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PRR5, a Novel Component of mTOR Complex 2, Regulates Platelet-derived Growth Factor Receptor β Expression and Signaling*[S]

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The protein kinase mammalian target of rapamycin (mTOR) plays an important role in the coordinate regulation of cellular responses to nutritional and growth factor conditions. mTOR achieves these roles through interacting with raptor and rictor to form two distinct protein complexes, mTORC1 and mTORC2. Previous studies have been focused on mTORC1 to elucidate the central roles of the complex in mediating nutritional and growth factor signals to the protein synthesis machinery. Functions of mTORC2, relative to mTORC1, have remained little understood. Here we report identification of a novel component of mTORC2 named PRR5 (PRoline-Rich protein 5), a protein encoded by a gene located on a chromosomal region frequently deleted during breast and colorectal carcinogenesis (Johnstone, C. N., Castellvi-Bel, S., Chang, L. M., Sung, R. K., Bowser, M. J., Pique, J. M., Castells, A., and Rustgi, A. K. (2005) *Genomics* 85, 338–351). PRR5 interacts with rictor, but not raptor, and the interaction is independent of mTOR and not disturbed under conditions that disrupt the mTOR-rictor interaction. PRR5, unlike Sin1, another component of mTORC2, is not important for the mTOR-rictor interaction and mTOR activity toward Akt phosphorylation. Despite no significant effect of PRR5 on mTORC2-mediated Akt phosphorylation, PRR5 silencing inhibits Akt and S6K1 phosphorylation and reduces cell proliferation rates, a result consistent with PRR5 roles in cell growth and tumorigenesis. The inhibition of Akt and S6K1 phosphorylation by PRR5 knock down correlates with reduction in the expression level of platelet-derived growth factor receptor β (PDGFRβ). PRR5 silencing impairs PDGF-stimulated phosphorylation of S6K1 and Akt but moderately reduces epidermal growth factor- and insulin-stimulated phosphorylation. These findings propose a potential role of mTORC2 in the cross-talk with the cellular machinery that regulates PDGFRβ expression and signaling.

Cell growth relies on coordinated regulation of signaling pathways that integrate cellular physiological status in response to nutrient levels, growth factor signals, and environmental stress. Impairment of the coordinated regulation can lead to disastrous effects on cell physiology, resulting in cell death or uncontrolled growth. mTOR,[2] a member of the phosphatidylinositol kinase-related kinase family, has been known as a central player in the signaling pathway that regulates cell growth in response to a variety of cellular signals derived from nutrient levels, growth factors, and environmental stress (2–4). mTOR plays a central role in the signaling network that regulates a variety of cellular processes including ribosome biogenesis, protein synthesis, autophagy, and actin cytoskeleton organization; human diseases such as cancer, diabetes, obesity, and harmatoma syndrome are associated with defects in mTOR signaling (5–9).

Recent years have seen discoveries of several mTOR effectors and binding proteins. mTOR exists in two multiprotein complexes, mTORC1 and mTORC2. mTORC1 consists of mTOR, raptor, GβL, and PRAS40, and it functions to regulate protein synthesis and cell growth in response to nutrient levels and growth factor signals (10–14). mTORC1 regulates phosphorylations of at least two regulators of protein synthesis, S6K1 and 4E-BP1, and mediates nutrient and insulin signals to the cell growth machinery (2, 15). mTORC1 is regulated by TSC-Rheb (tuberous sclerosis complex-Ras homolog-enriched in brain) signaling (16–19).

mTORC2 consists of mTOR, rictor, GβL, and Sin1, and it does not likely bind rapamycin-FK506-binding protein 12 complex, which makes mTORC2 distinctive from mTORC1 (13, 20, 21). *Saccharomyces cerevisiae* TORC2 consists of TOR2, LST8, AVO1 (Sin1 ortholog), and AVO3 (rictor ortholog) and two other components, AVO2 and BIT61, whose homologues have not been identified in higher eukaryotes (13, 22, 23). Functions and regulatory mechanisms of mTORC2 remain largely unknown. Recent studies showed that mTORC2 regulates protein kinase C α phosphorylation, actin cytoskeleton organization, and Akt phosphorylation at Ser-473 (20, 21, 24, 25). Recognizing the complex relationship between mTOR, S6K1, and

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

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[2] The abbreviations used are: mTOR, mammalian target of rapamycin; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; raptor, regulatory-associated protein of mTOR; rictor, rapamycin-insensitive companion of mTOR; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PRAS40, proline-rich Akt substrate 40 kDa; GST, glutathione *S*-transferase; HEK, human embryonic kidney; HA, hemagglutinin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; shRNA, short hairpin RNA.

Akt and knowing that we have not yet identified mammalian homologues of AVO2 and BIT61, we hypothesized that other unidentified mTOR-binding effector proteins may provide clues to the mechanism underlying mTORC2 signaling.

In this study, we identified a novel component of mTORC2 named PRR5, a protein having an implicative function in tumorigenesis (1). We determined that PRR5 specifically interacts with rictor, but not raptor, and the interaction is tighter than the rictor-mTOR interaction and independent of mTOR. We identified PRR5 and rictor residues crucial for the PRR5-rictor interaction and determined that PRR5 is important for PDGFRβ expression and PDGF signaling to Akt and S6K1.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Anti-mTOR (sc-1549), epidermal growth factor receptor (EGFR) (sc-03), Fas (sc-20140), p21 (sc-397), tubulin (sc-12462), PDFGR (sc-432), glyceraldehyde-3-phosphate dehydrogenase (sc-25778), and 14-3-3 (sc-732) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies specific to human PRR5 were generated against full-length PRR5 fused with glutathione *S*-transferase and an epitope peptide near the C terminus containing a sequence RGSGMSDLEGSGGR from YenZym antibodies (Burlingame, CA). Raptor- and rictor-specific antibodies were described in our previous report (14). Antibodies against S6K1 (9202), phospho-S6K1 Thr-389 (p-S6K1; 9205), Akt (9272), phospho-Akt Ser-372 (p-Akt; 9271), 4E-BP1 (9452), phospho-4E-BP1 (2855), and insulin receptor β (3025) antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit IgG TrueBlot (18–8816) used to detect PRR5 in immunoprecipitates was obtained from eBioscience (San Diego, CA). Anti-HA antibody (HA.11) was from Covance (Berkeley, CA). Anti-Myc 9E10 and growth factors EGF and PDGF were purchased from EMD Biosciences (San Diego, CA). Porcine insulin was purchased from Sigma. Glutathione 4B beads were from GE Healthcare.

