PTK6 Inhibits Down-regulation of EGF Receptor through Phosphorylation of ARAP1

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PTK6 (also known as Brk) is a non-receptor-tyrosine kinase containing SH3, SH2, and catalytic domains, that is expressed in more than 60% of breast carcinomas but not in normal mammary tissues. To analyze PTK6-interacting proteins, we have expressed Flag-tagged PTK6 in HEK293 cells and performed co-immunoprecipitation assays with Flag antibody-conjugated agarose. A 164-kDa protein in the precipitated fraction was identified as ARAP1 (also known as centaurin δ-2) by MALDI-TOF mass analysis. ARAP1 associated with PTK6 in an EGF/EGF receptor (EGFR)-dependent manner. In addition, the SH2 domain of PTK6, particularly the Arg105 residue that contacts EGFR in HEK293 cells and performed co-immunoprecipitation assays with Flag antibody-conjugated agarose. A 164-kDa protein in the precipitated fraction was identified as ARAP1 (also known as centaurin δ-2) by MALDI-TOF mass analysis. ARAP1 associated with PTK6 in an EGF/EGF receptor (EGFR)-dependent manner. In addition, the SH2 domain of PTK6, particularly the Arg105 residue that contacts the phosphate group of the tyrosine residue, was essential for the association. Moreover, PTK6 phosphorylated residue Tyr231 in the N-terminal domain of ARAP1. Expression of ARAP1, but not of the Y231F mutant, inhibited the down-regulation of EGFR in HEK293 cells expressing PTK6. Silencing of endogenous PTK6 expression in breast carcinoma cells decreased EGFR levels. These results demonstrate that PTK6 enhances EGFR signaling by inhibition of EGFR down-regulation through phosphorylation of ARAP1 in breast cancer cells.

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3 The abbreviations used are: PTK, protein tyrosine kinase; ARAP1, Arf-GAP, Rho-GAP, ankyrin repeat, and pleckstrin homology domain-containing protein; EGFR, EGF receptor; MDC, monodansyl cadaverine; PH, pleckstrin homology; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PR, proline-rich; SAM, sterile α motif.

PTK6 (also known as Brk) is a non-receptor-tyrosine kinase containing SH3, SH2, and catalytic domains, that is expressed in more than 60% of breast carcinomas but not in normal mammary tissues. To analyze PTK6-interacting proteins, we have expressed Flag-tagged PTK6 in HEK293 cells and performed co-immunoprecipitation assays with Flag antibody-conjugated agarose. A 164-kDa protein in the precipitated fraction was identified as ARAP1 (also known as centaurin δ-2) by MALDI-TOF mass analysis. ARAP1 associated with PTK6 in an EGF/EGF receptor (EGFR)-dependent manner. In addition, the SH2 domain of PTK6, particularly the Arg105 residue that contacts the phosphate group of the tyrosine residue, was essential for the association. Moreover, PTK6 phosphorylated residue Tyr231 in the N-terminal domain of ARAP1. Expression of ARAP1, but not of the Y231F mutant, inhibited the down-regulation of EGFR in HEK293 cells expressing PTK6. Silencing of endogenous PTK6 expression in breast carcinoma cells decreased EGFR levels. These results demonstrate that PTK6 enhances EGFR signaling by inhibition of EGFR down-regulation through phosphorylation of ARAP1 in breast cancer cells.

Protein tyrosine kinase-6 or PTK62 (also known as breast tumor kinase or Brk; Src-related intestinal kinase, or Sik in mice) is a non-receptor tyrosine kinase that is closely related to, but distinct from, the Src family (1–4). PTK6 expression is low or undetectable in normal mammary tissues or in benign lesions. However, approximately two-thirds of examined breast tumors express significant levels of PTK6 (3). Elevated expression of PTK6 has also been reported in other cancers, including colon carcinomas (5), prostate carcinomas (6), head and neck tumors (7), oral squamous cell carcinoma (8), and in lymphocytes (9). Expression of PTK6 makes mammary epithelial cells more sensitive to the mitogenic effects of EGF (10). PTK6 increases anchorage-independent proliferation (11), and enhances PI3-kinase pathway signaling through ErbB3 phosphorylation (12).

