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Phorbol 12-myristate 13-acetate (PMA)-induced migration of glioblastoma cells is mediated via p38MAPK/Hsp27 pathway

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ABSTRACT

We investigated the mechanism of phorbol 12-myristate 13-acetate (PMA)-induced migration of glioblastoma cells focusing on the p38 mitogen-activated protein kinase (MAPK)/heat shock protein 27 (Hsp27) pathway. PMA-induced cell migration and activation of p38MAPK in A172 glioblastoma cells. PMA-induced formation of lamellipodia and focal complexes was blocked by inhibiting p38MAPK with SB203580 or small interfering RNA (siRNA). Furthermore, activation of p38MAPK resulted in phosphorylation of an F-actin polymerization regulator, Hsp27. Immunohistochemical analysis showed that upon PMA stimulation, both unphosphorylated and phosphorylated Hsp27 were translocated to the lamellipodia. SB203580 or p38MAPK siRNA blocked these phenomena, indicating that Hsp27 phosphorylation and translocation from cytosol to membrane were mediated by p38MAPK. To address the question of whether endogenous Hsp27 participates in PMA-induced migration, we inhibited the expression of Hsp27 using Hsp27 siRNA. Although knockdown of Hsp27 by siRNA had little effect on p38MAPK activation, lamellipodia and focal complex formation was markedly inhibited. Migration was also abolished in Hsp27 siRNA-transfected cells. In conclusion, p38MAPK activation followed by Hsp27 phosphorylation was required for PMA-induced migration. Furthermore, Hsp27 itself played critical roles in PMA-induced migration. Our data provide substantial evidence for a model elucidating the molecular mechanisms of regulation of actin dynamics and migration by PMA-activated protein kinase C in glioblastoma cells.

1. Introduction

Glioblastoma cells migrate into surrounding normal brain tissue distinct from the primary site. This characteristic makes glioblastoma difficult to surgically resect and cure. Therefore, regulation of the invasiveness of glioblastoma cells would make it possible to develop effective new treatments for glioblastomas.

Protein kinase C (PKC) comprises a family of serine–threonine kinases that catalyze numerous biochemical reactions critical to the function of many cellular constituents [1,2]. The total PKC expression and activity levels are significantly higher in normal nervous tissue compared with non-neuronal tissue, suggesting that this enzyme system plays a fundamental role in normal central nervous system physiology [3]. PKCs have been implicated in the regulation of astrocyte

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; Hsp, heat shock protein; siRNA, small interfering RNA; PKC, protein kinase C; BIS, bisindolylmaleimide I; DMSO, dimethyl sulfoxide

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growth [4]. Furthermore, PKC activity levels have been found to be much higher in neoplastic astrocytes than normal glial cells [5,6]. Recently, it was reported that PKCs are also involved in the invasion of glioma cells [7].

Heat shock proteins (Hsp) are highly conserved proteins that are expressed in response to various forms of stress and environmental challenges. Some Hsps are also expressed under non-stress conditions and act as molecular chaperones [8]. In tumor cells, Hsps are associated with tumorigenicity, multidrug resistance and apoptosis [9,10]. Malignant gliomas express several Hsps in vivo, including members of the small Hsp family, Hsp27, Hsp72 and Hsp90 [8]. We previously reported that geldanamycin, an inhibitor of Hsp90, inhibited migration in glioblastoma cells [11]. This drug induces the degradation of Chk1 protein, which is chaperoned by Hsp90, through a ubiquitin-proteasome pathway [12].

A correlation between Hsp27 expression and grade of malignancy has been shown in astrocytic tumors [13,14]. Hsp27 is a molecular chaperone that protects cells from external stimuli and can contribute to the high resistance of solid tumors to chemotherapeutic agents [9]. Hsp27 is a substrate for the mitogen-activated protein kinase-activated protein (MAPKAP) kinases 2 and 3, which are phosphorylated by p38 mitogen-activated protein kinases (MAPK) α and β in response to various stimuli [15–18]. It is well known that PKC activates members of the MAPK family, p38MAPK, extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK). Furthermore, previous reports showed that phosphorylation of Hsp27 induced by MAPKAP kinase 2 was essential for the modulation of actin dynamics in smooth muscle [18] and endothelial cells [19].

Although several reports about PKC and glioblastoma migration have been published [7,20–22], the mechanism of PKC-induced migration of glioblastoma cells has not been completely clarified. To investigate the molecular mechanism underlying the PKC-induced migration, phorbol 12-myristate 13-acetate (PMA) was used to activate PKC. In this study, we showed that PMA-induced activation of the p38MAPK/Hsp27 cascade followed by formation of lamellipodia and focal complexes, and finally increased the migratory activity. Knockdown of endogenous p38MAPK or Hsp27 by small interfering RNA (siRNA) resulted in inhibition of PMA-induced formation of lamellipodia and focal complexes as well as decreased migratory ability. These results showed that PKC activation by PMA was involved in glioblastoma cell migration through the p38MAPK/Hsp27 pathway.

2. Materials and methods

2.1. Reagents and antibodies

PMA and bisindolylmaleimide I (BIS) were purchased from Sigma–Aldrich Corporation (St. Louis, MO). SB203580, a SAPK/p38 inhibitor, was purchased from Chemicon Int. (Temecula, CA). PMA, BIS and SB203580 were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO).

The primary antibodies used were mouse anti-p38MAPK monoclonal antibody; rabbit anti-phospho-p38MAPK (Thr180/Tyr182) polyclonal antibody; mouse anti-Hsp27 monoclonal antibody; rabbit anti-phospho-Hsp27 (Ser82) polyclonal antibody (Cell Signaling Technology, Beverly, MA); rabbit anti-phospho-Hsp27 (Ser15) polyclonal antibody; and rabbit anti-phospho-Hsp27 (Ser78) polyclonal antibody (Stressgen, Victoria, Canada).

2.2. Cell lines

A172 glioblastoma cells were obtained from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Bio Whittaker, Rockland, ME) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Cells were passaged every 3–4 days to ensure logarithmic growth.

2.3. Stimulation of PKC by a specific PKC inducer

PMA was used to activate PKC. The glioblastoma cells were incubated in serum-free medium for 24 h. Then PMA was added to the culture medium and the cells were cultured for 0–120 min.

2.4. Inhibition of kinase activity by specific inhibitors

BIS and SB203580 were used for the inhibition of PKC and p38MAPK activity, respectively. Initially, glioblastoma cells were cultured in serum-free medium for 24 h. Then BIS or SB203580 was added to the culture medium to the concentration of 5 or 20 μM 30 min prior to the addition of PMA. The cells treated with inhibitors were incubated with PMA for 60 min and subjected to further analysis.