Identification of PRR5—The strategy that we described in our previous study (14) was used with modifications in steps of immunoprecipitation and preparation of trypsinized samples. mTOR immunoprecipitate was prepared from HEK293T cells as described previously (14) using a lysis buffer containing 40 mM Hepes, pH 7.4, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 10 mM β-glycerophosphate, 0.3% Chaps, and EDTA-free protease inhibitors (Roche Applied Science). mTOR immunoprecipitates were washed four times with the lysis buffer and twice with the lysis buffer without the detergent. mTOR-binding proteins were eluted from the immunoprecipitate in a buffer containing 0.075% SDS. The eluate was diluted with a trypsin digestion buffer (25 mM ammonium bicarbonate, 2.5 mM $CaCl_2$, pH 8.0) and incubated with trypsin (2 μg) overnight. The trypsinized sample was diluted with 0.1% formic acid to obtain a pH below 3.0 and loaded onto a mixed mode cation exchange cartridge (MCX cartridge; Waters Inc., Milford, MA) to remove salt and detergent from the samples. Peptides bound to the resin were eluted with 5% ammonium hydroxide in methanol and lyophilized. Lyophilized samples were dissolved in 0.1% formic acid and analyzed by microcapillary electrospray tandem mass spectrometry on an electrospray linear ion trap mass spectrometer (ThermoElectron, Waltham, MA). Tandem mass spectrometry spectral data were analyzed as described in our previous study (14). Peptide sequence matches were filtered using a probabilistic scoring algorithm called Peptide Prophet (26, 27) that assigns a value between 0 and 1 to peptide sequence matches, with a score of 1 representing the highest confidence match.

Plasmid Constructions and Mutagenesis—PRR5 cDNAs for isoforms 1, 2, and 3 of human origin kindly provided by Dr. C. Johnstone and Dr. A. Rustgi at the University of Pennsylvania were cloned into prk5-myc and prk5-HA expression vectors by use of a PCR amplification kit (Roche Applied Science). The PRR5 and rictor DNA fragments used in Fig. 4, *C* and *D*, were generated by PCR amplification and subcloned into mammalian expression vector prk5-myc, and all the clones were confirmed by sequencing. pLKO shRNA vector (provided by Dr. S. Stewart, Washington University) was used for knockdown experiments. Target sequences were 5′-catgctgcaggccatcttcta-3′ (sh-PRR5 4), 5′-ggacaagattcgcttctatga-3′ (sh-PRR5 15), 5′-aaccctgcctttgtcatgcct-3′ (sh-mTOR), 5′-caccaccaaagcaacctatag-3′ (sh-rictor), and 5′-aacgtacgcggaatacttcga-3′ (scrambled shRNA). All other constructs used in the experiments have been previously described (10, 11, 14).

Cell Culture and Transfection—HEK293T, HeLa, HT1080, HepG2 cells, and other cancer cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C in 5% CO_2. For transient expression, HEK293T cells were transfected with recombinant DNAs or shRNA plasmids using FuGENE 6 (Roche Applied Science) following the manufacturer's protocol. Cells were harvested 2 days post-transfection for co-immunoprecipitation assay.

Recombinant Protein Production—GST-tagged PRR5 isoforms 1, 2, and 3 cloned in pGEX6T-2 (Amersham Biosciences) were expressed in BL21(DE3) cells (EMD Biosciences) by induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h and purified with glutathione-Sepharose 4B beads according to a standard protocol.

Co-immunoprecipitation and Western Blotting—For co-immunoprecipitation studies, whole-cell extracts were prepared in 0.3% Chaps buffer and immunoprecipitated with the anti-mTOR, anti-raptor, anti-rictor, anti-PRR5, anti-HA, or anti-Myc antibodies. Precipitated proteins were washed four times in 0.3% Chaps buffer, loaded onto 8% Tris-glycine gels (Invitrogen), transferred for 4 h onto immunoblot polyvinylidene difluoride membranes (Bio-Rad), and detected with ECL Western blotting detection reagents (Perkin-Elmer).

Lentiviral Preparation, Viral Infection, and Stable Cell Generation—A pLKO-shRNA plasmid encoding an shRNA that targets PRR5 or a scrambled sequence was transduced into HEK293T cells with lentiviral packaging vectors pHR′8.2ΔR and pCMV-VSV-G (provided by Dr. S. Stewart, Washington University) using FuGENE 6. Viruses were collected from the medium 60 h after transfection, and target cells were infected with the collected viruses four times over 15 h in the presence of polybrene. Cells were harvested 3 to 5 days post-infection or selected under puromycin for several days.

Cell Proliferation Assay—HeLa cells transduced with lentiviral shRNAs were split into 6-cm plates at 20% confluence; the next

