An Intramolecular Interaction between SH2-Kinase Linker and Kinase Domain Is Essential for the Catalytic Activity of Protein-tyrosine Kinase-6*

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Protein-tyrosine kinase-6 (PTK6, also known as Brk) is a non-receptor tyrosine kinase that contains SH3, SH2, and catalytic (Kinase) domains. We have identified an intramolecular interaction between the linker (Linker) region connecting the SH2 and Kinase domains and the Kinase domain. Residue Trp-184 within the Linker region is essential for the Linker-Kinase interaction but not for the Linker-SH3 interaction. A recombinant PTK6 Kinase domain connected to the Linker region had catalytic activity in terms of auto-phosphorylation, phosphorylation of a PTK6 substrate, BKS, and phosphorylation of an oligopeptide substrate, whereas the Kinase domain itself, or one connected to a Linker region containing a W184A substitution, did not. The introduction of the W184A mutation into PTK6 also abrogated autophosphorylation and phosphorylation of another PTK6 substrate, Sam68, as well as phosphorylation of intracellular proteins. It also abolished the ability of PTK6 to promote proliferation and prevent apoptosis of HEK 293 cells, as well as to permit anchorage-independent colony formation. Therefore, unlike Src family members, in which the Linker-Kinase interaction inhibits catalytic activity, in PTK6 this interaction has an essential positive role.

Protein-tyrosine kinase (PTK)-6, also known as Brk, is a non-receptor PTK. A partial cDNA of PTK6 was first identified during an extensive survey of PTK mRNAs expressed in human melanocytes (1). A full-length human cDNA was cloned from breast carcinoma cells (2), and a cDNA for its mouse homolog, Sik, which has 80% amino acid identity to PTK6, was cloned from mouse intestinal crypt cells (3).

PTK6 and Sik are expressed in differentiating epithelial cells of the gastrointestinal tract and skin (3–6). Although PTK6 is not expressed in normal mammary gland epithelial cells and melanocytes (1, 7), elevated expression has been reported in breast carcinomas (2, 7, 8), melanomas (9), and colon carcinomas (6, 10). In some cases, the location of PTK6 changes during tumorigenesis. It is found in the nucleus in normal prostate epithelia and well differentiated prostate carcinomas and in both the nucleus and cytoplasm of normal oral epithelial cells; however it is mainly present in the cytoplasm in poorly differentiated prostate and oral squamous cell cancers (11, 12). The presence of PTK6 renders mammary epithelial cells more sensitive to the mitogenic effects of EGF and increases anchorage-independent proliferation (13). It also permits EGF-dependent phosphorylation of ErbB3 and subsequent activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (14). In addition, a complex of PTK6 and Akt dissociates in response to EGF stimulation in keratinocytes and COS-1 cells but not in T-47D breast carcinoma cells (15). It has therefore been suggested that PTK6 may function as a signaling molecule whose kinase activity normally limits the activity of Akt in unstimulated cells. Chen et al. (16) have shown that PTK6 can promote cell motility and invasion by phosphorylating paxillin. These findings suggest that deregulation of PTK6 via its inappropriate expression and subcellular localization may contribute to tumorigenesis.

The PTK6 polypeptide deduced from the cDNA sequence contains an SH3 domain, an SH2 domain, and a tyrosine kinase catalytic (Kinase) domain (2). PTK6 has homology to Src family members. However, it shares only 46% amino acid identity with Src and lacks an N-terminal consensus sequence for acylation and membrane association (2). Furthermore, the genomic structure of PTK6 is quite distinct from that of Src family kinases (5). Therefore, based on the exon structure, the existence of a unique family of non-receptor-type PTKs that includes PTK6/Sik, Srm, Frk, and Drosophila Src42A/Dsrc41 has been proposed (17).