2.5. Migration assay

The cells were seeded in 12-well culture dishes (BD Sciences) and cultured until they became subconfluent. The cells were then incubated with serum-free culture medium for 24 h, and were scraped with a 200 μl micro-pipet tip and washed with PBS. The cells were incubated with serum-free culture medium for an additional 16 h with PMA or vehicle. When specific inhibitors were used, an appropriate concentration of each inhibitor was added to the culture medium 30 min before PMA treatment. After 16 h of incubation, the cells were photographed, and the migrated area was measured using NIH Image software.

2.6. Transfection of siRNA into glioblastoma cells

siRNA for p38MAPK was purchased from Upstate Inc. (Chicago, IL). siRNA for Hsp27 was purchased from Cell Signaling Technology. For control experiments, control siRNA-A (Santa Cruz) was used. Reagents for transfection (Lipofectamine 2000 and Opti-MEM I reduced serum medium) were obtained from Invitrogen (Carlsbad, CA). Transfection of siRNA into the glioblastoma cells was done according to the manufacturer's instructions. After a 48 h incubation of the cells with siRNA, the cells were cultured for an additional 24 h

with serum-free medium. The transfected cells were incubated with PMA or vehicle for 60 min and then subjected to Western blot analysis or fluorescence immunohistochemistry.

2.7. Cytosolic and membrane fractions of cells

Western blot analysis of cytosolic and membrane fractions of cells was carried out as described [23]. The cells treated with PMA for 0–60 min were washed with PBS and harvested in ice-cold homogenization buffer (20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 0.25 M sucrose and protease inhibitors). The cells were homogenized on ice and centrifuged at 15,000 rpm for 45 min. The supernatant was recovered as the cytosolic fraction. The pellet was resuspended in homogenization buffer containing 1% Triton X-100 and incubated on ice for 30 min. Then the suspension was centrifuged at 15,000 rpm for 45 min, and the supernatant was recovered as the membrane fraction.

2.8. Western blot analysis

Total cells were harvested from each culture condition at the appropriate time intervals and washed with ice-cold PBS. The total protein was then extracted using lysis buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 2 μg/ml pepstatin. The samples were centrifuged at 15,000 rpm for 30 min at 4 °C, and the extracts were stored at −80 °C until use. Protein concentration was determined using the BCA assay (Pierce, Rockford, IL).

For Western blot analysis, equal amounts of protein (20–30 μg) were electrophoresed on an SDS-PAGE gel, transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium 0.45 micron, Bio-Rad, Hercules, CA) and stained with Ponceau S (Sigma). After confirmation of protein transfer, proteins were detected with specific antibodies. All primary antibodies were used at 1:1000 dilution. Actin protein was detected as a control with mouse anti-human β-actin monoclonal antibody (Chemicon) used at 1:10,000 dilution. Sheep anti-mouse IgG or donkey anti-rabbit IgG horseradish peroxidase-linked secondary antibodies (Amersham, Piscateway, NJ) at 1:4000 dilution were used as secondary antibodies. Protein detection was performed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and signals were visualized using Hyperfilm ECL (Amersham).

2.9. Fluorescence immunohistochemistry

Cells were plated in 8-well chamber slides and incubated overnight. After attachment of the cells to the slides, the cells were incubated with serum-free culture medium for 24 h. After a 30 min pre-treatment with specific inhibitors or vehicle, 100 nM PMA was added to the culture medium without serum and the cells were incubated for 60 min. Then, the cells were fixed with 3.7% paraformaldehyde for 20 min and washed with PBS three times. The cells were permeabilized with 0.2% Triton X-100 for 5 min and washed with PBS three times. After blocking with 2% BSA, the cells were incubated at room temperature with specific primary anti-

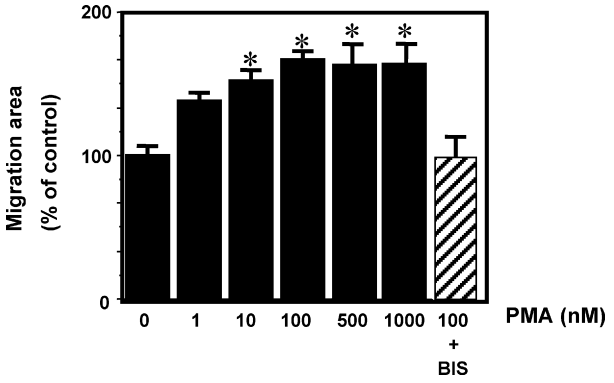

Fig. 1 – PMA-induced cell migration dose-dependently in A172 glioblastoma cells. Serum-starved cells were wounded and treated with various concentrations of PMA with or without BIS. The cells were photographed and the migrated area was measured 16 h after wounding. Results from three independent experiments are presented. Migration is expressed as the percentage of unstimulated cells. *$p < 0.05$, compared to the control, Student's t-test.

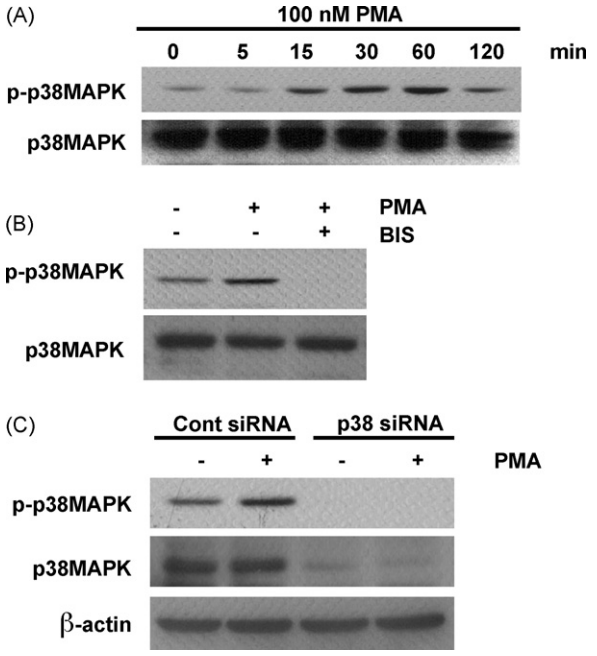

Fig. 2 – Effect of PMA stimulation and inhibitors on expression and phosphorylation of p38MAPK. (A) The cells were treated with 100 nM PMA for the indicated times. (B) For inhibition experiments, BIS was added to serum-starved cells 30 min prior to PMA stimulation. (C) For transfection experiments, the cells were transfected with p38MAPK or control siRNA. The siRNA-transfected cells were incubated without serum for 24 h. Then the cells were stimulated with PMA or vehicle for 60 min. Expression and phosphorylation of p38MAPK were examined by Western blot analysis. p38MAPK activation was evaluated with an anti-phospho-p38MAPK-specific antibody. The amount of total p38MAPK was determined by reprobing the membrane with anti-p38MAPK antibody after stripping.

