis after the nuclear envelope has broken down.[19] In our experiments, IMR90 cells were synchronized in G_1/S by double thymidine arrest and SP600125 was added 5.5–6 hr after the release, when the cells were mainly in G_2 (verified by FACS, data not shown), to exclude the potential effects of JNK inhibition to other cell cycle stages. SP600125-treated cells showed a significant delay in Cdc2 dephosphorylation and cyclin A degradation, while both events were apparent at 10 hr after release in control cells. These observations indicate that JNK activity is necessary for mitotic entry in unstressed cells (Fig. 1D).

Histone H3 phosphorylation at S10 is a well documented marker of late G_2 and early M for the initiation of chromosome condensation.[1] To further validate this finding that mitotic entry is delayed by inhibition of basal JNK activity, we tested the effect of SP600125 on histone H3 phosphorylation at S10. We also examined the effect of SP600125 on the nuclear translocation of cyclin B1, which occurs prior to nuclear envelope break down (NEBD) and is considered to be an early marker of mitotic entry.[20] IMR90 cells were synchronized, released for 5.5 hrs, treated with 20 μM SP600125 or DMSO for 1.5 hrs, and co-immunostained with antibodies against cyclin B1 and phosphorylated histone H3 (pH3) at S10. In control cells, the intensity of pH3 staining roughly correlated with the localization of cyclin B1: weak pH3 staining (G_2 phase) correlated with cytoplasmic cyclin B1, moderate pH3 staining (early prophase) correlated with both cytoplasmic and nuclear cyclin B1, and strong pH3 staining (late prophase) with nuclear cyclin B1 (Fig. 1E, left).[17] Most of the control cells exhibiting cyclin B1 nuclear translocation showed either moderate or strong pH3 staining (Fig. 1E, graph). However, most SP600125-treated cells showed little to no pH3 staining under these conditions with cyclin B1 being dispersed throughout the cytoplasm. These results support the finding that inhibition of basal JNK activity blocks mitotic entry and they strongly suggest that, during G_2/M transition, JNK activity is directly correlated with histone H3 phosphorylation at S10.

G_2/M cell cycle arrest by SP600125 was also confirmed in mouse nontransformed fibroblast NIH3T3 cells. When NIH3T3 cells were synchronized, released and treated with SP600125, very few pH3-positive cells were observed. This implies that the function of basal JNKs during G_2/M transition may not be cell-type specific (Fig. 1F).

Basal JNK activity is necessary for histone H3 phosphorylation during G_2/M phase. In previous experiments, we mainly used SP600125 to selectively block the activity of JNKs. However, SP600125 has been reported to inhibit global histone H3-S10 phosphorylation independently of JNK pathways.[21] Therefore, to confirm that the inhibition of histone H3 phosphorylation observed in Figure 1E was due to the inhibition of JNKs, rather than SP600125 itself, we examined histone H3 phosphorylation in the presence of other JNK-specific inhibitors, JNK inhibitor I and JNK inhibitor III. JNK inhibitor I blocks JNK signaling by preventing interactions between JNK and JNK-interacting protein-1 (JIP-1).[22] JNK inhibitor III selectively disrupts c-Jun/JNK complex formation and consequently, activation of c-Jun.[23] To further confirm whether the inhibition of histone H3-S10 phosphorylation is specifically due to the inhibition of JNKs, we examined histone H3-S10 phosphorylation in the presence of selective p38 inhibitor, SB203580. IMR90 cells were synchronized, released for 5.5 hrs, and incubated with SP600125, JNK inhibitor I, JNK inhibitor III, SB203580, or DMSO for 1.5 hrs. As shown in Figure 2A, both JNK inhibitor I and inhibitor III decreased the number of pH3-positive cells, although inhibitor I was less effective than inhibitor III. Both inhibitors were also less effective than SP600125 (Fig. 2A). These differences in efficacy are likely based on the mechanism of each inhibitor.[22,23] Although both JNKs and p38 are stress-activated protein kinases (SAPKs), SB203580-treated cells did not exhibit any decrease in mitotic histone H3-S10 phosphorylation compared to control cells (Fig. 2A). Taken together, these observations demonstrate that basal JNKs specifically control histone H3-S10 phosphorylation during G_2/M phase.