PTK6 is expressed in normal epithelial tissues, including the gastrointestinal tract and skin (13–14), prostate (6), and oral epithelium (8). PTK6 does not promote proliferation of non-transformed cells, but plays a role in the regulation of apoptosis (15). PTK6 tends to be located in the nucleus in normal epithelial cells or well-differentiated tumors, but is cytoplasmic in malignant carcinomas (6, 8). We have recently shown that the targeting of PTK6 to the plasma membrane enhances its oncogenic ability, while nuclear localization does not, demonstrating the importance of its association with the plasma membrane during tumorigenesis (16).

Molecular details of the function of PTK6 have been revealed through the identification of interacting proteins and substrates of PTK6. The nuclear RNA-binding protein Sam68 is phosphorylated by and interacts with PTK6, and phosphorylation of Sam68 attenuates its RNA binding ability (17). SLM-1 and SLM-2, which have similar functions as Sam68, have also been identified as substrates of PTK6 (18). An adaptor protein, BKS (also known as STAP-2), which modulates the activity of the signal transducer and activator of transcription (STAT) family (19) is phosphorylated by PTK6 (20). PTK6 associates with Akt and phosphorylates a tyrosine residue, which inhibits Akt activity and growth promoting oncogenic activities (21). Phosphorylation of paxillin by PTK6 promotes cell motility and invasion, which contributes to oncogenesis (22). PTK6 and insulin receptor substrate 4 interact through the SH3 and SH2 domains of PTK6 after stimulation by IGF-1, and this interaction increases the tyrosine phosphorylation and enzymatic activity of PTK6 (23). PTK6 also interacts with ErbB2 and enhances ErbB2-induced cell proliferation by inducing prolonged activation of the Ras/MAP kinase pathway and activation of the cyclin E/CDK2 complex (24). Furthermore, substrates of PTK6, including STAT3, STAT5b, kinesin-associated protein 3A, and P190Rho-GAP, stimulate cellular proliferation (25–29).

To further understand the role of this kinase in tumorigenesis, we screened for novel interacting proteins of PTK6. One PTK6-interacting protein, Arf-GAP, Rho-GAP, ankyrin repeat, and pleckstrin homology (PH) domain-containing protein 1 (ARAP1; also known as centaurin δ-2), was further analyzed for the effects of serum-depletion, EGF stimulation, and PTK6 catalytic activity on interactions with PTK6. In addition, domains

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importantly for the interaction of PTK6 and ARAP1 were assigned, and the phosphorylation site of ARAP1 by PTK6 was determined. Finally, the effect of ARAP1 phosphorylation by PTK6 on down-regulation of EGFR receptor (EGFR) was studied.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-ARAP1 antibody has been previously described (30). Anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Billerica, MA). Anti-Myc agarose, anti-Brk (PTK6), anti-phospho-Erk1/2, anti-Erk2, anti-phospho-EGFR (Tyr1173), anti-β-actin, and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag M2 antibody, anti-Flag M2 antibody-conjugated agarose, and protein A-Sepharose were purchased from Sigma. Anti-EGFR, anti-phospho-Akt (Ser473), anti-phosphotyrosine antibody (4G10), and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). Mouse TrueBlot™-horseradish peroxidase-conjugated anti-mouse IgG was from eBioscience (San Diego, CA). AG1478 and G418 were purchased from A.G. Scientific (San Diego, CA). EGF, puromycin, and monodansyl cadaverine (MDC) were obtained from Sigma.

Construction of Expression Vectors—To generate an expression construct for Flag-PTK6-SH3-SH2, a cDNA encoding the translation initiation codon, Flag tag, and 3–174 amino acid residues of PTK6 was amplified by PCR using pcDNA3.1-Flag-PTK6 (16) as template and the primer pair listed in supplemental Table S1. To construct expression vectors for Flag-PTK6-SH2 and Flag-PTK6-Linker-Kinase, cDNAs encoding 76–174 and 171–451 residues of PTK6 were PCR-amplified using PBS-PTK6-MR (2) as template and primer pairs that included the Flag tag sequence (supplemental Table S1), respectively. The amplified fragments were digested with HindIII and EcoRI, and ligated into pcDNA3.1 (Invitrogen, Carlsbad, CA). To make an expression construct for a variant of PTK6 containing an SH3 domain and a novel C-terminal proline-rich sequence (PTK6-SH3-PR), which is encoded from an alternatively spliced form of PTK6 transcript (3), a fragment of PTK6 cDNA (nucleotide positions 272–393 of GenBank™ NM_005975) was deleted by site-directed mutagenesis using pcDNA3.1-Flag-PTK6 as template and the primer pair listed in supplemental Table S1. The R105A mutation was introduced according to the manufacturer’s protocol (Stratagene, La Jolla, CA).

