PTK6 (protein tyrosine kinase 6; also known as Brk (breast tumour kinase)) is a non-receptor tyrosine kinase, closely related to Src, but evolutionarily distinct, that is up-regulated in various cancers, including breast cancer. Hsp90 (heat-shock protein 90) was identified as a PTK6-interacting protein in HEK (human embryonic kidney)-293 cells overexpressing PTK6. Hsp90 interacted with the PTK6 tyrosine kinase catalytic domain, but catalytic activity was not required for the interaction. Geldanamycin, an Hsp90 inhibitor, significantly decreased the PTK6 protein level through proteasome-dependent degradation, but did not affect the level of Src. Geldanamycin treatment also decreased phosphorylation of PTK6 substrates due to reduced amounts of PTK6. Moreover, overexpression of CHIP [C-terminus of Hsc70 (heat-shock cognate 70)-interacting protein], a chaperone-dependent E3 ligase, enhanced proteosomal degradation of PTK6. Geldanamycin increased the interaction of PTK6 with CHIP, but decreased the interaction of PTK6 with Hsp90. We also found that endogenous PTK6 associated with Hsp90 and geldanamycin decreased expression of endogenous PTK6 in breast carcinoma cells. Finally, we report that silencing endogenous CHIP expression in breast carcinoma cells inhibited geldanamycin-induced PTK6 reduction. These results demonstrate that Hsp90 plays an essential role in regulating PTK6 stability and suggest that Hsp90 inhibitors may be useful as therapeutic drugs for PTK6-positive cancers, including breast cancer.

Key words: C-terminus of Hsc70 (heat-shock cognate 70)-interacting protein (CHIP), heat-shock protein 90 (Hsp90), proteosomal degradation, protein stability, PTK6 protein tyrosine kinase 6 (PTK6), tyrosine kinase.

INTRODUCTION

PTK6 (protein tyrosine kinase 6; also known as Brk (breast tumour kinase)) is an intracellular tyrosine kinase that was first identified in human melanocytes [1]. PTK6 contains an SH (Src homology) 3 domain, an SH2 domain and a catalytic tyrosine kinase domain. It is closely related to Src family members, but evolutionarily distinct in several aspects, including lack of an N-terminal myristoylation signal, unique exon organization and low homology with Src family members [2]. Although PTK6 is not expressed in normal mammary tissues or in benign lesions, it is up-regulated in various cancers, including breast tumours [3,4]. With PTK6 being detected in over 80% of human invasive ductal breast tumours [5], PTK6 up-regulation has been identified as the most common aberration in ductal breast tumours. Expression of PTK6 makes mammary epithelial cells more sensitive to the mitogenic effects of EGF (epidermal growth factor) and increases anchorage-independent proliferation [6,7]. Down-regulation of PTK6 reduces proliferation in breast carcinoma cells [8] as well as decreasing EGFR- or HER2-dependent migration and invasion. Akt is a direct substrate of PTK6 and its phosphorylation promotes proliferation by enhancing their transcriptional activity.

The interaction of PTK6 with the ErbB (v-erb-b2 erythroblastic leukaemia viral oncogene homologue) family members [EGFR (EGF receptor), ErbB2, ErbB3 and ErbB4] is involved in tumour development. PTK6 enhances PI3K (phosphoinositide 3-kinase)/Akt signalling by increasing ErbB3 phosphorylation and promotes ErbB2-dependent Ras/MAPK signalling. In addition, Akt is a direct substrate of PTK6 and its phosphorylation promotes oncogenic signalling. Phosphorylation of paxillin and p190RhoGAP by PTK6 contributes to cell migration and invasion. EGFR signalling is enhanced by inhibiting EGFR down-regulation through PTK6-dependent phosphorylation of ARAP1 [ArfGAP with RhoGAP domain, ankryin repeat and PH (pleckstrin homology) domain 1] [10].

