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Molecules and Cells

Peroxiredoxin 6 Promotes Lung Cancer Cell Invasion by Inducing Urokinase-Type Plasminogen Activator via p38 Kinase, Phosphoinositide 3-Kinase, and Akt

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The peroxiredoxin family of peroxidase has six mammalian members (Prx 1-6). Considering their frequent up-regulation in cancer cells, Prxs may contribute to cancer cells' survival in face of oxidative stress. Here, we show that Prx 6 promotes the invasiveness of lung cancer cells, accompanied by an increase in the activity of phosphoinositide 3-kinase (PI3K), the phosphorylation of p38 kinase and Akt, and the protein levels of uPA. Functional studies reveal that these components support Prx 6-induced invasion in the sequence p38 kinase/PI3K, Akt, and uPA. The findings provide a new understanding of the action of Prx 6 in cancer.

INTRODUCTION

Peroxiredoxins (Prxs) are a family of proteins showing peroxidase activities that degrade hydrogen peroxide (H_2O_2) and alkyl hydroperoxides. Six members of the family, Prxs 1-6, have been identified in mammalian tissues. They have either one (1-Cys Prx; Prx 6) or two (2-Cys Prx; Prxs 1-5) conserved cysteine residues in the catalytic center that are directly involved in peroxidase catalysis, undergoing peroxide-dependent oxidation and thiol-dependent reduction (Immenschuh and Baumgart-Vogt, 2005; Rhee et al., 2001; 2005; Wood et al., 2003). Due to the Prxs' ability to remove oxidants, it is generally accepted that Prxs act as cytoprotective antioxidant enzymes; this idea is supported by the observation that knockout of the genes for Prx 1 (Neumann et al., 2003), Prx 2 (Lee et al., 2003), or Prx 6 (Wang et al., 2003; 2006; 2008) results in enhanced oxidative damage in the specified cells and tissues of animals tested to date.

Prxs have recently received increasing attention in the field of cancer biology. Analyses of cancer samples obtained from patients have revealed the increased expression of Prxs in malignancies of various organs and tissues, including lung (Lehtonen et al., 2004), breast (Karihtala et al., 2003; Li et al., 2006), skin (Lee et al., 2002), thyroid (Yanagawa et al., 1999), and pleural mesothelium (Kinnula et al., 2002). While the up-regulation of Prx 1 (Chen et al., 2002; Kim et al., 2007), Prx 2 (Chung et al., 2001; Park et al., 2000), Prx 4 (Smith et al., 2007), Prx 5 (Kropotov et al., 2006), or Prx 6 (Castagna et al., 2004) may contribute to cancer cells' resistance to chemotherapy and radiotherapy, additional functions of the Prxs have also been suggested. For example, the expression of Prx 6 in breast cancer cells was significantly associated with their lymph-node metastasis (Li et al., 2006). Direct analysis of Prx 6 function via its overexpression and RNA interference has consistently revealed that Prx 6 enhances the invasiveness and thus the metastatic potential of breast cancer cells (Chang et al., 2007). Therefore, Prx 6 appears to influence the efficacy of cancer therapy not only by supporting the resistance of cancer cells but also by promoting their invasiveness and metastasis. The mechanism underlying this latter function of Prx 6 is poorly understood at present, despite the fact that such information is essential for a better understanding of tumor biology and the development of new treatment strategies.

Cancer cell invasion of tissues requires the actions of proteinases that degrade the extracellular matrix, such as urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs). Indeed, these enzymes are frequently up-regulated in cancer cells as a result of constitutive activation of signaling components that promote cell invasiveness (Duffy, 2002; Egeblad and Werb, 2002; Orlichenko and Radisky, 2008). Phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases are examples of such signaling components (Bae et al., 2006; Huang et al., 2004; Jiang and Liu, 2008). Therefore, in this study, we investigated the cellular components involved in Prx 6-induced invasion of cancer cells. Human lung cancer cells were employed as a model, in view of the importance of Prx 6 in the biology and cancer of lungs (Chang et al., 2007; Lehtonen et al., 2004).