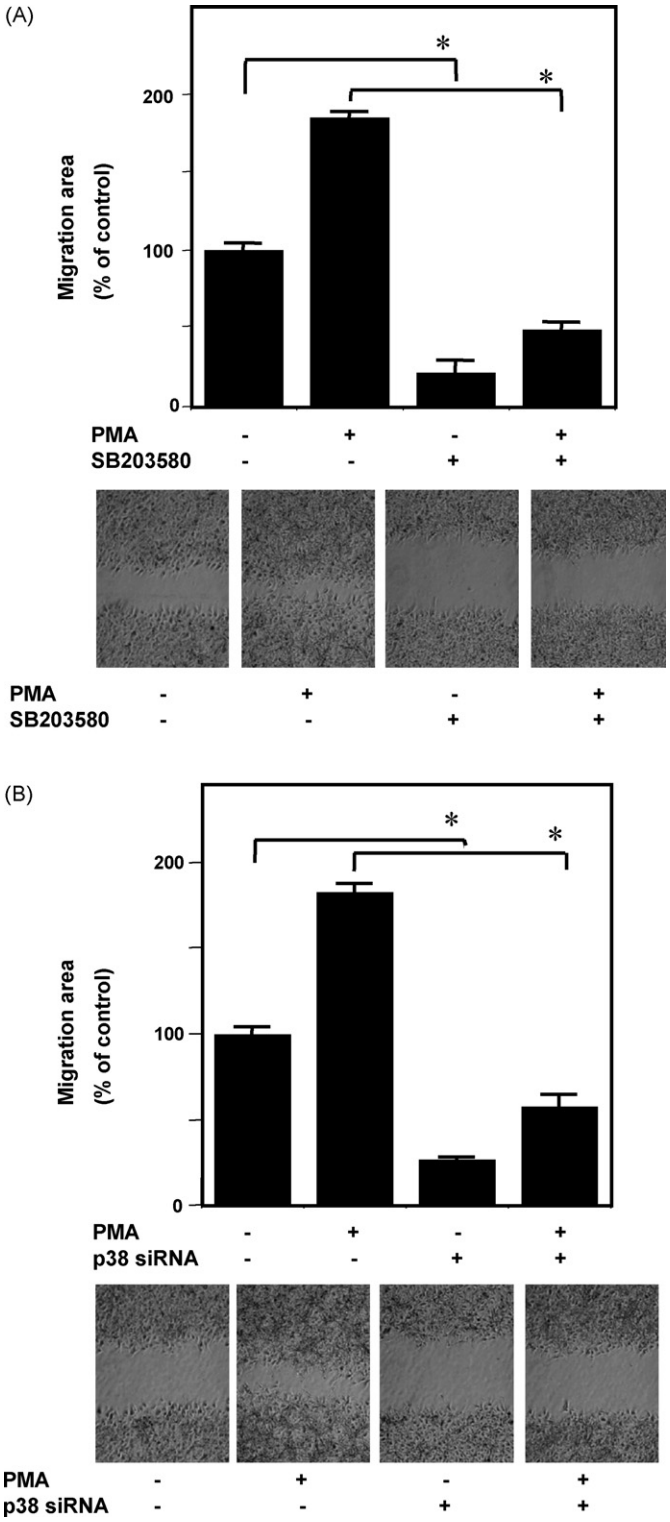

Fig. 3 – Effect of PMA stimulation and inhibitors on migration of glioblastoma cells. (A) For the migration assay, serum-starved cell cultures were wounded. After wounding, the cells were pretreated with SB203580 or vehicle for 30 min prior to stimulation with PMA (100 nM). (B) p38MAPK siRNA-transfected cells were incubated without serum for 24 h and the cells were wounded. Then the cells were stimulated with PMA. After 16 h, migration was evaluated. Results from three independent experiments are presented. Migration is expressed as the percentage of unstimulated cells. *$p < 0.05$, Student's *t*-test.

bodies at a dilution of 1:100. Then the cells were washed with PBS three times and were incubated at room temperature with fluorescence-labeled secondary antibodies at 1:200 dilution. After washing with PBS, the samples were mounted. Alexa fluor 488 phalloidin (Invitrogen) was used for detection of F-actin, which appeared green under a fluorescence microscope. The secondary antibodies used were Alexa fluor 594 goat anti-rabbit antibody and Alexa fluor 594 goat anti-mouse antibody (Invitrogen). Both secondary antibodies appeared red under a fluorescence microscope.

2.10. Statistical analysis

All experiments were performed at least three times. The data are expressed as the mean ± standard error of the mean (S.E.M.). Probability (P) was calculated using a Student's *t*-test. *p*-Values lower than 0.05 were considered significant.

3. Results

3.1. PMA-induced migration of glioblastoma cells via PKC activation

Firstly, to investigate whether PKC induces migration of glioblastoma cells, the cells were treated with various concentrations of a PKC stimulator, PMA. Migration was analyzed using A172 glioblastoma cells. As shown in Fig. 1, cell migration was induced by PMA dose-dependently. To confirm that the PMA-induced migration of glioblastoma cells was dependent on PKC activation, a PKC-specific inhibitor, BIS, was used [24,25]. The results showed that PMA-induced migration was abrogated by BIS. These results indicated that PMA-induced migration of A172 glioblastoma cells was mediated via PKC activation.

3.2. PMA-induced migration via p38MAPK activation in glioblastoma cells

Since p38MAPK is involved in migration, we analyzed the effect of PMA stimulation on p38MAPK in glioblastoma cells. We examined the phosphorylation of p38MAPK upon PMA stimulation. As shown in Fig. 2A, phosphorylation of p38MAPK was induced after 15 min of PMA stimulation and increased up to 60 min. To examine whether the phosphorylation of p38MAPK was induced by PKC, BIS was added to the culture medium prior to PMA stimulation (Fig. 2B). BIS abolished the phosphorylation of p38MAPK. The results indicated that PMA-induced p38MAPK activation was dependent on PKC activation.

