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Atypical Protein Kinase Cλ Is Critical for Growth Factor Receptor-induced Dorsal Ruffle Turnover and Cell Migration*

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Background: Growth factors induce actin cytoskeletal reorganization and cell migration in fibroblast cells.
Results: Genetic inactivation of aPKCλ in mouse embryonic fibroblast cells inhibits PDGF-induced dorsal ruffle turnover and cell migration.
Conclusion: Our results demonstrate a critical role for aPKCλ in PDGF-induced dorsal ruffle turnover and cell migration.
Significance: These data will advance our understanding of the regulation of cell morphology induced by growth factors.

$G\alpha_{13}$, a member of the heterotrimeric G proteins, is critical for actin cytoskeletal reorganization and cell migration. Previously we have shown that $G\alpha_{13}$ is essential for both G protein-coupled receptor and receptor tyrosine kinase-induced actin cytoskeletal reorganization such as dynamic dorsal ruffle turnover and cell migration. Ric-8A, a non-receptor guanine nucleotide exchange factor for some heterotrimeric G proteins, is critical for coupling receptor tyrosine kinases to $G\alpha_{13}$. Here, we show that PDGF can induce phosphorylation of Ric-8A. Atypical protein kinase Cλ (aPKCλ) is required for Ric-8A phosphorylation. Furthermore, aPKCλ is required for PDGF-induced dorsal ruffle turnover and cell migration as demonstrated by both down-regulation of aPKCλ protein levels in cells by RNA interference and by studies in aPKCλ knock-out cells. Moreover, phosphorylation of Ric-8A modulates its subcellular localization. Hence, aPKCλ is critical for PDGF-induced actin cytoskeletal reorganization and cell migration.

Heterotrimeric G proteins ($G\alpha$ and $G\beta\gamma$ subunits) are essential for the transmembrane signaling by G protein-coupled receptors (GPCRs)[2] (1). Based on the sequence and functional homologies, $G\alpha$ subunits are grouped into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ (2). Among these four subfamilies of G proteins, the physiological function of $G\alpha_{12}$ subfamily is less well understood. In this family, there are two members, $G\alpha_{12}$ and $G\alpha_{13}$. $G\alpha_{12}$ knock-out mice appeared normal (3). $G\alpha_{13}$ knock-out mice displayed embryonic lethality (around embryonic day 9.5) (4). Deficiency of $G\alpha_{13}$ impaired cell migration induced by $G\alpha_{13}$-coupled GPCRs (3–5). We previously made a surprising discovery that $G\alpha_{13}$ is also required for receptor tyrosine kinases (RTK)-induced cell migration (5). This $G\alpha_{13}$ function is independent of GPCRs (5). Therefore, $G\alpha_{13}$ plays a critical role in cell migration induced by both GPCRs and RTKs (5, 6). However, the molecular mechanisms by which RTKs signal to $G\alpha_{13}$ are not completely understood.

Growth factors induce massive actin cytoskeletal remodeling in stimulated cells (7). One major event in actin reorganization is the formation and breakdown of dorsal ruffles (the dorsal ruffle turnover process). Growth factors initiate rapid formation of dorsal ruffles, and these ruffles move along the dorsal side of the cells, contract, close, and eventually disassemble. The physiological functions of these dorsal ruffles include cell migration, macropinocytosis, plasma membrane recycling, and others (8).

We have previously reported that, in mouse embryonic fibroblast (MEF) cells, dorsal ruffles formed within ~5 min after PDGF treatment (9). These dorsal ruffles started to disassemble ~10 min after PDGF treatment. Dorsal ruffles formed only one time after PDGF stimulation. After the disassembly of these dorsal ruffles, protrusion of large peripheral membrane ruffles was observed (9). In $G\alpha_{13}$-deleted MEF cells, the formation of dorsal ruffles was normal after PDGF stimulation. However, the disassembly of dorsal ruffles was disrupted (~10 times slower), identifying $G\alpha_{13}$ as the first regulator of dorsal ruffle disassembly (9).

Furthermore, we have shown that Ric-8A, a non-GPCR guanine nucleotide exchange factor for some $G\alpha$ proteins, is critical in relaying RTK signals to $G\alpha_{13}$ (10). Down-regulation of Ric-8A in cells impaired PDGF-induced dorsal ruffle turnover and decreased PDGF-initiated cell migration. Deficiency of Ric-8A impaired the translocation of $G\alpha_{13}$ to the cell cortex. Therefore, Ric-8A is involved in the PDGFR-$G\alpha_{13}$ pathway and possibly functions as the guanine nucleotide exchange factor for GPCR-independent activation of $G\alpha_{13}$ in this PDGF-initiated pathway.

Atypical protein kinase Cs (aPKCs) include protein kinase ζ and λ isoforms. Different from other PKC isoforms, the activation of aPKCs does not require Ca^{2+} or diacylglycerol. Instead, the aPKCs are regulated by the Par complex (11). This complex

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; aPKCλ, atypical protein kinase Cλ; MEF, mouse embryonic fibroblast; BIM-1, bisindolylmaleimide-1.

regulates cell polarity, cell division, and cell migration. Here, we have studied the potential role of aPKC in mediating PDGF-induced actin cytoskeletal reorganization and cell migration. We have shown that aPKC is involved in PDGF-induced dorsal ruffle turnover and is required for PDGF-initiated cell migration.

EXPERIMENTAL PROCEDURES

RNA Interference—RNA interference of aPKCλ was performed in MEF cells as described previously (12). Four siRNA target sequences of aPKCλ used were 5′-ACAUGUGAUACAGAGUGA-3′, 5′-AUGGAUUUCACUCUGUAU-3′, 5′-AGCAGACCAAUAAGUGCA-3′, and 5′-CACGCACUGCACUUAUUG-3′ (Integrated DNA Technologies). Control siRNA targeted a LacZ sequence. Transfection of siRNAs was performed using Lipofectamine 2000 (Invitrogen). Briefly, siRNAs and Lipofectamine were diluted and mixed in Opti-MEM I to allow the formation of siRNA-liposome complexes. After 20 min of incubation at room temperature, the mixture was overlaid onto cells cultured in growth medium without antibiotics. The transfected cells were usually analyzed 48 h after transfection.

In Vitro Wound-healing Cell Migration Assay—Wild type and aPKCλ$^{-/-}$ MEF cells in DMEM (Invitrogen) containing 10% FBS were seeded into wells of 24-multiwell plates (Becton-Dickinson) (13, 14). After starvation for 15 h, wounds were made with sterile pipette tips. Cells were washed with PBS and refreshed with medium containing 20 ng/ml platelet-derived growth factor (PDGF-BB). After ~16 h of incubation at 37 °C, cells were fixed and photographed (with 100× magnification).

