J. Biochem. 2009;146(1)133–139
doi:10.1093/jb/mvp050

Oncogenic Functions of PTK6 are Enhanced by Its Targeting to Plasma Membrane But Abolished by Its Targeting to Nucleus

Han Ie Kim and Seung-Taek Lee*

Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

Received December 13, 2008; accepted March 12, 2009; published online March 20, 2009

PTK6 (also known as Brk) is an intracellular tyrosine kinase whose expression is up-regulated in several tumour types. Because localization of protein tyrosine kinases plays an important role in the development of cancers, we investigated the relationship between subcellular localization of PTK6 and its oncogenic properties. PTK6 was targeted to the plasma membrane or the nucleus of HEK 293 cells using the Src myristoylation signal (Myr) or SV40 T-antigen nuclear localization signal (NLS), respectively. The profile of cellular proteins phosphorylated by Myr-PTK6 was quite different from those phosphorylated by NLS-PTK6. Localization of PTK6 to the plasma membrane enhanced the ability of PTK6 to promote proliferation, cell survival and migration and to permit anchorage-independent colony formation. In contrast, nuclear localization of PTK6 impaired these functions. Our results demonstrate that recruitment of PTK6 to the plasma membrane is required for oncogenic function.

Key words: Brk, myristoylation, nuclear localization, oncogene, PTK6, subcellular localization, tyrosine kinase.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; Myr, myristoylation signal; NLS, nuclear localization signal; OSCC, oral squamous cell carcinoma; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling.

PTK6 (also known as Brk) is an intracellular tyrosine kinase containing an SH3 domain, an SH2 domain and a catalytic tyrosine kinase domain (*1*). Expression of PTK6 is up-regulated in breast carcinomas (*1–3*), melanomas (*4*) and colon carcinomas (*5*, *6*), whereas PTK6 is not expressed in normal mammary gland epithelial cells and melanocytes (*3*, *7*).

PTK6 is present in both the cytosol and nucleus (*2*, *8*, *9*); however, its subcellular location appears to vary between cell types. PTK6 is located in the cytosol in some cancer cell lines, such as breast carcinoma T-47D cells (*2*). In addition, PTK6 was detected in the nucleus in normal prostate epithelia and well-differentiated prostate carcinomas, but mainly in the cytosol in poorly differentiated and highly tumorigenic prostate carcinoma PC3 cells (*9*). Similarly, PTK6 is present in both the cytosol and nucleus in normal oral epithelial cells, mainly in the cytosol in moderately differentiated oral squamous cell carcinomas (OSCC) cells, and in perinuclear regions in poorly differentiated OSCC cells, with a correlation between reduced PTK6 expression and decreased differentiation (*10*). These findings suggest that abnormal subcellular localization of PTK6 may contribute to tumorigenesis.

N-myristoylation is a cotranslational modification of proteins, in which myristate is covalently attached to the amino-terminus of various cellular proteins, viral proteins and oncoproteins (*11*). *N*-myristoylation targets proteins to membranes and plays an essential role in many physiological and pathological events such as signal transduction, carcinogenesis and viral replication and assembly. Proteins destined for the nucleus contain a nuclear localization signal (NLS) sequence; this sequence is recognized by cytosolic nuclear transport receptors that direct proteins to the nucleus and confine them there (*12*).

To examine the relationship between subcellular localization and oncogenic properties of PTK6, we constructed variants of PTK6 that were targeted to the plasma membrane by the Src myristoylation signal (Myr) (Myr-HA-PTK6) or to the nucleus by the SV40 T-antigen NLS sequence (NLS-HA-PTK6), and expressed these in HEK 293 cells. Tyrosine-phosphorylation of cellular proteins and cell proliferation, survival, migration and anchorage-independent colony formation were examined in HEK 293 cells expressing Flag-PTK6 (wild-type), Myr-HA-PTK6, or NLS-HA-PTK6.

MATERIALS AND METHODS

Expression Constructs—To construct a mammalian expression vector expressing Flag-tagged PTK6 (Flag-PTK6), a DNA cassette containing a translation initiating methionine, a Flag-tag sequence and sticky ends corresponding to Asp718I at the 5′ end and NcoI at the 3′ end, was made by annealing the primer pair 5′-GTACCGGCCGCCACC**ATG**GATTACAAGGATGACGACGATAAGCT-3′ and 5′-CATGAGCTTATCGTCGTCATCCTTGTAATC**CAT**GGTGGCGGCCG-3′ (the translation start codon is shown in bold and the FLAG-tag sequence is underlined). A fragment containing PTK6 cDNA was

*To whom correspondence should be addressed. Tel: +82-2-2123-2703, Fax: +82-2-362-9897, E-mail: stlee@yonsei.ac.kr

© The Authors 2009. Published by Oxford University Press on behalf of the Japanese Biochemical Society. All rights reserved.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

isolated by digestion of pBS-PTK6-MR (*13*) with NcoI and BamHI, and subcloned with the Flag-tag cassette into pcDNA3.1 (Invitrogen) that was digested with Asp718I and BamHI.

To make a construct for expression of Myr-HA-PTK6 in mammalian cells (pcDNA3.1-Myr-HA-PTK6), a fragment containing the Myr sequence of Src cDNA (*14*) was PCR amplified using mouse Src cDNA (pLNCX-mSrc) as template and the primer pair 5′-GGGGTACCG GCCGCGCCACCATGGGCAGCAACAGAGCAAGCCCAA GGAC-3′ and 5′-GGACCTCATGAG*AGCGTAATCTGGA ACATCGTATGGGTAAGC*GTTTTCCGAGGGCTCCAG-3′ (the Myr sequence is underlined and the HA-tag sequence is denoted by italics). The PCR product was digested with Asp718I and BspHI. A fragment containing full-length PTK6 cDNA was isolated by digestion of pBS-PTK6-MR with NcoI and BamHI and these two fragments were subcloned into pcDNA3.1 that was digested with Asp718I and BamHI.