In Src family members, the inactive conformation of the enzyme is stabilized by intramolecular interactions between the C-terminal phosphotyrosine and the SH2 domain and between proline residues in the SH2-Kinase linker (Linker) and the SH3 domain (18–24). Such intramolecular interactions also negatively regulate the catalytic activity of PTK6 (25–27). In Src family members, these interactions also lock the Kinase domain in an inactive conformation by pinning the Linker region against the N lobe of the Kinase domain (20–22). A tryptophan residue in the Linker region, which is highly conserved among PTKs (e.g. Trp-260 in chick c-Src), interacts with the C-terminal end of the αC helix in the N lobe of the Kinase domain (21, 22, 28). This is particularly important for maintaining the inactive state of the Kinase domain. In addition, a
conserved hydrophobic residue in the Linker region (e.g. Leu-255 in chick c-Src) projects into a hydrophobic pocket on the back of the N lobe and stabilizes the inactive conformation (29, 30).

We have examined the interaction between the Linker region and Kinase domain of PTK6. Site-directed mutagenesis and surface plasmon resonance analysis showed that Trp-184 of PTK6, which is analogous to Trp-260 of chick c-Src, is important for the interaction. We analyzed the biochemical and functional properties of the Trp-184 residue and report that it plays a very different role to the analogous residues in Src family members.

EXPERIMENTAL PROCEDURES

Expression Constructs—To generate constructs expressing the glutathione S-transferase (GST)-fused SH3 domain (GST-PTK6-SH3), GST-TPK6-Kinase, GST-PTK6-Linker(ΔN)-Kinase, and GST-PTK6-Linker-Kinase (Fig. 1) in Escherichia coli, cDNA encoding residues 3–72, 189–451, 180–451, and 171–451 of PTK6 were amplified by PCR using primer pairs, 5′-GGGATCCGAGGAGCATGCTCG-3′ and 5′-GAAATCCGATCTCCCTCGCCAGG-3′, 5′-GGGAATTCACGTCTCCCTCGCCAGGGA-3′, and 5′-GAAATTCACGTCTCCCTCGCCAGGGA-3′, respectively, and pBS-PTK6-Kinase (5) as the template. The amplified fragment encoding PTK6-SH3 was digested with BamHI and EcoRI and ligated into pGEX-4T3 (Amersham Biosciences) to generate pGEX-4T3-PTK6-SH3. The other amplified digested with BamHI and EcoRI, ligated into pGEX-4T3-PTK6-Kinase, pGEX-4T3-Linker(ΔN)-Kinase, and pGEX-4T3-Linker-Kinase, respectively. To produce a construct expressing GST-BSK in E. coli, a cDNA encoding BSK (31) was amplified by PCR using the primer pair 5′-GGGATCCGAGGAGCATGCTCG-3′ and 5′-GAAATCCGATCTCCCTCGCCAGGGA-3′ and a random cDNA mixture from the breast carcinoma cell line MCF-7 as the template. The PCR product was digested with BamHI and EcoRI and subcloned into pGEX-4T3 to generate pGEX-4T3-PTK6-BRK kinase domain of PTK6. Site-directed mutagenesis was performed by primer-mediated site-directed mutagenesis with a QuickChange kit (Stratagene). The identity of all the constructs was verified by DNA sequencing to avoid PCR errors.

Production of Recombinant Fusion Proteins—GST fusion proteins were expressed in E. coli XL1-blue and purified with glutathione-Sepharose 4B resin (Amersham Biosciences) as described (32). Trx-fusion proteins containing a hexahistidine (His) tag were expressed in E. coli BL21(DE3)pLyssN (Novagen) and purified with Ni2+-nitrilotriacetic acid-agarose beads (Qiagen) following the manufacturer’s instructions.

Pull-down Assays—for pull-down assays using GST-fused proteins, cell lysates containing ~2 μg of GST-fused proteins were incubated at 4 °C for 2 h with glutathione-Sepharose 4B resin pre-equilibrated in phosphate-buffered saline buffer. The resin was washed three times, and the GST-fused proteins bound to it were incubated with 2 μg of purified Trx-fused proteins at 4 °C for 8 h. The resin was washed again, and the bound proteins were eluted by boiling after adding SDS sample buffer and subjected to Western blot analysis.