To make an expression construct for Myc-tagged ARAP1ΔSAM, the Flag tag of pC1-Flag-ARAP1ΔSAM (30) was changed to a Myc tag by site-directed mutagenesis with a primer pair listed in supplemental Table S2. An expression construct for Myc-ARAP1 was generated through amplification of the N-terminal region of ARAP1 containing the Myc tag using pC1-Flag-ARAP1 (31) as template and a primer pair (supplemental Table S2) and ligation into the XhoI and XbaI sites of pC1-Flag-ARAP1. Myc-ARAP1-Y23F, Y231F, and Y288F were generated by site-directed mutagenesis using pC1-Myc-ARAP1 as template and primer pairs described in supplemental Table S2. All constructs were confirmed by DNA sequencing.

Cell Culture and Cell Lines—Human embryonic kidney 293 (HEK293) and human breast carcinoma cell lines, BT-474 and T-47D, were maintained in Dulbecco’s Modified Eagle’s medium supplemented with 5 and 10% fetal bovine serum, respectively. For transient expression, HEK293 cells or HEK293 cells stably expressing Flag-PTK6 (HEK293-Flag-PTK6) were transfected with each of the expression constructs using WelFect-EXT™ PLUS (WelGENE, Daegu, Korea), according to the manufacturer’s recommendations. For stable expression, HEK293 cells were transfected with each expression construct using the calcium phosphate method, followed by selection with 1,200 μg/ml of G418. After 2 weeks, G418-resistant colonies were cloned and expanded.

Enrichment of PTK6-interacting Proteins—Subconfluent HEK293-Flag-PTK6 cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 0.05% protease inhibitor mixture (Cat. No. P8340, Sigma)) on ice for 10 min, and then centrifuged at 10,000 × g for 10 min. The cell lysate containing Flag-PTK6 was incubated with anti-Flag M2 agarose that had been equilibrated in PBS buffer at 4 °C for 4 h. The resin was washed in PBS buffer three times. The precipitated Flag-tagged proteins were boiled in SDS sample buffer containing 100 mM β-mercaptoethanol, and separated by SDS-PAGE. For proteomic analysis of the proteins, the gel was stained with a colloidal Coomassie staining solution (17% ammonium sulfate, 3% phosphoric acid, 34% methanol, and 0.1% Coomassie Brilliant Blue G-250).

In-gel Trypsin Digestion, Peptide Extraction, MALDI-TOF Mass Spectrometry, and Peptide Mass Fingerprinting—Interesting bands in the stained gel were subjected to in-gel digestion with trypsin, mass spectrometry, and mass fingerprinting, as described previously (32). Mass analysis was performed on a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) in the reflect mode. For protein identification, measured monoisotopic masses of peptides were analyzed using the MASCOT search program (Matrix Science, Boston, MA) with the MSDB data base.

Western Blot Analysis and Pull-down Assays—For EGFR stimulation, subconfluent cells were starved in a serum-free medium for 24 h and, if necessary, pretreated with a drug for 30 min. Then the cells were stimulated with EGF (50 ng/ml) for the indicated time intervals. For Western blot analysis, cell lysates mixed with SDS sample buffer containing 100 mM β-mercaptoethanol were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The immunoreactive proteins were detected with primary antibody, horseradish peroxidase-conjugated secondary antibody, and enhanced chemiluminescent detection kit (Millipore Corp., Billerica, MA). For pull-down assays, cell lysate was incubated with anti-Myc antibody or anti-Flag M2 antibody-conjugated agarose equilibrated in PBS buffer at 4 °C for 1 h or 4 h, respectively. For PTK6 immunoprecipitation, cell lysate was incubated with anti-PTK6 antibody at 4 °C overnight, and then with protein A-Sepharose for 2 h.

The resin was washed in PBS buffer three times. Proteins bound to the resin were mixed with SDS sample buffer containing 100 mM β-mercaptoethanol, resolved by SDS-PAGE, and
analyzed by Western blot analysis. For quantification of EGFR level, chemiluminescence was detected by LAS-3000 (Fujifilm, Tokyo, Japan) and quantified by Multi Gauge V2.2 software (Fujifilm). Statistical analysis was performed by Student’s t test.