An expression construct for NLS-HA-PTK6 (pcDNA3.1-NLS-HA-PTK6) was made by primer-mediated site-directed mutagenesis with a Quickchange kit (Stratagene) using pcDNA3.1-Myr-HA-PTK6 as template and the following primers: 5′-CTTGGTACCGGCCGCGC CACCATGGGCCCAAAAAAGAAGAGAAAGGTAGAAGC TTACCCATACGATGTTCCAGATTAC-3′ and 5′-GTAAT CTGGAACATCGTATGGGTAAGCTTCTACCTTTCTCTT CTTTTTTGGGCCCATGGTGGCGCGGCCGGTACCAAG-3′ (the NLS sequence of the SV40 T-antigen is underlined) (*15*). All constructs were verified by DNA sequencing.

Cell Culture and Transfection—HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone). For transient expression, transfection of constructs into HEK 293 cells was carried out with WelFect-EX PLUS (WelGENE Inc.) following to the manufacturer's instructions, and the cells were analysed 48 h after transfection. For stable expression, transfection was performed with calcium phosphate followed by selection with 1.2 mg/ml G418 (*16*). G418-resistant cell colonies were pooled and expanded for further analysis.

Western Blot Analysis and Immunoprecipitation—Western blot analysis and immunoprecipitations were carried out as described previously (*17*) using the following primary antibodies: anti-Flag (Sigma), anti-HA (Sigma), anti-PTK6 (Santa Cruz Biotechnologies), anti-phospho-tyrosine (4G10; Upstate Biotechnology), anti-Stat3 (Santa Cruz Biotechnologies), anti-paxillin (Santa Cruz Biotechnologies), anti-Sam68 (Santa Cruz Biotechnologies) and anti-β-actin (Sigma).

Immunostaining and Confocal Microscopy—Immunostaining of cells was performed as described previously (*18*). Cells were incubated with anti-Flag or anti-HA antibody and stained with Rhodamin Red-X-conjugated goat anti–mouse IgG. After washing the cells, the nucleus was stained with 4′-6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline for 3 min at room temperature. Cells were washed, mounted with Vectashield mounting medium (Vector Laboratories) containing the anti-fading agent p-phenylenediamine and analysed by confocal microscopy.

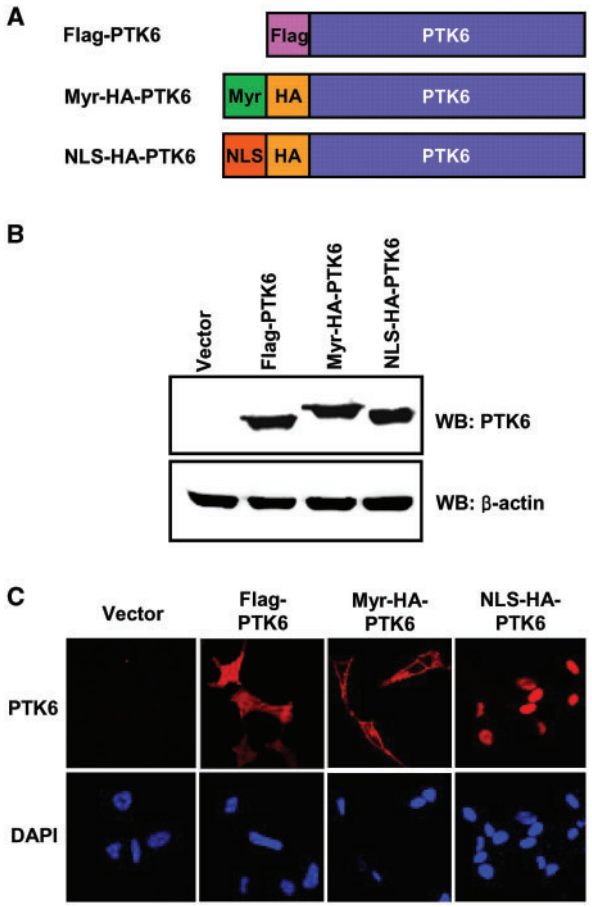


Fig. 1. **Expression of PTK6 and its variants in HEK 293 cells.** (A) Schematic diagram of wild-type Flag-PTK6, plasma membrane-targeted Myr-HA-PTK6, and nuclear-targeted NLS-HA-PTK6. (B) HEK 293 cells were transiently transfected with vector (pcDNA3.1) alone, or constructs expressing Flag-PTK6, Myr-HA-PTK6, or NLS-HA-PTK6. Expression of PTK6 and its variants was measured by western blot analysis with anti-PTK6 antibody. (C) HEK 293 cells transiently expressing PTK6 and its variants were subjected to immunofluorescence staining with anti-Flag or anti-HA antibody and examined by confocal microscopy.

Cell Proliferation Assay—Subconfluent cells were depleted in serum-free DMEM for 24 h and incubated in DMEM containing 1% FBS and 50 ng/ml EGF in the presence of [^{3}H]thymidine for the indicated time intervals. Cell proliferation was determined by the amount of [^{3}H]thymidine incorporated, as described previously (*19*).