Then we assessed the effect of the p38MAPK activity on PMA-induced cell migration. As shown in Fig. 3A, SB203580 significantly decreased the PMA-induced migration of the cells. To achieve more specific inhibition, the expression of endogenous p38MAPK was inhibited by siRNA. As shown in Fig. 2C, we confirmed by Western blot analysis that siRNA inhibited the endogenous p38MAPK protein expression. The phosphorylation of p38MAPK was also inhibited by the siRNA. Fig. 3B shows that p38MAPK siRNA reduced PMA-induced migration to 31% of the control level. These results indicated that p38MAPK was required for the PMA-induced migration of glioblastoma cells.

3.3. PMA-induced phosphorylation of Hsp27 via p38MAPK activation

Previous reports showed that phosphorylation of Hsp27 played an important role in cell migration [18,19,26]. We analyzed the phosphorylation status of three serine residues of Hsp27 (Ser15, −78 and −82) after PMA stimulation. As shown in Fig. 4A, upon PMA stimulation, all three serine residues were rapidly phosphorylated. In contrast, the expression of unphosphorylated Hsp27 was constant after PMA stimulation.

Activation of p38MAPK resulted in activation of MAPKAP kinases 2/3 and phosphorylation of Hsp27 [15–18]. To

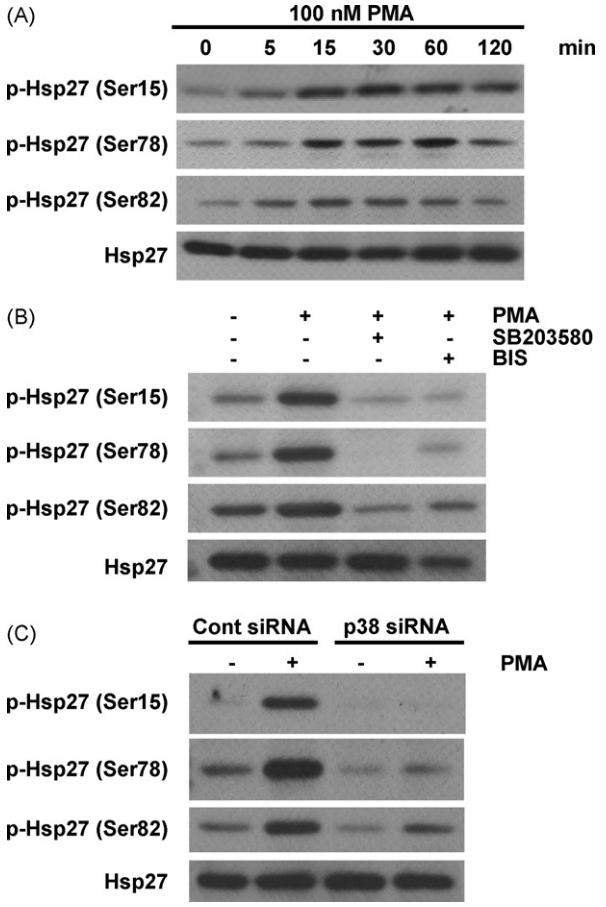

Fig. 4 – Effect of PMA and inhibitors on expression and phosphorylation of Hsp27. (A) The cells were treated with 100 nM PMA for the indicated times. (B) For inhibition experiments, BIS or SB203580 was added to serum-starved cells 30 min prior to PMA stimulation. (C) The cells transfected with p38MAPK or control siRNA were incubated without serum for 24 h. Then the cells were stimulated with PMA or vehicle for 60 min. Expression and phosphorylation of Hsp27 were examined by Western blot analysis. Hsp27 phosphorylation was evaluated with anti-phospho-Ser15, −78, and −82 specific antibodies. The amount of total Hsp27 was determined with an anti-Hsp27 antibody.

