G Proteins G\textsubscript{12} and G\textsubscript{13} Control the Dynamic Turnover of Growth Factor-induced Dorsal Ruffles\textsuperscript{*}

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Growth factors induce massive actin cytoskeletal remodeling in cells. These reorganization events underlie various cellular responses such as cell migration and morphological changes. One major form of actin reorganization is the formation and disassembly of dorsal ruffles (also named waves, dorsal rings, or circular ruffles). Dorsal ruffles are involved in physiological functions including cell migration, invasion, macropinocytosis, plasma membrane recycling, and others. Growth factors initiate rapid formation (within 5 min) of circular membrane ruffles, and these ruffles move along the dorsal side of the cells, constrict, close, and eventually disassemble (~20 min). Considerable attention has been devoted to the mechanism by which growth factors induce the formation of dorsal ruffles. However, little is known of the mechanism by which these ruffles are disassembled. Here we have shown that G proteins G\textsubscript{12} and G\textsubscript{13} control the rate of disassembly of dorsal ruffles. In G\textsubscript{12}/-\textbackslash G\textsubscript{13}/- fibroblast cells, dorsal ruffles induced by growth factor treatment remain visible substantially longer (~60 min) than in wild-type cells, whereas the rate of formation of these ruffles was the same with or without G\textsubscript{12} and G\textsubscript{13}. Thus, G\textsubscript{12}/G\textsubscript{13} critically regulate dorsal ruffle turnover.

The earliest ultrastructural 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 (1–6). The physiological functions of dorsal ruffles, including macropinocytosis, cell migration, and invasion, are continually expanding (7–10). It has been suggested that one major function of dorsal ruffles is to reorganize the actin cytoskeleton to prepare a static cell for motility (11).

In serum-starved fibroblasts, platelet-derived growth factor (PDGF)\textsuperscript{2} induces at least two types of membrane ruffles, peripheral membrane ruffles (or lamellipodia) and dorsal ruffles (12). In addition to fibroblast cells, dorsal ruffle formation in response to growth factors has been reported in glial cells, endothelial cells, hippocampal neurons, kidney epithelial cells, and tumor cells (1, 2, 11, 13, 14). In addition, other stimuli can induce dorsal ruffles such as activation of IgE receptors in mast cells (15). Dorsal ruffles are dynamic structures. They form and disassemble rapidly (11, 16, 17). Intensive investigations have revealed several critical regulators of the dorsal ruffle formation in response to growth factors such as Rac, PAK1, and WAVE1 (12). However, not much is known about the control of the rapid disassembly of dorsal ruffles. Here we have demonstrated a critical role for G proteins G\textsubscript{12} and G\textsubscript{13} in this turnover process.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Reagents—}Anti-HA monoclonal antibody and anti-Myc monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. PDGF-BB was purchased from Oncogene. Texas Red Phalloidin and Alexa Fluor 488 F(ab\textsuperscript{1})\textsubscript{2} fragment of goat anti-mouse IgG(H+L) were purchased from Molecular Probes. ROCK inhibitor Y-27632 and Src family inhibitor PP2 were purchased from BD Biosciences. The PCR products of chicken Src constitutively active mutant SrcE378G and SrcY527F cDNAs were subcloned into pcDNA3.1/hyg/myc. HA-Rac1G12V and HA-Rac1T14N were obtained from Guthrie Research Institute.


\textbf{Cell Culture and Transfection—}G\textsubscript{12}/-\textbackslash G\textsubscript{13}/- MEF cells were provided by J. Gu and M. Simon (California Institute of Technology) and have been previously described (18, 19). Cells were grown in DMEM containing 10% fetal bovine serum and penicillin/streptomycin. The day before transfection, cells were plated onto glass coverslips coated with poly-D-lysine and cultured in DMEM containing 10% fetal bovine serum overnight. Cells were washed once with DMEM and incubated with 3 \mu g of plasmid DNA mixed with 5 \mu l of Lipofectamine-2000/well of a 6-well plate for 5 h. The medium was replaced with DMEM containing fetal bovine serum and cultured overnight. Cells were then starved for 24 h. For stimulation, cells were treated with 20 ng/ml PDGF-BB for 20 min. In some cases, cells were treated with different kinds of inhibitors as indicated (10 \mu M ROCK inhibitor Y-27632 for 10 min and 10 \mu M Src family inhibitor PP2 for 30 min) followed by PDGF-BB treatment. Cells were fixed and stained.