To test whether JNKs are responsible for histone H3 phosphorylation at G_2/M phase, we measured histone H3-S10 phosphorylation in the presence and absence of JNK inhibitors by immunoblotting. IMR90 cells were synchronized by double thymidine arrest, released for 5.5 hrs, and treated with SP600125 or JNK inhibitor III for 1.5 or 3.5 hrs (i.e., 7 or 9 hr after the release). Inhibition of JNK activity by SP600125 or JNK inhibitor III was confirmed by immunoblotting with anti-p-JNK/SAPK antibody (Fig. 2B). Neither inhibitor affected the expression of JNKs (data not shown). In control cells, histone H3-S10 phosphorylation increased from 1.5 to 3.5 hrs (Fig. 2B). However, the phosphorylation of histone H3-S10 was not observed both in SP600125-treated cells (at both time points) and in the JNK inhibitor III-treated cells (at 3.5 hr; Fig. 2B).

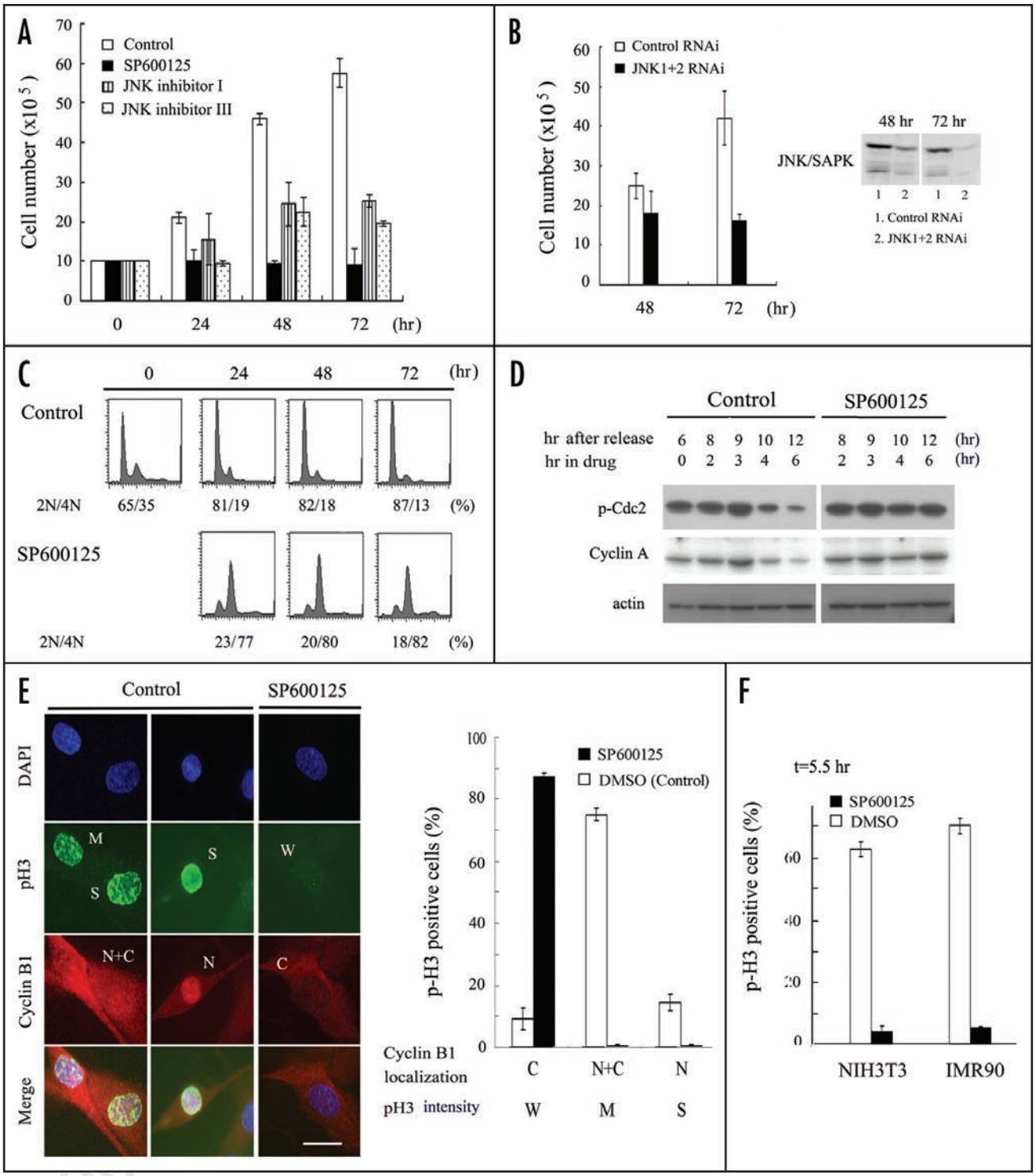

Figure 1. SP600125 inhibits cell proliferation and induces G_2/M cell cycle arrest. (A) Effects of SP600125 (20 μM), JNK inhibitor I (10 μM) and JNK inhibitor III (10 μM) on the proliferation of IMR90 cells. Data are shown as the mean (±SD) of three independent experiments. (B) Effect of the knock-down of JNK1 and 2 by RNAi on the proliferation of IMR90 cells. Depletion of JNK/SAPK expression was evaluated by immunoblots after RNAi oligonucleotides