Surface Plasmon Resonance Analysis—Binding of PTK6-Linker or the Trp-184 mutant form of PTK6-Linker (PTK6-Linker(W184A)) to PTK6-Kinase was analyzed by surface plasmon resonance using a Bia-core (BIAcore 3000 BIACORE) as described (32), with a minor modification. The surface of the sensor chip, CM5, was activated by injecting a mixture of 0.2 μl N-ethyl-N'-dimethylaminopropyl-carbodiimide and 0.05 μl N-hydroxysuccinimide. Thereafter, GST-PTK6-Kinase (ligand) in coupling buffer (10 mM sodium acetate, pH 4.0) was injected and immobilized on the surface of the chip. Activated surface that was not immobilized was blocked by 1 μl ethanolamine-HCl (pH 8.5). A running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20) was used during the immobilization and binding analysis. In typical experiments, the immobilization levels of GST and GST-PTK6-Kinase were ~4,550 and 4,450 response units, respectively.

Various concentrations of analyte (Trx-PTK6-Linker or Trx-PTK6-Linker(W184A)) were injected so as to pass through the flow cell with immobilized GST followed by the flow cell with immobilized GST-PTK6-Kinase in 2 min at a flow rate of 5 μl/min. For regeneration, proteins bound to ligands were removed by 1-min pulse injections of 50 mM NaOH and 1 mM NaCl into the GST-PTK6-Kinase flow cell. This regeneration condition retained more than 95% of the original capacity for binding Trx-PTK6-Linker. To correct for bulk shift, data from the reference flow cells were subtracted from the experimental data. The equilibrium constant (Kd) was calculated from the rate constants for association (k+a) and dissociation (k−a) and the equation Kd = k+a/k−a.

Cell Culture and Transfection—HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin. For transient expression, transfected into HEK 293 cells was carried out with FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions (33), and the cells were harvested 48 h after transfection. For stable expression, transfection was performed with calcium phosphate followed by selection with 1,200 μg/ml G418 (34). After 2 weeks, G418-resistant colonies were cloned and expanded.

Cell Proliferation and Cell Survival Assays—Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA) with 10 μl/ml protease inhibitor mixture (Sigma) and 1 mM Na3VO4 on ice for 30 min. The lysates were cleared by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant was determined by the BCA assay (Pierce). For Western blot analysis, 40-μg samples of lysate protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Immunoreactive proteins were visualized with anti-His tag (Qiagen), anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-PTK6 (Santa Cruz Biotechnologies), anti-Sam68 (Santa Cruz Biotechnologies), and anti-β-actin (Sigma) primary antibodies, horseradish peroxidase-conjugated secondary antibody, and an enhanced chemiluminescence detection kit (Amersham Biosciences). For immunoprecipitation experiments, the PTK6 protein was immunoprecipitated with anti-PTK6 antibody at 4 °C for 5 h followed by 30 μl of protein A beads (1 ml slurry, Sigma) for 1 h. The beads were washed three times with lysis buffer, and the immunoprecipitated proteins were subjected to in vitro kinase assay or Western blot analysis.

In Vitro Kinase Assay—To examine autophosphorylation of PTK6 and phosphorylation of BKS by PTK6, purified GST-PTK6-Kinase (Fig. 1) was added in pET-32c(-)-PTK6-Linker, GST-PTK6(LN,W184A)-Kinase (207 ng), or GST-PTK6-Linker-Kinase (215 ng) was incubated alone or with 1 μg of GST-BKS in the presence of 100 μM [γ32P]ATP (specific radioactivity, 300–400 cpm/pmol) in 25 μl of kinase reaction buffer (20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 1 mM MnCl2, 50 μM Na3VO4) for 30 min at 30 °C. The reactions were terminated by spotting onto phospho-cellulose paper (Whatman), washed in 5% trichloroacetic acid and 0.5% H3PO4 four times for 10 min for each wash, followed by ethanol once, and then dried. Radioactivity was quantified by scintillation counting.

