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Receptor-dependent RhoA Activation in G_{12}/G_{13} -deficient Cells

GENETIC EVIDENCE FOR AN INVOLVEMENT OF G_q/G_{11} *

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Stephan Vogt‡, Robert Grosse‡, Günter Schultz§, and Stefan Offermanns‡¶

From the ‡Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany
and the §Institute of Pharmacology, Free University Berlin, Thielallee 69-73, 14195 Berlin, Germany

The small GTPase RhoA is involved in the regulation of various cellular functions like the remodeling of the actin cytoskeleton and the induction of transcriptional activity. G-protein-coupled receptors (GPCRs), which are able to activate G_q/G_{11} and G_{12}/G_{13} are major upstream regulators of RhoA activity, and G_{12}/G_{13} have been shown to couple GPCRs to the activation of Rho by regulating the activity of a subfamily of RhoGEF proteins. However, the possible contribution of G_q/G_{11} to the regulation of RhoA activity via GPCRs is controversial. We have used a genetic approach to study the role of heterotrimeric G-proteins in the activation of RhoA via endogenous GPCRs. In pertussis toxin-treated $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient as well as in $G_{\alpha_q}/G_{\alpha_{11}}$ -deficient mouse embryonic fibroblasts (MEFs), in which coupling of receptors is restricted to G_q/G_{11} and G_{12}/G_{13} , respectively, receptor activation results in Rho activation. Rho activation induced by receptor agonists via G_q/G_{11} occurs with lower potency than Rho activation via G_{12}/G_{13} . Activation of RhoA via G_q/G_{11} is not affected by the phospholipase-C blocker U73122 or the Ca^{2+} -chelator BAPTA, but can be blocked by a dominant-negative mutant of the RhoGEF protein LARG. Our data clearly show that G_{12}/G_{13} as well as G_q/G_{11} alone can couple GPCRs to the rapid activation of RhoA. G_q/G_{11} -mediated RhoA activation occurs independently of phospholipase C- β and appears to involve LARG.

The small GTPase RhoA plays a central role in the organization of the cellular actin cytoskeleton through its ability to stimulate the formation of actomyosin-based structures and to regulate their contractility (1). In addition to its role in the regulation of the actin cytoskeleton, RhoA has also been involved in various other cellular processes like the regulation of microtubule dynamics or transcriptional activity (1, 2). Analogous to other regulatory guanine nucleotide-binding proteins Rho functions as a molecular switch by cycling between an inactive GDP-bound form and an active GTP-bound form. In the active state RhoA relays extracellular signals to a number of downstream effectors. These include protein kinases like Rho kinase or citron kinase, lipid kinases like phospholipase D, or phosphatidylinositol 4-phosphate 5-kinase as well as non-

kinase proteins like rhothekin, rhotekin, or diaphanous (3). RhoA is activated through various receptors including those coupled to heterotrimeric G-proteins (4, 5). Activation of RhoA through G-protein-coupled receptors (GPCRs)¹ is involved in a variety of physiological regulatory processes (6). One of the best described cellular paradigms for GPCR-mediated RhoA activation is the RhoA-dependent actin stress fiber formation in fibroblasts activated by various GPCR agonists like lysophosphatidic acid or thrombin. However, a GPCR/Rho-mediated regulation of actin-based structures has also been shown to occur in many other eukaryotic cells. For instance, in neuronal cells activation of Rho through lysophosphatidic acid or thrombin receptors leads to the formation of contractile actomyosin filaments thereby inducing neurite retraction and cell rounding (7, 8). In vascular smooth muscle cells, the Rho-mediated pathway has been shown to contribute to the vasoconstrictor-induced actomyosin-based cell contraction (9, 10), and the same pathway appears to be centrally involved in the platelet shape change response (11).