\textbf{Microinjection—}Cells were maintained in DMEM supplemented with 10% fetal bovine serum in a 37 °C incubator.
humidified with 5% CO₂. Cells were seeded onto heat-sterilized 25-mm-round coverslips and grown for 36 h prior to microinjection. Cell nuclei were pressure microinjected with cDNAs prepared in HKCl microinjection buffer (10 mM HEPES, 140 mM KCl, pH 7.4). β-actin-GFP, G₁₂, and G₁₃ 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 cDNAs were injected, the final concentration of each cDNA in the injection needle was kept at 20 μg/ml. After injection, cells were maintained at 37 °C in a humidified CO₂ environment for 90 min to allow for expression of injected cDNAs. Cells were transferred to Recording Medium (Hanks Balanced Salt Solution, without phenol-red, supplemented with 20 mM HEPES, 1% fetal bovine serum, 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 β-actin-GFP at 32 °C. We routinely image the cells at 32 °C rather than 37 °C to prevent focal drift because of the temperature differential between the recording chamber and the microscopy room.

**Time-lapse Fluorescence Microscopy**—After microinjection, cells were transferred to a hybrid perfusion chamber consisting of a Sykes-Moore coverslip holder (Bellco Glass Inc., Vineland, NJ) and a thermal-controlled incubator (Harvard Apparatus, Holliston, MA) mounted on a Nikon TE-2000U inverted microscope (Nikon Inc., Melville, NY). The cells and recording medium were allowed to equilibrate to 32 °C for 5 min prior to addition of PDGF and beginning the time-lapse recordings. β-actin-GFP fluorescence was imaged directly with a fluorescein/GFP filter cube (HyQ DM 505; Chroma Technology Corp., Rockingham, VT). Images were collected using an ORCAII-ER, cooled CCD camera (Hamamatsu Corp, Bridgewater, NJ) and were transferred to a computer workstation running the MetaMorph imaging software (Universal Imaging/Molecular Devices, West Chester, PA). Formation of dorsal ruffles in the presence of added PDGF (20 ng/ml) was monitored using a ×20 objective (plan fluor, with phase contrast, NA 0.5; Nikon), and images were collected at 15-s intervals for ~60 min after addition of PDGF. In experiments where cells were co-injected with β-actin-GFP and G₁₂ and/or G₁₃, cells were removed from the chamber and fixed after recordings for detection of exogenously expressed...

![FIGURE 1. Slow turnover of dorsal ruffles in G₁₂⁻/⁻ G₁₃⁻/⁻ fibroblast cells. 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 or peripheral membrane ruffles. Data are representative of 17 recorded cells. B, without PDGF treatment, no dorsal ruffles were observed in wild-type fibroblasts. Data are representative of 10 recorded cells. C, actin-GFP plasmid DNA was injected into G₁₂⁻/⁻ G₁₃⁻/⁻ MEF cells. Images were taken after the addition of PDGF. Data are representative of 51 recorded cells. D, in the absence of PDGF, no dorsal ruffles were observed in G₁₂⁻/⁻ G₁₃⁻/⁻ cells. Data are representative of 10 recorded cells.](image-url)
**G12 and G13 Regulate Dorsal Ruffle Turnover**

Ga12 and Ga13 by indirect immunofluorescence analysis. Post-acquisition analysis and processing of images was performed using MetaMorph.