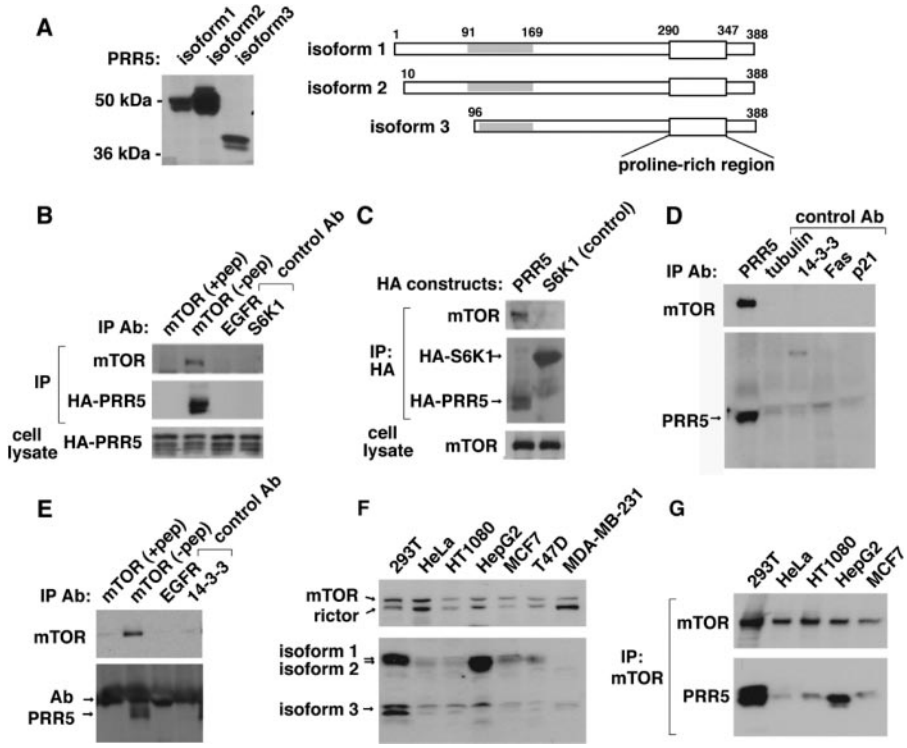

FIGURE 1. **PRR5 interacts with the rictor-mTOR complex.** *A*, expression of cloned PRR5 isoforms 1 (1–388 residues), 2 (10–388 residues), and 3 (96–388 residues) in 293T cells. The proline-rich region between the 290th and 347th residues, indicated by a *rectangular box*, contains 15 prolines among 58 amino acids. The *gray-colored* region shares sequence similarity with the HbrB domain. *B*, recombinant PRR5 is immunoprecipitated by mTOR-specific antibody. mTOR immunoprecipitate was obtained from 293T cells expressing recombinant PRR5 (*HA-PRR5*) in the presence (+*pep*) or absence (−*pep*) of an mTOR antibody epitope peptide and analyzed using an HA antibody on Western blots. *C*, endogenous mTOR is immunoprecipitated with HA-PRR5. 293T cells were transiently transfected with a plasmid encoding HA-PRR5 or HA-S6K1; 2 days post-transfection HA immunoprecipitates were obtained and endogenous mTOR was analyzed on immunoblot. *D*, PRR5 immunoprecipitate contains endogenous mTOR. Immunoprecipitates were obtained from 293T cells using antibodies specific to PRR5, tubulin, 14-3-3, Fas, and p21, and the amount of mTOR was analyzed on immunoblots. *E*, endogenous PRR5 is immunoprecipitated by mTOR antibody. mTOR immunoprecipitates obtained from 293T cells in the presence (+*pep*) or absence (−*pep*) of mTOR antibody epitope peptides were analyzed using a PRR5-specific antibody. *F*, PRR5 expression level is various in several cell lines. An equal amount of total protein from each cell line was loaded on SDS-PAGE, and the amounts of mTOR, rictor, and PRR5 were analyzed by Western blotting. *G*, PRR5 interacts with mTOR in various cell lines. mTOR immunoprecipitates were prepared from each cell line, and mTOR and PRR5 were analyzed by Western blotting.

day cells were trypsinized and diluted ten times with Dulbecco's modified Eagle's medium. One ml of diluted cell culture was loaded on a ViCell analyzer (Beckman Coulter Inc., Fullerton, CA).

Real-time PCR Analysis—Total RNA was prepared from HeLa cells transduced by lentiviral shRNAs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 5 μg of total RNA using the iScript cDNA synthesis kit for real-time PCR (Bio-Rad) and resuspended in diethylpyrocarbonate-treated water. PCR products were generated by PCR amplification using Lightcycler Faststart DNA Master^{plus} SYBR Green 1 (Bio-Rad). Amplification of human PDGFRβ cDNA was performed using a forward primer, 5′-tgtgacggagagtgtgaatgac-3′, paired with a reverse primer, 5′-agggtgcggttgtctttgaac-3′. Amplification of TATA box-binding protein cDNA was performed using a forward primer, 5′-taatcccaagcggtttgctg-3′, paired with a reverse primer, 5′-gcacaccattttcccagaactg-3′.

RESULTS

Identification of PRR5 as an mTOR-binding Protein—In our recent study, we described an approach combining the electrospray linear ion trap mass spectrometer and mTOR immunoprecipitation to identify mTOR-binding proteins (14). This approach, without relying on SDS-PAGE separation of proteins, increased the sensitivity of detection and led us to identify PRAS40 and Sin1 that were barely detectable on Coomassie-stained gels. We modified sample preparative conditions for mass spectrometry as detailed under "Experimental Procedures." In the new preparation, we identified three peptides of high scores of P value, a parameter of fidelity for MS/MS matches, that were detected from proteins isolated specifically in mTOR immunoprecipitate but not in control immunoprecipitates (supplemental Table 1) (14). The three identified peptides were derived from PRR5, a proline-rich protein that has an implicative role in tumorigenesis (1). The PRR5 gene is located on chromosome 22q13.31, a region that is frequently deleted during human breast and colorectal carcinogenesis. PRR5 was previously shown to exist as several isoforms of splicing variants (1). PRR5, containing a high content of proline residues (28 among 388 amino acids, 7.2%), is conserved in higher eukaryotes. BLAST search revealed that human PRR5 shares 33–86% identity with genes from amphibians, fishes, rodents, and primates but does not show similarity to genes from *Drosophila melanogaster* and *Caenorhabditis elegans* as well as BIT61 and AVO2, two TOR2-interacting proteins whose mammalian homologues have not been found (supplemental Fig. S1). PRR5 residues 91–169 share sequence similarity with the HbrB domain, a domain found in proteins involved in hyphal growth and polarity (28). The longest isoform (isoform 1) contains 388 amino acids with a proline-rich region near the C terminus (Fig. 1*A*). Isoforms 2 and 3 are 9 and 95 amino acids shorter than isoform 1 at the N terminus, respectively.

To confirm that mTOR specifically interacts with PRR5, mTOR immunoprecipitate was obtained from HEK293T cells and the amount of transiently expressed PRR5 isoform 2, a highly expressed form of the three isoforms (Fig. 1*A*), was analyzed by Western blotting. Supporting the specific interaction between mTOR and PRR5, PRR5 was detected only in mTOR immunoprecipitate purified in the absence of an mTOR antibody-blocking peptide but not in the presence of the blocking peptide or in immunoprecipitates obtained using control antibodies (Fig. 1*B*). Consistent with the specific interaction between mTOR and PRR5, endogenous mTOR was isolated in

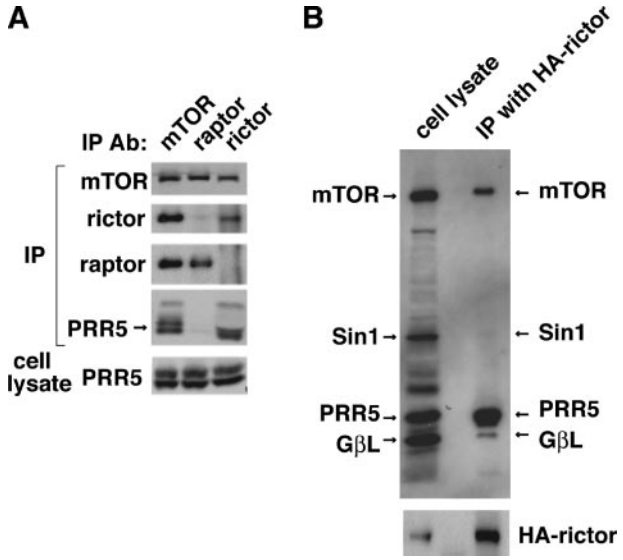

FIGURE 2. **PRR5 is a constituent of mTORC2.** *A*, PRR5 is detected in mTOR and rictor immunoprecipitates but not in raptor immunoprecipitates. Anti-mTOR, raptor, and rictor polyclonal antibodies were used for immunoprecipitation to isolate mTOR-, raptor-, and rictor-binding proteins from 293T cells. *B*, PRR5 binds rictor and mTOR in a stoichiometric manner. Myc-tagged mTOR, Sin1, PRR5, and GβL were expressed together with HA-rictor in 293T cells. Two days post-transfection, HA immunoprecipitates were obtained and analyzed for the amounts of the Myc-tagged recombinant proteins on immunoblots.