The catalytic activity of PTK6 or its derivatives was determined with a random copolymer of glutamate and tyrosine (poly(Glu,Tyr), Sigma) as substrate. GST-PTK6-Kinase, GST-PTK6-Linker(ΔN)-Kinase, GST-PTK6-Linker(ΔN,W184A)-Kinase, and GST-PTK6-Linker-Kinase were expressed in E. coli and purified as described above. PTK6 and its mutants were transiently expressed in HEK 293 cells by transfection and immunoprecipitated with anti-PTK6 antibody. For the reactions we used 200 ng of purified GST fusion protein or a half of the protein immunoprecipitated with 1 μg of antibody. In vitro kinase assays were performed as described above, except for the addition of 0.5 mg/ml poly(Glu,Tyr) as substrate. Aliquots (17 μl) of the reaction mixtures were spotted onto phospho-cellulose paper (Whatman), washed in 5% trichloroacetic acid and 0.5% H3PO4 four times for 10 min for each wash, followed by ethanol once, and then dried. Radioactivity was quantified by scintillation counting.

Cell Proliferation and Cell Survival—To assay cell proliferation, subconfluent cells were starved in serum-free DMEM for 24 h and incubated in DMEM containing 1% fetal bovine serum and 50 ng/ml EGF for 24 h. For inhibition experiments, cell proliferation was determined by the amount of [3H]thymidine incorp­orated, as described previously (35). For cell survival assays, subconfluent cells were incubated with serum-free DMEM containing various concentrations of H2O2 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 (36).

Anchor-independent Colony Formation—Anchor-independent
colony formation was measured as described previously (37), with minor modifications. A bottom layer of 0.5% low melting point agarose (SeaPlaque, FMC Corp.) in DMEM with 10% fetal bovine serum was poured into 6-well dishes and allowed to harden at 4 °C. Cells (1×10^4/well) of the appropriate stable cell line were suspended in DMEM-10% fetal bovine serum plus 0.35% agarose at 42 °C and poured onto the solidified base layer. After 5 weeks of incubation, the numbers of colonies formed were counted with a microscope. All assays were performed three times in duplicate.

RESULTS

The Linker Region Interacts with the Kinase Domain of PTK6—To see whether an intramolecular interaction occurs between the Linker region and the Kinase domain of PTK6, we expressed GST-PTK6-Kinase and Trx-PTK6-Linker as soluble proteins in E. coli (Fig. 1). The Trx-PTK6-Linker was pulled down by GST-PTK6-Kinase, but not by GST, and Trx on its own did not bind to GST-PTK6-Kinase (Fig. 2). In addition, when GST-PTK6-Kinase or GST was pulled down with the Trx-PTK6-Linker or Trx bound to Ni2⁺-nitrilotriacetic acid resin, GST-PTK6-Kinase bound specifically to the Trx-PTK6-Linker (data not shown). These results indicate that the Linker region and Kinase domain are bound to each other in PTK6.

The Trp-184 Residue in the Linker Region Is Important for the Linker-Kinase Interaction but Not for the Linker-SH3 Interaction—A tryptophan residue, Trp-184, in the C-terminal region of the Linker in PTK6 is conserved among PTK6, Src, and Csk family members (Fig. 3). In addition, leucine residue Leu-178 of PTK6 is located close to a hydrophobic amino acid (Leu or Trp) that is conserved in Src and Csk family members.

To see which amino acid residue(s) in the Linker region plays an important role in the intramolecular interaction between the Linker region and the Kinase domain, various mutations were introduced into Trx-PTK6-Linker, and the mutant polypeptides were expressed as soluble proteins in E. coli (Fig. 1). As expected, changing the conserved tryptophan 184 to alanine (W184A) abolished the interaction (Fig. 4A), whereas neither mutation of the nearby tryptophan 181 to alanine (W181A) nor mutations of leucine 178 to alanine (L178A) or glycine (L178G) affected the interaction. Nor was it affected by substitution of the three proline residues (Pro-175, Pro-177, and Pro-179) that are important for binding to the SH3 domain (27) or of the other proline (Pro-187) in the Linker region.