Knockdown of PTK6—PTK6 shRNA constructs (MISSION™ TRC shRNA, Sigma) were screened for the ability to knockdown PTK6 expression. PTK6-shRNA 1064 (TRCN000021552) and 1866 (TRCN000021549), which most efficiently decreased PTK6 expression, were transfected into BT-474 cells using WelFext-EXT™ PLUS and selected with 1/9262 g/ml of puromycin. Puromycin-resistant cells were pooled and expanded.

Biotinylation and Precipitation of Cell-surface Proteins—After washing twice with ice-cold PBS, cell-surface proteins were biotinylated with 0.1 mg/ml Sulfo-NHS-LC-Biotin (Pierce) for 30 min on ice. Unreacted biotin was quenched and removed by washing twice with ice-cold PBS containing 0.1 M glycine and twice with ice-cold PBS. For precipitation of surface biotinylated proteins, cells were lysed with lysis buffer and the cell lysate incubated with NeutrAvidin Plus UltraLink Resin (Pierce), equilibrated in lysis buffer at 4 °C for 1 h. The resin was washed with lysis buffer twice. Levels of cell-surface EGFR were analyzed by Western blot.

RESULTS

Survey of Proteins Interacting with PTK6—To find interacting proteins of PTK6, Flag-tagged PTK6 (Flag-PTK6) expressed in HEK293 cells was pulled down with anti-Flag-agarose, and the precipitated proteins were then visualized in SDS gels by Coomassie Brilliant Blue Staining (Fig. 1A). Several bands were detected in the pull-down proteins from HEK293 cells expressing PTK6 compared with those from HEK293 cells transfected with empty vector (Fig. 1B). By in-gel digestion with trypsin and MALDI-TOF MS, and peptide fingerprinting

| Band number | Protein name | MW (kDa) | NCBI accession No. | Coverage | Score |
|-------------|--------------|----------|-------------------|----------|-------|
| 1           | ARAP1        | 162.09   | NP_001035207      | 30       | 85    |
| 2           | Heat shock protein 90-α | 84.48 | P07900            | 24       | 117   |
| 3           | Sam68        | 48.20    | NP_006550         | 32       | 67    |
| 4           | PTK6         | 51.80    | NP_005966         | 41       | 207   |

TABLE 1 Identification of pull-down proteins by trypsin in-gel digestion, MALDI-TOF MS, and peptide fingerprinting

As Flag-PTK6 was expressed at higher levels in HEK293 cells through transfection of increasing amounts of PTK6 expression vector, the amount of ARAP1 co-immunoprecipitated with PTK6 was increased (Fig. 1C). These results strongly suggest that an interaction occurs between PTK6 and ARAP1.

Serum- and EGF-dependent Binding of PTK6 to ARAP1—An interaction of ARAP1 with PTK6 was observed in cells cultured in serum-supplemented medium (Fig. 1B and C). To test the effect of serum on the interaction, HEK293 cells expressing Flag-PTK6 or vector were either starved for 24 h or cultured normally. The binding of ARAP1 to PTK6 was greatly decreased in the serum-depleted condition compared with the serum-supplemented normal culture condition (Fig. 2A).
EGFR is known to activate the catalytic activity of PTK6 (10). To test whether the interaction between PTK6 and ARAP1 is dependent on the activation of EGFR, serum-depleted HEK293 cells expressing Flag-tagged PTK6 were stimulated with EGF. Upon EGF stimulation, PTK6 interacted with ARAP1. The interaction was maintained in a pattern similar to that of EGFR phosphorylation (Fig. 2B). Moreover, when an inhibitor of EGFR tyrosine kinase, AG1478, was used to treat Flag-PTK6-expressing HEK293 cells, the association of PTK6 with ARAP1 was blocked (Fig. 2C). Thus, PTK6 interacts with ARAP1 in a manner that depends on the activation of EGFR catalytic activity.

Requirement of Catalytic Activity of PTK6 for the Interaction of PTK6 and ARAP1—To investigate whether ARAP1 binding to PTK6 requires the catalytic activity of PTK6, binding of ARAP1 to Flag-PTK6 or, a catalytically inactive mutant, Flag-PTK6-K219M (11), was measured after stimulation with EGF. Loss of catalytic activity of PTK6 markedly diminished, but did not abolish, binding to ARAP1 (mean and S.D. referred to the wild-type PTK6 was 25.9 ± 8.9%) (Fig. 3). These results indicate that the catalytic activity of PTK6 is required for the full interaction with ARAP1.