transfected cells were incubated for 48 or 72 hrs (right). (C) Effect of SP600125 (20 μM) on cell cycle progression, as measured by flow cytometric analysis. The fraction of cells with 2N and 4N DNA content is indicated. (D–F) G_2/M arrest by SP600125. IMR90 or NIH3T3 cells were treated with SP600125 (20 μM) following synchronization by double thymidine arrest and release for 5.5–6 hrs. (D) Immunoblot analysis for cyclin A, phosphorylated Cdc2 (Y15), and actin (loading control). (E) Immunofluorescence microscopy for Ser-10-phosphorylated histone H3 (pH3) and cyclin B1 performed 1.5 hr after SP600125 exposure (Scale bar, 10 μm). Nuclei were counterstained with DAPI. (E, graph) The number of pH3-positive cells was counted and sorted based on pH3 fluorescence intensity (W, weak; M, moderate; S, strong) and on the location of cyclin B1 (C, cytoplasm; CN, cytoplasm and nucleus; N, nucleus). Only cells in G_2 or prophase prior to nuclear envelope breakdown were counted. (F) The percent of pH3-positive cells following treatment of IMR90 or NIH3T3 cultures with SP600125 for 1.5 hr and immunostaining with anti-pH3 antibody and DAPI. For each condition, 200 cells were counted, and the percentage of pH3-positive cells was determined from the total number of DAPI-stained cells. Data represent the mean (±SD) of three independent experiments.