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MATERIALS AND METHODS

Antibodies and materials

Antibodies used were anti-Prx 6 (purchased from Lab Frontier, Korea); anti-uPA, -MMP-2, and -MMP-9 (Calbiochem, USA); anti-phospho-Akt, -ERK, -phospho-ERK, -p38 kinase, -phospho-p38 kinase (Cell Signaling, USA); anti-phospho-JNK (Biosource, USA); anti-JNK (BD Pharmingen, USA); anti-Akt, -α-tubulin, -hemagglutinin (HA) (Santa Cruz Biotechnology, USA); anti-Flag (Sigma-Aldrich, USA); anti-Myc, -p85 subunit of PI3K (Upstate Biotechnology, USA). The small interfering RNAs (siRNAs) of Prx 6 and uPA were obtained from Ambion (USA) and Santa Cruz, respectively. Calbiochem provided all the synthetic inhibitors used in this study.

Cell culture and transfection

Human A549 and H460 lung cancer cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (50 μg/ml). The expression constructs used include Flag-tagged Prx 6 cloned into pCR3 vectors (generous gifts from Dr. Sang Won Kang, Ewha Womans University), HA-tagged dominant negative mutant of PI3K (DN-PI3K) in pCMV6 vectors (Lee et al., 2005a), Myc-tagged DN-Akt in pUSEamp vectors (Lee et al., 2005b), and DN-p38 kinase in pCMV5 vectors (Park et al., 2003). The indicated constructs and siRNAs were introduced into cells using Lipofectamine 2000 (Invitrogen, USA). Where necessary, transfected cells were selected using 1 mg/ml G418 sulfate.

Invasion assay

Cells (5×10^4) were suspended in 200 μl of medium containing 0.1% BSA, which was seeded onto the upper surfaces of Matrigel-coated polycarbonate filters (BD Biosciences, USA). Filters were placed in modified Boyden chambers (Corning, USA), and the lower compartment of the chambers filled with 1 ml of medium supplemented with 10% FBS. After incubation for 24 h at 37°C, the cells migrating to the lower surface of the filter were fixed and stained using Diff-Quick kit (Fisher Scientific, USA), and counted under a microscope (Bae et al., 2006).

Zymography

Conditioned media were prepared by incubating cells in serum-free medium for 24 h. Where indicated, media were supplemented with specific inhibitors. Equal volumes of conditioned media were subjected to 10% SDS-PAGE on gels containing either 0.1% gelatin for MMPs or 2.5% casein and 10 μg/ml plasminogen for uPA. Gels were stained, and enzyme activities were visualized as clear bands (Bae et al., 2006; Park et al., 2008).

Western blot analysis

Proteins in conditioned media or cell lysates prepared using a previously reported method (Kim et al., 2001) were separated by SDS-PAGE, electrotransferred to Immobilon membranes (Millipore, USA), subsequently blotted using the specified antibodies, and visualized with the ECL detection system (Amersham, Sweden). Where indicated, proteins in membranes were stained with Ponceau S to confirm equal loading of samples.

PI3K assay

Cells were lysed as described previously (Lee et al., 2005a). Equal amounts of lysate proteins (400 μg) were immunoprecipitated with anti-p85. Immune complexes were washed and resolved in reaction buffer containing γ-[^{32}P]ATP. L-α-phosphatidylinositol (Sigma-Aldrich) was added to initiate the kinase reactions, which were quenched by adding 1 M HCl after 20 min incubation. Reactions were analyzed with thin-layer chromatography.

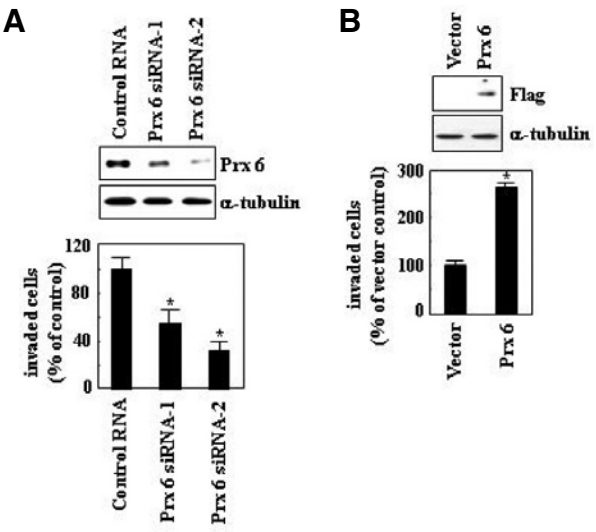

Fig. 1. Prx 6 promotes invasion of A549 cells. (A) Top, control and Prx 6 siRNAs were introduced into A549 cells. After 36 h of incubation, cellular levels of Prx 6 were compared by Western blotting using α-tubulin as a loading control. Bottom, A549 cells treated with indicated siRNAs were seeded onto Matrigel-coated polycarbonate filters. Cells were incubated for 24 h in modified Boyden chambers, and cells migrating through the filters were stained and then counted under a light microscope. Data are presented as means and standard deviations of migrating cell numbers. (B) Top, A549 cells were stably transfected with empty pCR3 vector or vectors containing Flag-tagged Prx 6. Expression patterns of the introduced genes were analyzed by Western blotting. Bottom, control and Prx 6 transfectants were analyzed for invasiveness. *, $p < 0.05$ versus controls, $n = 3$.