Binding of the PTK6 SH2 Domain to ARAP1—To identify the ARAP1-interaction domain of PTK6, HEK293 cells were transfected with various PTK6 constructs (Fig. 4A), including Flag-PTK6-SH3-SH2, Flag-PTK6-SH3-PR containing an SH3 domain and a unique proline-rich region, which is the product of an alternatively spliced transcript, Flag-PTK6-SH2, and Flag-PTK6-Linker-Kinase, or Flag-PTK6 as a positive control. Immunoprecipitates with Flag antibody from EGF-stimulated HEK293 cell lysates were subjected to Western blot analysis with anti-Flag and anti-ARAP1 antibodies. Flag-PTK6-Linker-Kinase and Flag-PTK6-SH3-PR did not bind ARAP1, while Flag-PTK6-SH3-SH2 and Flag-PTK6-SH2 did (Fig. 4B), indicating that the SH2 domain of PTK6 includes the binding site for ARAP1.

Arg175 within the SH2 domain of v-Src makes contact with the phosphate group on a tyrosine residue (33) and its mutation to Leu abrogates phosphotyrosine binding (34). Arg 105 of PTK6, which corresponds to Arg175 of v-Src, was mutated to Ala (R105A). The R105A mutant disrupted the interaction between PTK6 and ARAP1 (Fig. 4C). This result suggests that the interaction involves the Arg105 residue in the SH2 domain of PTK6 and the phospho-tyrosine residue(s) of ARAP1.

Phosphorylation and Binding of Tyr231 of ARAP1 to PTK6—To identify the PTK6 interaction domain of ARAP1, wild-type Myc-ARAP1 or N-terminal sterile (SAM), and proline-rich (PR) domain-deleted Myc-ARAP1 (Myc-ARAP1ΔSAM) were transiently transfected into HEK293 cells either transfected with vector or stably expressing PTK6 (Fig. 5A). Upon stimulation with EGF, wild-type Myc-ARAP1, but not the N-terminal-deleted ARAP1, was phosphorylated and interacted with PTK6 (Fig. 5B). Phosphorylation of ARAP1 and interaction between ARAP1 and PTK6 by EGF stimulation were analyzed by immunoprecipitation and Western blotting. Wild-type Myc-ARAP1 and Phospho-tyrosine residue(s) of ARAP1.

PTK6 Inhibits EGFR Down-regulation via ARAP1 Phosphorylation

FIGURE 2. Serum and EGF-dependent binding of PTK6 to ARAP1. A, serum-dependent binding of PTK6 to ARAP1. HEK293 cells stably expressing Flag-PTK6 or empty vector were incubated with serum-free medium (−) or 5% FBS-supplemented medium (+) for 24 h. B, EGF-dependent binding of PTK6 to ARAP1. HEK293 cells stably expressing Flag-PTK6 were starved and then stimulated with 50 ng/ml EGF for indicated time periods. C, effect of EGFR inhibitor on binding of PTK6 with ARAP1. After pretreatment with an EGFR inhibitor, AG1478 (1 μM), for 30 min, EGF (50 ng/ml) was added for 5 min. Flag-PTK6 was pulled down with anti-Flag antibody and interaction of ARAP1 with Flag-PTK6 was analyzed by Western blot analysis using anti-ARAP1 antibody (A–C). To monitor EGF-induced signaling, phosphorylation levels of EGFR (B) and Erks (C) were examined by Western blot.

FIGURE 3. Requirement of PTK6 catalytic activity for interaction of PTK6 with ARAP1. HEK293 cells stably expressing Flag-PTK6 or the K219M mutant, which is catalytically inactive, were stimulated with 50 ng/ml EGF for 5 min. Pull-down of Flag-PTK6 and its interaction to ARAP1 were analyzed, as described above.

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sis. When Tyr^{231} of ARAP1 was mutated, ARAP1 was not phosphorylated and did not interact with PTK6 (Fig. 5C). Therefore, the N-terminal region of ARAP1, particularly Tyr^{231}, is important for phosphorylation by, and interaction with, PTK6.