Moreover, in a condition that Trx-PTK6-Linker bound specifically to GST-PTK6-SH3 (Fig. 1), the W184A mutation did not affect the Linker-SH3 interaction (Fig. 4B).

We determined the kinetic and equilibrium constants of the interaction between PTK6-Kinase and PTK6-Linker by surface plasmon resonance using a Biacore 2000. A dilute solution of
Trx-PTK6-Linker or the W184A mutant (Trx-PTK6-Linker(W184A)) as analyte was allowed to bind to immobilized GST-PTK6-Kinase. As shown in the sensorgrams of Fig. 5, Trx-PTK6-Linker bound to GST-PTK6-Kinase with a dissociation constant (KD) of 1.10 × 10⁻⁷ M and Trx-PTK6-Linker(W184A) did not bind.

The C-terminal Half of the Linker Region Including the Trp-184 Residue Is Essential for PTK6 Activity—Recombinant PTK6 derivatives were expressed in E. coli (Fig. 1), and their catalytic activities were analyzed by examining autophosphorylation and phosphorylation of a PTK6 substrate, BKS, and an oligopeptide substrate. GST-PTK6-Kinase (Fig. 1), which does not contain any of the Linker region, neither autophosphorylated nor phosphorylated GST-BKS. On the other hand, GST-PTK6-Linker-Kinase, which contains the Linker region (but not the SH2 and SH3 domains) (Fig. 1), phosphorylated itself and GST-BKS. When the N-terminal half of the

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Linker region, which contains the 3 proline residues important for binding to the SH3 domain (27), was removed (GST-PTK6-Linker(ΔN) - Kinase) (Fig. 1), its catalytic activity was unchanged. However, introduction of the W184A mutation into this construct (GST-PTK6-Linker(ΔN,W184A)-Kinase) resulted in complete loss of catalytic activity (Fig. 6B). This result demonstrates that the C-terminal half of the Linker region, including Trp-184, is required for catalytic activity.

W184A Mutation Eliminates the Catalytic Activity of PTK6 in Mammalian Cells—To examine whether the Linker-Kinase interaction is essential for catalytic activity of the PTK6 polypeptide expressed in mammalian cells, the W184A mutation and the ATP-binding site mutation K219M (13) were introduced into the constructs expressing wild type PTK6 and mutant forms (W184A, K219M, Y447F, W184A/Y447F, and K219M/Y447F). A, phosphorylation of cellular proteins by PTK6. Lysates were analyzed by Western blotting with anti-phosphotyrosine antibody (upper panel), anti-PTK6 antibody for transfection efficiency (middle panel), and anti-β-actin antibody to normalize levels of loading (bottom panel). B, autophosphorylation of PTK6. PTK6 and its derivatives were immunoprecipitated (IP) from lysates with anti-PTK6 antibody and analyzed by Western blotting with anti-phosphotyrosine antibody (upper panel) and anti-PTK6 antibody (bottom panel). C, phosphorylation of Sam68 by PTK6. Sam68 was immunoprecipitated with anti-Sam68 antibody and analyzed by Western blot analysis with anti-phosphotyrosine antibody (upper panel) and anti-Sam68 antibody (bottom panel). The results in A–C are representative of three to five independent experiments.

D, phosphorylation of an oligopeptide substrate by PTK6. PTK6 and its derivatives were immunoprecipitated with anti-PTK6 antibody, and catalytic activity was examined by the methods described in the legend to Fig. 6. Each value is the mean ± S.D. of three independent determinations of duplicate experiments.