Cell Survival and Apoptosis Assays—To measure cell survival, subconfluent cells were starved in serum-free DMEM containing various concentrations of H_2O_2 for 48 h. Viable cells were determined using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (*20*). To detect apoptotic cells, subconfluent cells were incubated with serum-free DMEM containing 0 or 500 μM H_2O_2 for 24 h. Apoptotic cells were detected by fluorescence microscopy using the In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics) for Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling (TUNEL)

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

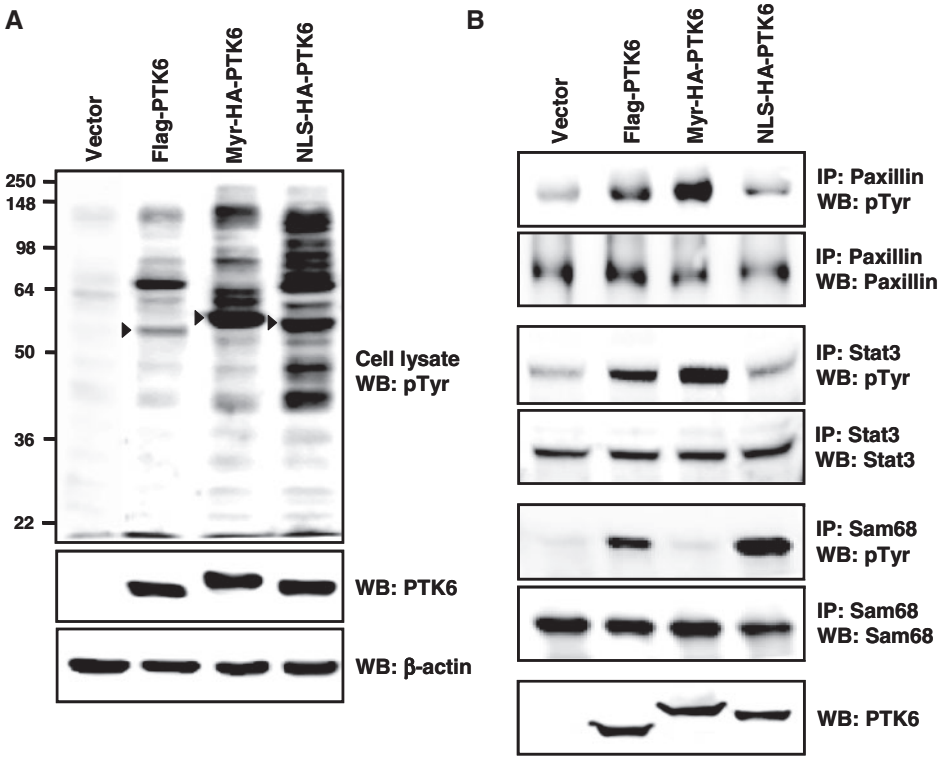


Fig. 2. **Protein phosphorylation in HEK 293 cells stably expressing PTK6 or its variants.** (A) Phosphorylation of cellular proteins by PTK6 and its variants. Lysates from HEK 293 cells stably expressing Flag-PTK6, Myr-HA-PTK6, or NLS-HA-PTK6 were analysed by western blot analysis using anti-phospho-tyrosine antibody (upper panel), anti-PTK6 antibody (middle panel) and anti-β-actin antibody to normalize levels of loading (bottom panel). Arrowheads in the upper panel denote phosphorylated Flag-PTK6, Myr-HA-PTK6 and NLS-HA-PTK6. (B) Phosphorylation of paxillin, Stat3 and Sam68 by PTK6 and its variants. Paxillin, Stat3 and Sam68 were immunoprecipitated from the cell lysates described above. Immunoprecipitates were analysed by western blot analysis with anti-phospho-tyrosine antibody, and antibodies against Stat3, paxillin and Sam68, respectively. PTK6 and its variants were detected in the lysates by western blotting with anti-PTK6 antibody.

assay according to the manufacturer's instructions. Double-stranded DNA in nuclei was counterstained with DAPI.

Anchorage-Independent Colony Formation Assay—Anchorage-independent colony formation was measured as described previously (*17*). Cells (1×10^4/well) were suspended in DMEM-10% FBS containing 0.35% low melting point agarose (SeaPlaque, FMC Corp.) at 42°C and overlaid onto the solidified 0.5% agarose layer containing DMEM-10% FBS. After 5 weeks of incubation, the numbers of colonies formed were counted with a microscope.

Migration Assay—Cell migration was assayed as described previously (*21*, *22*) using modified Boyden chambers (8-μm pore size, Costar Transwell filters, Corning-Costar Corp.) coated with 10 μl 0.1% gelatin. Confluent cells were detached using trypsin and resuspended in DMEM supplemented with 1% FBS. Typically, 100,000 cells were added to the upper chamber and allowed to migrate to the lower chamber for 12 h in DMEM medium containing 1% FBS. Cells were fixed and stained with Gill's Hematoxylin No. 3 (Fisher) and Eosin-Y. Cells that had migrated through the membrane were observed using bright-field microscopy (200x, IX51 inverted microscope, Olympus). All assays were performed three times in duplicate.

RESULTS

Expression of PTK6 and Its Variants in HEK 293 Cells—To examine the effect of subcellular localization on oncogenic properties of PTK6, wild-type PTK6 (Flag-PTK6) and modified PTK6 targeted to the plasma membrane by a myristoylation signal (Myr-HA-PTK6) or to the nucleus by an NLS (NLS-HA-PTK6) (Fig. 1A) were transiently expressed in HEK 293 cells. The molecular weights of Flag-PTK6, Myr-HA-PTK6 and NLS-HA-PTK6 were ~53, 57 and 56 kDa, respectively (Fig. 1B). The subcellular location of PTK6 and its variants was examined by immunofluorescence microscopy. Flag-PTK6 was detected in both cytoplasm and nucleoplasm. In contrast, Myr-HA-PTK6 was located mainly in the plasma membrane and NLS-HA-PTK6 predominantly in the nucleus (Fig. 1C).