Inhibition of EGFR Down-regulation through PTK6-mediated Phosphorylation of ARAP1—ARAP1 has an Arf-GAP domain, which plays a role in endocytosis (30, 35). Thus, we first analyzed the effect of ARAP1 phosphorylation on the down-regulation of overexpressed Flag-EGFR (36) in normal culture medium supplemented with serum. Expression of either wild-type ARAP1 or the Y231F mutant in HEK293 cells that did not express PTK6 led to slight differences in EGFR levels. However, the expression of wild-type ARAP1 resulted in much higher EGFR levels than the expression of ARAP1-Y231F in HEK293 cells with PTK6 (Fig. 6, A and B). These results indicate that phosphorylation of ARAP1 by PTK6 is important for the inhibition of EGFR internalization and degradation.

To test the effect of ARAP1 phosphorylation on EGFR internalization, an inhibitor of clathrin-dependent receptor endocytosis, MDC, was added to HEK293-PTK6 cells that were transiently expressing Flag-EGFR and either wild-type ARAP1 or the Y231F mutant. Interestingly, upon MDC treatment, EGFR levels were similar in both cells expressing ARAP1 and those with ARAP1-Y231F (Fig. 6, C and D). Thus, phosphorylation of ARAP1 by PTK6 inhibits the down-regulation of EGFR, largely by blocking clathrin-mediated endocytosis.

We next analyzed whether the EGF-induced phosphorylation of ARAP1 affects endogenous EGFR levels and its downstream signaling in serum-depleted HEK293-PTK6 cells that were transiently expressing Flag-EGFR and either wild-type ARAP1 or the Y231F mutant. Interestingly, upon MDC treatment, EGFR levels were similar in both cells expressing ARAP1 and those with ARAP1-Y231F (Fig. 6, C and D). Thus, phosphorylation of ARAP1 by PTK6 inhibits the down-regulation of EGFR, largely by blocking clathrin-mediated endocytosis.

We next analyzed whether the EGF-induced phosphorylation of ARAP1 affects endogenous EGFR levels and its downstream signaling in serum-depleted HEK293-PTK6 cells that were transiently expressing either wild-type ARAP1 or the Y231F mutant. Under starvation conditions, basal levels of surface and total EGFR in HEK293-PTK6 cells expressing ARAP1-Y231F were

**FIGURE 4.** Determination of the ARAP1-binding domain in PTK6. A, schematic representation of PTK6 and its domains. B, PTK6 domain interacting with ARAP1. Flag-tagged PTK6 or its various domains was stably expressed in HEK293 cells, and then pulled down with anti-Flag antibody after treatment with 50 ng/ml EGF for 5 min. Interaction of PTK6 or its domains with ARAP1 was assayed by Western blot analysis using anti-ARAP1 antibody. C, involvement of Arg{sup 105} of PTK6 for binding to ARAP1. HEK293 cells were transfected with a construct expressing either Flag-PTK6 SH2 or Flag-PTK6 SH2 R105A. Pull-down of Flag-PTK6-SH2 and interaction with ARAP1 were analyzed, as described above.

**FIGURE 5.** Determination of PTK6-binding site in ARAP1. A, schematic view of ARAP1 and ARAP1{sup ΔSAM}. B, importance of the N-terminal domain of ARAP1 for association with PTK6. HEK293 cells stably expressing PTK6 were transiently transfected with an expression construct for either Myc-ARAP1 or Myc-ARAP1{sup ΔSAM}. After treatment with 50 ng/ml EGF for 5 min, Myc-tagged proteins were pulled down. PTK6-dependent phosphorylation of Myc-ARAP1 was analyzed by Western blot using anti-phosphotyrosine antibody. Interaction between PTK6 and Myc-ARAP1 was determined by Western blot analysis using anti-Myc (ARAP1) antibody and anti-PTK6 antibody. C, determination of PTK6-dependent phosphorylation site in ARAP1. Tyrosine residues in the N-terminal domain (amino acid residues 1–314) of ARAP1 were changed to phenylalanine by site-directed mutagenesis. HEK293 cells stably expressing PTK6 were transiently transfected with constructs expressing Myc-ARAP1, Myc-ARAP1-Y23F, Myc-ARAP1-Y231F, and Myc-ARAP1-Y288F. Myc-ARAP1 and mutant proteins were immunoprecipitated by anti-Myc antibody and analyzed by Western blot analysis using anti-phosphotyrosine, anti-Myc (ARAP1), or anti-PTK6 antibody.
similar to the levels in the cells expressing wild type ARAP1 (0 min in Fig. 7). Upon EGF stimulation, cells expressing ARAP1-Y231F decreased their levels of surface EGFR as well as total EGFR more rapidly, and displayed activation of Akt and Erks more transiently and weakly, than cells expressing wild-type ARAP1 (Fig. 7). These results indicate that EGF-induced phosphorylation of ARAP1 via PTK6 inhibits EGFR and its downstream signaling by delaying EGFR down-regulation.