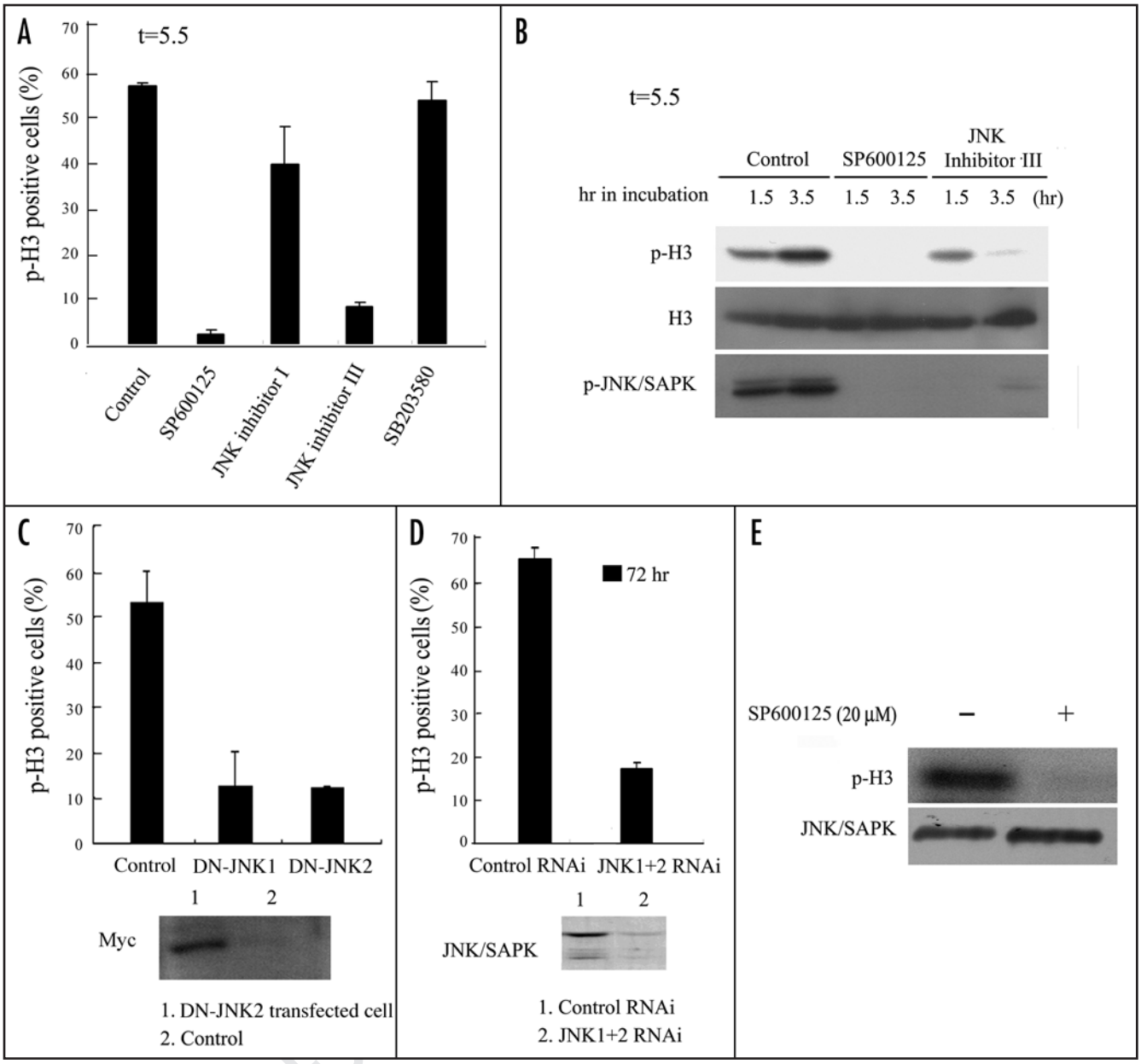

Figure 2. JNK inhibition blocks the phosphorylation of histone H3 at serine 10. (A and B) IMR90 cells were synchronized by double thymidine arrest, released for 5.5 hrs (t = 5.5), and treated with SP600125 (20 μM), JNK inhibitor I (10 μM), JNK inhibitor III (10 μM), SB203580 (20 μM), or DMSO as indicated. (A) Cells were treated with each inhibitor for 1.5 hrs and processed for Ser-10-phosphorylated histone H3 (pH3) immunofluorescence staining. The mean (±SD) percentage of pH3-positive cells was determined in three independent experiments, based on the total number of DAPI-stained cells. (B) Immunoblot analysis of pH3, histone H3, and phosphorylated JNK/SAPK. (C) IMR90 cells transfected with DN-JNK1 or DN-JNK2 were synchronized as described in Materials and Methods, released for 7 hr, and processed for pH3 immunofluoresence staining. Expression of DN-JNK2 was confirmed by anti-Myc immunoblotting. (D) IMR90 cells where the expression of JNK1 and 2 was knocked down by RNAi as described in Materials and Methods, were immunostained with pH3 and DAPI. JNK/SAPK depletion was shown by an immunoblot (lower). (C and D) The mean percentage (±SD) of pH3-positive cells was determined as in (A). (E) In vitro kinase assay of JNKs immunoprecipitated from the cells synchronized by double thymidine arrest and released for 7 hrs. Kinase activity was assayed in the presence or absence of 20 μM SP600125. ^{32}P-labeled histone H3 was detected by autoradiography (upper). Immunoprecipitated JNKs were detected by immunoblot (lower).