FIG. 6. Effect of PTK6-Linker and the W184A mutation on the catalytic activity of PTK6-Kinase expressed in E. coli. A, auto-phosphorylation and phosphorylation of BKS by PTK6-Kinase and its derivatives. GST-PTK6-Kinase, GST-PTK6-Linker(ΔN)-Kinase, GST-PTK6-Linker(ΔN,W184A)-Kinase, or GST-PTK6-Linker-Kinase were incubated with or without GST-BKS in the presence of [γ-32P]ATP in kinase reaction buffer. The products were analyzed by SDS-PAGE and autoradiography. The result is representative of three independent experiments. L, Linker; K, Kinase. B, phosphorylation of an oligopeptide substrate by PTK6-Kinase and its derivatives. Purified enzymes were incubated with the oligopeptide substrate, poly(Glu,Tyr), in the presence of [γ-32P]ATP. The products were spotted onto phosphocellulose paper and washed as described under “Experimental Procedures.” Radioactivity was quantified by scintillation counting. Activities are expressed as the percent of the activity of GST-PTK6-Linker-Kinase. Each value is the mean ± S.D. of three independent determinations of duplicate experiments.

FIG. 7. Effect of the W184A mutation on the catalytic activity of PTK6 expressed in HEK 293 cells. HEK 293 cells were transfected with vector alone and constructs expressing wild type PTK6 and mutant forms (W184A, K219M, Y447F, W184A/Y447F, and K219M/Y447F). A, phosphorylation of cellular proteins by PTK6. Lysates were analyzed by Western blotting with anti-phosphotyrosine antibody (upper panel), anti-PTK6 antibody for transfection efficiency (middle panel), and anti-β-actin antibody to normalize levels of loading (bottom panel). B, autophosphorylation of PTK6. PTK6 and its derivatives were immunoprecipitated (IP) from lysates with anti-PTK6 antibody and analyzed by Western blotting anti-phosphotyrosine antibody (upper panel) and anti-PTK6 antibody (bottom panel). C, phosphorylation of Sam68 by PTK6. Sam68 was immunoprecipitated with anti-Sam68 antibody and analyzed by Western blot analysis with anti-phosphotyrosine antibody (upper panel) and anti-Sam68 antibody (bottom panel). The results in A–C are representative of three to five independent experiments.

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To quantify the catalytic activity of wild type PTK6 and its mutants, they were immunoprecipitated from the HEK 293 cells with anti-PTK6 antibody and tested with the oligopeptide substrate (Fig. 7D). With the catalytic activity of the wild type set at 100%, those obtained with vector, W184A mutant, K219M mutant, Y447F mutant, W184A/Y447F mutant, and K219M/Y447F mutant were 7.86 ± 0.01, 28.13 ± 1.45, 28.93 ± 1.39, 189.06 ± 1.27, 27.74 ± 0.13, and 28.13 ± 1.73%, respectively. The slightly higher catalytic activities with the W184A, K219M, W184A/Y447F, and K219M/Y447F mutants than with the vector control were probably due to co-immunoprecipitated endogenous tyrosine kinases.

The W184A Mutation Abolishes the Proliferative, Anti-apoptotic, and Anchorage-independent Activities of PTK6—To examine the biological consequences of the W184A mutation, HEK 293 cells stably expressing the constitutively active Y447F mutant and its derivatives, W184A/Y447F and K219M/Y447F mutants, were constructed. To assess overall catalytic activity, the level of phosphotyrosine-containing proteins in the cell lysates was analyzed by Western blotting with anti-phosphotyrosine antibody (Fig. 8A). A strong increase in tyrosine phosphorylation of cellular proteins was detected in the cell line stably expressing the constitutively active Y447F mutant but not in those expressing the W184A/Y447F mutant or the K219M/Y447F mutant. These results are consistent with those obtained by transient expression (Fig. 7A).