**DISCUSSION**

We have screened for interacting proteins of PTK6 using a pull-down assay with Flag-tagged PTK6, followed by proteomic analysis of the co-precipitated proteins. ARAP1 that is made up of SAM, PR, Arf-GAP, Rho-GAP, RA, and five PH domains (30–31, 37) was identified as one of the PTK6-interacting proteins. An earlier study was done with an alternatively spliced variant of ARAP1 that does not encode the SAM and PR domains (ARAP1ΔSAM) (30). In that report, it was shown that ARAP1ΔSAM associates with the Golgi and inhibits cell spreading via Arf-GAP and Rho-GAP activity (30). Thus, ARAP1 was proposed to be a point of convergence for Arf, Rho, and phosphoinositides, and to serve as a node in a signaling network that coordinates the membrane and actin remodeling involved in cell movement.

PTK6-interacting proteins differ in their requirement for PTK6 catalytic activity. For example, the adaptor protein BKS requires the catalytic activity of PTK6 for binding (20), whereas the nuclear RNA-binding protein Sam68 does not (17). It is known that EGF induces PTK6 to interact with EGFR and activates it (12). We found that stimulation of EGFR by EGF induces the interaction of PTK6 with ARAP1, and that an EGFR inhibitor, AG1478, blocks this association. Moreover, the kinase-dead PTK6 K219M mutant did not bind to ARAP1 as well as the wild-type Flag-PTK6. Thus, these results indicate...
that the catalytic activity of PTK6 contributes to the PTK6-ARAP1 interaction. Among PTK6-interacting proteins, Sam68, paxillin, and IRS-4 require both the SH2 and SH3 domains of PTK6 for interaction. Interestingly, we found that only the SH2 domain, and more specifically the phosphotyrosine recognition site, of PTK6 is involved in the interaction with ARAP1. Because the Tyr231 residue of ARAP1 was determined to be the major site phosphorylated by, and associated with, PTK6, we are sure that the SH2 domain of PTK6 binds to the Tyr231 of ARAP1.

The literature related to the down-regulation of EGFR is complex. Although it is agreed that the first step is internalization of the receptor, multiple mechanisms have been reported, including a clathrin-dependent endocytic pathway (38, 39), a caveolin-dependent pathway (40, 41), and a clathrin- and caveolin-independent pathway (42). The results of our experiment using a clathrin-mediated endocytosis inhibitor, MDC, suggest that the down-regulation of EGFR in our system is largely mediated by clathrin-dependent endocytosis.

Arf proteins are major regulators for the formation of intracellular vesicles. ARAP1 is reported to be an Arf-GAP that preferentially uses Arf5, but can also use Arf1 and Arf6 (30, 43–44). Arf6 has been implicated in multiple pathways of endocytosis (45). Whereas Arf1 plays a central role in the secretory pathway (46), it has recently been found to regulate endocytosis (42). Arf5 has not been reported to be a regulator of endocytosis. Thus, it is possible that the effects of ARAP1 are mediated through an effect on Arf.

The effect of ARAP1 on EGFR endocytosis is still being discovered. Yoon et al. (31) found that ARAP1 colocalizes with EGFR upon EGF stimulation. Reduction of ARAP1 expression accelerates the association of EGF/EGFR with EEA1-positive endosomes and the degradation of EGFR, and reduces the propagation of Erk and JNK signals (31). Daniele et al. (47) also reported that ARAP1 is involved in the trafficking of EGFR. In contrast to the report by Yoon et al. (31), they found that overexpression of ARAP1 increases the degradation of EGFR in peripheral endosomal compartments. In addition, the ARAP1 knockdown accumulates EGFR in sorting/late endosomal compartments and delays EGFR degradation and Erk inactivation (47).