Statistic analysis

Results were analyzed for statistical significance with the Student's *t*-test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Prx 6 enhances invasiveness of A549 cells

In order to determine whether Prx 6 influences the invasiveness of lung cancer cells, A549 cells were used as a model. Analysis on Matrigel-coated membrane filters revealed that these cells' invasiveness decreased following a decrease in Prx 6 levels via siRNA (Fig. 1A), and consistently increased upon expression of exogenous Prx 6 (Fig. 1B). This finding confirms the ability of Prx 6 to promote the invasion of A549 cells. In view of the recent similar finding reported using breast cancer cells (Chang et al., 2007), we hypothesize that Prx 6 exerts the effect in malignant cells found in multiple organs.

uPA is required for Prx 6-induced invasion

The cellular components involved in Prx 6-induced invasion have not been directly investigated previously. To determine such components, we utilized the ectopic expression system of Prx 6, in preference to RNA interference, because the former modulated invasion-associated events more clearly. Zymography and Western blot analyses revealed that Prx 6 transfection

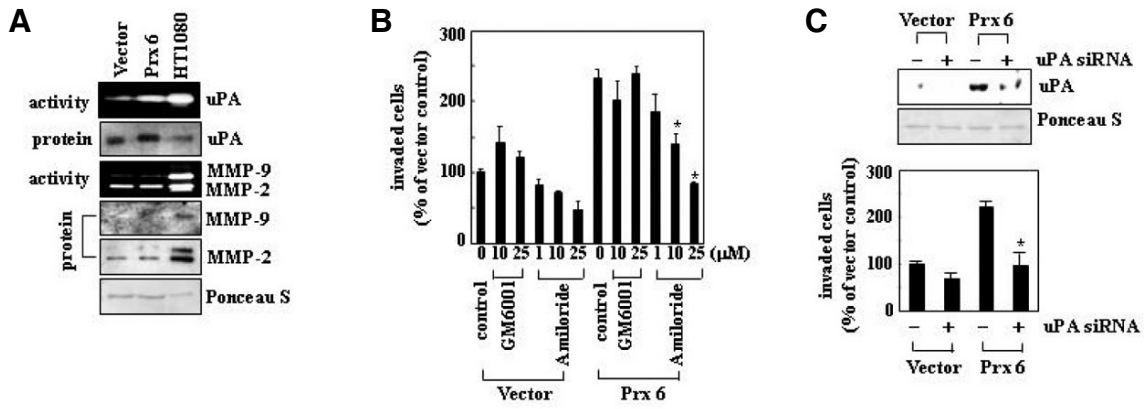

Fig. 2. Prx 6 promotes cell invasion by inducing uPA. (A) Conditioned media were prepared by incubating the control and Prx 6 transfectants in serum-free media for 24 h. Samples were analyzed by zymography and Western blotting to compare activities and protein levels of uPA, MMP-2 and MMP-9. HT-1080 fibrosarcoma cells were used as a positive control. Protein loading of conditioned media was verified via Ponceau S staining of blot filters. (B) Matrigel invasion assays were performed in the presence or absence of the indicated concentrations of amiloride and GM6001. (C) uPA siRNA was introduced into A549 cells. Top, conditioned media prepared and analyzed for uPA levels using Western blotting. Bottom, comparison of cellular invasiveness. *, $p < 0.05$ versus untreated Prx 6 control, $n = 3$.