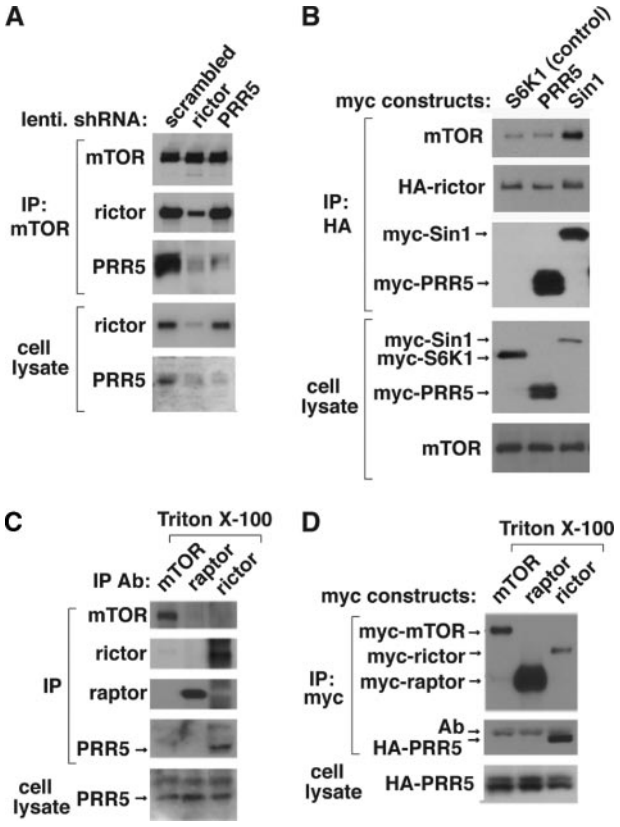

FIGURE 3. **PRR5 is not required for the rictor-mTOR interaction and interacts with rictor independently of mTOR.** *A*, rictor is important for the stability or expression of PRR5. 293T cells were transduced by a lentiviral shRNA specific to rictor or PRR5 or a scrambled shRNA. Three days after the viral infection, the amounts of mTOR, rictor, and PRR5 in mTOR immunoprecipitates were analyzed on immunoblots. *B*, PRR5 is not important for the integrity of the mTOR-rictor interaction. Myc-tagged S6K1 (*control*), PRR5, or Sin1 was expressed together with HA-rictor in 293T cells. Two days post-transfection, HA-rictor immunoprecipitates were obtained and the amounts of mTOR and Myc-tagged proteins were analyzed on immunoblots. *C*, PRR5 interacts with rictor independently of mTOR. 293T cells were lysed in a buffer containing Triton X-100, a condition that disrupts the interaction between mTOR, raptor, and rictor (10, 20), and anti-mTOR, raptor, and rictor polyclonal antibodies were used for immunoprecipitation to isolate mTOR-, raptor-, and rictor-binding proteins. *D*, the PRR5-rictor interaction survives a condition that disrupts the interaction between mTOR and rictor. Myc-tagged mTOR, raptor, or rictor was expressed in 293T cells; 2 days post-transfection, Myc immunoprecipitates were obtained and the amount of PRR5 in the immunoprecipitates was analyzed on immunoblots.

immunoprecipitates of recombinant PRR5 but not in control immunoprecipitates (Fig. 1*C*). We generated polyclonal antibodies specific to human PRR5 using GST fusion PRR5 full-length protein or an epitope peptide near the C terminus as an antigen. The latter antibody was able to pull down endogenous PRR5 that is associated with endogenous mTOR (Fig. 1*D*). Using the PRR5-specific antibody, we confirmed that endogenous PRR5 is purified specifically by anti-mTOR immunoprecipitation but not control antibodies (Fig. 1*E*).

Tissue distribution of human PRR5 mRNA had been reported previously (1). PRR5 mRNA is most abundant in kidney and liver. It is also highly detected in brain, spleen, testis, and placenta. Northern blot analysis had shown multiple different-sized bands evident in tissues including spleen, testis, and heart. We observed that PRR5 is expressed in different amounts in several human cell lines such as 293T, HeLa, HepG2, human fibrosarcoma cell line HT1080, and human breast cancer cell lines MCF-7, T47D, and MDA-MB-231 (Fig. 1*F*). In these cells, we observed that PRR5 is immunoprecipitated by mTOR antibody (Fig. 1*G*). MDA-MB-231 expresses little amount of isoforms 1 and 2, a result consistent with reverse transcription PCR data (1). PRR5 is most highly expressed in 293T cells, a cell line derived from the kidney where PRR5 mRNA level is most abundant (1).

PRR5 Is a Component of mTORC2—Knowing that PRR5 is an interacting protein of mTOR, we questioned which mTOR complex contains PRR5. Importantly, PRR5 was detected in mTOR and rictor immunoprecipitates, but not in raptor immunoprecipitates, suggesting that PRR5 specifically targets mTORC2 (Fig. 2*A*). Supporting that a large proportion of the mTOR-rictor complex contains PRR5, a higher amount of Myc-tagged PRR5 was recovered bound to rictor than Myc-mTOR, although both Myc-tagged proteins were expressed at similar levels (Fig. 2*B*).

We thought that a stronger association of rictor with PRR5 than mTOR might support a role of rictor in the mediation of the PRR5-mTOR interaction. To test the possibility that PRR5 binding to mTOR requires rictor, we knocked down rictor in 293T cells through a lentiviral shRNA transduction and determined the amount of PRR5 associated with mTOR in mTOR immunoprecipitate. Rictor silencing led to a significant reduction in the amount of PRR5 not only in mTOR immunoprecipitate but also in cell lysate, indicating that rictor is important for the stability of PRR5 (Fig. 3*A*). Sin1, another component of mTORC2, has been shown to be important for the mTOR-rictor interaction (14, 29–31). Unlike Sin1 silencing, PRR5 silencing did not lead to a change in the affinity of the interaction between mTOR and rictor, supporting that PRR5 is not important for the rictor-mTOR interaction (Fig. 3*A*). Consistent with this result, overexpression of PRR5 did not alter the affinity of the mTOR-rictor interaction, an