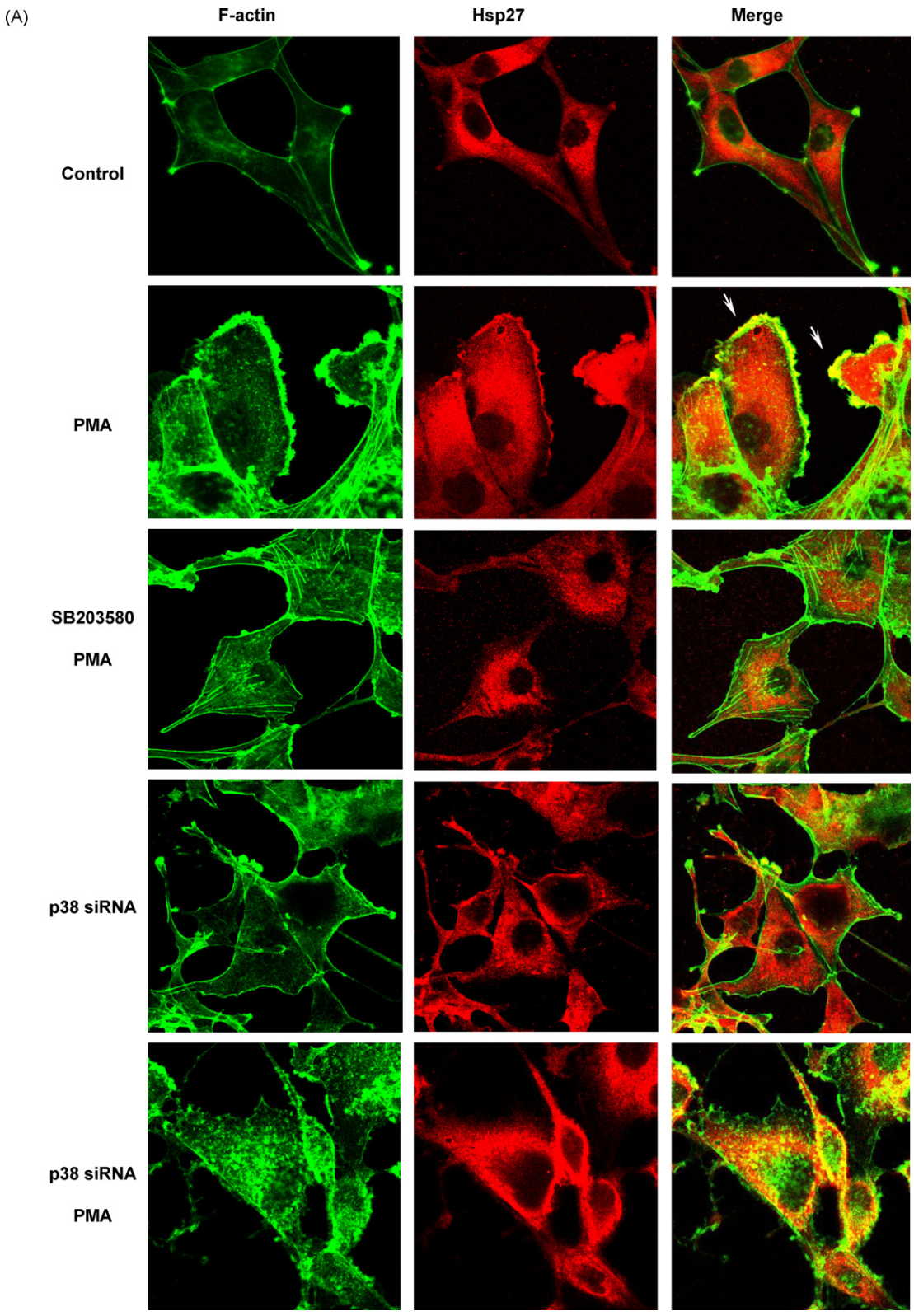

Fig. 5 – Distribution of F-actin and Hsp27 analyzed by fluorescence immunohistochemistry. The cells incubated in serum-free medium for 24 h were pre-treated with SB203580 or vehicle for 30 min and then treated with PMA (100 nM) for 60 min. For siRNA experiments, the cells were transfected with p38MAPK siRNA. After serum-starvation, the transfected cells were treated with PMA or vehicle for 60 min. After fixation, the cells were double-labeled for F-actin and unphosphorylated (A) or phosphorylated (B) Hsp27. F-actin and both forms of Hsp27 were labeled green and red, respectively. Yellow fluorescence shows the region of colocalization of F-actin and unphosphorylated (A) or phosphorylated (B) Hsp27. Arrows indicate the lamellipodia.

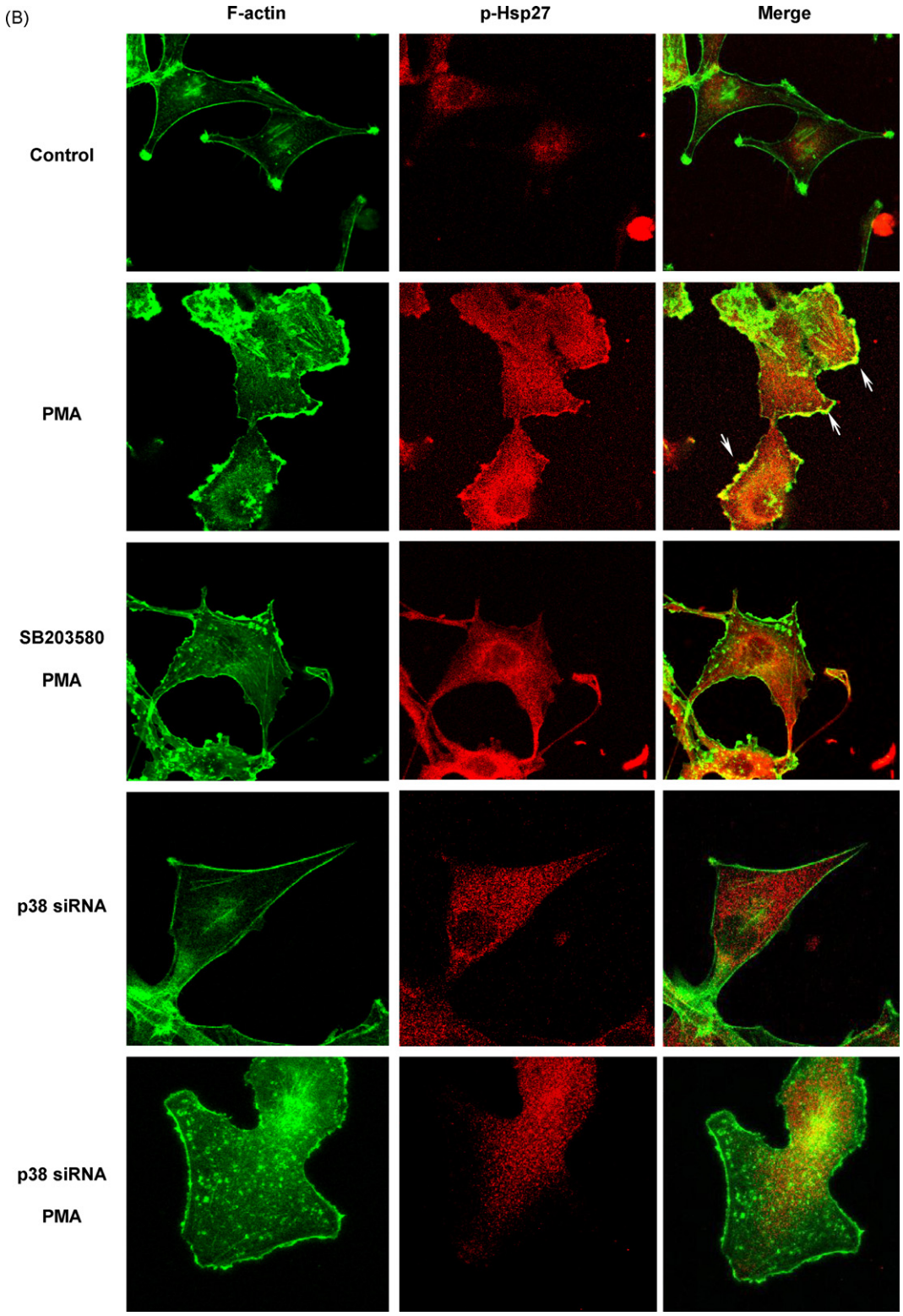

Fig. 5. (Continued).

investigate whether the phosphorylation of Hsp27 was dependent on p38MAPK activation, SB203580 was added to the cells prior to PMA stimulation. In addition, to confirm that phosphorylation of Hsp27 is a consequence of PKC activation, BIS was used. Fig. 4B shows that BIS or SB203580 diminished the phosphorylation of Hsp27.