In an attempt to understand the roles of PTK6, we screened interacting proteins of PTK6 using a proteomic approach [10]. One of the PTK6-interacting proteins we identified was Hsp90 (heat-shock protein) 90. Hsp90 is a main functional component of the important cytoplasmic chaperone complex and plays an important role in the folding of newly synthesized proteins as well as in the stabilization and refolding of denatured proteins after stress [11,12]. An inducible form, Hsp90α, and a constitutive form, Hsp90β, are predominantly present as homodimers and are localized in the cytoplasm [12]. Other Hsp90 family members...
were identified in other cellular compartments, e.g. Grp94 (94 kDa glucose-regulated protein; also known as Hsp90B1) in the endoplasmic reticulum and TRAP-1 [TNF (tumour necrosis factor) receptor-associated protein 1] in mitochondria [11,12]. Hsp90 is involved in the conformational maturation of Hsp90 clients, including proteins involved in signal transduction, such as kinases and hormone receptors [13].

In the present paper, we report the role of Hsp90 as an interacting protein of PTK6. We determined which domain of PTK6 binds to Hsp90 and whether the catalytic activity of PTK6 is necessary for their interaction. Using an Hsp90 inhibitor, we examined a role for Hsp90 in the stability and function of PTK6. We also analysed the molecular mechanism for PTK6 degradation in the presence of the Hsp90 inhibitor. On the basis of our findings, we discuss the potential of Hsp90 inhibitors as anti-cancer agents for PTK6-positive malignancies.

EXPERIMENTAL

Antibodies and reagents
Anti-Hsp90α and anti-Hsp90β antibodies were purchased from Stressgen. Anti-PTK6 (Brk), anti-CHIP [C-terminus of Hsc70 (heat-shock cognate 70)-interacting protein], anti-Hsp70, anti-β-actin, anti-STAT3, anti-Sam68, anti-paxillin, anti-Myc and anti-HA (haemagglutinin) antibodies were purchased from Santa Cruz Biotechnology. Anti-Src antibody was purchased from Cell Signaling Technology. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was purchased from AbFrontier. Anti-(FLAG M2) antibody and anti-(FLAG M2) antibody-conjugated agarose were purchased from Sigma. G418 (Geneticin), geldanamycin and MG132 were purchased from A.G. Scientific.

Expression constructs
The human PTK6 expression vectors, pcDNA3.1-FLAG-PTK6 and pcDNA3.1-PTK6, have been described previously [7,14]. The PTK6 kinase-dead mutant (FLAG-PTK6-K219M), constitutively active mutant (FLAG-PTK6-Y447F), SH3-binding-defective mutant (termed FLAG-PTK6-3PA, which harbours P175A/P177A/P179A) and a hyperactive mutant (FLAG-PTK6-3PA/Y447F) were generated in FLAG–PTK6 expression plasmids by site-directed mutagenesis with the QuikChange® kit (Stratagene) according to the manufacturer’s protocol. FLAG-PTK6-SH3-SH2 and FLAG-PTK6-Linker-Kinase constructs were described previously [10]. Expression constructs for CHIP, Myc–CHIP and its mutants, and HA–ubiquitin were as described previously [15]. All constructs were confirmed by DNA sequencing.

Cell culture and transfection
HEK (human embryonic kidney)-293 cells and the human breast carcinoma cell lines T-47D and BT-474 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transient expression, HEK-293 cells or HEK-293 cells stably expressing PTK6 were transfected with each of the indicated expression constructs using Lipofectamine® (Invitrogen) according to the manufacturer’s recommendations. For stable expression, HEK-293 cells were transfected with each expression construct using the calcium phosphate method, followed by selection with 1200 μg/ml of G418. After 2 weeks, G418-resistant colonies were cloned and expanded.

Preparation of cell lysates
Subconfluent HEK-293-FLAG–PTK6 cells were washed twice with ice-cold PBS. Unless specified otherwise, cells were lysed on ice for 10 min in lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate and 0.05% protease inhibitor cocktail (catalogue number P8340, Sigma)] and centrifuged at 10000 g for 10 min at 4°C. To obtain whole-cell lysates, cells were lysed in SDS sample buffer [50 mM Tris/HCl (pH 6.8), 2% SDS, 0.1% Bromophenol Blue and 10% glycerol].