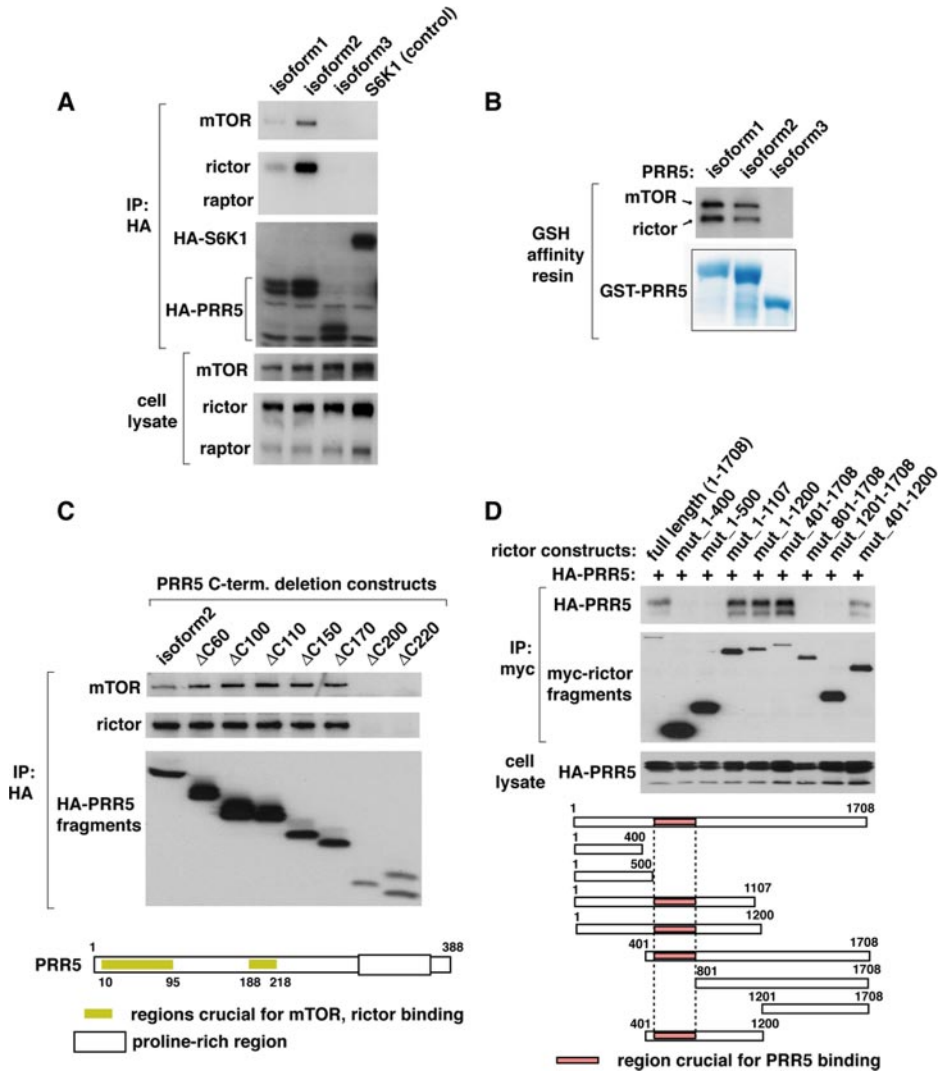

FIGURE 4. **Identification of PRR5 and rictor residues crucial for the PRR5-rictor interaction.** *A*, PRR5 isoforms 1 and 2, but not isoform 3, bind mTOR and rictor. 293T cells were transiently transfected with a plasmid encoding isoform 1, 2, or 3, and the amounts of endogenous mTOR, raptor, and rictor in Myc immunoprecipitates were analyzed on Western blots. *B*, the interaction of PRR5 isoforms 1 and 2 with mTOR and rictor does not require post-translational modifications or associated proteins of PRR5. GST fusion PRR5 isoforms 1, 2, and 3 were expressed in *E. coli* and purified using glutathione (*GSH*) affinity resin. The same amounts of cell lysates obtained from 293T cells were mixed with each isoform bound to glutathione resin for 2 h, and the amounts of endogenous mTOR and rictor recovered in glutathione-bound fraction were analyzed on immunoblots. The amounts of GST fusion PRR5 were visualized by Coomassie blue staining. *C*, the N-terminal half of PRR5 contains residues important for binding rictor and mTOR. HA-tagged deletion constructs of PRR5 were expressed in 293T cells, and the amounts of endogenous mTOR and rictor bound to the deletion mutants were analyzed on immunoblots. *D*, identification of rictor residues crucial for the PRR5-rictor interaction. Myc-tagged deletion mutants of rictor were coexpressed with HA-PRR5 in 293T cells, and the amounts of HA-PRR5 recovered with Myc constructs were analyzed on immunoblots.

interaction stabilized by Sin1 overexpression (Fig. 3*B*). Furthermore, the PRR5-mTOR interaction, but not the PRR5-rictor interaction, was destabilized in a lysis buffer containing Triton X-100 (Fig. 3, *C* and *D*), indicating that the PRR5-rictor interaction is resistant to the detergent condition that disrupts the mTOR-rictor interaction (10). These results demonstrate that PRR5 binds rictor preferentially and independently of mTOR and rictor is important for the mTOR-PRR5 interaction.

PRR5 Residues 10–95 and 188–218 Are Crucial for Binding Rictor—Knowing that PRR5 interacts with mTOR and rictor, we questioned whether all the isoforms interact with mTOR and rictor isoforms. We expressed HA-tagged isoforms in 293T cells and analyzed the amount of endogenous mTOR and rictor recovered with HA-PPR5 isoforms in HA immunoprecipitate. Supporting that isoforms 1 and 2 interact with mTOR and rictor, we observed that endogenous mTOR and rictor are immunoprecipitated with HA-tagged isoforms 1 and 2, but not with isoform 3 (Fig. 4*A*). Confirming the specific interaction of isoforms 1 and 2 with mTOR and rictor, only isoforms 1 and 2, but not isoform 3, expressed as GST fusion proteins in *Escherichia coli* pulled down endogenous mTOR and rictor (Fig. 4*B*). These results suggest that the N-terminal 95 amino acids contain residues important for binding mTOR and rictor. The N-terminal region of PRR5 overlaps with the residues conserved among higher eukaryotic genes (supplemental Fig. S1), supporting that the interaction with rictor is likely important during the evolution of higher eukaryotes.

To search for C-terminal residues important for binding mTOR and rictor, we made C-terminal-truncated mutants of PRR5 and tested the mutants for their ability to bind mTOR and rictor. Deletion of C-terminal 200 amino acids (ΔC200), but not 170 amino acids (ΔC170), led to disruption of the interaction of PRR5 with mTOR and rictor, indicating that a region between residues 188 and 218 is important for PRR5 binding to mTOR and rictor (Fig. 4*C*). ΔC200 and ΔC220 mutants were expressed at lower levels compared with other mutants despite high amounts of plasmids used for transfection, indicating that the deleted residues are important for the stability of the protein. We also observed that ΔC220 is expressed as two molecular-sized forms on SDS-PAGE, indicating that the mutant might undergo post-translational modification. Although we could not exclude a possibility that ΔC200 or ΔC220 might weakly interact with mTOR and rictor, our observation supports that C-terminal 170 amino acids are not important for PRR5 binding to mTOR and rictor and that possibly a region of residues 188–218 is crucial for mTOR and rictor binding.