Protein Phosphorylation by PTK6 or Its Variants—We next examined the profile of protein phosphorylation in HEK 293 cells stably expressing Flag-PTK6, Myr-HA-PTK6 and NLS-HA-PTK6. Wild-type and variant PTK6 proteins were expressed at similar levels (Fig. 2A, middle panel). Expression of PTK6 and its variants increased the level of phosphorylation of cellular proteins (Fig. 2A, upper panel), confirming that these proteins are catalytically active as tyrosine kinases. Interestingly, the

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

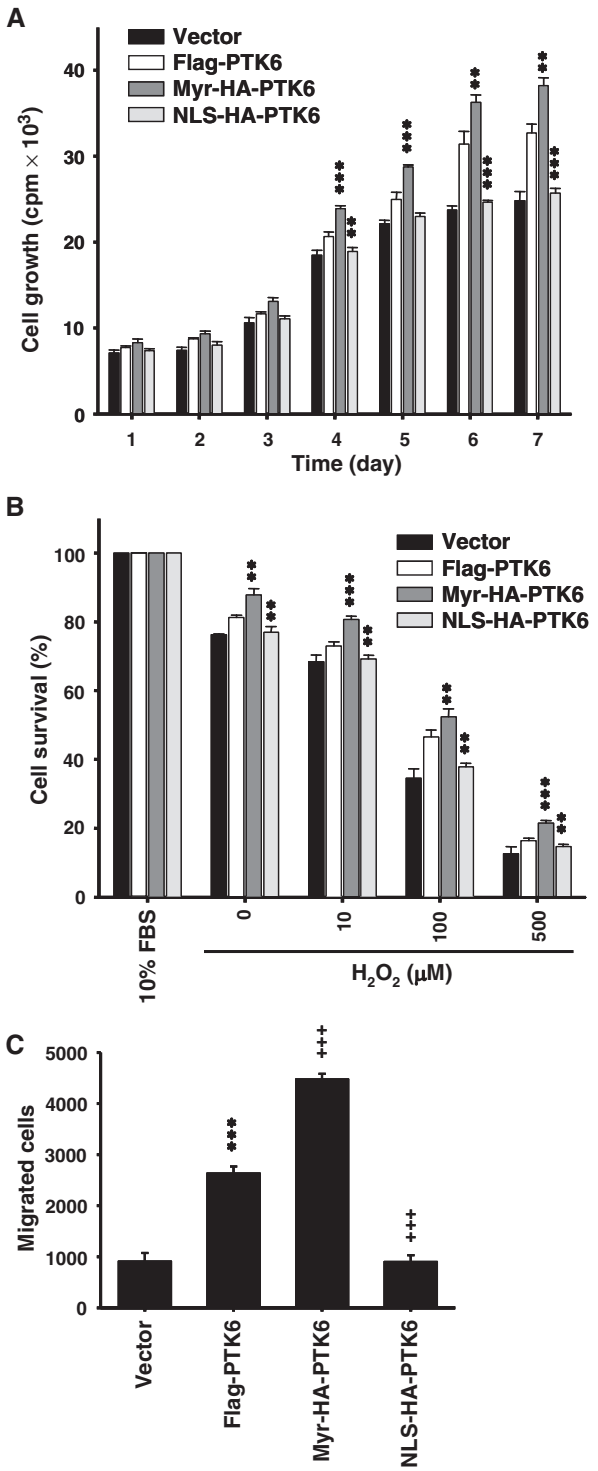


Fig. 3. **Cell proliferation, survival and chemotactic migration in HEK 293 cells stably expressing PTK6 and its variants.** (A) Effect on cell proliferation. HEK 293 cells stably expressing Flag-PTK6, Myr-HA-PTK6, or NLS-HA-PTK6 were grown for 7 days in 1% FBS. Cell proliferation was determined by [^{3}H]thymidine incorporation. (B) Effect on cell survival. HEK 293 cells stably expressing PTK6 or its variants were incubated in serum-free DMEM containing various concentrations of H_2O_2 for 48 h. Surviving cells were measured by MTT assay. pcDNA3.1 vector alone (black), Flag-PTK6 (white), Myr-HA-PTK6 (dark grey), NLS-HA-PTK6 (light grey). Statistical comparison of cell growth or survival of Myr-HA-PTK6 or NLS-HA-PTK6 cells compared with Flag-PTK6 cells was performed using Student's t test. $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Effect on chemotactic cell migration. HEK 293 cells stably expressing PTK6 or its variants were plated onto Transwell chambers coated with 10 μl 0.1% gelatin and were allowed to migrate into the lower chamber containing 1% FBS for 12 h. Cells that had migrated to the lower side of the chamber were counted. Statistical comparisons of the cell migration of Flag-PTK6 cells compared with HEK 293 vector cells (*), and of Myr-HA-PTK6 or NLS-HA-PTK6 cells compared with Flag-PTK6 cells (+), were performed using Student's t test. *** or $^{+++}P < 0.001$. The error bars represent standard deviations (SD). Each value is the mean ± SD of three independent determinations of duplicate experiments.

pattern of phosphorylation of cellular proteins by Myr-HA-PTK6 was quite different from that of NLS-HA-PTK6, suggesting that there are substrates specific to the plasma membrane-anchored PTK6 or the nuclear PTK6.