According to our findings, wild-type ARAP1, which is phosphorylated by PTK6 on Tyr231, delayed the down-regulation of EGFR, compared with the unphosphorylatable ARAP1 mutant (Y231F), in a HEK293 cell system. Also, knockdown of PTK6 in breast carcinoma BT-474 cells, which express endogenous PTK6, ARAP1, and EGFR, markedly increased the down-regulation of EGFR. This suggests that phospho-Tyr231 ARAP1 has enhanced Arf-GAP activity for the inactivation of Arf-GTP. One possible explanation for this regulation is that Tyr231 phosphorylation relieves the inhibition of Arf-GAP activity of ARAP1 caused by the SAM domain. The SAM domain may negatively regulate Arf-GAP activity by holding the GAP

FIGURE 7. **Effect of ARAP1 phosphorylation on EGFR levels and Akt and Erk activation.** HEK293 cells stably expressing PTK6 were transiently transfected with constructs expressing wild-type Myc-ARAP1 (white bar) or the Y231F mutant (black bar). Starved cells were stimulated with 50 ng/ml EGF for indicated time periods. Surface proteins were biotinylated and precipitated with avidin resin. Surface proteins were analyzed by Western blotting with anti-EGFR antibody and, as a control for contaminating cytosolic proteins, with anti–β-actin antibody. Cell lysates were analyzed with anti-EGFR, anti-Myc (ARAP1), anti-phospho-Akt, and anti-phospho-Erk1/2 antibodies (A). For quantification, surface (B) and total (C) EGFR levels were normalized to PTK6 levels. EGFR levels of HEK293-PTK6 cells expressing wild-type Myc-ARAP1 were defined as 100%. Each value is the mean ± S.D. (n = 3).

FIGURE 8. **Interaction of endogenous PTK6 with ARAP1.** Human breast carcinoma cells, BT-474 and T-47D, which endogenously express EGFR, PTK6, and ARAP1, were starved and then stimulated with 50 ng/ml EGF for 5 min. Cell lysates were immunoprecipitated with anti-PTK6 antibody or control antibody (IgG). The immunoprecipitates were analyzed by Western blot analysis using anti-PTK6 and anti-ARAP1 antibodies.
domain in an auto-inhibitory conformation (44). It is possible that Tyr231 phosphorylation of ARAP1 by PTK6 and its binding to PTK6 destroys an auto-inhibited conformation of ARAP1 and leads to the inactivation of Arf-GTP. Another possibility for the regulation of ARAP1 activation via Tyr231 phosphorylation is through a change of localization of ARAP1. Campa et al. (43) has shown that ARAP1 is recruited to the plasma membrane independent of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and that subsequent production of PIP3 triggers Arf-GAP activity. Thus, we presume that ARAP1 might be phosphorylated by and recruited to PTK6 at the plasma membrane through its SH2 domain, prior to activation of Arf-GAP through the contact of its nearby PH domain with membrane PIP3.

Interestingly, it was found that the kinase-dead PTK6 also interacts with ARAP1 following EGF stimulus, though with a lower extent than the catalytically active PTK6. Moreover, overexpression of EGFR in HEK293 cells slightly increases phosphorylation of ARAP1 in the absence of PTK6 (data not shown). These results suggest that EGFR itself, or PTKs downstream of EGFR, phosphorylates Tyr231 of ARAP1 to a lesser extent. Among the PTKs, the Src family members are most closely related to PTK6 (2–4) and are involved in endosomal trafficking of receptor-type tyrosine kinases, such as EGFR (48, 49). Src is also known to phosphorylate ARAP1 (31). We analyzed whether Src family members, Src and Lck, have an effect on EGFR internalization through the phosphorylation and interaction of ARAP1. Src and Lck phosphorylate the Y231F mutant of ARAP1, as well as wild-type ARAP1, indicating that Tyr231 and other residues of ARAP1 are phosphorylated by Src and Lck. In addition, phosphorylation of ARAP1 by these kinases seems not to depend on EGF stimulation and phosphorylated ARAP1 does not interact with Src and Lck (data not shown). Moreover, unlike PTK6, Src, and Lck did not have detectable effects on the down-regulation of EGFR (supplemental Fig. S1).

In conclusion, we have identified ARAP1 as a novel interacting protein of PTK6. PTK6 phosphorylates ARAP1 and binds to
the phosphorylated ARAP1 through its SH2 domain. PTK6-dependent ARAP1 phosphorylation inhibits EGFR internalization, increases the duration of EGFR/EGFR signaling, and in turn, increases oncogenic abilities of the cells expressing PTK6. Novel regulatory mechanisms of ARAP1 by PTK6 may reveal how PTK6 potentiates EGFR/EGFR signaling and tumorigenesis.

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