Rictor Residues 500–800 Are Crucial for Binding PRR5—Following the dissection study on PRR5, we analyzed which regions of rictor are important for binding PRR5. We generated

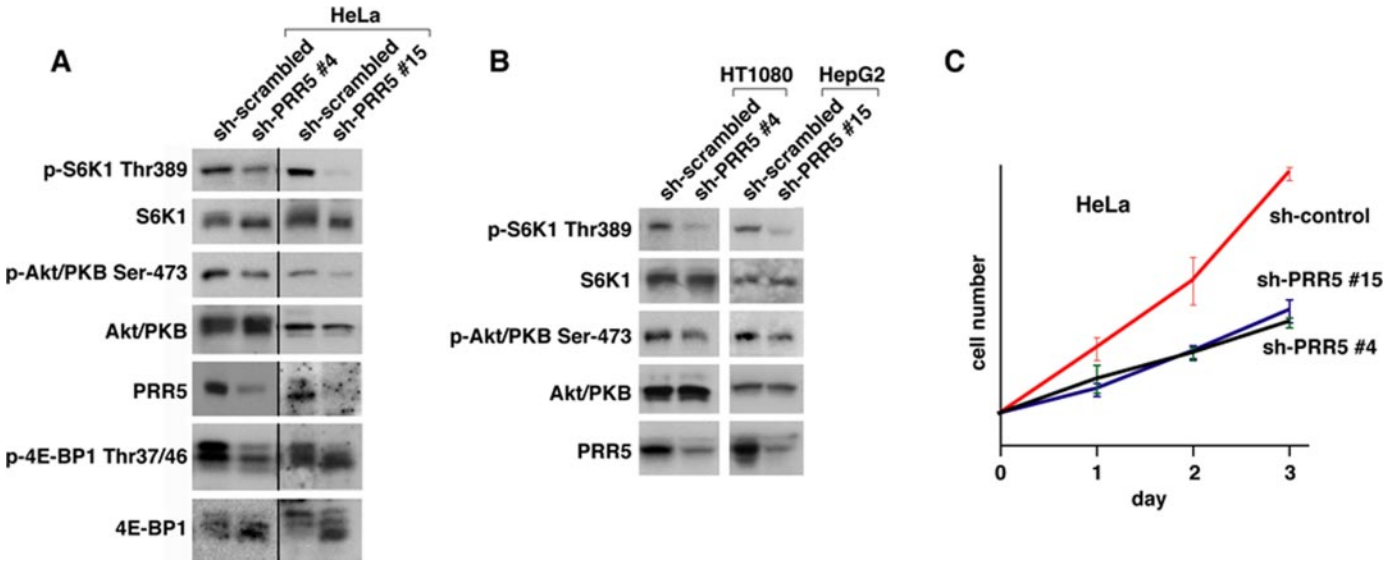

FIGURE 5. **PRR5 silencing inhibits Akt, S6K1, and 4E-BP1 phosphorylation and cell proliferation.** *A*, HeLa cells were transduced by a lentiviral shRNA targeting PRR5 (number 4 or 15) or a scrambled shRNA. Four days post-infection cell lysates were obtained and the phosphorylation status and expression levels of S6K1, Akt, and 4E-BP1 were analyzed. *B*, cell lysates were obtained from HT1080 and HepG2 cells and analyzed as described in *panel A*. *C*, PRR5 silencing inhibits cell proliferation rates. HeLa cells were transduced by a lentiviral PRR shRNA (number 4 or 15) or a scrambled shRNA. Lentiviral-transduced cells were seeded on 6-cm plates at 10–20% confluence at day 0 and analyzed for cell number at days 1, 2, and 3 using the Beckman ViCell analyzer. Standard deviations were obtained from three independent measurements.

several deletion constructs of rictor and analyzed the ability of the mutant proteins to interact with PRR5 in 293T cells. Fragments containing N-terminal 400 (mut_1–400) or 500 amino acids (mut_1–500) or a fragment with deletion of N-terminal 800 amino acids (mut_801–1708) were unable to interact with PRR5, indicating that residues deleted in the mutants are important for the interaction between PRR5 and rictor (Fig. 4*D*). Supporting that rictor N-terminal 400 amino acids are not crucial for binding PRR5, a fragment with deletion of N-terminal 400 amino acids (mut_401–1708) interacted with PRR5 to a similar extent as wild type rictor. Knowing that C-terminal 600 amino acids are not important for binding rictor, we made a mutant, mut_401–1107, that does not contain both N-terminal 400 amino acids and C-terminal 600 amino acids. This mutant could still bind rictor, indicating that the N-terminal 400 and C-terminal 600 amino acids are not crucial for binding PRR5. These results led us to identify a region of residues 501–800 that is required for rictor to bind PRR5. Further dissection might help to identify a smaller fragment of rictor that is necessary and sufficient to bind PRR5.

PRR5 Silencing Impairs S6K1 and Akt Phosphorylations—To determine the functional consequence of the PRR5-mTOR interaction in mTOR signaling, we analyzed the phosphorylation states of Akt Ser-473 and S6K1 Thr-389 in PRR5-silenced HeLa cells. Because PRR5 is a component of mTORC2, we expected that PRR5 silencing might lead to reduction in the phosphorylation of Akt. We observed that PRR5 silencing moderately reduces the level of Akt phosphorylation (Fig. 5*A*). Interestingly, a more significant reduction was observed in the level of S6K1 phosphorylation than Akt phosphorylation when PRR5 was silenced. The severe effect of PRR5 silencing on S6K1 phosphorylation was also observed in HT1080 and HepG2 cells (Fig. 5*B*). S6K1 is phosphorylated at multiple residues and the regulation of the phosphorylations is complex (34–36). The more severe effect of PRR5 silencing on S6K1 phosphorylation than Akt phosphorylation likely supports that PRR5 is involved in the regulation of S6K1 phosphorylation by other than Akt (14). PRR5 silencing also reduced levels of phosphorylated states of 4E-BP1, another substrate of mTORC1, supporting that PRR5 silencing has inhibitory effects on mTORC1 signaling (Fig. 5*A*).

Several studies have shown that rictor silencing does not alter S6K1 phosphorylation and under certain conditions could even lead to an increase in its phosphorylation (20, 21). Sin1 silencing inhibited the phosphorylation of Akt but not S6K1 (28–30). These results have raised the question of why reduced Akt phosphorylation does not lead to a reduction in S6K1 phosphorylation. A possible interpretation is a shift of the equilibrium from the complex formation of mTORC2 to mTORC1 when rictor or Sin1 was silenced. Unlike rictor or Sin1 silencing, PRR5 silencing does not disturb the interaction between mTOR and rictor (Fig. 2) and therefore PRR5 silencing would not cause any alteration in the stability of mTORC1 and mTORC2.

We then questioned whether PRR5 silencing might have effects on *in vitro* mTORC2 activity toward Akt phosphorylation at Ser-473. mTORC2 purified from PRR5-silenced cells exhibited a kinase activity toward Akt to a similar extent as mTORC2 purified from scrambled shRNA-transduced control cells (data not shown). This result indicates that the inhibitory effects of PRR5 silencing on the phosphorylation of Akt is not likely to be due to reduced kinase activity of mTORC2 toward Akt.