Western blot analyses and immunoprecipitation assays
For Western blot analyses, cell lysates were mixed with SDS sample buffer containing 100 mM 2-mercaptetoethanol, resolved by SDS/PAGE (10% gel) and transferred on to PVDF membranes. The immunoreactive proteins were detected with primary antibody, horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (Millipore). For immunoprecipitation assays, the soluble fraction of the cell lysate was incubated with the anti-(FLAG M2) antibody-conjugated agarose equilibrated in PBS at 4°C for 4 h. The resin was washed in PBS three times. The proteins bound to the resin were mixed with SDS sample buffer containing 100 mM 2-mercaptetoethanol, resolved by SDS/PAGE (10% gel) and analysed by Western blot analysis. For quantification of PTK6 level, chemiluminescence was detected by LAS-3000 (Fujifilm) and quantified by Multi Gauge v2.2 software (Fujifilm). Statistical analyses were performed using Student’s t test.

In vivo ubiquitination assays
HEK-293 cells stably expressing FLAG–PTK6 were transfected with HA–ubiquitin and/or CHIP expression vectors, followed by treatment with 1 μM geldanamycin and 1 μM MG132. The cell lysate was incubated with the anti-(FLAG M2) antibody-conjugated agarose, followed by Western blotting with the anti-HA antibody to detect ubiquitinated PTK6.

Knockdown of CHIP
The CHIP siRNA (short interfering RNA; QIAGEN; target sequence, 5′-CCCGCUUGGUGCCGGUGAUU-3′) [15] was used. The CHIP siRNA or scrambled siRNA was transfected into T-47D and BT-474 cells using Lipofectamine®. After 48 h of transfection, cells were treated with 1 μM geldanamycin and subjected to Western blotting using anti-PTK6 and anti-CHIP antibodies.

RESULTS

The PTK6 and Hsp90 interaction requires the Linker-Kinase domain of PTK6 but not its catalytic activity
In a previous study, we identified Hsp90 as an interacting protein of PTK6 [10]. To confirm the interaction of Hsp90 with PTK6 and analyse the effect of PTK6 catalytic activity on their binding, HEK-293 cells were stably transfected with wild-type FLAG-PTK6, FLAG-PTK6-3PA/Y447F or FLAG-PTK6-K219M. FLAG-PTK6-3PA/Y447F is a catalytically hyperactive mutant which has three proline residues changed to alanines (3PA) in a linker region binding to the SH3 domain and a Y447F mutation at the C-terminal tail [7,16]. FLAG-PTK6-K219M is...
A mutant that destroys the tyrosine kinase ATP-binding site and thus is catalytically inactive [7] (Supplementary Figure S1 at http://www.BiochemJ.org/bj/447/bj4470313add.htm). FLAG–PTK6 was immunoprecipitated by anti-FLAG agarose and the precipitates were subjected to Western blot analyses with anti-FLAG, anti-Hsp90α, and anti-Hsp90β antibodies. From these experiments, we found that PTK6 interacts with both Hsp90α and Hsp90β (Figure 1). Interestingly, both hyperactive and catalytically inactive mutants of PTK6 showed higher binding to Hsp90α and Hsp90β when compared with the wild-type PTK6 (Figure 1). This result suggests that any mutation that distorts the normal tertiary structure of PTK6, rather than loss or increase of catalytic activity, may increase its interaction with Hsp90.

To identify which domain of PTK6 interacts with Hsp90, HEK-293 cells were transfected with FLAG–PTK6-SH3-SH2, FLAG–PTK6-Linker-Kinase [which has a catalytic domain of tyrosine kinase (Kinase domain) with a linker (Linker) between the SH2 and Kinase domains and a short C-terminal tail] or FLAG–PTK6 as a positive control (Supplementary Figure S1). FLAG–PTK6-SH3-SH2 did not interact with Hsp90α and Hsp90β, but the FLAG–PTK-Linker-Kinase mutant did (Supplementary Figure 2A at http://www.BiochemJ.org/bj/447/bj4470313add.htm). Therefore the Linker-Kinase domain of PTK6 is essential for binding with Hsp90.