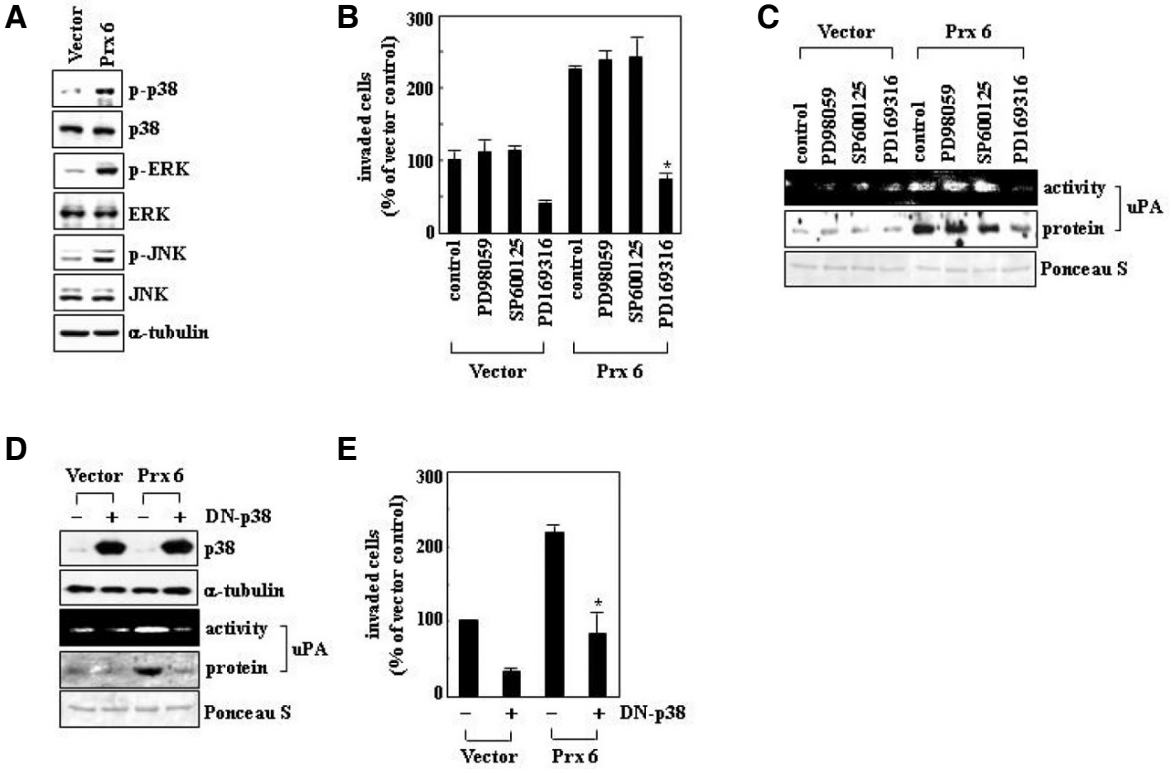

Fig. 3. p38 kinase mediates the induction of uPA by Prx 6. (A) Control and Prx 6 transfectants were lysed, and the levels of the indicated proteins and phosphorylation were compared by Western blotting. (B) Transfectants were incubated in the presence or absence of inhibitors for ERK (PD98059, 10 μM), JNK (SP600125, 10 μM), and p38 kinase (PD169316, 10 μM), and degrees of invasiveness were compared. (C) Conditioned media were prepared under the indicated conditions. Levels of uPA activity and protein were compared. (D) DN-p38 kinase was introduced into the specified transfectants. Levels of p38 protein as well as uPA activity and expression were compared. (E) Comparison of invasiveness of the indicated transfectants. *, $p < 0.05$ versus untreated Prx 6 control, $n = 3$.

resulted in increased activity and protein levels of uPA but not of MMP-2 or MMP-9 (Fig. 2A). To determine whether uPA is required for Prx 6-induced invasion, assays were performed in the presence or absence of amiloride, an inhibitor of uPA. Amiloride abolished the ability of Prx 6 to trigger invasion. This effect was not observed using the MMP inhibitor, GM6001 (Fig. 2B). Consistent with the effect of amiloride, Prx 6 failed to induce cell invasion upon suppression of the levels of uPA via RNA interference (Fig. 2C). Our results suggest that Prx 6 promotes the invasion of A549 cells by increasing uPA levels.