The negative regulation of Akt and S6K1 phosphorylations in PRR5-silenced cells prompted us to examine whether PRR5 silencing can inhibit cell proliferation. We transduced HeLa cells with a lentiviral shRNA targeting PRR5 or a scrambled shRNA as a control. Knock down of PRR5 inhibited proliferation rates of HeLa cells by ~2- to 2.2-fold relative to the rate of

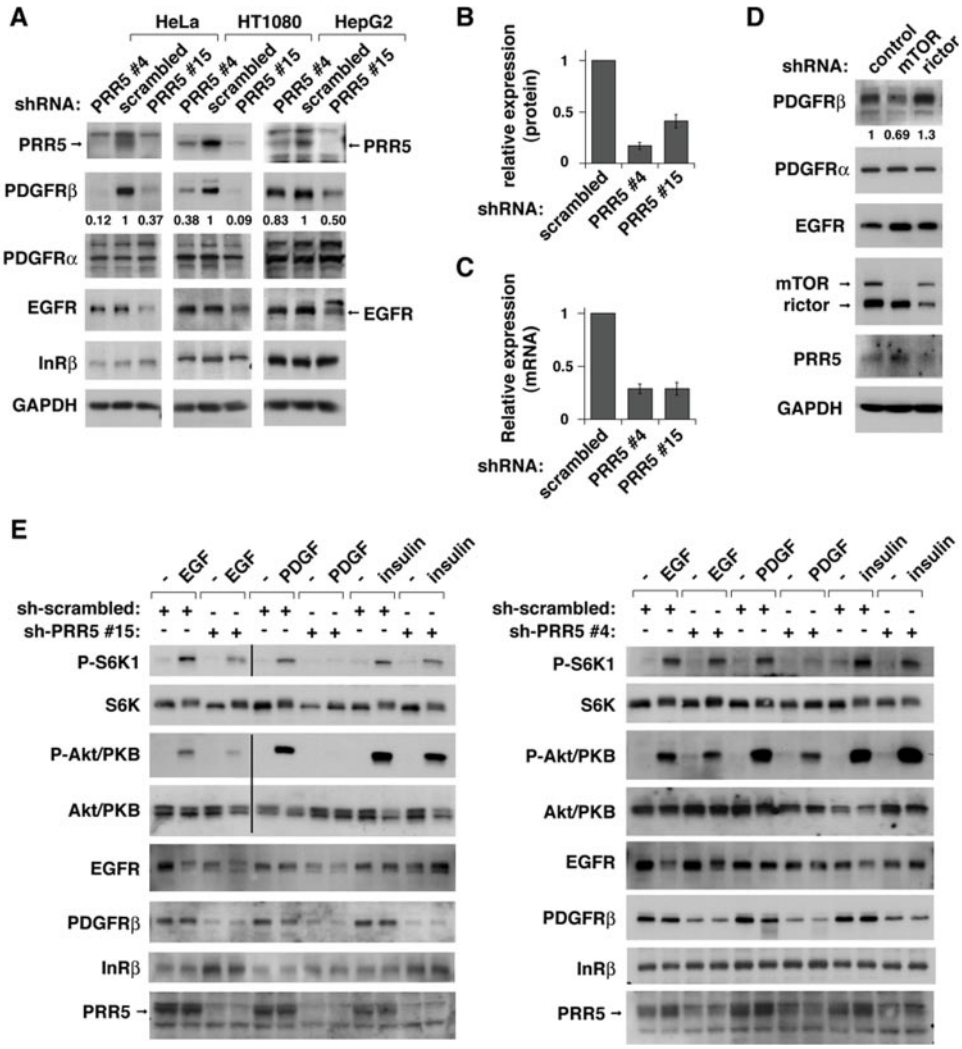

FIGURE 6. **PRR5 silencing inhibits PDGFRβ expression and signaling.** *A*, HeLa, HT1080, and HepG2 cells were transduced by a lentiviral shRNA targeting PRR5 (number 4 or 15) or a scrambled shRNA. Four or 5 days post-infection, cell lysates were obtained and expression levels of the indicated proteins were analyzed on immunoblots. *B*, the graph shows means ± S.D. ($n = 3$) for PDGFRβ expression levels from the experiment performed as in *panel A* using HeLa cells. *C*, PRR5 silencing reduces mRNA levels for PDGFRβ. Real-time quantitative reverse transcription PCR analysis of PDGFRβ mRNA levels was performed for HeLa cells transduced with PRR5 shRNA number 4 or 15 or a scrambled shRNA. mRNA levels for TATA box-binding protein were measured for a control. The graph shows means ± S.D. ($n = 2$) for PDGFRβ mRNA levels relative to those for TATA box-binding protein for each shRNA-transduced cell. *D*, mTOR or rictor silencing has a marginal effect on PDGFRβ expression. HeLa cells were transduced by a lentiviral shRNA targeting either mTOR or rictor or by a scrambled shRNA for a control. *E*, PRR5 silencing inhibits PDGFRβ signaling. PRR5-silenced or scrambled shRNA-transduced HeLa cells were starved of serum for 24 h and treated with EGF, PDGF, or insulin for 10 min. Cell lysates were obtained and phosphorylation status and expression levels of indicated proteins were analyzed on immunoblots.

the control cells (Fig. 5*C*). The reduction in cell proliferation rate was significant to $p < 0.001$ at day 3 for both PRR5 shRNA number 4- and number 15-transduced cells.

PRR5 Is Important for PDGFRβ Expression—Regarding the mechanism underlying the inhibition of Akt and S6K1 phosphorylation in PRR5-silenced cells, we thought it possible that PRR5 silencing might have negative effects on components upstream of both Akt and S6K1. A lesson learned from mTORC1 signaling is that mTORC1 and its effector S6K1 regulate the stability of insulin receptor substrate-1 negatively through a feedback loop that inhibits Akt signaling (7, 32). We considered the possibility that PRR5 silencing leads to reduced expression or inactivation of growth factor receptors coupled to Akt and S6K1 signaling. To test this possibility, we analyzed expression levels of growth factor receptors PDGFR, EGFR, and insulin receptor in PRR5-silenced and scrambled shRNA-transduced HeLa cells. Among the tested receptors, PRR5 silencing reduced only the expression level of PDGFRβ (Fig. 6, *A* and *B*). PRR5 silencing led to moderate or marginal effects on expression of EGFR, which depended upon silencing efficiency of PRR5 shRNAs, and it barely altered levels of PDGFRα and insulin receptor β. PRR5 silencing did not alter the expression level of glyceraldehyde-3-phosphate dehydrogenase that was measured as a control. These effects were reproducibly observed in HT1080 and HepG2 cell lines, confirming PRR5 functions related to the regulation of PDGFRβ expression.

The down-regulation of PDGFRβ is likely due to reduction in its mRNA level. PRR5 silencing decreased mRNA level for PDGFRβ by ~4-fold for both PRR5 shRNAs (Fig. 6*C*). We then investigated whether PRR5 silencing-induced down-regulation of the receptor is due to mTORC2 functional loss. Interestingly, rictor silencing led to a marginal increase in expression level of PDGFRβ whereas mTOR silencing marginally reduced it (Fig. 6*D*). Silencing of either gene did not significantly alter expression of other growth factor receptors such as EGFR and PDGFRα. The specificity of rictor silencing on PDGFRβ might support that the effect of PRR5 silencing on PDGFRβ expression is related to mTORC2 functions. The opposite effects between rictor and PRR5 silencing on PDGFRβ expression likely imply a potential role of PRR5 as a negative regulator of mTORC2 for PDGFRβ expression.