PTK6 substrates include Stat3, paxillin and Sam68 (*23–25*). To examine whether phosphorylation of specific PTK6 substrates is dependent on PTK6 localization, Stat3, paxillin and Sam68 were immunoprecipitated from HEK 293 cell lysates and their phosphorylation status was analysed by western blotting with anti-phospho-tyrosine antibody. Compared with cells expressing Flag-PTK6, a strong increase in tyrosine phosphorylation of Stat3 and paxillin was detected in cells stably expressing Myr-HA-PTK6, but not in cells expressing NLS-HA-PTK6 (Fig. 2B, first and second panels). Conversely, phosphorylation of Sam68 was increased in cells expressing NLS-HA-PTK6 but not in cells expressing Myr-HA-PTK6 (Fig. 2B, third panel). These results demonstrate that the phosphorylation substrates of PTK6 are determined by its subcellular location.

Effect of Intracellular Location of PTK6 on Cell Proliferation, Anti-Apoptosis, Migration and Anchorage-Independent Growth—To examine the biological consequences of different subcellular localizations of PTK6, HEK293 cells stably expressing Flag-PTK6, Myr-HA-PTK6 or NLS-HA-PTK6 were assayed for several properties associated with oncogenesis. First, we analysed cell proliferation by measuring [^{3}H]thymidine incorporation after exposure to EGF (Fig. 3A). Compared with control vector-transfected cells, expression of wild-type Flag-PTK6 markedly stimulated [^{3}H]thymidine incorporation after six or more days of EGF stimulation. Expression of Myr-HA-PTK6 enhanced cell proliferation over that induced by Flag-PTK6, whereas no further stimulation was seen in cells expressing NLS-HA-PTK6. We next analysed apoptosis of cells after 1- or 2-day incubation in serum-free medium containing various concentrations of H_2O_2; qualitatively by TUNEL assay (Fig. 4) or quantitatively by MTT assay (Fig. 3B). TUNEL assay showed that appearance of apoptotic cells were reduced by expression of Flag-PTK6, markedly in cells incubated with 500 μM H_2O_2 (Fig. 4). Similarly, MTT assay showed that expression of Flag-PTK6 inhibited induction of cell death by serum depletion irrespective of the presence of H_2O_2, but most markedly at 100 μM H_2O_2 (Fig. 3B). Expression of Myr-HA-PTK6 enhanced the anti-apoptotic activity of PTK6, whereas expression of NLS-HA-PTK6

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

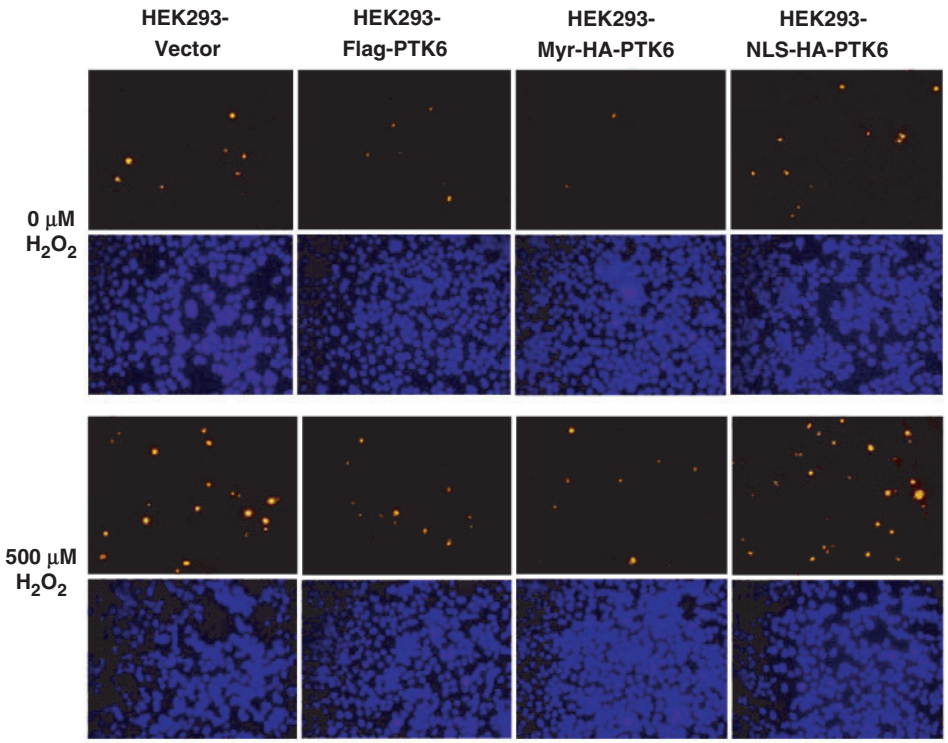


Fig. 4. **Detection of apoptosis in HEK 293 cells expressing PTK6 and its variants.** HEK 293 cells stably expressing Flag-PTK6, Myr-HA-PTK6, or NLS-HA-PTK6 were incubated in serum-free medium containing 0 or 500 μM H_2O_2 for 24 h. Apoptotic cells were detected by TUNEL staining (orange) as described under MATERIAL AND METHODS section. Nuclei were counterstained with DAPI (blue).