**An Hsp90 inhibitor, geldanamycin, decreases PTK6 expression**

To understand the significance of Hsp90 binding to PTK6, geldanamycin, an Hsp90 inhibitor, was used to treat HEK-293 cells stably expressing wild-type FLAG–PTK6 or its various mutants. The mutant constructs used in these experiments were FLAG–PTK6-K219M (the kinase-dead mutant), FLAG–PTK6-3PA/Y447F (the hyperactive mutant), FLAG–PTK6-3PA (the SH3-binding defective mutant) and FLAG–PTK6-Y447F (the constitutively active mutant) (Supplementary Figure S1). Geldanamycin decreased expression of the wild-type PTK6 in a time-dependent manner. The K219M, 3PA/Y447F and 3PA mutants of PTK6 were more rapidly degraded than the wild-type PTK6 (Figure 2). However, the constitutively active Y447F mutation at the C-terminal tail did not show rapid degradation like the other mutants, but was very similar to the wild-type PTK6. Therefore these data suggest that the geldanamycin-induced reduction in PTK6 expression may result from decreased protein stability rather than from changes in catalytic activity.

We then analysed which PTK6 domain is required for the geldanamycin-induced reduction of PTK6. Expression of PTK6–Linker–Kinase, as well as of the wild-type PTK6, was decreased upon geldanamycin treatment, but the level of PTK6–SH3–SH2 was not (Supplementary Figure 2B). This result demonstrates that the Linker-Kinase domain of PTK6 is a target for Hsp90.

To measure the half-life of PTK6 in HEK-293 cells, we added a protein synthesis inhibitor, cycloheximide, along with geldanamycin, to HEK-293 cells stably expressing FLAG–PTK6. The half-life of PTK6 in the absence of geldanamycin was approximately 24 h, but in the presence of geldanamycin it was decreased to approximately 10 h (Supplementary Figure S3 at http://www.BiochemJ.org/bj/447/bj4470313add.htm). These results suggest that Hsp90 ensures the stability of PTK6 by interacting with its Linker-Kinase domain.

**The geldanamycin-induced reduction in PTK6 expression involves proteosome-dependent protein degradation**

It is known that geldanamycin-induced dissociation of Hsp90 and its interacting proteins enhances degradation of the interactors through the ubiquitin–proteasome pathway [17]. To
Figure 3 The geldanamycin-induced decrease in PTK6 involves proteasomal degradation

(A) Flag–PTK6 was stably expressed in HEK-293 cells. The cells were treated with 1 μM geldanamycin (GA) and 1 μM MG132 for the indicated time periods and lysed in SDS sample buffer. PTK6 expression in whole cell lysates was assayed by Western blot (IB) using the anti-PTK6 antibody. (B) HEK-293 cells stably expressing Flag–PTK6 were transiently transfected with HA–ubiquitin (Ub) and treated with 1 μM geldanamycin and 1 μM MG132 for 6 h. Flag–PTK6 was immunoprecipitated (IP) with the anti-FLAG antibody. Ubiquitination of Flag–PTK6 was analysed by Western blotting using the anti-HA antibody. β-Actin and GAPDH were used as protein loading controls.

examine whether the proteasome mediates the geldanamycin-induced decrease of PTK6 expression, a proteasome inhibitor, MG132, along with geldanamycin, was added to HEK-293 cells stably expressing FLAG–PTK6. When cells were lysed with SDS sample buffer containing 2% SDS, we found that MG132 prevents the geldanamycin-induced decrease in PTK6 expression (Figure 3A). Next, to investigate whether PTK6 is ubiquitinated during geldanamycin-induced degradation, HEK-293 cells stably expressing FLAG-PTK6 were transiently transfected with HA–ubiquitin (Ub) and treated with 1 μM geldanamycin and 1 μM MG132 for 6 h. Flag–PTK6 was immunoprecipitated (IP) with the anti-FLAG antibody. Ubiquitination of Flag–PTK6 was analysed by Western blotting using the anti-HA antibody. β-Actin and GAPDH were used as protein loading controls.

Figure 4 Role of Hsp90 on the stability of endogenous PTK6 in breast carcinoma cells

(A) Endogenously expressed PTK6 in T-47D and BT-474 cells was immunoprecipitated (IP) with the anti-PTK6 antibody. Interaction of Hsp90α and Hsp90β with endogenous PTK6 was analysed by Western blotting (IB) using the anti-Hsp90α and anti-Hsp90β antibodies. (B and C) T-47D and BT-474 cells were treated with 1 μM geldanamycin (GA) for the indicated time periods. Expression of PTK6 and Src was examined by Western blotting using the anti-PTK6 and anti-Src antibodies. β-Actin and GAPDH were used as protein loading controls in T-47D and BT-474 cells respectively.

at http://www.BiochemJ.org/bj/447/bj4470313add.htm). These results indicate that the geldanamycin-induced reduction of PTK6 via proteasomal degradation inhibits phosphorylation of PTK6 substrates, further reflecting the importance of Hsp90 for the cellular function of PTK6.