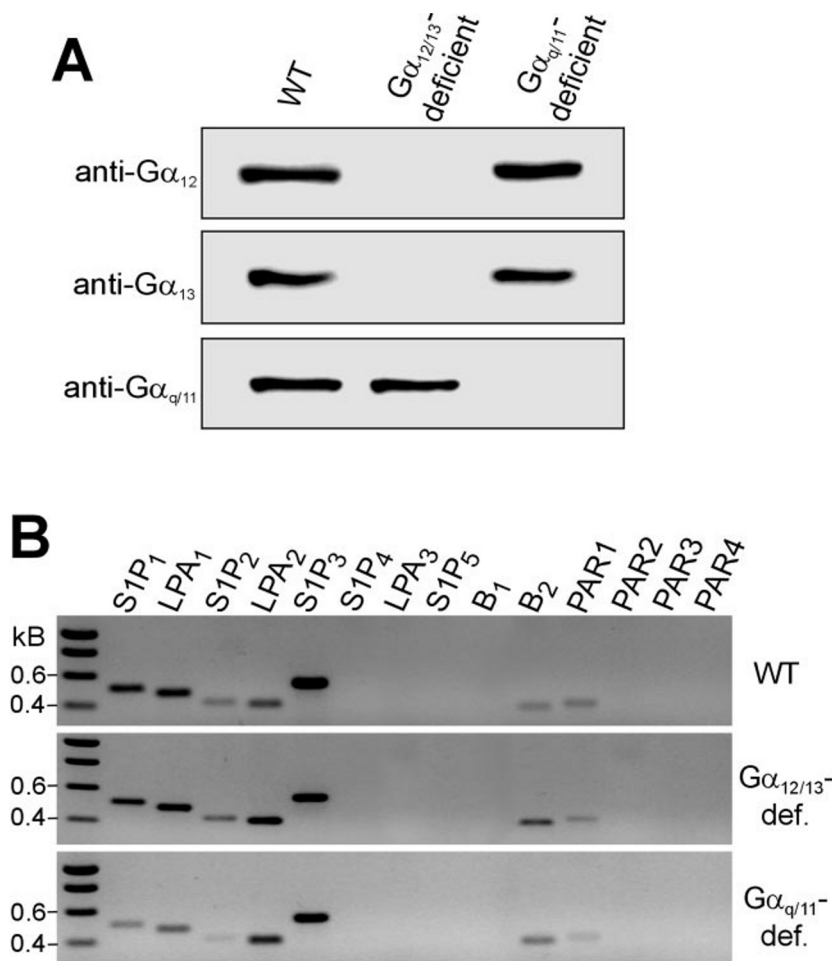
It is well established that G-proteins of the G_{12} -family, G_{12} and G_{13} , can couple GPCRs to the activation of RhoA. Constitutively active mutants of $G_{\alpha_{12}}$ and $G_{\alpha_{13}}$ have been shown to induce actin stress fiber formation as well as other RhoA-dependent cellular effects (Ref. 12; for review see Ref. 6). Recent studies in reconstituted or co-transfected systems have demonstrated that a group of RhoGEF proteins, consisting of p115 RhoGEF, PDZ-RhoGEF, and LARG, interact with $G_{\alpha_{12}}$ and $G_{\alpha_{13}}$ through their RGS domains, thereby stimulating RhoA activity (13–16). Receptors, which activate G_{12}/G_{13} also couple to G_q and G_{11} . It has been a controversial issue, whether G_q/G_{11} -mediated signaling contributes to the activation of RhoA via GPCRs. While various reports show Rho-dependent effects of constitutively active G_{α_q} mutants (Refs. 8 and 18; for review see Ref. 6) other studies demonstrated that active mutants of G_{α_q} are not able to induce Rho-mediated processes (12, 17). The recent development of methods for the precipitation of the activated form of RhoA from cell lysates allowed to directly determine the effects of different G-protein α -subunits on Rho activity. It could be confirmed that constitutively active mutants of $G_{\alpha_{12}}/G_{\alpha_{13}}$ can induce RhoA activation (19, 20). However, again, conflicting data exist with regard to the potential role of G_q/G_{11} in GPCR-mediated RhoA activation. In NIH3T3 and HEK293T cells, expression of constitutively active G_{α_q} -family members results in an increased level of active RhoA (21–23). In contrast, expression of mutant G_{α_q} in COS-7 cells

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¶ To whom correspondence should be addressed: Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany. Tel.: 49-6221-54-8247; Fax: 49-6221-54-8549; E-mail: Stefan.Offermanns@urz.uni-heidelberg.de.

¹ The abbreviations used are: GPCR, G-protein-coupled receptors; GST, glutathione-S-transferase; RBD, Rho-binding domain; PTX, pertussis toxin; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; PLC, phospholipase C; RhoGEF, Rho guanine nucleotide exchange factor; SRE, serum response element; MEF, mouse embryonic fibroblast; RGS, regulator of G-protein signaling; GST, glutathione S-transferase.