Fluorescence Microscopy—Cells were washed once with phosphate-buffered saline and fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 min at room temperature.
Cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 5 min and then blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h. Cells were incubated with diluted anti-Myc primary antibody (1:200) for 1 h. Texas Red Phalloidin was used to detect F-actin polymers. Alexa Fluor 488 F(ab′)2 fragment of goat anti-mouse IgG(H+L) was used to detect the Myc tag. Stained cells were mounted and observed under a fluorescence microscope.

Src Kinase Assay—The immunocomplex kinase assay was done as previously described (22). HEK293 cells were transfected with plasmid DNAs of Src(E378G), Src(Y527F), or Src(K295M) (all in pcDNA3-Myc-His vector). After the preparation of whole cell extracts, Myc and His6-tagged Src (E378G), Src(Y527F), and Src(K295M) immunoprecipitation was done with anti-Myc antibody. Src kinase assays were done with 5 μg of the purified glutathione S-transferase (GST) and the cytoplasmic domain of human erythrocyte band 3 fusion protein (GST-CDB3) as substrate (22).

FRET Imaging and Analysis—The Rac fluorescent resonance energy transfer (FRET) probe was kindly provided by Dr. Michiyuki Matsuda (Osaka University, Osaka, Japan) and has been described previously (20). Fibroblast cells were transfected with the FRET probe and imaged after 48 h on an Axiovert (Carl Zeiss, Jena, Germany) inverted microscope with a × 40 (Fig. 4, B and C) or ×63 (Fig. 4A) objective. Images were acquired using an Orca-C4742-80 camera (Hamamatsu Photonix). For dual imaging of the FRET probe, a Dual-View wavelength slitter (Optical Insights, Tucson, AZ) with an OI-05-Ex/40 (length splitter (Optical Insights, Tucson, AZ) with an OI-05-Em emission filters (480 nm) for the excitation filter (436 ± 20 nm; Chroma, Brattleboro, VT) and OI-05-Em emission filters (480 ± 30 nm for CFP and 535 ± 40 nm for YFP; Chroma), were used. The ratio image of YFP/CFP was generated using Openlab (Improvision, Lexington, MA) after background subtraction and was used to represent FRET efficiency.

RESULTS AND DISCUSSION

Deficiency of G12 and G13 Reduces the Rate of the Breakdown of Dorsal Ruffles—The G12 family (consisting of G12 and G13) of heterotrimeric G proteins is known to regulate actin cytoskeletal reorganization (21, 22). In G12, G13 knock-out MEF cells, neither G protein-coupled receptors for thrombin or lysophosphatic acid nor receptor tyrosine kinases for PDGF or epidermal growth factor induced migration of these cells (18, 19, 23). To visualize directly the effect of G12 and G13 on actin cytoskeletal reorganization, we utilized live cell imaging techniques to compare the dynamics of actin cytoskeletal reorganization in wild-type and G12, G13 knock-out MEF cells. We injected plasmid DNA encoding actin-GFP into MEF cells and G12, G13 knock-out cells. After expression for 1–2 h, we treated the cells with PDGF-BB (20 ng/ml) and monitored the subcellular distribution of actin polymers. In MEF cells, dorsal ruffles formed within 5 min (4.33 ± 2.17 min, n = 17) after PDGF treatment (Fig. 1A and supplemental Movie S1). These dorsal ruffles were disassembled ~20 min (21.42 ± 6.90 min, n = 17) after PDGF treatment (Fig. 1A). Multiple dorsal ruffles were often observed in a single cell, but these dorsal ruffles formed only one time after PDGF stimulation. After the disassembly of these dorsal ruffles, protrusion of large peripheral membrane ruffles was observed (Fig. 1A). Without PDGF treatment, actin-GFP was uniformly distributed in MEF cells (Fig. 1B and supplemental Movie S2). On the other hand, in G12, G13 cells, dorsal ruffles formed within 5 min (4.86 ± 3.88 min, n = 51) after PDGF-BB treatment (Fig. 1C and supplemental Movie S3). However, these dorsal ruffles then took much longer (57.10 ± 16.87 min, n = 51) to disassemble (Fig. 1C). After dorsal ruffle disassembly, small, but not large, peripheral membrane ruffles were observed. Formation of dorsal ruffles in G12, G13 cells was dependent on PDGF treatment (Fig. 1D and supplemental Movie S4). These data demonstrate that G12 and/or G13 control the rate of the breakdown of dorsal ruffles.