Boyden Chamber Cell Migration Assay—Cell migration was assayed using Boyden chambers (8.0 μm pore size polyethylene terephthalate membrane, FALCON cell culture insert (Becton-Dickinson)) (9, 13–15). Cells were starved for 15 h, trypsinized, and counted. 200 μl of 5–10 × 10^4 cells in serum free medium were added to the upper chamber, and 500 μl of appropriate medium with 20 ng/ml PDGF-BB were added to the lower chamber. PKC inhibitors bisindolylmaleimide-1 (BIM-1) and Gö 6983 were purchased from TOCRIS. The concentration of BIM-1 used was 1 μM. The concentration of Gö 6983 was 6 μM. Transwells were incubated for 6 h at 37 °C. Cells on the upper surface of the membrane were removed with a cotton swab, and cells on the undersurface of the membrane were fixed with 3.7% formaldehyde and stained with crystal violet. Photographs of three random regions (10× objective) were taken, and the number of cells was counted to calculate the average number of cells that had transmigrated per field.

Fluorescence Microscopy—Staining and observation of actin filaments were performed as described previously (16). Cells were plated onto coverslips coated with gelatin. Cells were then fixed with 3.7% formaldehyde. The fixed cells were then permeabilized in 0.1% Triton X-100 for 5 min. After washing in PBS, phalloidin conjugated to Alexa Fluor 488 (Molecular Probes) in a solution containing PBS and 1% BSA was added to stain actin filaments. After incubation for 30 min at room temperature, the cells were washed extensively to reduce nonspecific interactions. The coverslips were then fixed onto slides and imaged using a Zeiss AXIOVERT 200 fluorescence microscope. Post-acquisition analysis and processing of images were performed using MetaMorph.

Protein Purification—GST-tagged Ric-8A and GST-tagged Ric8A(S501A) proteins were purified from *Escherichia coli* as described previously (5). BL21 (DE3) *E. coli* cells harboring pGEX-4T-1-Ric-8A or pGEX-4T-1-Ric-8A(S501A) plasmids were grown to A_{600} = 0.5–0.6 in LB medium at 37 °C and induced with 25 μM isopropyl 1-thio-β-D-galactopyranoside at 18 °C overnight (17). Cells were harvest and lysed in 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1% Triton X-100, proteinase inhibitors, and purified with glutathione agarose beads (Thermo Scientific).

In Vitro aPKCλ Phosphorylation Assay—2 μg of purified recombinant Ric-8A proteins or Ric-8A(S501A) mutant proteins were incubated with 0.15 μg of purified recombinant active aPKCλ protein in kinase buffer (35 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM $CaCl_2$,) containing 5 μCi (100 μM) of [γ-^{32}P]ATP for 30 min at 30 °C in a final volume of 40 μl. Reactions were terminated by addition of concentrated sample buffer and separated by SDS-PAGE followed by exposure to x-ray film and quantitation.

ELISA Assay—384-well plates were coated with 100 μl/well of 100 ng of Ser-501-phosphorylated peptide LIQPMGMSpPRGHLTS or unphosphorylated peptide LIQPMGMSpPRGHLTS overnight at 4 °C. After extensive wash, anti-pSer-501-Ric-8A polyclonal antibody or anti-Ric-8A monoclonal antibody (NewEast Biosciences) was added at different dilutions and incubated for 1 h at room temperature. After washes, HRP-conjugated secondary antibodies were added and incubated for another 1 h. Thirty minutes after the addition of substrate solutions, the optical density at 450 nm was recorded.

Microinjection—Cells were maintained in DMEM supplemented with 10% fetal bovine serum in a 37 °C incubator humidified with 5% CO_2. Cells were seeded onto heat-sterilized 25-mm round coverslips and grown for 36 h prior to microinjection. Cell nuclei were pressure microinjected with plasmid DNAs prepared in HKCl microinjection buffer (10 mM HEPES, 140 mM KCl, pH 7.4). β-Actin-GFP, Ric-8A(S501A)-GFP, together with actin-mRFP, or Ric-8A(S501D)-GFP together with actin-mRFP were injected at a concentration of 20 μg/ml using back-loaded glass capillaries and a Narishige micromanipulator (Narishige, Greenvale, NY). For experiments in which multiple plasmid DNAs were injected, the final concentration of each plasmid DNA in the injection needle was kept at 20 μg/ml. After injection, cells were maintained at 37 °C in a humidified CO_2 environment for 90 min to allow for expression of injected plasmid DNAs. Cells were transferred to Recording Medium (Hanks Balanced Salt Solution, without phenol red, supplemented with 20 mM HEPES, 1% dextran-charcoal stripped FBS (Atlanta Biologicals), 4.5 g/liter glucose, essential and non-essential amino acids) and the formation of dorsal ruffles in the presence of added PDGF (20 ng/ml) was monitored by time-lapse fluorescence microscopy of actin-GFP or actin-mRFP at 37 °C.

Time-lapse Fluorescence Microscopy—After microinjection cells were transferred to a perfusion chamber consisting of a Sykes-Moore coverslip holder (Bellco Glass Inc, Vineland, NJ) and a thermal-controlled microincubator (Harvard Apparatus,