Hsp90 stabilizes endogenously expressed PTK6 in breast carcinoma cells

Because the role of Hsp90 on PTK6 stability was studied in the HEK-293 cell system overexpressing PTK6, we additionally analysed it in the human breast carcinoma cell lines, T-47D and BT-474, which endogenously express PTK6. Consistent with the results using overexpressed PTK6, endogenous PTK6 also interacted with Hsp90α and Hsp90β in T-47D and BT-474 cells (Figure 4A). Geldanamycin also decreased endogenous PTK6 expression in a time-dependent manner (Figure 4B). However, geldanamycin did not affect endogenous Src levels in T-47D and BT-474 cells (Figure 4C). Therefore PTK6 is more dependent on Hsp90 than Src to maintain its stability in human breast carcinoma cells.

Geldanamycin-induced PTK6 degradation is enhanced by CHIP expression

A chaperone-dependent E3 ligase, CHIP, has been reported to induce the ubiquitination of Hsp90-interacting proteins, including ErbB2 [18,19]. To examine whether CHIP is involved in the geldanamycin-induced decrease in PTK6, CHIP was

Phosphorylation of protein substrates by PTK6 is decreased in the presence of geldanamycin

PTK6 is a protein tyrosine kinase and phosphorylates protein substrates including Sam68, paxillin and STAT3 [4,9]. Thus we evaluated whether geldanamycin affects PTK6 phosphorylation of protein substrates. As expected, treatment of geldanamycin decreased phosphorylation of cellular proteins by PTK6, including decreased phosphorylation of STAT3, Sam68 and paxillin (Supplementary Figure S4...
Hsp90 rescues PTK6 from proteasomal degradation

Figure 5 Effect of CHIP expression on geldanamycin-induced PTK6 degradation

(A and B) HEK-293 cells stably expressing PTK6 were transiently transfected with the CHIP expression vector. After the addition of 1 μM geldanamycin (GA) for the indicated time periods, PTK6 expression was examined by Western blot analysis (IB) using the anti-PTK6 antibody. (A) A representative immunoblot. (IB) For quantification, PTK6 expression was normalized to β-actin levels. PTK6 expression at 0 h was defined as 100%. Results are means±S.D. from three independent experiments. (C) HA–ubiquitin (Ub/ub) and CHIP were transiently expressed in HEK-293 cells stably expressing FLAG–PTK6. The cells were treated with 1 μM geldanamycin and 1 μM MG132 for 6 h and FLAG–PTK6 was immunoprecipitated (IP) with the anti-FLAG antibody. FLAG–PTK6 ubiquitination was analysed by Western blotting using the anti-HA antibody.

transiently expressed in HEK-293 cells stably expressing PTK6. Overexpression of CHIP enhanced the geldanamycin-induced decrease in PTK6 levels (Figures 5A and 5B). To examine whether CHIP is involved in the ubiquitination of PTK6, CHIP and HA–ubiquitin were co-expressed in HEK-293 cells stably expressing FLAG–PTK6. Indeed, CHIP enhanced the geldanamycin-induced polyubiquitination of PTK6 in the presence of geldanamycin and MG132 (Figure 5C). These findings suggest that CHIP plays a role as an E3 ligase in the geldanamycin-induced decrease in PTK6 expression.

CHIP contains an N-terminal TPR (tetra-tricopeptide repeat) domain, required for binding to chaperones, and a C-terminal U-box domain involved in ubiquitin ligase activity [20]. To analyse which domain of CHIP is involved in PTK6 degradation, wild-type CHIP, the TPR domain mutant (CHIP-K30A) or the U-box domain mutant (CHIP-H260Q) were transiently expressed in HEK-293 cells stably expressing FLAG–PTK6. After geldanamycin treatment, degradation of PTK6 was enhanced by wild-type CHIP expression, but not by the CHIP-K30A or CHIP-H260Q mutants (Figures 6A and 6B). Thus both the TPR domain and U-box domain of CHIP are necessary for the geldanamycin-induced degradation of PTK6.