To further verify these results, we examined the effect of dominant negative (DN) JNK1 or 2 on histone H3-S10 phosphorylation in IMR90 cells. Immunoblotting was performed to confirm the expression of DN-JNK1 (data not shown) and DN-JNK2 (Fig. 2C). As shown in Figure 2C, the number of pH3-positive cells in DN-JNK1- or DN-JNK2-transfected cultures was much lower than that in control (untransfected) cultures.

The control of histone H3 phosphorylation by basal JNKs at the G_2/M was also verified in cells where the expression of JNK1 and 2 was knocked down by RNAi. 72 hrs after the RNAi oligonucleotides were transfected, the expression of JNK1 and 2 was vastly diminished as shown by an immunoblot (Fig. 2D). In these cells, the histone H3 phosphorylation at S10 was decreased (Fig. 2D). These results by the block of basal JNK activity using dominant negative JNKs or RNAi

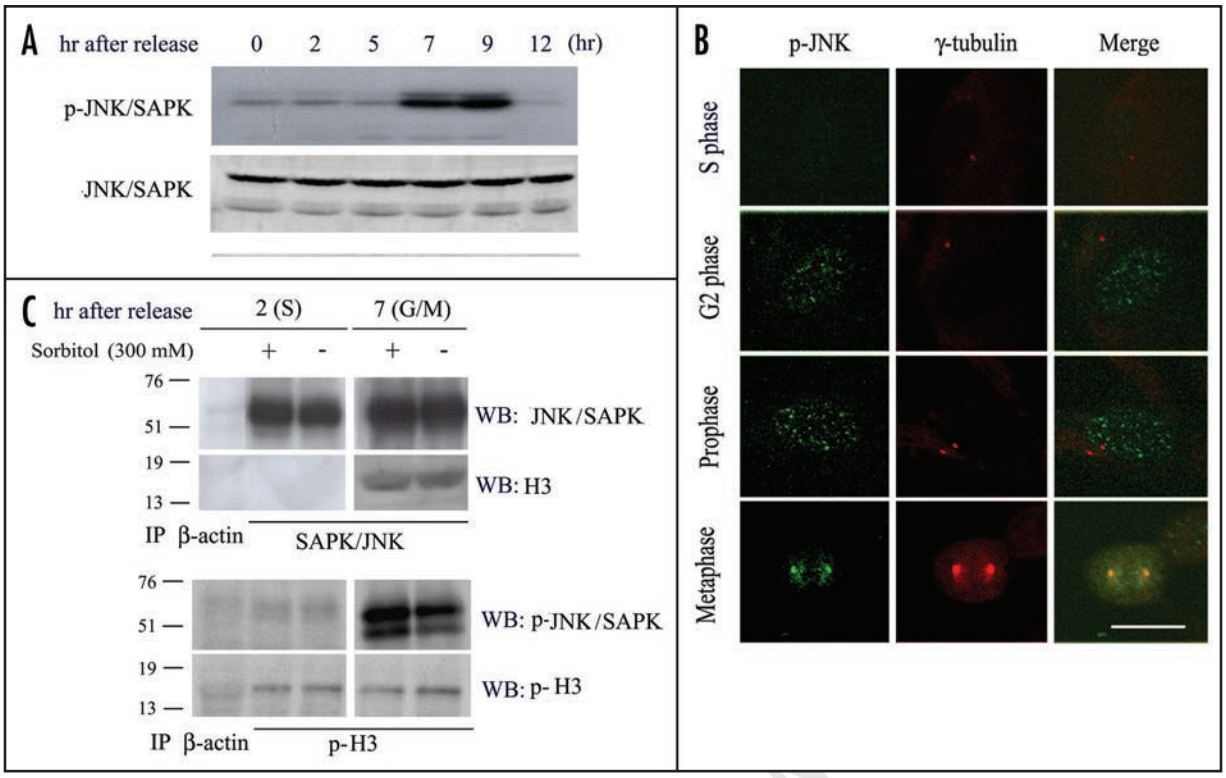

Figure 3. Cell cycle-dependent activation and localization of basal JNKs. (A) IMR90 cells synchronized by double thymidine arrest, harvested at the indicated time after release, and lysates were subjected to immunoblot analysis for phosphorylated and total JNK/SAPK. (B) Alternatively, to observe the localization of phosphorylated JNK1/2 at various stages of the cell cycle, cells were processed for immunofluorescence staining of phosphorylated JNK1/2 and γ-tubulin. (Scale bar, 10 μm) (C) Cells released for 1.5 or 6.5 hrs were exposed to osmotic stress (300 mM sorbitol) for 30 min or left untreated (thus, 2 or 7 hr after the release in each case). From these cells, JNK/SAPK was immunoprecipitated and probed for histone H3 (upper) and, in reverse, pH3 was immunoprecipitated and probed for p-JNK/SAPK (lower) in western blots. Immunoprecipitation using anti-β-actin antibody served as a negative control.

are consistent with those obtained by using selective JNK inhibitors, and demonstrate that basal JNK1/2 regulates mitotic entry by controlling histone H3-S10 phosphorylation.