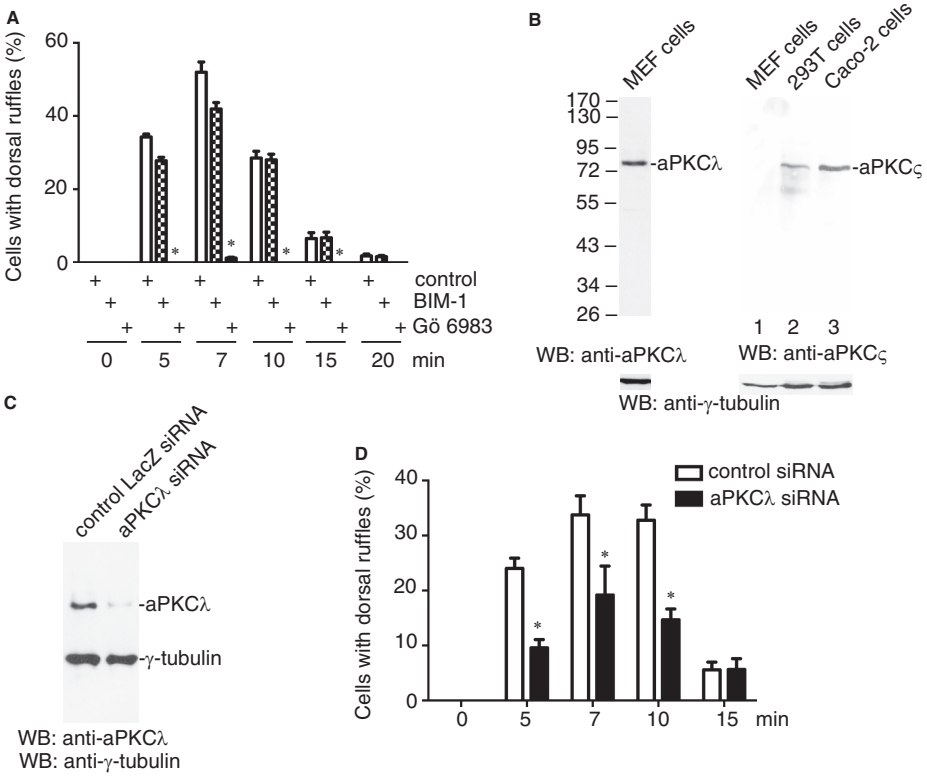

FIGURE 1. **aPKCλ is required for PDGF-BB-induced dorsal ruffle turnover.** *A*, effect of PKC inhibitors on PDGF-induced dorsal ruffle formation. In wild-type MEF cells, PDGF-BB (20 ng/ml) induced the formation of dorsal ruffles. Although BIM-1 (an inhibitor of typical PKCs) did not affect the PDGF-BB induced turnover of dorsal ruffles in MEF cells, Gö 6983 (an inhibitor of all PKCs) abolished the PDGF-BB induced turnover of dorsal ruffles. *Empty bars*, control; *cross-hatched bars*, treated with BIM-1; *Black bars*, treated with Gö 6983 (at the time points of 0, 5, 10, 15, and 20 min; the value was 0). *B*, Western blot (*WB*) shows that MEF cells only expresses aPKCλ, but not aPKCζ (*top panels*). *Bottom panels*, Western blot with anti-tubulin antibody to show the whole-cell lysate loading. *C*, Western blot shows that aPKCλ siRNAs decrease the protein level of aPKCλ in MEF cells, LacZ siRNAs do not. Western blot with anti-tubulin antibody shows the whole-cell lysate loading. *D*, effect of aPKCλ siRNAs on PDGF-BB-induced dorsal ruffle formation. Data are representative of three to five experiments. *Error bars*, mean ± S.E. *, $p < 0.05$.

Holliston, MA) mounted on a Nikon TiE inverted microscope equipped with Perfect Focus™ (Nikon, Inc., Melville, NY). The cells and recording medium were allowed to equilibrate at 37 °C for 5 min prior to addition of PDGF and beginning time-lapse recordings. Actin-GFP or actin-mRFP fluorescence was imaged directly with a fluorescein/GFP or an mCherry filter cube (Chroma Technology Corp, Bellows Falls, VT). Images were collected using an Andor Neo, cMOS camera (Andor Technology, Belfast, Ireland) and were transferred to a computer work station running the Nikon Elements™ software. Formation of dorsal ruffles in the presence of added PDGF (20 ng/ml) was monitored using a 20× objective (plan apoλ, NA 0.75) and images were collected at 1-min intervals for ~60 min after addition of PDGF. Post-acquisition analysis and processing of images was performed using Elements™.

Statistical Analysis—Data are expressed as mean ± S.E. and analyzed by the Student *t* test with significance defined as $p < 0.05$.

RESULTS

aPKCλ Is Involved in PDGF-BB-induced Dorsal Ruffle Turnover—Previously, we have shown that G protein $G\alpha_{13}$ is essential for RTK-induced dorsal ruffle turnover and cell migration (5, 9, 10). The signals from these RTKs (including PDGFRs) are relayed to $G\alpha_{13}$ via a non-GPCR guanine nucleotide exchange factor Ric-8A (10). To investigate the signaling pathway from PDGFR to Ric-8A, we first examined the protein modification of Ric-8A in MEF cells after PDGF-BB treatment. Serum-starved MEF cells were treated with 20 ng/ml PDGF-BB for 5 min. Ric-8A proteins from treated and untreated cells were immunoprecipitated and separated by SDS-PAGE. The bands representing Ric-8A proteins were cut out from the gel, and the proteins were analyzed by mass spectrometry. One of the protein modifications increased by PDGF-BB stimulation was the phosphorylation of Ser-501 on Ric-8A (data not shown). Based on the surrounding amino acid sequences $RVIQPMGMS_{501}PR$, the potential kinases for this phosphorylation include CDK1 and aPKCs (18). Given the short time (5 min) of stimulation by PDGF-BB, we focused on aPKCs in this study.

First, we investigated whether aPKCλ is involved in PDGFR-induced dorsal ruffle formation and cell migration. The earliest ultra-structural changes of cells treated with growth factors are the intensive bursts of ruffling of the dorsal surface plasma membranes as seen under the phase-contrast microscope (7, 19, 20). The physiological functions of dorsal ruffles, including macropinocytosis, cell migration and invasion, are continually expanding (21–24). It has been suggested that one major function of dorsal ruffles is to reorganize the actin cytoskeleton to prepare a static cell for motility (25). We used three different and complementary approaches to investigate the role of aPKCλ in growth factor-induced actin cytoskeletal reorganiza-