To evaluate the interaction between PTK6 and CHIP, HEK-293 cells stably expressing FLAG–PTK6 were transiently transfected with wild-type CHIP, the CHIP-K30A mutant or the CHIP-H260Q mutant. The CHIP-K30A mutant, which has a defect in binding Hsp proteins, did not interact with PTK6 whereas wild-type CHIP and CHIP-H260Q both interacted with PTK6 in the presence of geldanamycin (Figure 6C). These data indicate that the TPR domain of CHIP is required for its interaction with PTK6 and its function as an E3 ligase in PTK6 degradation.

PTK6 interacts with Hsp90 in the absence of geldanamycin or with Hsp70 and CHIP in the presence of geldanamycin

Another important chaperone, Hsp70, is also involved in the proper folding and stability of client proteins through complex formation [13, 17]. To understand the PTK6–chaperone complex involved in the stability of PTK6, the interaction of PTK6 with Hsp90, Hsp70 and CHIP was examined. In the absence of geldanamycin, PTK6 associates with Hsp90α and Hsp90β, with no observed association between Hsp70 and CHIP. However, in the presence of geldanamycin, PTK6 associates with Hsp70 and CHIP, but not with Hsp90 (Figure 7). These results indicate that, under normal cellular conditions, PTK6 interacts with Hsp90, but not with Hsp70. However, in the presence of geldanamycin, PTK6 is released from Hsp90 and forms a complex with Hsp70 and CHIP for ubiquitin-dependent degradation.

CHIP is required for the geldanamycin-induced PTK6 degradation in breast carcinoma cells

To confirm the role of CHIP in PTK6 stability in breast carcinoma cells, endogenous CHIP was knocked down using CHIP siRNA in T-47D and BT-474 cells. Endogenous PTK6 was degraded by geldanamycin treatment in both T-47D and BT-474 cells. However, knockdown of CHIP clearly blocks the geldanamycin-induced degradation of endogenous PTK6 (Figure 8). These results support our hypothesis that CHIP plays a role in the geldanamycin-induced degradation of endogenous PTK6 in human breast carcinoma cells.

DISCUSSION

In our previous study, Hsp90 was determined to be one of the PTK6-interacting proteins [10]. Hsp90, an abundant molecular chaperone, is involved in protein folding and stabilization and the refolding of denatured proteins [11]. Client proteins of Hsp90 include many protein kinases, as determined by co-precipitation of v-Src, Fes, Fps and Yes with Hsp90 [21,22]. The stability and activation of many protein kinases depends on their interaction with the Hsp90 molecular chaperone system [23]. From these findings, we hypothesized that Hsp90 might have a role in the folding and stability of PTK6. As expected, we
found that PTK6 interacts with Hsp90 and that the Linker-Kinase domain of PTK6 is essential for the PTK6–Hsp90 interaction, though its catalytic activity is not required. An Hsp90 inhibitor, geldanamycin, disrupts the interaction between Hsp90 and its client proteins by inhibiting Hsp90 ATPase activity [24]. We found that geldanamycin not only abolishes the interaction of Hsp90 with PTK6, but also decreases the intracellular level of PTK6.

In addition, in breast carcinoma T-47D and BT-474 cell lines, endogenously expressed PTK6 was significantly decreased by geldanamycin. Although Src is also an Hsp90 client [23,25], geldanamycin did not affect the Src level. Therefore PTK6 is more dependent on Hsp90 than Src in maintaining its stability in human breast carcinoma cells. These findings suggest that PTK6 is less stable and thus more dependent on Hsp90 chaperone function than Src.