Proliferation of the stable transformants was analyzed by [3H]thymidine incorporation after exposure to EGF (Fig. 8B). Compared with the vector-transfected cells, expression of the Y447F mutant in the HEK 293 cells stimulated [3H]thymidine incorporation, especially after 6 or more days of EGF stimulation. No such stimulation was seen in cells expressing either of the W184A/Y447F and K219M/Y447F mutant forms. Apoptosis was analyzed by MTT assays of cells incubated in serum-free medium with various concentrations of H2O2 for 48 h (Fig. 8C). Expression of the Y447F mutant inhibited apoptosis in response to serum depletion regardless of the presence of H2O2, although most markedly in the presence of 100 µM H2O2. Introduction of either of the W184A and K219M mutations abolished this anti-apoptotic effect.

The transforming potential of the Y447F mutant and its derivatives was also evaluated by examining the capacity of the transfected HEK 293 cells for anchorage-independent growth. Expression of the Y447F mutant resulted in a significant increase in the number (Table I) and size (data not shown) of stably expressing the constitutively active Y447F mutant but not in those expressing the W184A/Y447F mutant or the K219M/Y447F mutant. These results are consistent with those obtained by transient expression (Fig. 7A).

DISCUSSION

The Linker region and Kinase domain of many non-receptor type PTKs, especially members of the Src family, are known to interact intramolecularly (21, 22, 24, 28–30, 38). This interaction, along with intramolecular interactions between the C-terminal phosphotyrosine residue and the SH2 domain and between proline residues in the Linker region and the SH3 domain, plays a role in autoinhibition of catalytic activity. Similarly, interactions between Tyr(P)-447 and the SH2 domain, and between proline residues in the Linker region and the SH3 domain, inhibit the catalytic activity of PTK6 (25–27, 39). In this study, we have identified an additional intramolecular interaction between the Linker region and the Kinase domain of PTK6 and have analyzed its effect on the catalytic and biological activity of PTK6.

Although the Linker-Kinase interaction inhibits catalytic activity in Src family members, our results demonstrate that...
this interaction is absolutely required for catalytic activity of PTK6. This conclusion is based on two observations. First, in vitro kinase assays of the purified proteins showed that both GST-fused PTK6-Linker-Kinase and GST-fused PTK6-Linker(KD)-Kinase have catalytic activity but GST-fused PTK6-Kinase and GST-fused PTK6-Linker(KD,W184A)-Kinase do not. Second, expression of PTK6 in HEK 293 cells caused autophosphorylation and phosphorylation of Sam68, as well as of intracellular proteins, but the W184A mutant, which disrupts the Linker-Kinase interaction, had none of these activities. In addition, even when the W184A mutation was introduced into the Y447F mutant that shows higher catalytic activity than wild type, these activities were not detected at all. We thus believe that the W184A mutation completely destroys catalytic activity of PTK6. Because the interaction between the Linker region and the Kinase domain is fairly strong (K_D = 1.10 × 10^{-7} M), it was interesting to see whether the Linker region may activate the catalytically inactive Kinase domain in trans. However, the catalytic activity of GST-PTK6-Kinase in the presence of a 1000 times molar excess of Trx-PTK6-Linker was only 0.34% to that of GST-PTK6-Linker-Kinase, although it was dose dependently increased by addition of Trx-PTK6-Linker. Our data thus suggest that the interaction between the Linker region and the Kinase domain should be in cis for its catalytic activity.

The expression of PTK6 increases sensitivity to the mitogenic effects of EGF and permits anchorage-independent proliferation of mouse embryonic fibroblast NIH3T3 and breast epithelial HB4 cells (13). It also stimulates ErbB3 phosphorylation and subsequent recruitment of phosphatidylinositol 3-kinase and Akt in mammary luminal cells (14). The effect of PTK6 on the regulation of phosphatidylinositol 3-kinase and Akt activity raises the possibility that breast tumors expressing PTK6 may acquire resistance to pro-apoptotic signals (14).

We confirmed that high catalytic activity of PTK6 increases proliferation, anchorage-independent colony formation, and cell survival, especially under conditions of oxidative stress, in the HEK 293 cell system. However, these effects are abolished by the W184A mutation. These results show a good correlation with the effect of the W184A mutation on catalytic activity of PTK6.