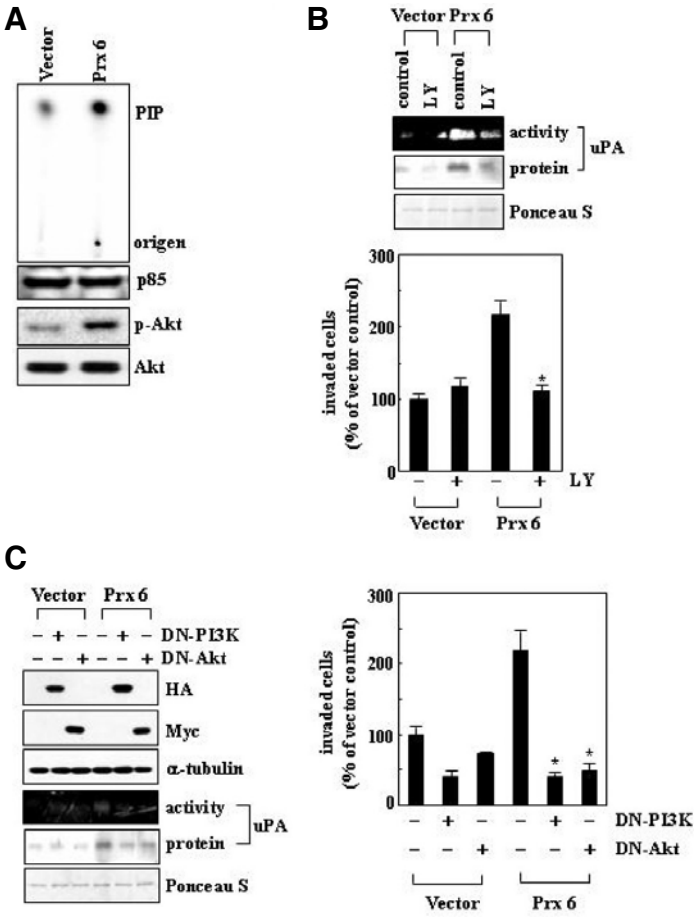

Fig. 4. Role of PI3K and Akt in Prx 6-induced uPA accumulation. (A) A549 transfectants were lysed, and PI3K activities in the lysates were then analyzed with *in vitro* kinase assays using L-α-phosphatidylinositol as a substrate. Levels of the p85 subunit of PI3K, Akt, and Akt phosphorylation were compared by Western blotting. (B) A549 transfectants were cultured in the presence or absence of LY (10 μM) for 24 h. Top, culture media were collected and analyzed for uPA. Bottom, comparison of invasiveness of transfectants. (C) HA-tagged DN-PI3K or Myc-tagged DN-Akt was introduced into A549 transfectants. Left, cell lysates were prepared and analyzed for expression by Western blotting. Alternatively, conditioned media were prepared and analyzed for uPA. Right, comparison of invasiveness of transfectants. *, $p < 0.05$ versus untreated Prx 6 control, $n = 3$.

p38 kinase mediates Prx 6-induced uPA accumulation

Since mitogen-activated protein kinases influence cell invasion under diverse experimental settings (Huang et al., 2004; Reddy et al., 2003), their roles in this system were investigated. Prx 6 transfection enhanced the phosphorylation levels of the three members of the kinase family; p38 kinase, ERK, and JNK (Fig. 3A). This is suggestive of their activation. However, Prx 6-induced invasion was abolished only by a p38 kinase inhibitor (PD169316) and not by inhibitors of ERK (PD98059) or JNK (SP600125) (Fig. 3B). While PD98059 and SP600125 consistently failed to affect the ability of Prx 6 to induce uPA, this was efficiently attenuated using PD169316 (Fig. 3C). To further confirm the effects of PD169316, a dominant-negative mutant of p38 kinase (DN-p38 kinase) was introduced into cells. This treatment abolished the ability of Prx 6 to induce uPA (Fig. 3D), and, consequently, to promote cell invasion (Fig. 3E). The data indicate that p38 kinase mediates the induction of uPA by Prx 6.

Role of PI3K and Akt in Prx 6-induced invasion

Prx 6 transfection was additionally associated with increases in PI3K activity and Akt phosphorylation (Fig. 4A), suggesting activation of the PI3K-Akt pathway, which promotes cell invasion under various experimental conditions (Bae et al., 2006; Jiang and Liu, 2008; Samuels and Ericson, 2006). Indeed, LY294002 (LY), an inhibitor of PI3K, abolished the ability of Prx 6 to induce uPA and promote cell invasion (Fig. 4B). Similar results were obtained upon introducing DN-PI3K or DN-Akt into cells (Fig. 4C), suggesting that PI3K and Akt act as additional mediators of Prx 6-induced uPA accumulation. Given that p38 kinase can phosphorylate Akt, thus acting upstream of Akt in a manner similar to that of PI3K (Cabane et al., 2004; Rane et al., 2001; Zhang et al., 2001), this hierarchical relationship was investigated in the present system. In fact, Prx 6-induced phosphorylation of Akt was attenuated by LY and PD169316 (Fig. 5), confirming that both PI3K and p38 kinase act upstream of Akt in the Prx 6-induced signaling pathway. Similar hierarchical relationship of these components was reported under other experimental settings (Cabane et al., 2004; Rane et al., 2001; Zhang et al., 2001).