We then checked whether JNKs can directly phosphorylate histone H3 in vitro. IMR90 cells at G_2/M phase were obtained by double thymidine arrest and release for 7 hrs. JNKs immunoprecipitated from these cells were then assayed for kinase activity. The result revealed that JNKs purified from G_2/M cells directly phosphorylated histone H3 (Fig. 2E). Moreover, this phosphorylation was completely inhibited in the presence of SP600125 (Fig. 2E).

Basal JNKs become activated and directly interact with histone H3 at G_2/M phase. Our data up to now showed that basal JNKs promote mitotic progression by controlling histone H3 phosphorylation during the G_2/M phase of the cell cycle. To further prove the function of basal JNKs for mitotic entry, we investigated whether basal JNK1/2 becomes activated, independently of any stresses, during cell cycle progression. We observed that the phosphorylation and activation of basal JNKs is cell cycle-dependent. Immunoblot analysis of phosphorylated JNK1/2 revealed that these active forms of the protein were detected only between 7 and 9 hr after the release from double thymidine arrest, when most cells were in G_2/M or M phase (Fig. 3A). Total levels of basal JNKs remained unaltered throughout the cell cycle (Fig. 3A).

In addition to immunoblot analysis, we performed immunofluorescence staining of phosphorylated JNK1/2. Cells were co-stained for γ-tubulin, which served as a marker for centrosomes and provided a reference for each cell cycle stage. Consistent with immunoblot results, no phosphorylated JNK1/2 was detected in S phase (Fig. 3B). However, phosphorylated JNK1/2 began to localize to nuclei at late G_2 phase. This localization became more intense in prophase, and in metaphase, phosphorylated JNK1/2 was concentrated at centrosomes (Fig. 3B). This pattern of localization during G_2/M phase agrees well with the proposed function of JNK1/2 in promoting mitotic entry via histone H3 phosphorylation. It is also consistent with the localization of other mitotic kinases that have been reported to control histone H3 phsophorylation. For example, NIMA and aurora A kinases have been shown to be present in the nucleus during late G_2 and concentrated at centrosomes in early mitosis.[4,24] Also, aurora B kinase has been shown to co-localize with the phosphorylated form of histone H3.[25]