To investigate the specific effect of p38MAPK on the phosphorylation of Hsp27, we transfected the cells with p38MAPK siRNA. As indicated in Fig. 4C, p38MAPK siRNA inhibited the PMA-induced phosphorylation of Hsp27 at all three serine residues, whereas the expression of unphosphorylated Hsp27 was stable. These findings indicated that

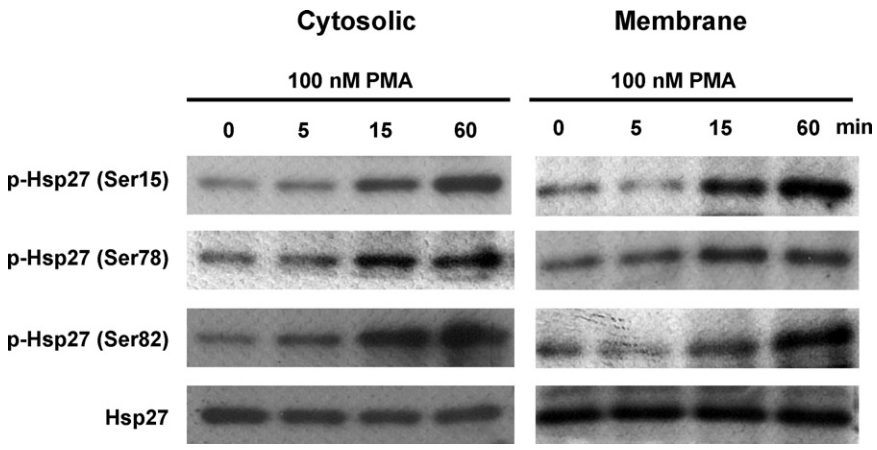

Fig. 6 – Distribution of F-actin and Hsp27 analyzed by Western blot analysis. The cytosolic and membrane protein fractions were obtained from PMA-treated cells. The distributions of unphosphorylated and phosphorylated Hsp27 were analyzed by Western blot analysis.

the PMA-induced phosphorylation of Hsp27 in glioblastoma cells was mediated via PKC/p38MAPK activation.

3.4. *Phosphorylated Hsp27 was observed in the lamellipodia whose formation was induced by PMA via p38MAPK*

When cells migrate, dynamic reorganization of the actin cytoskeleton occurs. Actin is polymerized at the leading edge of moving cells [15]. Phosphorylated Hsp27 is necessary for the formation of F-actin, and increases the rate and extent of actin polymerization in lamellipodia [27]. Based on these previous reports, we next investigated whether the phosphorylation of Hsp27 was involved in regulation of actin dynamics and migration in glioblastoma cells.

Initially, to examine the effect of PMA on the actin cytoskeleton, cells were stained with fluorescently labeled phalloidin to detect F-actin. As shown in the second panels of Fig. 5A and B, PMA-induced actin reorganization in the glioblastoma cells, and caused lamellipodia to be visible at the cell periphery. To examine the role of p38MAPK in PMA-induced lamellipodia formation, we used inhibitors. SB203580 (Fig. 5A and B, the third panels) or BIS (data not shown) largely abolished the lamellipodia formation. Furthermore, PMA-induced lamellipodia formation was markedly diminished by p38MAPK siRNA (Fig. 5A and B, the fifth panels). These findings indicated that PMA-induced lamellipodia formation and this phenomenon was mediated via PKC/p38MAPK in glioblastoma cells.

Next, to investigate the action of p38MAPK on Hsp27, the cells were double-stained with phalloidin and an antibody for unphosphorylated or phosphorylated Hsp27. In quiescent cells, unphosphorylated Hsp27 was preferentially localized in the perinuclear region of the cytoplasm, and phosphorylated Hsp27 was faintly observed in the perinuclear region (Fig. 5A and B, the first panels). Superimposed images showed that unphosphorylated Hsp27 and phosphorylated Hsp27 were present in lamellipodia 1 h after PMA stimulation (Fig. 5A and B, the second panels). The PMA-induced accumulation of both unphosphorylated and phosphorylated Hsp27 proteins in lamellipodia was largely abolished by SB203580 (Fig. 5A and B, the third panels), BIS (data not shown), or p38MAPK siRNA (Fig. 5A and B, the fifth panels). These findings indicated that the expression of phosphorylated Hsp27 in lamellipodia was induced by p38MAPK upon PMA-induced PKC activation.

In parallel, to confirm the subcellular distributions of Hsp27s, we carried out cell fractionation experiments. Western blot analysis indicated that unphosphorylated Hsp27 was present in both the cytosolic and membrane fractions in the control, and the subcellular distributions were not significantly changed after PMA stimulation (Fig. 6). On the other hand, phosphorylated Hsp27s were detected faintly in the cytosol and membrane in the control cells. Upon PMA stimulation, phosphorylated Hsp27 was significantly increased in not only the cytosolic but also the membrane fraction.

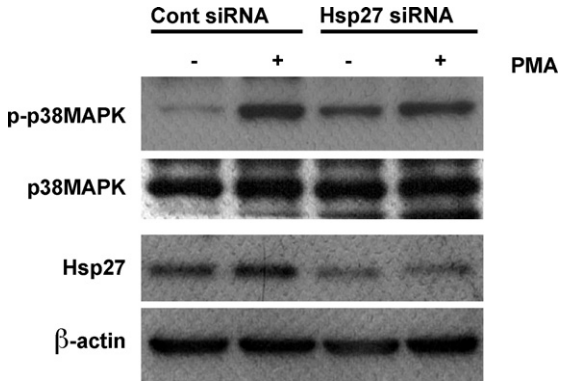

Fig. 7 – Effect of knockdown of Hsp27 by siRNA on expression and phosphorylation of p38MAPK and Hsp27. The cells transfected with Hsp27 or control siRNA were incubated without serum for 24 h. Then the cells were stimulated with PMA (100 nM) or vehicle for 60 min. Expression and phosphorylation of p38MAPK or Hsp27 were examined by Western blot analysis.

3.5. Hsp27 siRNA inhibited PMA-induced lamellipodia formation with little effect on p38MAPK activation

Although a relationship between Hsp27 expression and the malignancy grade of glioma has been reported, the relationship between Hsp27 expression itself and glioblastoma cell migration remains poorly understood. There are few studies describing the effect of Hsp27 gene knockdown on glioblastoma cell migration.

To investigate whether Hsp27 expression itself directly contributes to PMA-induced migration and lamellipodia formation of glioblastoma cells, endogenous Hsp27 expression was inhibited by siRNA. We confirmed by Western blot analysis that siRNA inhibited the endogenous Hsp27 protein expression (Fig. 7). Next, we examined the effect of the Hsp27 inhibition on p38MAPK expression. Knockdown of the Hsp27 gene had no effect on the basal expression (unphosphorylated form) of p38MAPK and had little effect on PMA-induced phosphorylation of p38MAPK (Fig. 7).

Then we analyzed the effect of Hsp27 on PMA-induced actin reorganization and lamellipodia formation by fluorescence immunohistochemistry. As shown in Fig. 8A, transfection of Hsp27 siRNA inhibited PMA-induced lamellipodia formation even under p38MAPK-activated conditions.