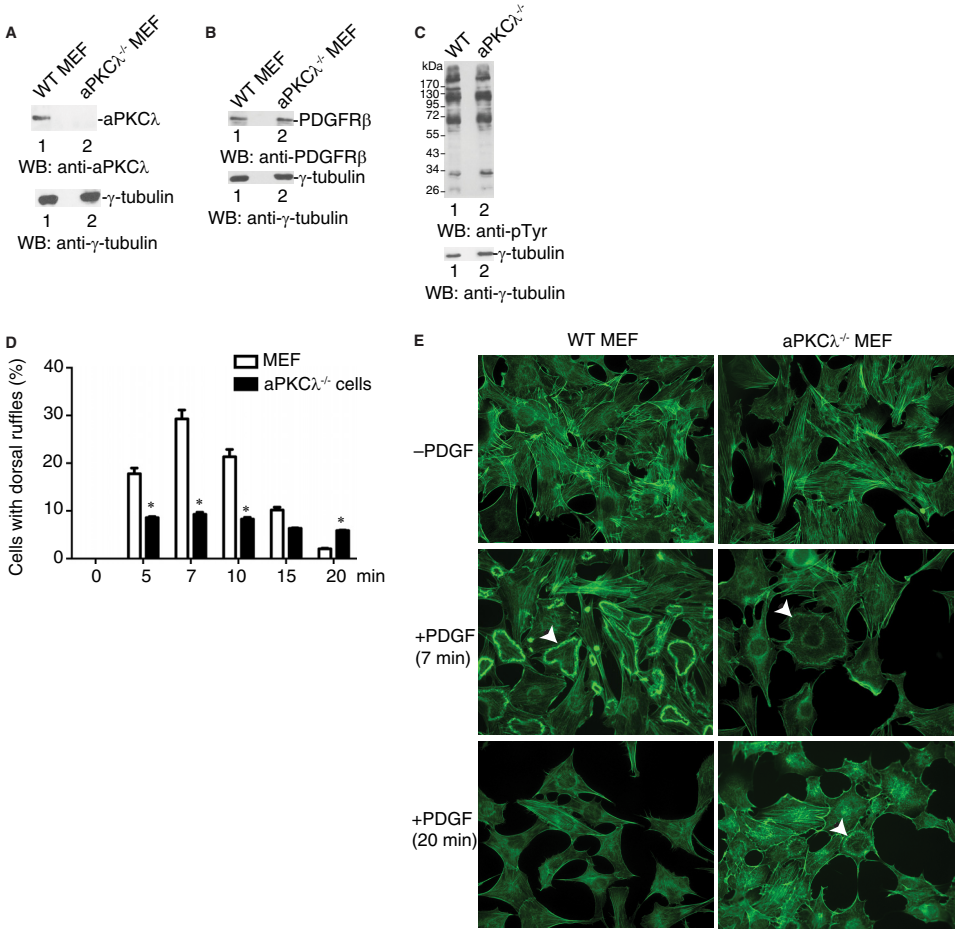

FIGURE 2. **Genetic deletion of aPKCλ impairs PDGF-BB-initiated dorsal ruffle turnover.** *A*, Western blot (*WB*) shows the absence of aPKCλ proteins in aPKCλ$^{-/-}$ MEF cells (*top panels*). *Bottom panels*, Western blot with anti-tubulin antibody to show the whole-cell lysate loading. *B*, Western blot shows that wild-type and aPKCλ$^{-/-}$ MEF cells have similar levels of PDGFRβ proteins. *C*, Western blot with anti-phosphotyrosine antibody shows that aPKCλ deletion does not markedly change the PDGF-induced protein tyrosine phosphorylation pattern. *D*, effect of aPKCλ genetic deletion on PDGF-BB-induced dorsal ruffle turnover. *E*, actin filament staining. Cells were fixed and stained with phalloidin-Alexa Fluor 488. *Arrowheads* indicate dorsal ruffles. Data are representative of three to five experiments. *Error bars*, mean ± S.E. *, $p < 0.05$.

tion and cell migration: aPKC inhibitors, aPKCλ siRNA knockdown, and aPKCλ$^{-/-}$ cells.

We started with a pharmacological approach. Although there are no specific aPKCλ inhibitors available, there are inhibitors (such as Gö 6983) that inhibit the activity of all PKCs and inhibitors (such as BIM-1) that inhibit the activity of typical PKCs (26, 27). The differential activity is attributed to that of aPKCs. In wild-type MEF cells, PDGF-BB (20 ng/ml) induced the formation of dorsal ruffles within 5 min (Fig. 1*A*). The dorsal ruffles started to disassemble after 7 min, and, by 20 min, most of the cells were without dorsal ruffles (dorsal ruffles were disassembled) (Fig. 1*A*). Although BIM-1 did not affect the PDGF-BB induced formation of dorsal ruffles in MEF cells, Gö 6983 abolished the PDGF-BB induced formation of dorsal ruffles (Fig. 1*A*), suggesting that the activity of aPKCs is required for PDGF-BB induced formation of dorsal ruffles.

There are two isoforms of aPKCs in mice: aPKCζ and aPKCλ. Using Western blots, we confirmed a previous report that MEF cells only expresses aPKCλ, but not aPKCζ (Fig. 1*B*) (28). RNA interference was then used to down-regulate the protein levels of aPKCλ in MEF cells (Fig. 1*C*). We transfected siRNAs either against aPKCλ or against LacZ (as control) into MEF cells. Although aPKCλ siRNAs decreased the protein level of aPKCλ in MEF cells, LacZ siRNAs did not (Fig. 1*C*). Decrease of aPKCλ protein levels had no effects on MEF cell proliferation within the first 48 h of cell culture after splitting the cells (data not shown). Although the control LacZ siRNAs did not affect PDGF-BB induced dorsal ruffle turnover, aPKCλ siRNAs decreased the PDGF-BB induced turnover of dorsal ruffles (Fig. 1*D*).

To genetically demonstrate a biological role for aPKCλ in PDGF-induced dorsal ruffle turnover, we used aPKCλ$^{-/-}$ MEF cells (Fig. 2*A*) (28). Genetic deletion of aPKCλ did not significantly change the expression levels of PDGFRβ and the PDGF-induced protein tyrosine phosphorylation patterns (Fig. 2, *B* and *C*). Consistent with the above data from PKC inhibitors and aPKCλ siRNAs, aPKCλ deletion impaired PDGF-induced dorsal ruffle turnover (Fig. 2*D*). The disassembly of dorsal ruffles was significantly delayed (Fig. 2*D*). For example, at 20 min after PDGF treatment, there were very few cells with dorsal ruffles in wild-type MEF cells, yet in aPKCλ$^{-/-}$ cells, dorsal ruffles remained in most of the cells forming dorsal ruffles (Fig. 2*D*). Dorsal ruffles contracted and disassembled slower in aPKCλ$^{-/-}$ cells compared with those in wild-type MEF cells (Fig. 2*E*). In aPKCλ$^{-/-}$ cells, after 20 min, dorsal ruffles could still be observed.