Both G12 and G13 Are Able to Accelerate the Turnover of Dorsal Ruffles—To determine whether G12 or G13 or both are essential for dorsal ruffle turnover, we re-expressed G12 or G13 in G12, G13 knock-out cells and examined the dynamics of dorsal ruffle turnover after PDGF treatment. Co-injection of actin-GFP and G12 into G12, G13 cells significantly shortened the duration of dorsal ruffles (Fig. 2A and supplemental Movie S5). In these cells, dorsal ruffles formed quickly (3.1 ± 0.7 min, n = 10) and disassembled within ~25 min (24 ± 7.3 min, n = 10), similar to the wild-type fibroblast cells. When G13 and actin-GFP were co-injected into G12, G13 cells, dorsal ruffles formed quickly (3.8 ± 1.9 min, n = 10) and also disassembled quickly (14.8 ± 6.2 min, n = 10) (Fig. 2B and supplemental Movie S6). These data demonstrate that both G13 (possibly the main one) and G12 have the capability to accelerate the turnover of dorsal ruffles.

We confirmed these observations with immunostaining of actin polymers 20 min after PDGF treatment in control cells and cells lacking G12 and G13. Under these conditions, dorsal ruffles were disassembled in most of the wild-type fibroblast cells but were persistent in G12, G13 cells, as observed in the time-lapse recordings described above. Indeed, 20 min after PDGF stimulation, only ~10% (n > 200) of the MEF cells plated on poly-D-lysine-coated glass coverslips displayed dorsal ruffles while the majority of these cells had already turned over the dorsal ruffles and only peripheral membrane ruffles

FIGURE 2. Both G12 and G13 control the turnover of dorsal ruffles. A, G12, G13 knock-out MEF cells were injected with actin-GFP plus G12 and imaged after the addition of 20 ng/ml of PDGF-BB. Arrowheads point to dorsal ruffles. Data are representative of 10 recorded cells. B, G12, G13 knock-out MEF cells were injected with actin-GFP plus G12 and imaged after the addition of 20 ng/ml of PDGF-BB. Arrowheads point to dorsal ruffles. Data are representative of 10 recorded cells. C, phalloidin staining of actin polymers of MEF cells after PDGF treatment for 20 min. PDGF induced the formation of peripheral membrane ruffles. D, phalloidin staining of actin polymers of G12, G13 cells with PDGF treatment for 20 min. PDGF induced the formation of dorsal ruffles in G12, G13 cells. E, expression of G13, QL inhibited dorsal ruffle formation in transfected cells. Transfected cells were stained by immunostaining with anti-Myc antibody (shown in insert). Actin polymers are shown with phalloidin staining. F, expression of G13, QL inhibited dorsal ruffle formation in all transfected cells. Transfected cells are shown by immunostaining with anti-Myc antibody (insert). G and H, expression of G13, QL (G) or G13, QL (H) had no effect on PDGF-induced dorsal ruffle formation in G12, G13 cells. Transfected cells are shown by immunostaining with anti-Myc antibody (inserts). * indicates transfected cells. Data are representative of at least ten experiments. I, summary of data from panels C–H. Data represent mean ± S.D. of ten experiments.
remained after PDGF-BB (20 ng/ml) treatment (Fig. 2C). On the other hand, ~90% \( (n > 200) \) of the \( \alpha_{12}^{-/-} \alpha_{13}^{-/-} \) fibroblast cells still had dorsal ruffles 20 min after PDGF stimulation (Fig. 2D). Next, we transiently transfected C-terminal Myc-tagged \( \alpha_{12} \) or \( \alpha_{13} \) into \( \alpha_{12}^{-/-} \alpha_{13}^{-/-} \) cells, and the transfected cells were identified by immunostaining with the anti-Myc antibody (shown in the inserts of the figures). When constitutively active \( \alpha_{12}Q227L \) or \( \alpha_{13}Q229L \) was transiently expressed in \( \alpha_{12}^{-/-} \alpha_{13}^{-/-} \) cells, no dorsal ruffles were seen in any transfected cells after PDGF treatment for 20 min (Fig. 2E and F), indicating that the re-introduction of \( \alpha_{12} \) or \( \alpha_{13} \) accelerated the turnover of dorsal ruffles. In addition to the \( \alpha_{12} \) family, we have tested other G proteins. Transient re-expression of constitutively active \( \alpha_{s}Q219L \) or \( \alpha_{q}Q209L \) had no effect on dorsal ruffle turnover (Fig. 2G and H). Again, these data demonstrate that \( \alpha_{12} \) and \( \alpha_{13} \) selectively regulate dorsal ruffle turnover.