We found that the half-life of PTK6 protein depends on Hsp90 chaperone activity. Inhibition of Hsp90 by geldanamycin rapidly increases degradation of unstable proteins. In the present study, we used several mutants important for catalytic activity and auto-inhibition of PTK6. The kinase-dead K219M mutation in the PTK6 molecule prevents binding of ATP at the ATP-binding site of tyrosine kinases. The SH3-binding-defective 3PA mutant changes three proline residues to alanines (3PA) within the Linker region and prevents binding of the Linker to the SH3 domain. The constitutively active Y447F mutation at the C-terminal tail prevents inhibitory binding of the phospho-tyrosine residue to the SH2 domain. Both the 3PA and Y447F mutations contribute to enhanced PTK6 signalling and thus the 3PA/Y447F mutation is the most hyperactive [7,16]. Interestingly, the kinase-dead K219M and hyperactive 3PA/Y447F mutants of PTK6 interacted with Hsp90 more than the wild-type PTK6. Moreover, K219M, 3PA/Y447F and the SH3-binding-defective 3PA mutants showed faster degradation in the presence of geldanamycin as compared with the wild-type PTK6 or the constitutively active PTK6-Y447F mutant. Previously, Zhang et al. [26] also showed that the Y447F mutant of PTK6 behaves more like the wild-type PTK6 and Y447F mutant. Previously, Zhang et al. [26] also showed that the Y447F mutant of PTK6 behaves more like the wild-type PTK6 and Y447F mutant. Previously, Zhang et al. [26] also showed that the Y447F mutant of PTK6 behaves more like the wild-type PTK6 and Y447F mutant. Therefore we hypothesize that subtle changes in the tertiary structure of PTK6, particularly in the Linker-Kinase domain, which is an essential unit for its catalytic activity, can modify the ability to recruit other proteins and thus change its fate, such as degradation or downstream signalling.

Degradation of Hsp90 client proteins is known to occur via the ubiquitin–proteasome pathway [17]. We also showed that geldanamycin-induced PTK6 reduction is dependent on
proteasomal degradation by analyses of soluble and insoluble fractions and ubiquitin binding after treatment with MG132. Previously, several E3 ubiquitin ligases containing chaperone-interacting motifs, such as CHIP, parkin and cullin 5, were identified [19,27,28]. Of these, CHIP is a 35-kDa E3 ubiquitin ligase identified by interacting with Hsc/Hsp70 [19]. The TPR domain of CHIP interacts with Hsc/Hsp70 and Hsp90. The U-box domain, which is closely related to a RING domain, has E3 ubiquitin ligase activity. Overexpression of CHIP promotes ubiquitination and degradation of many Hsp90 client proteins, including glucocorticoid receptor, ErbB2, p53 and FGFR3 (fibroblast growth factor receptor 3) [18,19,29,30]. Because CHIP is a well-known E3 ligase for Hsp90 clients, the role of CHIP in proteasomal degradation of PTK6 was analysed. We found that overexpression of CHIP enhanced geldanamycin-induced PTK6 degradation. In addition, using a TPR domain K30A mutant that disrupts interaction with Hsp90 or Hsc/Hsp70, and a U-box domain H260Q mutant that abolishes E3 ligase activity [18], we demonstrated that both the TPR and U-box domains of CHIP were required for PTK6 ubiquitination. From these results, we predicted that CHIP is one of the E3 ubiquitin ligases used in proteasome-mediated PTK6 degradation.

The ATP-dependent Hsp90 chaperone–client cycle has been studied extensively [17]. This cycle initiates upon binding of Hsp40/Hsp70 to a client, and Hsp90-HOP (60-kDa Hsp organizer protein) binds to the Hsp40/Hsp70–client early complex to form an intermediate complex. The intermediate complex releases Hsp40/Hsp70 and HOP by ATP hydrolysis of Hsp90 and thus becomes a mature complex of Hsp90 and the client protein with co-chaperones such as p23. An Hsp90 inhibitor, geldanamycin, dissociates the mature complex by inhibiting the ATPase activity of Hsp90. The released client protein is then degraded through the ubiquitin–proteasome pathway. As shown in the present study, it seems that a fraction of PTK6 interacts with Hsp90 and forms a mature chaperone–client complex to maintain a stable state. Disruption of this complex by geldanamycin leads to ubiquitin–proteasome-dependent degradation of PTK6 through its binding with CHIP and Hsp70.