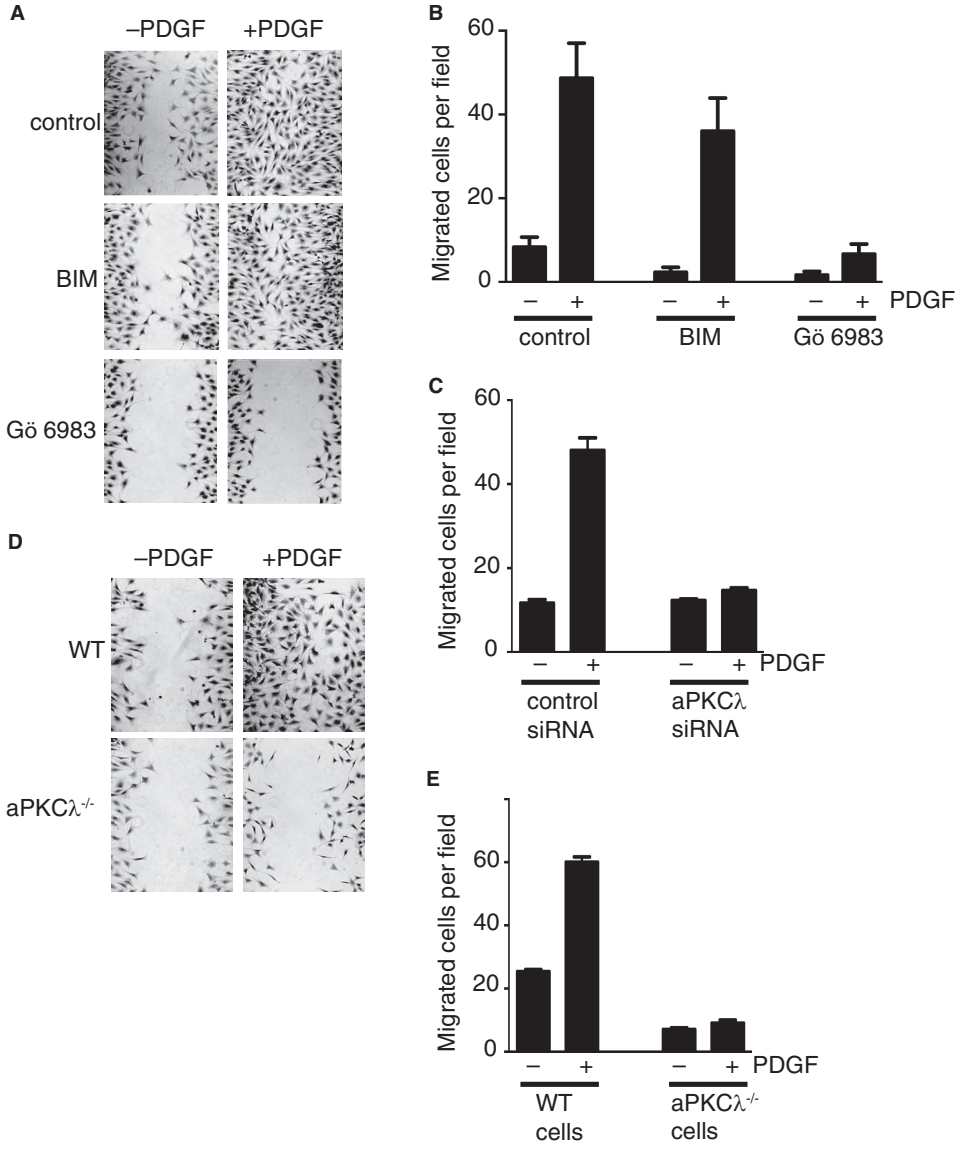

FIGURE 3. **aPKCλ is needed for PDGF-BB-initiated cell migration.** *A*, wound-healing assay of cell migration. MEF cells, BIM-treated cells, and Gö 6983-treated cells were assayed in the absence (starvation) or presence of 20 ng/ml of PDGF-BB for 16 h. Representative images are shown. *B*, Boyden chamber assay of cell migration of control, BIM- and Gö 6983-treated MEF cells in the absence or presence of 20 ng/ml PDGF-BB. *C*, Boyden chamber assay of cell migration of LacZ siRNA-treated and aPKCλ siRNA-treated MEF cells in the absence or presence of 20 ng/ml PDGF-BB. *D*, wound-healing assay of cell migration. MEF cells and aPKCλ$^{-/-}$ cells were assayed in the absence (starvation) or presence of 20 ng/ml of PDGF-BB for 16 h. Representative images are shown. *E*, Boyden chamber assay of cell migration of wild-type MEF cells and aPKCλ$^{-/-}$ cells in the absence or presence of 20 ng/ml PDGF-BB. Data are representative of three to five experiments. *Error bars*, mean ± S.E.

We should note that there were also less aPKCλ$^{-/-}$ cells forming dorsal ruffles. Together, all the above data demonstrate that aPKCλ is involved in PDGF-BB-initiated dynamic dorsal ruffle turnover.

aPKCλ Is Required for PDGF-BB-initiated Cell Migration—Next, we analyzed the role of aPKCλ in cell migration. Although some believe that dorsal ruffle turnover is part of the cell migration process and indeed required for cell migration, this notion is still under debate. Therefore, here, we treated these as two events of actin cytoskeletal reorganization. To investigate a possible role of aPKCλ in PDGF-BB-initiated cell migration, we used two approaches to compare the cell migration. One approach is the qualitative *in vitro* wound-healing assay, the other the quantitative Boyden chamber assay (13, 14). For the wound-healing assay, cells were grown to confluence. A wound (small scratch) was made in the middle of the tissue culture plate with a pipette tip. After ~16 h in the presence of PDGF-BB, control cells or cells treated with BIM-1 migrated and covered the wound, whereas Gö 6983-treated cells did not (Fig. 3*A*). Therefore, PDGF-BB-induced cell migration was sensitive to aPKC inhibition. These results were confirmed with Boyden chamber assays (Fig. 3*B*). In this chamber assay, MEF cells were loaded onto the top of the Boyden chamber. After ~6 h, cells migrated into the bottom of the chamber filter were counted. Although BIM-1 had no effect on PDGF-BB-induced migration, Gö 6983 significantly decreased PDGF-induced migration (Fig. 3*B*).

The role of aPKCλ in PDGF-induced cell migration was further demonstrated by RNA interference and by genetic deletion. Although control siRNA-treated MEF cells migrated in

response to PDGF-BB treatment, aPKCλ siRNA-treated cells showed reduced migration (Fig. 3*C*). Furthermore, although wild-type MEF cells had a normal response to PDGF-induced migration, the migration of aPKCλ$^{-/-}$ MEF cells was blocked in wound-healing assay (Fig. 3*D*) and in Boyden chamber assay (Fig. 3*E*). Together, these data demonstrate that aPKCλ plays a critical role in PDGF-induced cell migration.

aPKCλ Is Required for Ric-8A Phosphorylation—The above data demonstrate that aPKCλ is critical for PDGFR signal to dorsal ruffle turnover and cell migration. Previously, we have also shown that Ric-8A is essential for PDGF-BB-initiated dorsal ruffle turnover and cell migration. Furthermore, it has been shown that RTKs (including PDGFR) could increase the kinase activity of aPKCs in cells (29, 30). Thus, we examined the phosphorylation of Ric-8A, in the presence or absence of aPKCλ. First, we generated a rabbit polyclonal antibody specific for Ser-501-phosphorylated Ric-8A proteins using a short peptide containing phosphorylated Ser-501. In ELISA assays, this antigen peptide-affinity purified antibody (anti-pSer-501-Ric-8A antibody) specifically recognized the phosphorylated peptide but not the unphosphorylated peptide (Fig. 4*A*). As a control, we used a commercial anti-Ric-8A antibody, which recognizes both the phosphorylated and unphosphorylated Ric-8A proteins in the ELISA assay (Fig. 4*B*). The anti-Ric-8A antibody detected both the phosphorylated peptide as well as the unphosphorylated peptide (Fig. 4*B*). In MEF whole cell lysates, the anti-pSer-501-Ric-8A antibody recognized phosphorylated Ric-8A, and the signal was markedly reduced by pretreating the lysate with phosphatases (data not shown). Hence, this antibody specifically recognized the Ser-501-phosphorylated Ric-8A. Although PDGF induced Ric8A phosphorylation in wild-type MEF cells, this phosphorylation was absent in aPKCλ$^{-/-}$ cells (Fig. 4, *C* and *D*). The aPKCλ$^{-/-}$ cells expressed similar levels of total Ric-8A proteins as in wild-type MEF cells (Fig. 4, *C* and *D*). Therefore, aPKCλ is critical for Ric8A phosphorylation at Ser-501 in MEF cells. Furthermore, to demonstrate that aPKCλ could indeed directly phosphorylate Ric-8A proteins, we used purified full-length recombinant Ric-8A proteins, Ric-8A(S501A) proteins, and purified aPKCλ in an *in vitro* kinase assay (Fig. 4*E*). Although purified aPKCλ phosphorylated Ric-8A proteins, it did not phosphorylate Ric-8A(S501A) proteins (Fig. 4*E*). Together, these data demonstrate that aPKCλ is capable of and is required for phosphorylating Ric-8A at Ser-501.