**Rac and Src Mediate PDGF-induced Dorsal Ruffle Formation**—The absence of \( \alpha_{12} \) and \( \alpha_{13} \) slows down the turnover of dorsal ruffles, thus making this cell system advantageous for investigating the signaling molecules involved in dorsal ruffle formation and disassembly. Using this system, we confirmed an
essential role for Rac in PDGF-induced dorsal ruffle formation (9, 24, 25) (Fig. 3, A–C). Although transient expression of dominant-negative Rho mutant RhoA(T19N) had no effect on PDGF-induced dorsal ruffle formation (data not shown), expression of dominant-negative Rac mutant Rac1(T17N) blocked PDGF-induced dorsal ruffle formation (Fig. 3B). However, expression of constitutively active Rac1(G12V) by itself (in the absence of PDGF stimulation) only induced peripheral membrane ruffle formation in transfected cells (Fig. 3C). Thus, Rac is required, but not sufficient, for PDGF-induced dorsal ruffle formation.

Moreover, we uncovered a critical role for the activity level of tyrosine kinase Src in regulating dorsal ruffles versus podosomes. It is known that v-Src induces the formation of podosomes in transformed cells (26). These podosomes are actin-containing structures at the ventral (substratum-contacting) side of the cell. Given that these two structures contain many of the same molecules, it has been proposed that these two structures might be related, although evidence is lacking (12). As shown in Fig. 3, D and E, Src family tyrosine kinase inhibitor PP2 completely blocked PDGF-induced dorsal ruffle formation in Gα12/−Gα13/− cells. Furthermore, expression of a highly
activated Src mutant, Src(E378G), induced dorsal ruffle formation in ~20% \((n = 50)\) of transfected \(G_{12}\)/\(-G_{13}\)/ cells without PDGF treatment (Fig. 2F). On the other hand, expression of Src(Y527F) in \(G_{12}\)/\(-G_{13}\)/ cells led to the formation of podosomes in ~100% \((n > 100)\) of transfected cells (Fig. 3G). In ~20% \((n = 50)\) of Src(E378G)-transfected cells, we also observed podosome formation. Both Src(E378G) and Src(Y527F) mutants are constitutively active, although the kinase activity of Src(E378G) is higher than that of Src(Y527F) \((27)\). Additionally, these two Src mutants could differ in other aspects such as in interactions with other proteins. These data demonstrate that Src mediates PDGF-induced dorsal ruffle formation and that dorsal ruffles and podosomes are related and regulated by different levels of Src activity.