FIG. 1. Characterization of MEFs. *A*, absence of G_q/G_{11} and G_{12}/G_{13} in MEF cell lines. Shown are Western blots on lysates of the indicated MEF cells with antibodies specific for G_{12} , G_{13} , and $G_{q/11}$. *B*, detection of GPCRs expressed in MEF by RT-PCR. RNA was isolated from different MEF cell lines and reversely transcribed. Fragments of different receptor cDNAs were amplified using specific primers as described under "Materials and Methods." WT, wild-type MEFs; $G_{q/11}$ -defic., $G_{q/11}$ -deficient MEFs; $G_{12/13}$ -defic., $G_{12/13}$ -deficient MEFs.



does not induce activation of RhoA (24).

To study the role of different G-proteins under more physiological conditions we used G_q/G_{11} - and G_{12}/G_{13} -double deficient embryonic fibroblasts (MEFs) to determine their role in the activation of Rho via endogenous receptors. Our data clearly show that G_q/G_{11} can couple GPCRs to the rapid activation of RhoA. This process occurs in a phospholipase C- β -independent manner and appears to involve the RhoGEF protein LARG.

MATERIALS AND METHODS

Reagents—Thrombin (T3399), LPA (L7260), bradykinin (B3259), and U73122 were from Sigma-Aldrich (Dreieich, Germany). Pertussis toxin, BAPTA/AM, and Fura-2/AM were purchased from Calbiochem (Schwalbach, Germany).

Plasmids—HA- Δ DH/PH-LARG lacking the DH and the PH domain was generated by *Eco*NI and *Eco*RI digestion of full-length LARG and in-frame re-ligation using MungBean-nuclease and T_4 -DNA polymerase. Deletion was confirmed by sequencing (Li-cor 4200, Li-cor, Inc.). The modifying enzymes were purchased from New England Biolabs (Frankfurt, Germany), the ligase was obtained from Takara (Verviers, Belgium).

Cell Lines and Transfection—MEF cell lines were generated as described (25) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transiently transfected using LipofectAMINETM (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Serum starvation and pertussis toxin treatment were done for 24 h.

Western Blot Analysis—Lysates of MEFs were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis and visualized by chemiluminescence detection using sheep anti-mouse (Amersham Biosciences) or goat anti-rabbit antibodies (Cell Signaling, Frankfurt, Germany) coupled to horseradish peroxidase and were visualized using ECL reagent (Amersham Biosciences). Monoclonal antibodies against RhoA (26C4) or c-Myc (9E10) and rabbit polyclonal antibodies against

G_{13} (A-20) and $G_{q/11}$ (C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Anti- G_{12} rabbit polyclonal antibody was described previously (26). Monoclonal HA antibody was obtained from Covance Research Products. Antibodies raised in rabbit against p115-RhoGEF, PDZ-RhoGEF, and LARG were described previously (26).

RT-PCR—Cytoplasmatic RNA from MEFs was isolated using the Qiagen RNeasy Midi kit according to the manufacturer's protocol. Cytoplasmatic RNA was reversely transcribed using oligo-dT primers and the SuperScriptTM II System from Invitrogen. PCR was performed with specific primers hybridizing to specific regions of cDNAs of various G-protein-coupled receptors, which if possible were encoded by different exons using *Taq* polymerase (Invitrogen). The following primers were used: 5'-TCCACCGCCCATGTACTATTCA-3' and 5'-CTGCGACTG-CCTTTGGAGATGTT-3' (S1P₁); 5'-GCAGCTGCCTCTACTTCC-3' and 5'-ACGCGCCGGTTGCTCATTCC-3' (LPA₁); 5'-GCTGGAACCTGG-AGAAGTTT-3' and 5'-TGACTAGACAGCCGCACACCAA-3' (S1P₂); 5'-GGCGCCCCAAGATGTG-3' and 5'-GTAGCAACCCGACCCAGT-G-3' (LPA₂); 5'-GCCTCGGCCCTTCATCATCTTTG-3' and 5'-GCGT-GGCTGGAGCGGACTACA-3' (S1P₃); 5'-CATGGTGGGGTGGCTGA-GAGT-3' and 5'-CACGGCTGCGGAAGGAGTAGATGA-3' (S1P₄); 5'-G-CAACACAGACACAGCGG-ACGAGT-3' and 5'-GGCCACACCAGCA-GAATGAGC-3' (LPA₃); 5'-GGGGACCGCTGTTTCTCTTGCTAT-3' and 5'-CCCCTCCGTCGCTGGCTATTT-3' (S1P₅); 5'-GACGGCAAGCCCA-AGTGACCTG-3' and 5'-AAGAGCCCGCTGCAAAGACATAA-3' (B₁); 5'-TCTGGGGCTGTACTACTGCTTCTGA-3' and 5'-GTTGAGGCCGCT-GTTGCTGTAGG-3' (B₂); 5'-GCCCCGGCGCTTGCTGAT-3' and 5'-CA-CGCCCCGCTTCTTGACCTT-3' (PAR1); 5'-CTTCTGGCGGCCCTCGG-TCTCCTG-3' and 5'-GTTT-GCCTTCTTCTGCGGGGTGCC-3' (PAR2); 5'-ATGGAGCTGAGGGGAATCTACG-3' and 5'-GTGACCTCGCCAAA-TACCCAGTT-3' (PAR3); 5'-GACCCCCAGCATCTACGA-3' and 5'-GC-AGCCAGCAGCAACAC-3' (PAR4).