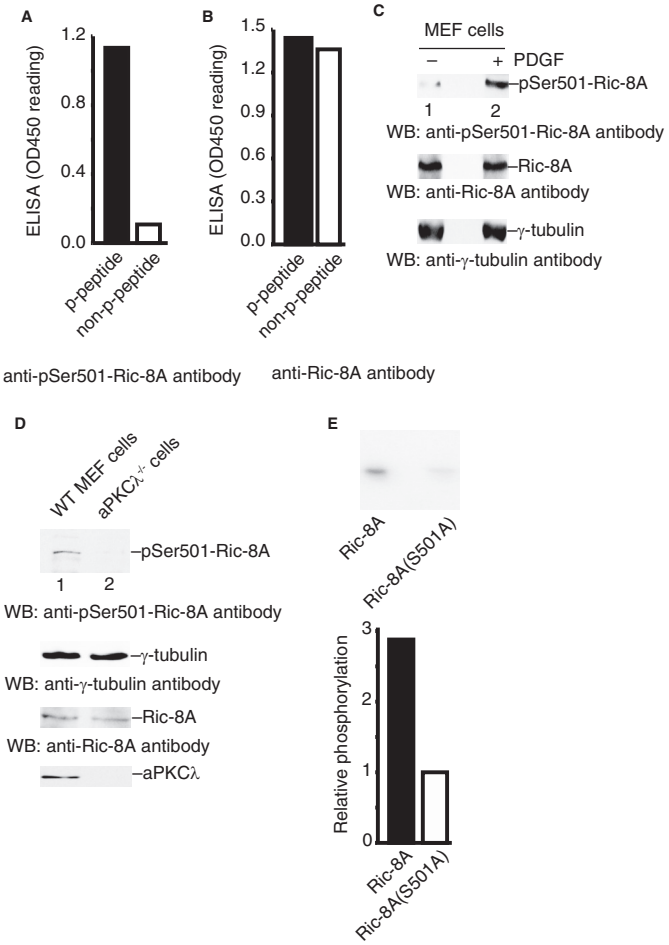

FIGURE 4. **aPKCλ is required for Ric-8A phosphorylation at Ser-501.** *A*, ELISA assay shows that the anti-pSer-501-Ric-8A antibody recognizes the pSer-501-containing peptide, but not the unphosphorylated peptide. *B*, ELISA assay shows that an anti-Ric-8A antibody recognizes both the phosphorylated and the unphosphorylated peptides. *C*, PDGF-BB increased the phosphorylation of Ric-8A at Ser-501 in wild-type MEF cells. *D*, PDGF-BB treatment increased the phosphorylation of Ric-8A at Ser-501 in the wild-type MEF cells (*lane 1*) but not in aPKCλ$^{-/-}$ cells (*lane 2*). *Middle panel*, both wild-type MEF cells and aPKCλ$^{-/-}$ cells have the same amounts of total Ric-8A proteins. *Bottom panel*, Western blot (*WB*) confirms the absence of aPKCλ proteins in aPKCλ$^{-/-}$ cells. *E*, aPKCλ is able to directly phosphorylate Ric-8A *in vitro*. Purified recombinant Ric-8A proteins or Ric-8A(S501A) mutant proteins were incubated with purified recombinant active aPKCλ protein in the presence of [γ-^{32}P]ATP. After SDS-PAGE, the phosphorylation was quantified. Representatives of three similar experiments were shown.

Effect of Ric-8A Mutations on Dorsal Ruffle Disassembly—To visualize directly the effect of Ric-8A Ser-501 phosphorylation on actin cytoskeletal reorganization, we utilized cell microinjection and live cell imaging techniques to compare the dynamics of actin cytoskeletal reorganization in Ric-8A(S501A) and Ric-8A(S501D) expressing MEF cells. First, we injected plasmid DNAs encoding actin-GFP into MEF cells and aPKCλ$^{-/-}$ cells. After expression for 90 min, we treated the cells with PDGF-BB and monitored the subcellular distribution of actin polymers. As we reported previously, in wild-type MEF cells, dorsal ruffles formed within 5 min (3.07 ± 0.07 min, n = 28) after PDGF treatment (Fig. 5*A*). These dorsal ruffles were disassembled ~10 min (11.36 ± 0.58 min, n = 28) after PDGF treatment (Fig. 5*A*). Without PDGF treatment, actin-GFP was uniformly distributed in MEF cells (9). However, in aPKCλ$^{-/-}$ cells, dorsal ruffles formed within 5 min (2.83 ± 0.22 min, n = 18) after PDGF-BB treatment (Fig. 5*B*). However, these dorsal ruffles then took much longer (24.56 ± 1.58 min, n = 18) to disassemble (Fig. 5*B*). These data confirm that, similar to Ric-8A and $G\alpha_{13}$, aPKCλ controls the rate of the breakdown of dorsal ruffles (9, 10).

If aPKCλ phosphorylation of Ric-8A is critical for Ric-8A function in dorsal ruffle turnover, we would expect different functional effects of Ric-8A(S501A) (which mimics the unphosphorylated form) and Ric-8A(S501D) (which mimics the phosphorylated form). We co-injected actin-mRFP and Ric-8A(S501A)-GFP or Ric-8A(S501D)-GFP plasmids into aPKCλ$^{-/-}$ cells (Fig. 5, *C–E*). In cells injected with Ric-8A(S501A), dorsal ruffle formation and disassembly were similar to those in aPKCλ$^{-/-}$ cells, as one would expect (formation