abolished the anti-apoptotic function (Figs 3B and 4). Thirdly, since PTK6 promotes EGF-induced migration (*24*), we used a chemotactic migration assay to determine the effect of PTK6 and its variants on migration of HEK 293 cells. Consistent with a previous report (*24*), chemotactic migration was stimulated in cells expressing Flag-PTK6 (Fig. 3C). The migration-stimulating activity of PTK6 was enhanced by targeting to the plasma membrane (Myr-HA-PTK6) but reduced to the level of the vector control by targeting to the nucleus (NLS-HA-PTK6). Lastly, the transforming potential of PTK6 and its variants was evaluated by examining the capacity of transfected HEK 293 cells for anchorage-independent growth. Compared with the vector control, expression of wild-type PTK6 stimulated colony formation in soft agar (Fig. 5). Expression of Myr-HA-PTK6 resulted in a significant further increase in the number and size of colonies formed, whereas expression of NLS-HA-PTK6 inhibited the colony-forming ability of PTK6 (Fig. 5). These findings suggest that targeting to the plasma membrane enhanced oncogenic functions of PTK6 in HEK 293 cells, whereas targeting to the nucleus suppressed such properties.

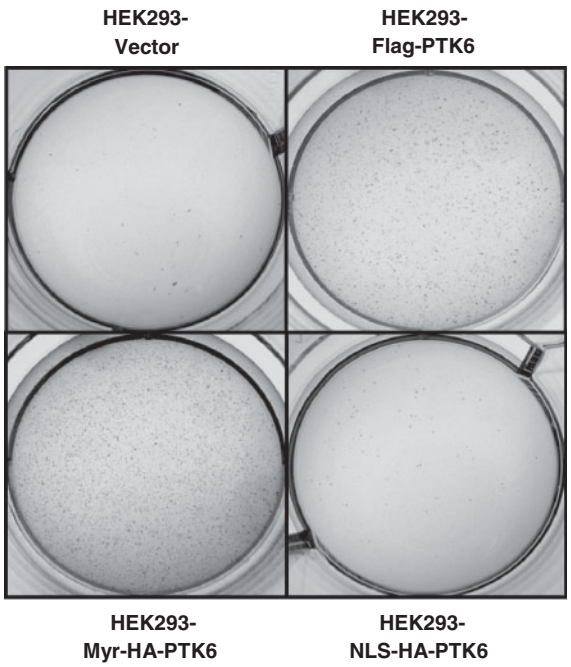
DISCUSSION

Although PTK6 shows strong homology to Src family members, it lacks an N-terminal myristoylation site (*1*, *13*), allowing localization in both the cytosol and nucleus. PTK6 is aberrantly expressed at higher levels in breast cancers and cultured breast tumour cells but not in normal mammary epithelial cells (*1*, *8*). In addition, subcellular localization of PTK6 differs between normal prostate epithelia and well-differentiated prostate tumours (*9*) and between normal oral epithelia and oral squamous cell carcinoma (*10*). These findings suggest that both the subcellular localization of PTK6 and its expression level may play a role in tumorigenesis. To address the impact of subcellular localization of PTK6 on tumorigenesis, we analysed the oncogenic properties of PTK6 using HEK 293 cell lines expressing either membrane-targeted (Myr-HA-PTK6) or nuclear-targeted (NLS-HA-PTK6) forms of PTK6.

The myristoylation signal of Src results in localization to the plasma membrane and is required for the transforming functions of Src (*14*, *26*). Inhibition of Src myristoylation by replacement of the N-terminal glycine with either alanine or glutamic acid abrogated its transforming potential (*27*). Although PTK6 lacks an N-terminal myristoylation site, in breast cancer cells PTK6 can be recruited to the plasma membrane upon activation of membrane-anchored signaling proteins, such as ErbB family members (*28–30*).

Expression of PTK6 sensitizes mammary epithelial cells to the mitogenic effects of EGF and permits anchorage-independent proliferation (*29*). PTK6 stimulates ErbB3 phosphorylation by association with epidermal growth factor receptor, which subsequently recruits PI3-kinase and Akt (*28*). Thus, it was suggested that breast cancers that express PTK6 might acquire

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012



Experiment	Number of colonies			
	HEK 293-Vector	HEK 293-Flag-PTK6	HEK 293-Myr-HA-PTK6	HEK 293-NLS-HA-PTK6
1	27	483	974	72
2	10	396	985	56
3	15	324	793	26
Mean ± SD	17 ± 9	401 ± 80	917 ± 108	51 ± 23

Fig. 5. **Anchorage-independent colony formation by HEK 293 cells stably expressing Flag-PTK6, Myr-HA-PTK6 and NLS-HA-PTK6.** HEK 293 cells transfected with pcDNA3.1 alone or stably expressing Flag-PTK6, Myr-HA-PTK6, or NLS-HA-PTK6 were plated on soft agar at a density of 1×10^4 cells/dish and grown for 5 weeks. Colonies larger than 50 μm in diameter were counted. Each experiment was performed in duplicate.

resistance to pro-apoptotic signals through regulation of PI3-kinase and Akt activity. PTK6 also promotes cell migration and invasion by EGF-dependent phosphorylation of paxillin and p190RhoGAP (*24*, *31*). In addition, a correlation between PTK6 and ErbB2 overexpression was found in tissue of invasive ductal breast carcinomas (*32*), and PTK6 increases the ErbB2-induced activation of Ras/MAPK signaling and cyclin E/cdk2 activity to induce cell proliferation in breast cancers (*30*). Consistent with the functional association of PTK6 with membrane-anchored signaling proteins, we found that targeted localization of PTK6 to the plasma membrane increases proliferation, migration, anchorage-independent colony formation, and cell survival, especially under conditions of oxidative stress, in the HEK 293 cell system.