Measurement of Intracellular $[Ca^{2+}]$ —For fluorescence-based imaging of $[Ca^{2+}]$ cells were cultured on glass coverslips. MEFs were loaded for 30 min with 2 μ M Fura-2/AM (Calbiochem) in HEPES-buffered saline (pH 7.4) containing 135 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM

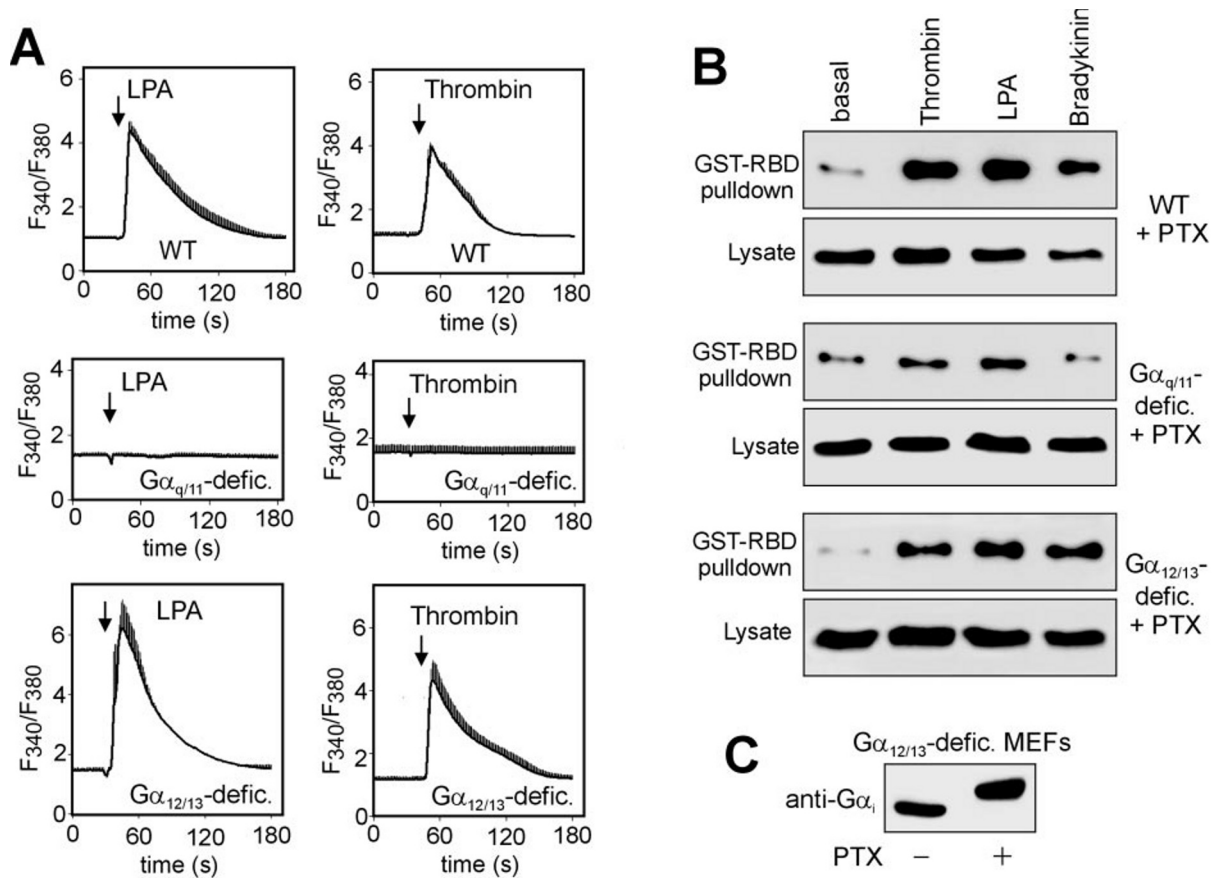


FIG. 2. Ca²⁺ transients and RhoA activation in wild type as well as Gα₁₂/Gα₁₃- and Gα_q/Gα₁₁-deficient cells. *A*, in Fura-2/AM-loaded MEF cells, the intracellular free Ca²⁺ concentration was recorded as described under "Materials and Methods," and the effects of LPA (5 μM; *left panels*) and thrombin (1 unit/ml; *right panels*) were determined. The time of addition of the stimuli is indicated by *arrows*. The *boldface lines* represent the means of 20 cells in a representative experiment. The *intra-assay variability* is given by *vertical lines* representing the S.D. over 20 cells in the experiment. *B*, serum-starved MEFs (WT, wild-type; Gα_{q/11}-defic., Gα_{q/11}-deficient; Gα_{12/13}-defic., Gα_{12/13}-deficient) were pertussis toxin-treated for 24 h, and the cells were stimulated via endogenous receptors by thrombin (1 unit/ml), LPA (5 μM) and bradykinin (10 μM), and activated RhoA was precipitated as described in under "Materials and Methods." Shown are Western blots using an anti-RhoA antibody on the GST-RBD precipitates as well as on corresponding lysates. *C*, to verify the complete ADP-ribosylation of G_i after pretreatment of cells with pertussis toxin, untreated, and PTX-treated (100 ng/ml; for 24 h) Gα₁₂/Gα₁₃-deficient cells were lysed, and lysates were analyzed by Western blotting with an antibody against G_i. Complete ADP-ribosylation after PTX treatment is indicated by the decreased mobility of G_i-type G-proteins in urea-containing SDS-polyacrylamide gels. Data shown are representative of at least three independently performed experiments.