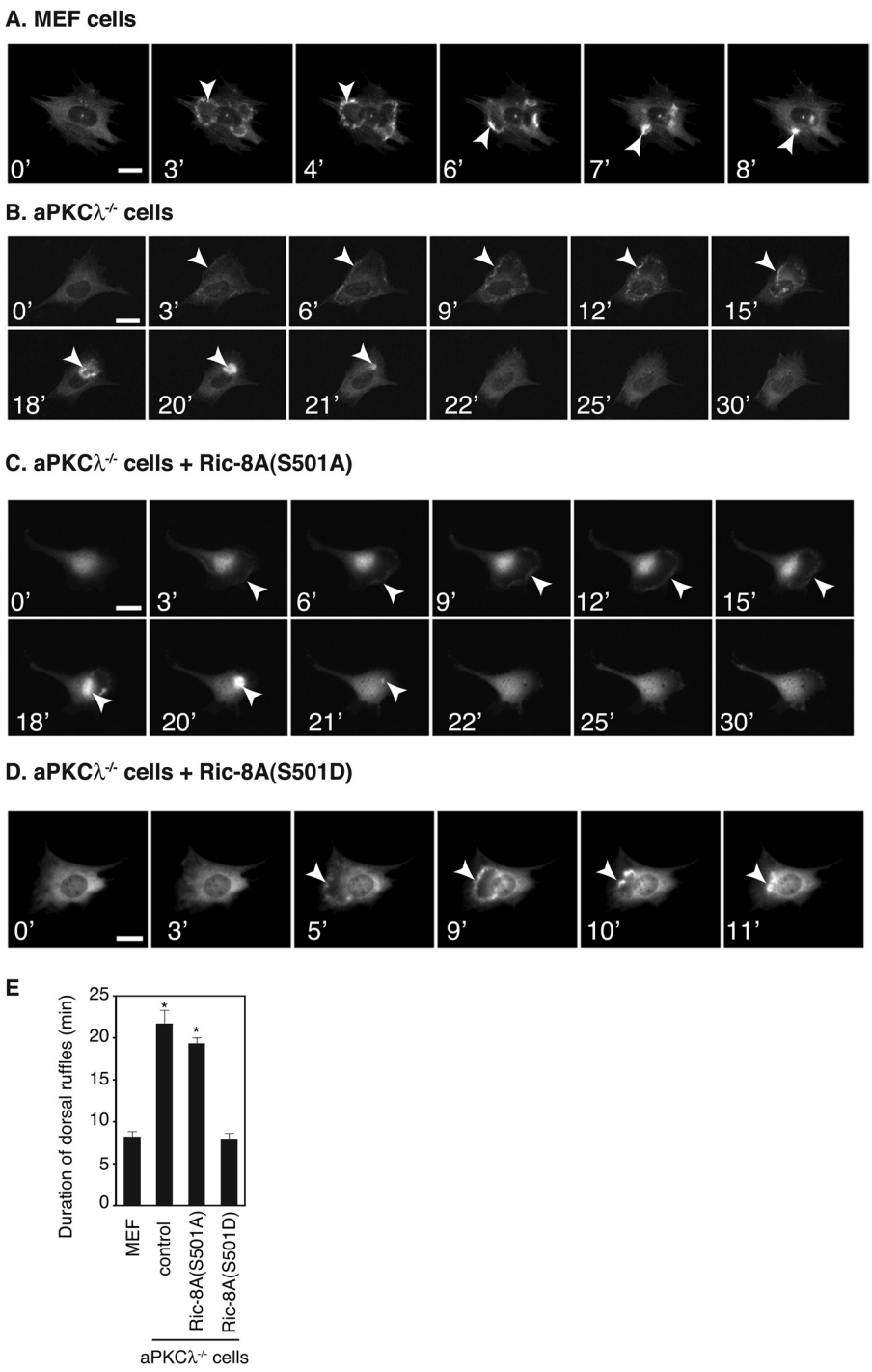

FIGURE 5. **Effect on dorsal ruffle turnover by Ric-8A mutations.** *A*, wild-type fibroblast cells were injected with actin-GFP and imaged after the addition of 20 ng/ml of PDGF-BB. *Arrowheads* point to dorsal ruffles. Data are representative of 28 recorded cells. *B*, actin-GFP plasmid DNA was injected into aPKCλ$^{-/-}$ MEF cells. Images were taken after the addition of PDGF. Data are representative of 18 cells. *C*, actin-mRFP and Ric-8A(S501A)-GFP plasmid DNAs were injected into aPKCλ$^{-/-}$ MEF cells. Images were taken after the addition of PDGF. The nuclear fluorescent staining was due to nucleus-localized Ric-8A(S501A)-GFP. Data are representative of 33 cells. *D*, actin-mRFP and Ric-8A(S501D)-GFP plasmid DNAs were injected into aPKCλ$^{-/-}$ MEF cells. Images were taken after the addition of PDGF. Data are representative of 18 cells. *E*, summary of data from *A–D*. Data represent mean ± S.E. of 18 to 33 cells. *Scale bars*, 20 μm. *, $p < 0.01$.

within 2.73 ± 0.09 min, $n = 33$; disassembled by 22.09 ± 0.73 min, $n = 33$) (Fig. 5, *C* and *E*). However, Ric-8A(S501D) significantly shorted the dorsal ruffle turnover in aPKCλ$^{-/-}$ cells (formation within 5.28 ± 0.24 min, $n = 18$; disassembled by 13.22 ± 0.7 min, $n = 18$) (Fig. 5, *D* and *E*). These data are consistent with our hypothesis that Ric-8A phosphorylation plays a role in its function in increasing dorsal ruffle turnover.

Phosphorylation-mediated Regulation of Ric-8A Subcellular Localization—To further investigate the effect of phosphorylation of Ric-8A on its cell biological function, we have determined the subcellular localization of Ric-8A(S501A) and Ric-8A(S501D) mutants. We transfected Ric-8A(S501A)-GFP or Ric-8A(S501D)-GFP plasmids into MEF cells. These cells were then observed by fluorescence microscopy. Although Ric-

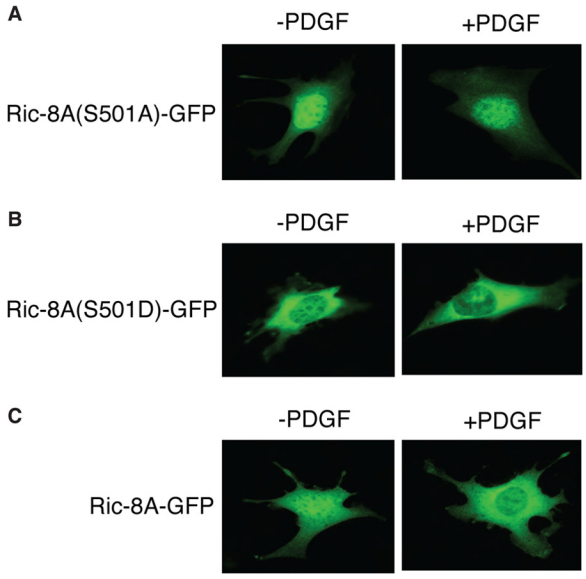

FIGURE 6. **Effect on subcellular localization by Ric-8A mutations.** *A*, Ric-8A(S501A)-GFP plasmid DNA was transfected into MEF cells. Cells were treated with or without 20 ng/ml of PDGF-BB for 10 min. *B*, Ric-8A(S501D)-GFP plasmid DNA was transfected into MEF cells. *C*, Ric-8A-GFP plasmid DNA was transfected into MEF cells. Cells were fixed and imaged by fluorescence microscopy. Data are representative of three similar experiments.