3.6. Effect of knockdown of p38MAPK or Hsp27 on focal adhesion formation

Next, we investigated the presence of focal adhesions in the glioblastoma cells by staining paxillin, a protein which forms focal adhesions and is recruited to the leading edge promptly upon the initiation of migration [28]. The results revealed significant formation of dot-shaped focal complexes under lamellipodia after PMA stimulation (Fig. 8B). SB203580 inhibited the PMA-induced formation of focal complexes in the leading edge of cells (data not shown). The same phenomena were observed in the cells transfected with siRNA for p38MAPK or Hsp27. These data indicated that the expression of both p38MAPK and Hsp27 might be involved in the PMA-induced formation of not only lamellipodia but also focal complexes.

3.7. Knockdown of Hsp27 inhibited PMA-stimulated migration even under p38MAPK-activated conditions

Finally, to address the question of whether Hsp27 is indeed involved in PMA-induced glioblastoma cell migration, we performed migration assays using cells transfected with Hsp27 siRNA. Hsp27 knockdown in the cells reduced PMA-induced migration to 51% of the control even under conditions of p38MAPK activation (Fig. 9). These findings indicated that

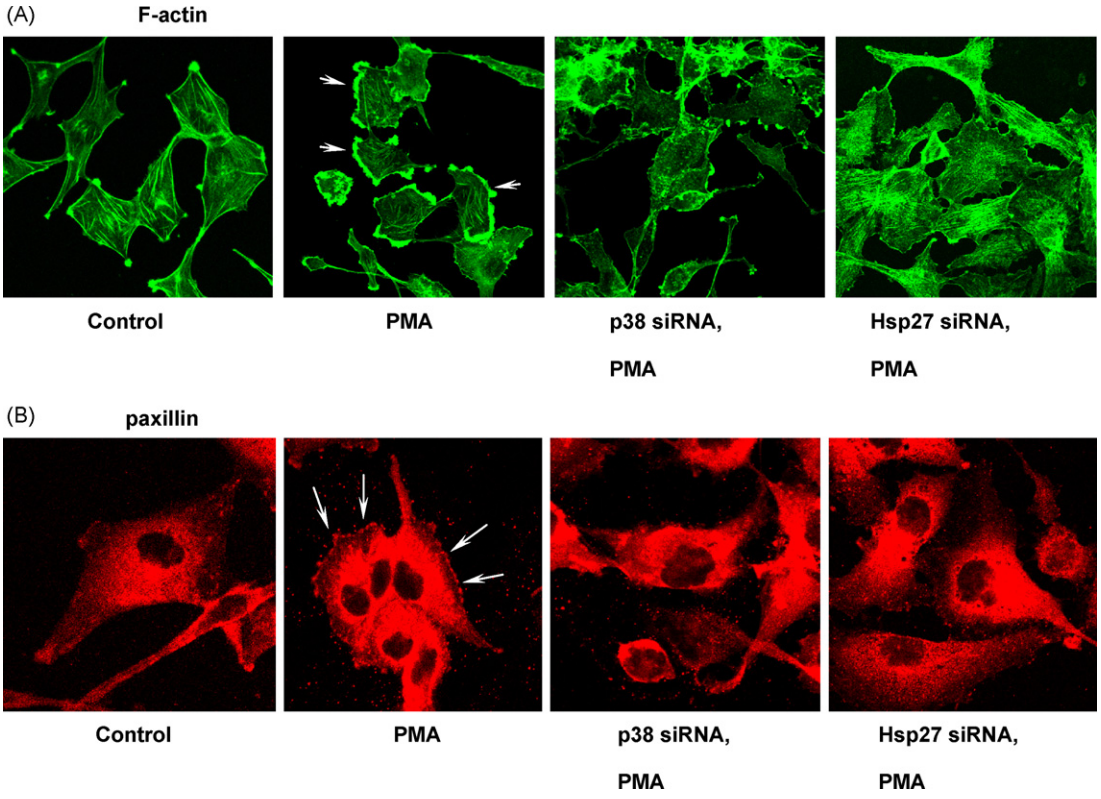

Fig. 8 – Effect of knockdown of p38MAPK or Hsp27 by siRNA on expression of F-actin and focal adhesions. The cells were transfected with siRNA for p38MAPK or Hsp27. After serum starvation, the cells were stimulated with PMA (100 nM) for 60 min. Then the cells were fixed and stained for F-actin (A) or paxillin (B). F-actin and paxillin are labeled green and red under fluorescence, respectively. Arrows (A) indicate the lamellipodia and long arrows (B) indicate the focal adhesions.