CaCl₂, 5.5 mM glucose, 10 mM HEPES, and 0.2% (w/v) bovine serum albumin. Coverslips were analyzed on a monochromator-equipped (Polychrome IV, TILL-Photonics, Germany) inverted microscope (Axiovert 135, Carl Zeiss, Germany). The fluorescence was recorded with a 12-bit CCD camera (IMAGO, TILL-Photonics) and TILLvision v4.0 software. Fura-2 fluorescence was excited at 340, 358, and 380 nm and filtered through a Fura-2 corresponding long-pass filter, and the 340/380 fluorescence ratio determined. After subtraction of background signals, the 340/380 fluorescence ratio was determined as described (27).

Immunofluorescence—Cells were fixed in 4% paraformaldehyde and stained as described previously (28). Antibody dilutions were: monoclonal anti-HA (Covance Research Products) 1:200, BODIPY FL phalloidin (B-607, Molecular Probes; Eugene) 1:150, TRITC-conjugated anti-mouse (Molecular Probes) 1:400. Images were generated using a fluorescence microscope (Leica) and a cooled CCD camera (Leica).

Rho Pull-down Assay—Activation of Rho was determined by a modified method described by Ren and Schwartz (29). MEF cells were seeded on 10-cm dishes and were either transfected with Myc-tagged RhoA and indicated plasmids or left untransfected for assessment of endogenous GTP-Rho. After serum starvation and pertussis toxin treatment for 24 h, cells were stimulated with different agonists and lysed in ice-cold lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 10 μg/ml leupeptin, and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were incubated for 45 min with glutathione-Sepharose beads (Amersham Biosciences) coupled with GST proteins fused to the Rho-binding domain (RBD) of rhotekin (29). Beads were washed four times with 600 μl of ice-cold Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10

mM MgCl₂, 10 μg/ml leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride, and samples were collected by the addition of Laemmli buffer and subsequently analyzed using SDS-PAGE. Endogenous RhoA and transfected Myc-RhoA was detected using a mouse monoclonal antibody (26C4) and anti-Myc antibody (9E10), respectively (Santa Cruz Biotechnology).