**FRET Analysis Shows a Prolonged Activation of Rac in the Absence of \(G_{12}\) and \(G_{13}\)**—Next we investigated the possible molecular mechanism by which \(G_{12}\) and \(G_{13}\) regulate dorsal ruffle disassembly. \(G_{12}\) and \(G_{13}\) could control the disassembly of dorsal ruffles by promoting the depolymerization of the F-actin polymers or by accelerating the inactivation of signaling components in the dorsal ruffle formation pathway, tilting the balance to a shorter duration of dorsal ruffles. Because expression of activated \(G_{12}\) and \(G_{13}\) mutants in fibroblast cells leads to actin stress fiber formation \((21, 22)\), it is unlikely that \(G_{12}\) and \(G_{13}\) actively promote F-actin depolymerization. Furthermore, actin stress fiber formation is not essential for the inhibitory effect of \(G_{13}\) on dorsal ruffle formation because Rho kinase inhibitor Y27632 blocked \(G_{13}\)/Q229L-induced actin stress fiber formation, but not \(G_{13}\)/Q229L-mediated dorsal ruffle disassembly \((data not shown)\). Therefore, we investigated the potential role of \(G_{12}\) and \(G_{13}\) in the inactivation of signaling components involved in dorsal ruffle formation. We used a FRET approach and investigated the temporal activation of one of these signaling molecules, Rac, in cells. A single-chain FRET probe \(i(n)\) Raichu-Rac1, which has Rac1, p21-binding domain, YFP (acceptor), and CFP (donor) encoded on a single cDNA, was developed to detect activated Rac in living cells \((20)\). Activation of Rac leads to the binding of the effector domain p21-binding domain, which brings YFP into close proximity of CFP and induces FRET. We transfected Raichu-Rac1 plasmids into wild-type fibroblasts and \(G_{12}\)/\(-G_{13}\)/ cells. These cells were stimulated with PDGF (20 ng/ml). Cells were imaged every minute for 1 h. As shown in Fig. 4A, in wild-type fibroblast cells Rac is activated within 1 min. On the other hand, Rac was activated within 10 min and lasted for more than 40 min in \(G_{12}\)/\(-G_{13}\)/ cells \((Fig. 4B)\). This defective turn-off of Rac activity was due to the absence of \(G_{12}\) and \(G_{13}\), because rescue by re-expression of \(G_{12}\) and \(G_{13}\) in \(G_{12}\)/\(-G_{13}\)/ cells shortened the duration of Rac activity to 1–4 min \((Fig. 4C)\). These data demonstrate that a potential molecular mechanism for \(G_{12}\) and \(G_{13}\) to control dorsal ruffle disassembly is to shorten the duration of activation of signaling molecules such as Rac that are involved in dorsal ruffle formation.

**CONCLUSION**

In summary, in the absence of G proteins \(G_{12}\) and \(G_{13}\), dorsal ruffles stay longer after their formation induced by growth factors. Dorsal ruffles share many components \(i(n)\) such as Rac, cortactin, dynamin, Arp2/3 complex, N-Wasp, and WAVE \(i(n)\) with other Rac-mediated actin-containing structures \(i(n)\) such as lamellipodia, podosomes, and invadopodia \((12)\). We propose that a delay in the disassembly of dorsal ruffles could slow the formation of other actin-based structures. Therefore, rapid disassembly of dorsal ruffles is essential for motility if cells form dorsal ruffles in response to stimuli. Given that formation of dorsal ruffles is controlled by Ras, Rac, and Rab5 small GTPases \((9, 17, 25, 28)\), one way to slow down the disassembly of dorsal ruffles \(i(n)\) in the absence of \(G_{12}\) and \(G_{13}\) \(w(n)\) could be to slow the conversion of Ras, Rac, or Rab5 from its active GTP-bound state to the inactive GDP-bound state, \(i.e.\) to slow the GTP hydrolysis. In other words, the presence of \(G_{12}\) and \(G_{13}\) could accelerate the conversion. It is interesting to note that we have previously shown that \(G_{12}\) could activate a specific Ras GTPase-activating protein, Gap1m, to shorten the duration of activated Ras in cells \((29)\). Furthermore, in a yeast two-hybrid assay, Ras-GAP III \(i(n)\) Gap1m \(i(n)\), related to Gap1m \((30)\), was shown to interact with \(G_{13}\). Moreover, Rap1GAP has also been shown to directly interact with \(G_{13}\), \(G_{12}\), and \(G_{13}\) \((4–6)\). Thus, \(G_{12}\) and \(G_{13}\) could act through a GAP for Ras, Rac, or Rab5 to accelerate the dorsal ruffle turnover.

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