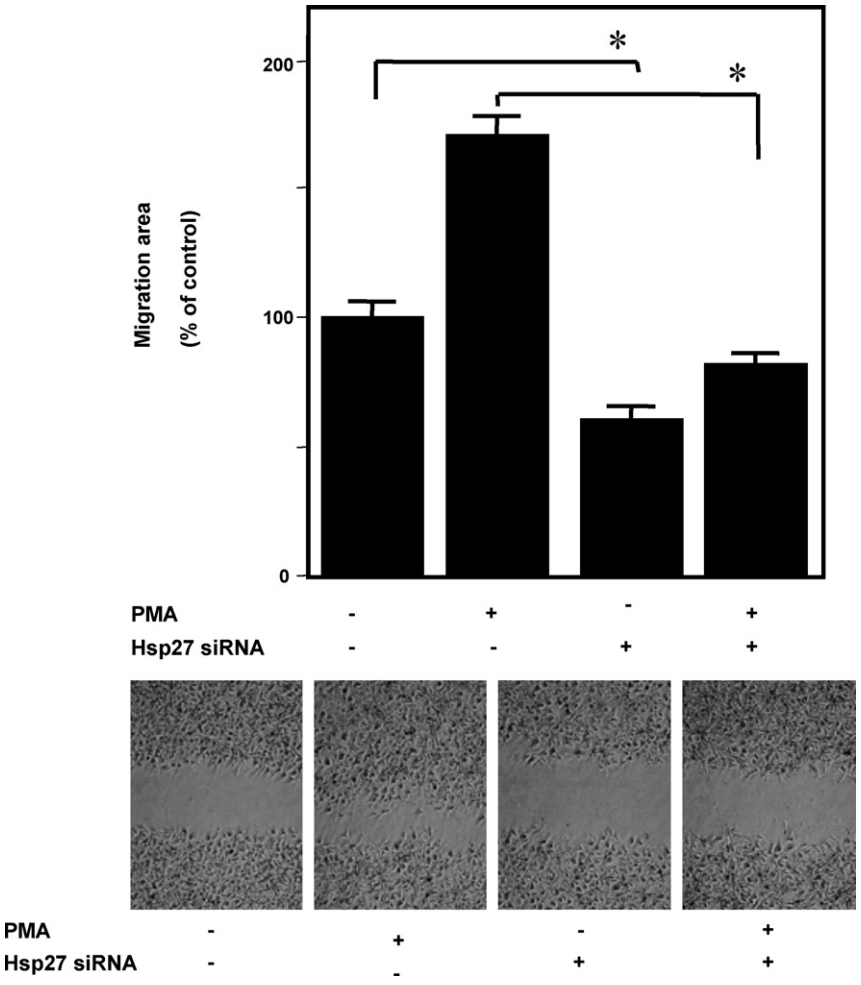

Fig. 9 – Effect of inhibition of Hsp27 by siRNA on PMA-induced migration. Hsp27 siRNA-transfected cells were incubated without serum for 24 h and the cells were wounded. Then the cells were stimulated with PMA (100 nM). After 16 h, migration was evaluated. Results from three independent experiments are presented. Migration is expressed as the percentage of unstimulated cells. *$p < 0.05$, Student's *t*-test.

Hsp27 acted as a downstream effector of p38MAPK, and Hsp27 expression itself might play critical roles in PMA-induced glioblastoma cell migration.

4. Discussion

One of the specific characteristics of glioblastomas is their ability to migrate and invade into the normal brain [29]. In glioblastoma cells, activation and over-expression of PKC have been reported [5]. In this study, we obtained the evidence that PMA-induced lamellipodia formation and subsequent migration and this phenomena required activation of the PKC/p38MAPK/Hsp27 pathway in A172 glioblastoma cells. We detected rapid p38MAPK activation in response to PMA stimulation (Fig. 2). Furthermore, p38MAPK-specific inhibitor and siRNA blocked PMA-stimulated lamellipodia formation and cell migration (Figs. 3 and 5). These results suggested that p38MAPK was necessary for the PMA-induced migration in the glioblastoma cells. To investigate the generality of our findings, we analyzed the effect of p38MAPK activity on PMA-stimulated migration in two other glioblastoma cell lines. PMA increased phosphorylation of p38MAPK and migratory activity, and PMA-induced migration was blocked by SB203580 in both LN18 and Hs683 glioblastoma cells (data not shown).

Recent studies showed that phosphorylated Hsp27, one of the downstream effectors of p38MAPK, is involved in the migration of VEGF-stimulated endothelial cells [19] or PDGF-stimulated smooth muscle cells [15]. Based on these results, we investigated the phosphorylation status of Hsp27 after PMA stimulation. The sites of phosphorylation of human Hsp27 have been mapped to Ser15, Ser78, and Ser82 [30]. Our findings showed that all 3 serine residues of Hsp27 were phosphorylated in response to PMA stimulation via p38MAPK activation (Fig. 4), and the phosphorylation was blocked by BIS. These results indicated that PMA-induced phosphorylation of Hsp27 was dependent on PKC/p38MAPK activation. And our data that the phosphorylation of Hsp27 was dependent on p38MAPK activation was consistent with the previous report [15].

Immunohistochemical analysis revealed that PMA induced the accumulation of phosphorylated Hsp27 in lamellipodia via

p38MAPK activation (Fig. 5B). The accumulation of phosphorylated Hsp27 in the membrane fraction was confirmed by fractionation experiments (Fig. 6). Previous reports showed that phosphorylated Hsp27 is involved in actin polymerization at the leading edge of cells [15], whereas unphosphorylated Hsp27 possesses an actin-capping activity and prevents the polymerization of actin filaments [31,32]. In this study, immunohistochemical analysis showed that unphosphorylated Hsp27 was also present in lamellipodia (Fig. 5A). One possible explanation is that the co-expressed unphosphorylated and phosphorylated forms of Hsp27 in the lamellipodia under PMA-stimulated conditions might indicate vigorous polymerization and depolymerization of actin in lamellipodia. p38MAPK inhibition decreased lamellipodia formation (Fig. 5A and B) and this phenomenon might be due to inhibition of Hsp27 phosphorylation. Expression of Hsp27 phosphorylation mutant was reported to inhibit growth factor-induced actin accumulation, normal lamellipodia formation, and migration [26,27]. These previous reports were consistent with our results that phosphorylation of Hsp27 might have crucial roles in PMA-induced cell migration.

It was reported that Hsp27 was up-regulated according to the malignancy grade in glioblastomas [8,33]. An important question that remains is whether basal Hsp27 expression contributes to PMA-induced glioblastoma cell migration. To address this question, we transfected the cells with Hsp27 siRNA to knockdown endogenous Hsp27 expression. We showed that Hsp27 itself was necessary for PMA-induced lamellipodia formation (Fig. 8A), and subsequent migration (Fig. 9). Interestingly, in Hsp27 siRNA-transfected cells, PMA-induced migration was reduced to 51% of the control level even under conditions of p38MAPK activation. Several studies indicated the ability of Hsp27 to increase the metastatic potential of tumor cells in nude mice [34–36]. Our data showing that Hsp27 expression might positively regulate PMA-induced glioblastoma cell migration are in accord with these studies.

We should note that there was no significant change in the formation of F-actin or focal adhesions after knockdown of p38MAPK or Hsp27 in non-PMA-stimulated cells (data not shown). However, under non-PMA-stimulated conditions, knockdown of p38MAPK by siRNA reduced the basal cell migration to 30% of that in the control siRNA-transfected cells. Moreover, knockdown of Hsp27 reduced the migration to 60% of the control. These results implied the possibility that endogenously expressed p38MAPK and Hsp27 themselves were involved in the migration of glioblastoma cells.

It is known that there are several downstream effectors of p38MAPK other than Hsp27 [37], and Hsp27 has widespread effects on cells not restricted to cell migration. Therefore, the molecular mechanisms of PKC/p38MAPK/Hsp27-modulated migration of glioblastoma cells should be further clarified.

In conclusion, PMA-activated PKC enhanced the formation of lamellipodia and focal complexes, and resulted in cell migration in A172 glioblastoma. We showed that PMA-activated PKC-induced migration of glioblastoma cells was mediated by the activation of p38MAPK followed by Hsp27 phosphorylation. Knockdown of Hsp27 even under p38MAPK-activated conditions also inhibited the migration of glioblastoma cells, indicating that Hsp27 expression might have a critical role in the PMA-induced migration. These results might lead us to develop novel therapeutic modalities for glioblastomas.

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