8A(S501A) proteins were mainly in the nucleus, Ric-8A(S501D) proteins were mainly in the cytoplasm (Fig. 6, *A* and *B*). As one would expect, PDGF treatment did not change the subcellular localization of these mutant proteins because they mimic either the unphosphorylated form or the phosphorylated form of Ric-8A. These data indicate that phosphorylation at Ser-501 of Ric-8A would change its subcellular localization. Furthermore, in wild-type Ric-8A-GFP-expressing MEF cells, before PDGF treatment, Ric-8A-GFP proteins were mainly localized in the nucleus with some staining in the cytoplasm (GFP signal in the nucleus over total GFP signal was ~50% even though the nuclear volume was smaller) (Fig. 6*C*). After PDGF stimulation for 20 min, Ric-8A-GFP proteins were mostly found in the cytoplasm (GFP signal in cytoplasm over total GFP signal was ~83%) (Fig. 6*C*). Moreover, these data are also consistent with the above data that the phosphorylated form of Ric-8A participates in the dorsal ruffle turnover, a cytoplasmic event.

DISCUSSION

Regulated actin cytoskeletal reorganization is a fundamental process in tissue morphogenesis and physiological cell migration (31). Dysregulation of the actin cytoskeleton is a hallmark of pathological processes such as tumor angiogenesis, cancer cell invasion, and metastasis (32). Previously, we have shown that $G\alpha_{13}$ controls the actin cytoskeletal reorganization, specifically the turnover of dorsal ruffles (5, 9). Indeed, $G\alpha_{13}$ was the first identified regulator of dorsal ruffle disassembly. Furthermore, we showed that Ric-8A links RTK signals to $G\alpha_{13}$ in this pathway (10). In the current study, we started to investigate the signaling components between RTKs and Ric-8A.

We demonstrate an essential role for aPKCλ in PDGFR-induced dorsal ruffle turnover and cell migration. We have shown that aPKCλ is required for the phosphorylation of Ric-8A at Ser-501. These data firmly put aPKCλ in the PDGFR/Ric-8A pathway. A previous biochemical study with a truncated Ric-8A mutant with deletion of the C-terminal residues from Arg-493 to the last residue Asp-530 (including Ser-501) showed an ~1.8-fold increase of the guanine nucleotide exchange activity of Ric-8A on $G\alpha_i$ proteins, when compared with the full-length Ric-8A protein (33). However, due to the fact that the recombinant full-length Ric-8A protein was not very soluble, a direct test of the effect of the phosphorylation of Ser-501 on the guanine nucleotide exchange activity of Ric-8A is technically challenging at the present time. However, the main mechanism by which the PAR3-PAR6-aPKC complex regulates cell function is to modulate the subcellular localization of downstream proteins. Therefore, we hypothesized that RTKs signal through aPKC to regulate the subcellular localization of Ric-8A, which in turn controls the subcellular translocation of $G\alpha_{13}$. We show here that Ser-501 phosphorylated Ric-8A is mainly localized in the cytoplasm, whereas the unphosphorylated Ric-8A is mainly in the nucleus.

Although the regulation of Ric-8A activity has not been studied before, Ric-8A has been genetically and biochemically shown to regulate G protein signaling. Ric-8 was originally identified in *Caenorhabditis elegans* through genetic analysis (34). Ric-8 functions upstream of $G\alpha_q$ in regulating neurotransmitter secretion (34). Ric-8 also acts upstream of $G\alpha_o$ and GPA16 during asymmetric cell division of one-cell stage *C. elegans* embryos (35–37). In *Drosophila*, Ric-8 is required for $G\alpha$-mediated spindle orientation and cell polarity during asymmetric cell division (38–40). In Ric-8 mutants, $G\alpha_i$ failed to localize at the plasma membrane (38–40). Ric-8 has also been genetically shown to play a role in gastrulation and is involved in the fog-concertina pathway (39). Concertina is the *Drosophila* homolog of $G\alpha_{13}$ (41). Fog (folded gastrulation) is an extracellular polypeptide growth factor (41). Thus, Ric-8 has been genetically demonstrated to be involved in $G\alpha_{13}$-mediated signaling in *Drosophila*. There are two distinct mammalian Ric-8-like genes, Ric-8A and Ric-8B (42). *In vitro* biochemical studies have shown that Ric-8A is a potent GEF for $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, $G\alpha_{12}$, and $G\alpha_{13}$ but not $G\alpha_s$ (42, 43). On the other hand, Ric-8B interacts with $G\alpha_s$ and $G\alpha_q$ (42, 43). Mechanistically, Ric-8A binds to GDP-bound $G\alpha$ proteins, promotes rapid GDP release, and forms a stable nucleotide free transition state complex with the $G\alpha$ that is disrupted upon GTP binding, thus leading to the formation of $G\alpha$-GTP. Furthermore, although Ric-8A mRNA is expressed in a variety of tissues, Ric-8B mRNA is mainly expressed in the olfactory epithelium (44). Moreover, Ric-$8A^{-/-}$ mouse embryos died in the early stages of embryonic development without blood vessels (45). Although Ric-8 has been implicated in receptor-independent activation of heterotrimeric G proteins in asymmetric division, our report was the first to reveal that Ric-8A functions downstream of a RTK (10). Thus, this might provide a molecular mechanism for linking RTKs to heterotrimeric G proteins. Downstream of Ric-8A signaling, how $G\alpha_{13}$ regulates actin cytoskeletal reorganization and cell migration is not clear. We will investigate the mechanisms in future studies.

Previously, we have shown that tyrosine kinase Src and small GTPase Rac (but not RhoA) are involved in RTK-induced dorsal ruffle formation (9). aPKCλ is part of the PAR3-PAR6-aPKC

complex which functions downstream of Cdc42. Deletion of Cdc42 impaired the signal relaying from PDGF to dorsal ruffles (46). Furthermore, we have shown previously that Rac is required, but not sufficient, for dorsal ruffle formation (9). However, higher Src activity is sufficient to drive dorsal ruffle formation (9). Thus, one possibility is that downstream of Src, both Rac and Cdc42 are needed for dorsal ruffle turnover and cell migration. Previously, we suggested that one potential molecular mechanism by which $G\alpha_{13}$ controls dorsal ruffle disassembly is to shorten the duration of activation of signaling molecules such as Rac that are involved in dorsal ruffle formation (9).

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