Here, we showed that wild-type PTK6 and its variants differ in their ability to phosphorylate the specific substrates Stat3, paxillin and Sam68. In cells expressing endogenous PTK6, Stat3 is transcriptionally activated and tyrosine-phosphorylated, and stimulates proliferation (*25*). PTK6 directly binds and phosphorylates paxillin at Y31 and Y118, and phosphorylated paxillin induces cell migration and invasion (*24*). A strong increase in tyrosine phosphorylation of Stat3 and paxillin was detected in cells stably expressing the plasma membrane-anchored Myr-HA-PTK6, supporting the proposal that recruitment of PTK6 to the plasma membrane enhances oncogenic activities such as proliferation and migration.

A nuclear localization signal has not been found in PTK6; however, PTK6 has been detected in the nucleus when associated with one of its substrates, Sam68 (*23*). PTK6 phosphorylates Sam68 in Sam68-SLM nuclear bodies and inhibits its RNA-binding activities (*33*). An RNA-binding-defective splice variant of Sam68 fails to promote cell proliferation, thus its RNA-binding functions appear to positively regulate cell growth (*34*). Therefore, the ability of PTK6 to phosphorylate and inhibit the RNA-binding activities of Sam68 is expected to decrease cell growth and promote differentiation. In our study, nuclear localization of PTK6 abolished the ability of PTK6 to promote proliferation and migration, to prevent apoptosis of HEK 293 cells, and to permit anchorage-independent colony formation, in strong contrast to plasma membrane-anchored PTK6. In addition, expression of NLS-HA-PTK6 markedly increased the phosphorylation level of Sam68 compared with expression of wild-type PTK6. This result explains, at least in part, our observation that nuclear localization of PTK6 decreases its oncogenic function and previous reports that PTK6 tends to be localized in the nucleus in normal epithelia and well-differentiated carcinomas, but not in poorly differentiated carcinomas (*9*, *10*).

We have shown that PTK6 performs contrasting roles in tumorigenicity depending on its intracellular localization, displaying increased oncogenic abilities when targeted to the plasma membrane and decreased oncogenicity when targeted to the nucleus, as a result of access to different substrates and signaling molecules. In this regard, determination of PTK6 at the plasma membrane could be a valuable prognostic indicator for progression of various tumours, whereas nuclear PTK6 may provide a prognostic marker for long-term survival in patients with PTK6-positive cancers. In addition, identification of substrates that are specifically phosphorylated by membrane- or nuclear-localized PTK6 may provide potential targets for therapeutic intervention in the treatment of cancer.

ACKNOWLEDGEMENTS

We thank Mr Hyun Min Kim for technical assistance with site-directed mutagenesis and establishment of stable cell lines. pLNCX-mSrc was generously provided by Dr Eok-Soo Oh (Ewha Womans University, Korea).