PTK6 is known to autophosphorylate its Tyr-342 and Tyr-447 residues (13, 26), and phosphorylation of Tyr-342 in the activation loop is required for catalytic activity (26). We showed that neither GST-fused PTK6-Linker(KD,W184A)-Kinase expressed in E. coli, nor PTK6(W184A) expressed in HEK 293 cells, autophosphorylated or phosphorylated substrates, whereas their wild type forms did. This result indicates that the interaction between the Linker region and the Kinase domain is a prerequisite for autophosphorylation of Tyr-342.

Because the W184A mutation in PTK6 abolishes the Linker and Kinase interaction and its catalytic activity, Trp-184 is likely to stabilize the active conformation of the catalytic domain. However, in Hck, the equivalent mutation, W260A, increases catalytic activity (28). Structural analysis showed that Trp-260 protrudes into a hydrophobic region of the N-terminal lobe of the catalytic domain and stabilizes the conformation of the αC helix that is not properly positioned for catalysis (21, 28). Also when a W260A mutant of Src was tested in Schizosaccharomyces pombe, it was as active as the wild type enzyme in phosphorylating yeast proteins in the absence of Csk (29), but the presence of the latter led to a drastic reduction in phosphorylation of the yeast proteins in the case of wild type Src but not of the W260A mutant. These observations demonstrate that the Linker-Kinase interaction is necessary for autoinhibition of catalytic activity in Src family members. It would therefore be interesting to clarify the structural basis of the difference between negative regulation by this interaction in Src family members and positive regulation in PTK6.

Qui and Miller (27) have recently examined the roles of the SH3 and SH2 domains in PTK6 regulation and substrate binding using a series of mutations that were predicted to disrupt the intracellular interactions involving the SH3 and SH2 domains. Because the W260A mutation of Hck is known to increase the accessibility of the SH2 and SH3 domains as well as catalytic activity (28), the W184A mutation of PTK6 was included. They showed that the W184A mutant expressed in HEK 293 cells had catalytic activity, although lower than that of wild type PTK6, somewhat inconsistent with our data. They also found that the mutation dramatically reduced autophosphorylation and gave no phosphorylation of Sam68. Based on their finding that the W184A mutant bound more efficiently to Sam68 than wild type PTK6, they suggested that the W184A mutation destabilizes the Linker-SH3 interaction and that the SH3 domain is accessible for binding to Sam68 (27). However, our finding demonstrates that the W184A mutation does not affect the Linker-SH3 interaction in vitro. The W184A mutant that has no catalytic activity will not autophosphorylate its Tyr-447 residue, and then the SH2 domain will be free for substrate binding. In addition, the absence of the interaction between SH2 domain and the C-terminal phosphotyrosine residue destabilizes the Linker-SH3 interaction in Src family members and PTK6 (27, 28, 41). The W184A mutant would thus have both SH2 and SH3 domains, which are accessible for substrate binding. Sam68 is known to bind both SH3 and SH2 domains of Ssk (25). Nevertheless, because Sam68 is not phosphorylated by the inactive W184A mutant, it cannot bind to the SH2 domain but only to the SH3 domain. Therefore, we assume that the increased binding of the W184A mutant to Sam68 would result from destabilization of the Linker-SH3 interaction indirectly rather than directly.

We have shown that the intramolecular interaction between the Linker region and the Kinase domain of PTK6 is essential for positive regulation of PTK6 activity. Our data provide important notions to understand molecular mechanisms of PTK6 regulation. In view of its oncogenic activity and its presence in various carcinomas including breast cancer, it has been proposed that PTK6 could be a therapeutic target in cancer (42).
Our recombinant PTK6 variants, such as PTK6-Linker(ΔN)-Kinas, which have catalytic activity but lack the ability to autoinhibit, could be useful for screening for inhibitors in vitro.

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An Intramolecular Interaction between SH2-Kinase Linker and Kinase Domain Is Essential for the Catalytic Activity of Protein-tyrosine Kinase-6
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