A potential problem associated with the silencing experiment is that silencing of rictor or mTOR, both of which are large-sized proteins, might cause destabilization of their partner proteins. This is likely the case, because we observed that reduced levels of either mTOR or raptor induced by its specific siRNA decrease the level of the other without affecting the amount of its mRNA (10). As we discussed earlier, rictor silencing may make mTOR more available to interact with raptor and therefore the effects of the gene silencing may be more complicated. Furthermore, we could not exclude the possibility that

PRR5 might regulate PDGFRβ expression independently of rictor.

PRR5 Is Important for PDGFRβ Signaling—Knowing that PRR5 silencing reduces the expression level of PDGFRβ, we investigated whether this reduction of the receptor level might explain the reduction of S6K1 and Akt phosphorylation observed in PRR5-silenced cells. We treated serum-starved PRR5-silenced or scrambled shRNA-transduced HeLa cells with EGF, PDGF, or insulin and analyzed the phosphorylation status of Akt and S6K1. Supporting the specific effects of PRR5 silencing on PDGFR signaling, PDGF-stimulated Akt and S6K1 phosphorylations were almost completely suppressed in PRR5-silenced cells (Fig. 6*E*). In contrast, PRR5 silencing had only a marginal effect on insulin-stimulated phosphorylation of Akt and S6K1. We confirmed the result using two PRR5 shRNAs (numbers 4 and 15) that have distinct target sequences. EGF-stimulated phosphorylations of Akt and S6K1 were reduced moderately when the PRR5 silencing effect was severe (shPRR5 15), which is likely due to a reduced amount of EGFR expressed in cells transduced by shPRR5 15 (Fig. 6, *A* and *E*). These results suggest that PRR5 plays a crucial role in the regulation of PDGFRβ expression and, as a consequence of alteration in PDGFRβ expression, PRR5 modulates PDGF signaling.

DISCUSSION

Our study demonstrated that PRR5 interacts with rictor to participate as a component of mTORC2. Recently, Pearce *et al.* (33) reported identification of a rictor-binding protein, Protor, the same protein as PRR5. The study elegantly demonstrated that Protor is a component of mTORC2 and the Protor-rictor interaction is resistant to a detergent condition that disrupts the mTOR-rictor interaction, a result consistent with our findings. Despite the demonstration of the interaction, the functional consequence of the interaction in the regulation of mTORC2 could not be clarified in both studies. We were unable to observe any effect of PRR5 on mTOR2 activity toward Akt phosphorylation at Ser-473. Knowing that mTORC2 regulates actin cytoskeleton organization, we investigated whether PRR5 is involved in the process. We observed that PRR5 silencing likely inhibits actin polymerization at focal adhesion (data not shown), but molecular events, involving paxillin and small GTPases, could not be clarified.

Without knowing a function of PRR5 related to mTORC2, the molecular mechanism through which PRR5 regulates mTOR signaling could not be clearly resolved. Because PRR5, like PRAS40, is a proline-rich protein, we might obtain important ideas on PRR5 functions from PRAS40 studies. Knowing that the proline-rich region of PRR5 is not important for binding rictor and mTOR (Fig. 4), we assume that the region might serve as binding sites for proteins containing Src homology 3 or WW domain or help assemble multielement signaling complexes. PRR5 may also play a role in the regulation of rictor-mediated recruitment of mTOR substrates or other signaling molecules. The latter possibility could be tested once we identify downstream targets of mTORC2. It might also be interesting to investigate whether PRR5, like PRAS40, is phosphorylated by mTOR (37).

Although we observed that rictor is important for the stability or expression of PRR5 (Fig. 3*A*), it seems likely that PRR5 has a function independent of rictor or mTOR. PRR5 is expressed most abundantly in 293T cells among the tested cell lines, but the expression level of rictor in 293T cells is lower than in HeLa cells (Fig. 1*F*). Among other cell lines, the expression level of PRR5 does not correlate with that of rictor. This suggests that there must be mTORC2 that does not contain PRR5 or that PRR5 has mTORC2-independent functions.

Another remaining question is whether PRR5 isoforms have functions distinctive between them. Nine amino acids at the N terminus are likely important for the stability of PRR5 or its mRNA, because we reproducibly observed that isoform 2 is always expressed at higher levels than isoform 1. The N-terminal 9 residues could be important for localization of PRR5 and mTORC2. Another possibility is that the extra residues might give differential effects on PRR5 regulation of mTORC2. Isoform 3, without having N-terminal residues crucial for rictor binding, is not likely involved in mTORC2. Nevertheless, we could not exclude a potential role of isoform 3 in the regulation of mTOR signaling, possibly through sequestering PRR5-binding proteins and thereby competing with isoforms 1 and 2. We reproducibly observed that PRR5 isoforms are expressed as two-sized forms on SDS-PAGE. We determined that this is not due to truncation at either end (data not shown), supporting that the isoforms likely undergo post-translational modifications.

Our study revealed that PRR5 has a specific role in the regulation of PDGFRβ expression. PDGF signaling, in contrast to EGF signaling, is known to be crucial in the activation of phosphoinositide-3 kinase signaling and specifically related to mTOR signaling (38, 39). A recent study showed that mTOR inhibition by rapamycin treatment leads to an increase in the expression level of PDGFRs, but not other growth factor receptors, and mTOR activation through disturbance of TSC-Rheb (tuberous sclerosis complex-Ras homolog-enriched in brain) signaling reversed the expression (39). We observed that mTOR silencing marginally reduces the expression level of PDGFRβ (Fig. 6*D*), a result opposite to what was observed with rapamycin treatment. mTOR silencing, unlike rapamycin that mainly targets mTORC1, disturbs both mTORC1 and mTORC2, and this disturbance might have complicated effects on the receptor expression.

PDGF signaling plays a crucial role for cell growth, tumorigenesis, and cell differentiation, and expression levels of PDGFRs are an important factor that determine the activity of PDGF signaling (40). How cells regulate expression of PDGFRs in response to cellular growth status has not been clearly understood. We assume that PRR5 or PRR5-containing mTORC2 might play an important role in this regulation. mTORC2 may regulate transcription factors that mediate mTOR signals to the gene expression machinery for PDGFRβ expression. Several proteins, including NF-Y, Myc, Sp1, and p73, are known as regulators of PDGFRβ gene expression (41–43), and it is possible that PRR5 or mTORC2 might regulate these molecules. Further studies involving PRR5 regulation of mTOR may increase our knowledge of mTORC2 functions and its regulation of PDFGRβ signaling activity.

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