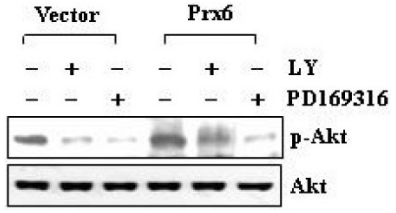

Fig. 5. p38 kinase acts upstream of Akt. A549 transfectants were treated with LY or PD169316 for 24 h. Cellular levels of Akt and its phosphorylation were analyzed by Western blotting.

Prx 6 action in other lung cancer cell types

To investigate the cell-type specificity of Prx 6 action, an alternative lung cancer cell line, H460, was analyzed. Similar to the data obtained with A549 cells, reduction of Prx 6 levels via RNA interference led to a decrease in the invasiveness of H460 cells

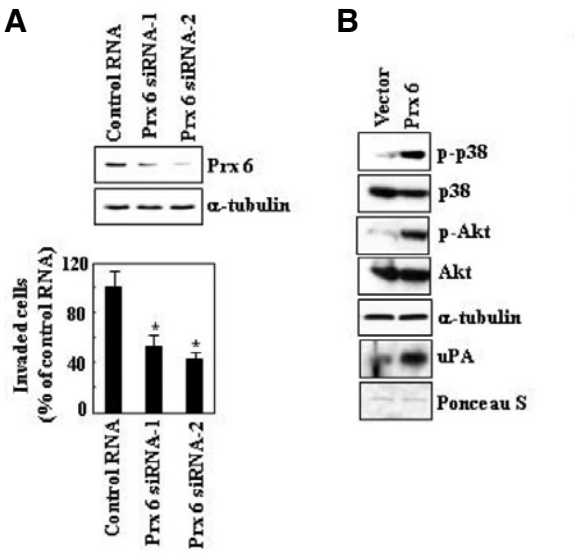

Fig. 6. Prx 6 enhances the invasiveness of H460 cells. (A) Top, control and Prx 6 siRNAs were introduced into H460 cells. After 36 h of incubation, cellular levels of Prx 6 were compared by Western blotting. Bottom, comparison of invasiveness of treated cells. *, $p < 0.05$ versus control RNA, $n = 3$. (B) H460 transfectants were analyzed for p38 kinase, Akt, and uPA. (C) Control and Prx 6 transfectants of H460 cells were incubated with or without LY (10 μM), PD169316 (10 μM), and amiloride (10 μM), and compared for invasiveness at 24 h of incubation. *, $p < 0.05$ versus untreated Prx 6 transfectants, $n = 3$.

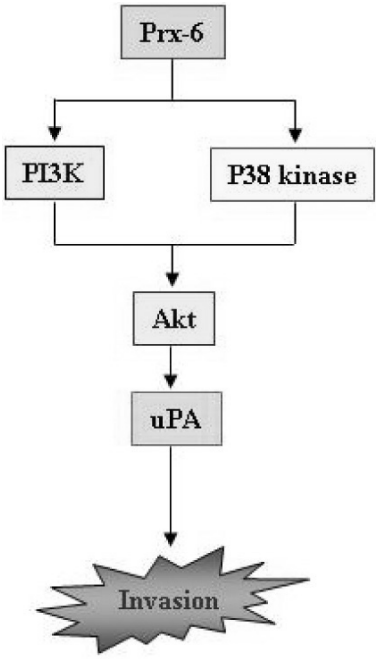

Fig. 7. Schematic model outlining the Prx 6-induced signaling pathway. Prx 6 appears to activate Akt via PI3K and p38 kinase: Akt, in turn, enhances the invasive potential of cells by inducing uPA.

(Fig. 6A). Moreover, Prx 6 transfection promoted phosphorylation of p38 kinase and Akt, uPA expression (Fig. 6B), and, consequently, cell invasiveness (Fig. 6C). Furthermore, invasion induced by Prx 6 was efficiently attenuated by inhibitors for PI3K, p38 kinase, and uPA. The data collectively suggest that the ability of Prx 6 to promote invasion via p38 kinase, PI3K, Akt, and uPA is not confined to a single cell type, but is applicable to multiple types of lung cancer cells.

In conclusion, we have shown that Prx 6 promotes invasion of lung cancer cells via cellular pathways sequentially involving p38 kinase/PI3K, Akt, and uPA (Fig. 7). These findings provide a significant step towards elucidating the actions of Prx 6 in cancer.

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