FUNDING

Seoul Research and Business Development Grant (10527); the Korea Science and Engineering Foundation through the Protein Network Research Center at Yonsei University; the Brain Korea 21 Trainee Program.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Mitchell, P.J., Barker, K.T., Martindale, J.E., Kamalati, T., Lowe, P.N., Page, M.J., Gusterson, B.A., and Crompton, M.R. (1994) Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours. *Oncogene* **9**, 2383–2390
2. Bae, J.-S. and Lee, S.-T. (2001) The human PTK6 interacts with a 23-kDa tyrosine-phosphorylated protein and is localized in cytoplasm in breast carcinoma T-47D cells. *J. Biochem. Mol. Biol.* **34**, 33–38
3. Brabek, J., Mojzita, D., Novotny, M., Puta, F., and Folk, P. (2002) The SH3 domain of Src can downregulate its kinase activity in the absence of the SH2 domain-pY527 interaction. *Biochem. Biophys. Res. Commun.* **296**, 664–670
4. Easty, D.J., Mitchell, P.J., Patel, K., Florenes, V.A., Spritz, R.A., and Bennett, D.C. (1997) Loss of expression of receptor tyrosine kinase family genes PTK7 and SEK in metastatic melanoma. *Int. J. Cancer* **71**, 1061–1065
5. Chen, W.S., Kung, H.J., Yang, W.K., and Lin, W.c. (1999) Comparative tyrosine-kinase profiles in colorectal cancers: enhanced arg expression in carcinoma as compared with adenoma and normal mucosa. *Int. J. Cancer* **83**, 579–584
6. Llor, X., Serfas, M.S., Bie, W., Vasioukhin, V., Polonskaia, M., Derry, J., Abbott, C.M., and Tyner, A.L. (1999) BRK/Sik expression in the gastrointestinal tract and in colon tumors. *Clin. Cancer Res.* **5**, 1767–1777
7. Lee, S.-T., Strunk, K.M., and Spritz, R.A. (1993) A survey of protein tyrosine kinase mRNAs expressed in normal human melanocytes. *Oncogene* **8**, 3403–3410
8. Barker, K.T., Jackson, L.E., and Crompton, M.R. (1997) BRK tyrosine kinase expression in a high proportion of human breast carcinomas. *Oncogene* **15**, 799–805
9. Derry, J.J., Prins, G.S., Ray, V., and Tyner, A.L. (2003) Altered localization and activity of the intracellular tyrosine kinase BRK/Sik in prostate tumor cells. *Oncogene* **22**, 4212–4220
10. Petro, B.J., Tan, R.C., Tyner, A.L., Lingen, M.W., and Watanabe, K. (2004) Differential expression of the non-receptor tyrosine kinase BRK in oral squamous cell carcinoma and normal oral epithelium. *Oral Oncol.* **40**, 1040–1047
11. Boutin, J.A. (1997) Myristoylation. *Cell Signal* **9**, 15–35
12. Melchior, F. and Gerace, L. (1995) Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.* **7**, 310–318
13. Lee, H., Kim, M., Lee, K.H., Kang, K.N., and Lee, S.-T. (1998) Exon-intron structure of the human PTK6 gene demonstrates that PTK6 constitutes a distinct family of non-receptor tyrosine kinase. *Mol. Cells* **8**, 401–407
14. Cross, F.R., Garber, E.A., Pellman, D., and Hanafusa, H. (1984) A short sequence in the p60src N terminus is required for p60src myristylation and membrane association and for cell transformation. *Mol. Cell. Biol.* **4**, 1834–1842
15. Kalderon, D., Roberts, B.L., Richardson, W.D., and Smith, A.E. (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39**, 499–509
16. Jordan, M., Schallhorn, A., and Wurm, F.M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* **24**, 596–601
17. Kim, H.I. and Lee, S.-T. (2005) An intramolecular interaction between SH2-kinase linker and kinase domain is essential for the catalytic activity of protein-tyrosine kinase-6. *J. Biol. Chem.* **280**, 28973–28980
18. Jung, J.W., Shin, W.S., Song, J., and Lee, S.-T. (2004) Cloning and characterization of the full-length mouse Ptk7 cDNA encoding a defective receptor protein tyrosine kinase. *Gene* **328**, 75–84
19. Park, M. and Lee, S.-T. (1999) The fourth immunoglobulin-like loop in the extracellular domain of FLT-1, a VEGF receptor, includes a major heparin-binding site. *Biochem. Biophys. Res. Commun.* **264**, 730–734
20. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63
21. Shin, W.S., Maeng, Y.S., Jung, J.W., Min, J.K., Kwon, Y.G., and Lee, S.-T. (2008) Soluble PTK7 inhibits tube formation, migration, and invasion of endothelial cells and angiogenesis. *Biochem. Biophys. Res. Commun.* **371**, 793–798
22. Kim, Y.M., Hwang, S., Kim, Y.M., Pyun, B.J., Kim, T.Y., Lee, S.-T., Gho, Y.S., and Kwon, Y.G. (2002) Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. *J. Biol. Chem.* **277**, 27872–27879
23. Derry, J.J., Richard, S., Valderrama-Carvajal, H., Ye, X., Vasioukhin, V., Cochrane, A.W., Chen, T., and Tyner, A.L. (2000) Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability. *Mol. Cell. Biol.* **20**, 6114–6126
24. Chen, H.Y., Shen, C.H., Tsai, Y.T., Lin, F.C., Huang, Y.P., and Chen, R.H. (2004) Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin. *Mol. Cell. Biol.* **24**, 10558–10572
25. Liu, L., Gao, Y., Qiu, H., Miller, W.T., Poli, V., and Reich, N.C. (2006) Identification of STAT3 as a specific substrate of breast tumor kinase. *Oncogene* **25**, 4904–4912
26. Shoji, S. and Kubota, Y. (1989) Function of protein myristoylation in cellular regulation and viral proliferation. *Yakugaku Zasshi* **109**, 71–85
27. Kamps, M.P., Buss, J.E., and Sefton, B.M. (1985) Mutation of NH2-terminal glycine of p60src prevents both myristoylation and morphological transformation. *Proc. Natl Acad. Sci. USA* **82**, 4625–4628
28. Kamalati, T., Jolin, H.E., Fry, M.J., and Crompton, M.R. (2000) Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation. *Oncogene* **19**, 5471–5476
29. Kamalati, T., Jolin, H.E., Mitchell, P.J., Barker, K.T., Jackson, L.E., Dean, C.J., Page, M.J., Gusterson, B.A., and Crompton, M.R. (1996) Brk, a breast tumor-derived non-receptor protein-tyrosine kinase, sensitizes mammary epithelial cells to epidermal growth factor. *J. Biol. Chem.* **271**, 30956–30963
30. Xiang, B., Chatti, K., Qiu, H., Lakshmi, B., Krasnitz, A., Hicks, J., Yu, M., Miller, W.T., and Muthuswamy, S.K. (2008) Brk is coamplified with ErbB2 to promote proliferation in breast cancer. *Proc. Natl Acad. Sci. USA* **105**, 12463–12468
31. Shen, C.H., Chen, H.Y., Lin, M.S., Li, F.Y., Chang, C.C., Kuo, M.L., Settleman, J., and Chen, R.H. (2008) Breast tumor kinase phosphorylates p190RhoGAP to regulate rho and ras and promote breast carcinoma growth, migration, and invasion. *Cancer Res.* **68**, 7779–7787
32. Born, M., Quintanilla-Fend, L., Braselmann, H., Reich, U., Richter, M., Hutzler, P., and Aubele, M. (2005) Simultaneous over-expression of the Her2/neu and PTK6 tyrosine kinases in archival invasive ductal breast carcinomas. *J. Pathol.* **205**, 592–596
33. Lukong, K.E., Larocque, D., Tyner, A.L., and Richard, S. (2005) Tyrosine phosphorylation of sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression. *J. Biol. Chem.* **280**, 38639–38647
34. Barlat, I., Maurier, F., Duchesne, M., Guitard, E., Tocque, B., and Schweighoffer, F. (1997) A role for Sam68 in cell cycle progression antagonized by a spliced variant within the KH domain. *J. Biol. Chem.* **272**, 3129–3132

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012