The localization of basal JNKs to the nucleus and centrosomes at G_2/M phase as well as their ability to phosphorylate histone H3 in vitro led us to examine whether JNKs directly interact with histone H3 in vivo to control mitotic entry. To do this experiment, IMR90 cells were synchronized by double thymidine arrest and released for either 2 hrs (S-Phase) or 7 hrs (G_2/M-Phase). JNKs or phosphorylated histone H3 were immunoprecipitated from these cells and then probed for histone H3 or p-JNK/SAPK, respectively. Histone H3 co-immunoprecipitated with JNKs and, in reverse, phosphorylated form of JNKJ/SAPK co-immunoprecipitated with phosphorylated histone H3 in cells that had been released for 7 hrs, but not in those released for 2 hrs (Fig. 3C). To understand whether

the stress-induced JNK activation alters this pattern of JNK-histone H3 interactions, we repeated these co-precipitation experiments in cells that had been treated with 300 mM sorbitol for 30 min to activate JNK/SAPK by osmotic stress. The results were identical to those obtained in non-stressed cells (Fig. 3C).

Discussion

JNKs are well-known for their ability to induce immediate-early gene expression in response to various extracellular stresses through histone H3 phosphorylation at S10. In this study, we have demonstrated a novel function of basal JNKs, which is separate from that of the stress-activated protein. Our data reveal that, in primary IMR90 cells, basal JNKs function at G_2/M phase to control histone H3 phosphorylation at S10 for mitotic onset. Previously, several studies have provided clues as to the function of JNKs in cell cycle regulation. For example, inactivation of MKK7 is lethal to mouse embryos, and *MKK7*$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibit impaired proliferation as well as G_2/M cell cycle arrest.[14] Also, inhibition of basal JNK activity in several cancer cell lines leads to G_2/M arrest.[15,16] Nonetheless, the mechanism by which JNKs control cell cycle progression and especially the G_2/M transition is not well understood. Our findings are consistent with these studies and strongly support a role for basal JNKs in promoting cell cycle progression and proliferation. Moreover, our results provide a possible mechanism by which JNKs promote G_2/M transition.

Although some studies have suggested that basal JNKs may play a role in cell proliferation,[15,16] the function of JNK signaling in the proliferation of cancer cell lines is controversial. For example, Kennedy et al. have shown that JNK1/2 attenuates cell proliferation after NIH3T3 cells are transformed by Ras.[26] We reasoned that, in transformed cell lines, mutations in oncogenic pathways may affect signaling pathways responsible for basal JNK activity. Thus, to understand the role of basal JNKs in cell cycle progression, we chose to study early-passage, non-transformed IMR90 cells in the absence of extracellular stress. In these cells, the role of JNKs in promoting mitotic entry via histone H3 phosphorylation is evident. However, the effect of JNKs on mitotic entry might vary in transformed cells depending on the genetic background of each cell line.

Our results clearly show that JNKs become activated only at G_2/M phase and that inhibition of JNK activity blocks histone H3-S10 phosphorylation. Although our data reveal that JNKs promote mitotic entry by phosphorylating histone H3, it is unclear if this phosphorylation event is direct or indirect. The fact that basal JNKs co-precipitate with H3 as well as the phosphorylated H3 co-precipitates with the phosphorylated active form of JNK/SAPK in cells exclusively at G_2/M phase and that basal JNKs from the cells at the G_2/M are able to directly phosphorylate H3 in vitro, suggest that basal JNKs directly interact with and phosphorylate histone H3 during G_2/M phase. Nevertheless, JNKs are localized to nuclei at late G_2 phase and in centrosomes at early mitotic stages. This cell cycle-dependent pattern of localization mirrors that of NIMA and aurora kinases, which are known to phosphorylate histone H3 for mitotic entry. Based on this, we cannot exclude the possibility that basal JNKs control H3 phosphorylation through NIMA and/or aurora kinases. Additional studies will be necessary not only to clarify this question, but also to elucidate the signaling pathway(s) that mediate activation of JNKs at G_2/M phase and ultimately, control mitotic entry.

Acknowledgements

This work was supported by a grant from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (NCCR-0620110-1, 2007-8-0734) and a grant from the Korean Research Foundation (MOEHRD) (KRF-2006-8-1284).

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