Hsp90 is expressed at higher levels in tumour cells than in normal cells [31]. Increased Hsp90 activity helps cancer progression, including breast cancer progression, by chaperoning mature oncogenic proteins such as ErbB2 and c-Src [32,33]. Currently, Hsp90 inhibitors such as benzoquinone ansamycin 17-allylaminio-17-desmethoxygeldanamycin are undergoing clinical trials in cancer treatment [34]. PTK6 is an important oncoprotein that induces proliferation, migration and anchorage-independent growth as well as inhibits apoptosis. In some types of cancers, including breast carcinoma and ovarian tumours, the role of PTK6 is more important because PTK6 expression is strongly related to tumour grade [5,35]. In the present study, we have shown that PTK6 is a new client molecule of Hsp90 and the expression and activity of PTK6 can be controlled by Hsp90 inhibitors. We also found that an Hsp90 inhibitor, geldanamycin, induces degradation of PTK6 without affecting Src, which is closely related to PTK6. Geldanamycin also inhibits phosphorylation of PTK6 substrates that are involved in tumorigenic signalling pathways. Thus our findings provide important clues for Hsp90 inhibitors that may be useful therapies for PTK6-positive malignant diseases, including breast cancer.

**AUTHOR CONTRIBUTION**

Shin-Ae Kang designed and performed the experiments, contributed to data analysis and wrote the paper; Hyun-Soo Cho, Jong Bok Yoon and In Kwon Chung contributed to data analysis and discussion; and Seung-Taek Lee directed the research and wrote the paper.

**FUNDING**

This work was supported by the National Research Foundation, Ministry of Education, Science, and Technology, Republic of Korea through the project for Studies on Ubiquitome Functions (grant number M1053301001-10B2301) and by Seoul Research and Business Development Korea (grant number 10527). S.-A.K. was a pre-doctoral trainee and a post-doctoral trainee of the Brain Korea 21 program from the Ministry of Education, Science and Technology, Republic of Korea.

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Received 14 May 2012/26 July 2012; accepted 31 July 2012
Published as BJ Immediate Publication 31 July 2012, doi:10.1042/BJ20120803
SUPPLEMENTARY ONLINE DATA

Hsp90 rescues PTK6 from proteasomal degradation in breast cancer cells

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Figure S1  Schematic representation of PTK6 and its point and deletion mutants

The SH3 domain, SH2 domain and tyrosine kinase catalytic domain (labelled Kinase) are shown in boxes. A linker region between the SH2 and Kinase domains is denoted as Linker.

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Figure S2  Analysis of PTK6 deletion mutants for interaction with Hsp90 (A) and expression upon treatment with geldanamycin (B)

Flag-tagged PTK6 or its deletion mutants were stably expressed in HEK-293 cells. (A) For analysis of the interaction between Hsp90 and PTK6, cell lysates were immunoprecipitated (IP) with the anti-Flag antibody and interactions between the PTK6 domains and Hsp90 were assayed by SDS/PAGE (12% gel) and Western blotting (IB) using the anti-Hsp90 antibody. (B) Expression of each mutant was examined after treatment of 1 μM geldanamycin (GA) for 24 h using SDS/PAGE (12% gel) and Western blot analysis (IB) with the anti-Flag antibody.

Figure S3  Effect of geldanamycin on the half-life of PTK6

HEK-293 cells stably expressing Flag–PTK6 were treated with 40 μM cycloheximide (CHX) and 1 μM geldanamycin (GA) for the indicated time periods. Cells were lysed in SDS sample buffer and the half-life of PTK6 was examined by Western blotting (IB) using the anti-PTK6 antibody. (A) A representative immunoblot. (B) For quantification, PTK6 expression was normalized to β-actin expression. PTK6 expression at 0 h was defined as 100%. Results are means ± S.D. from three independent experiments.
Hsp90 rescues PTK6 from proteasomal degradation

Figure S4 Effect of geldanamycin on PTK6 phosphorylation of its substrates

HEK-293 cells stably expressing PTK6 or empty vector were treated with 1 \( \mu \)M geldanamycin (GA) for 24 h. Cell lysates were immunoprecipitated with the anti-STAT3, anti-Sam68 or anti-paxillin antibodies. Phosphorylation of PTK6 substrates in cell lysates (A) and immunoprecipitates (IP) (B) was analysed by Western blotting (IB) with the anti-phospho-tyrosine antibody.

Received 14 May 2012/26 July 2012; accepted 31 July 2012
Published as BJ Immediate Publication 31 July 2012, doi:10.1042/BJ20120803