RESULTS

Mouse embryonic fibroblast cell lines were generated from wild-type embryos as well as from embryos double deficient for Gα_q/Gα₁₁ and Gα₁₂/Gα₁₃ (30, 31). Western blot analysis of lysates from these cell lines confirmed the absence of Gα_q/Gα₁₁ and Gα₁₂/Gα₁₃ in respective cells (Fig. 1A). We then determined, whether the different MEF cell lines express receptors for various ligands, which have been shown to induce the activation of G_q/G₁₁ as well as G₁₂/G₁₃, like the lysophospholipids lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), bradykinin, or proteases like thrombin (Fig. 1B). By using RT-PCR, we found that wild type as well as Gα_q/Gα₁₁- and Gα₁₂/Gα₁₃-deficient MEFs express receptors for sphingosine 1-phosphate (S1P₁, S1P₂, and S1P₃), LPA (LPA₁ and LPA₂), bradykinin (B₂) as well as for thrombin (PAR-1). No expression was found for S1P₄, S1P₅, or LPA₃ receptors as well as PAR-2, -3 or -4 receptors. S1P₂ and S1P₃ receptors have been shown to couple to G₁₂/G₁₃, G_q/G₁₁, as well as to G_i-type G-proteins (32), and there is good evidence that LPA₁ and LPA₂

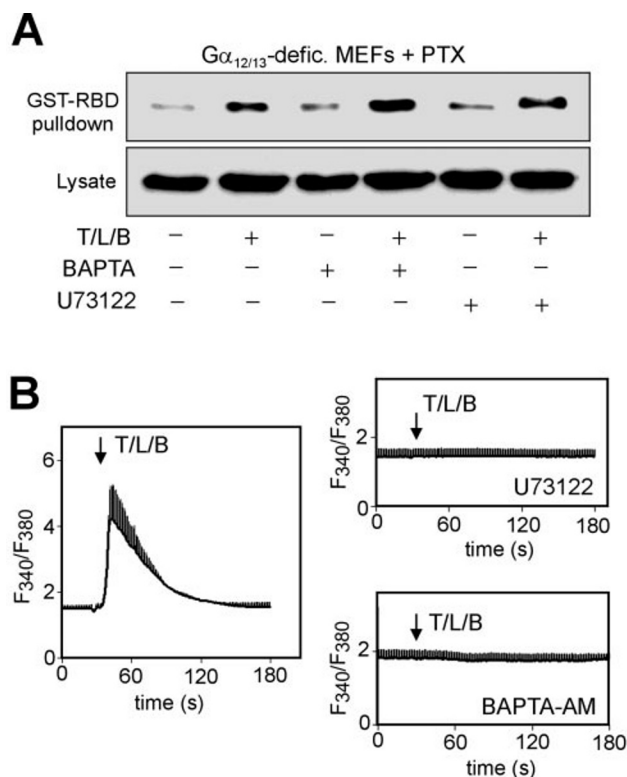


FIG. 3. RhoA activation in PTX-treated $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient MEFs is independent of phospholipase C and intracellular free Ca^{2+} . A, PTX-treated $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient cells were incubated without or with U73122 (10 μ M, 30 min) or the Ca^{2+} -chelator BAPTA/AM (10 μ M, 20 min). After stimulation with thrombin (1 unit/ml), LPA (5 μ M), or bradykinin (10 μ M) (L/T/B), RhoA activation was determined using a RhoA-pull-down assay as described under "Materials and Methods." Data shown are representative of at least three independently performed experiments. B, PTX-treated and Fura-2/AM-loaded $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient MEFs were incubated without or with U73122 (10 μ M, 30 min) or the Ca^{2+} -chelator BAPTA/AM (10 μ M, 20 min). Thereafter, thrombin (1 unit/ml), LPA (5 μ M), or bradykinin (10 μ M) (T/L/B) was added to the cells while the fluorescence ratio was recorded.

receptors have a very similar G-protein coupling pattern (33). Similarly, the thrombin receptor PAR-1 has also been shown to couple to all three G-protein families (11, 34–36). Notably, the pattern of expressed receptors did not differ between all three cell lines indicating that they are well suited for a comparative analysis of receptor-mediated effects.

In further experiments we used LPA, bradykinin and thrombin to stimulate cells through their endogenous receptors. To study the role of G_q/G_{11} and G_{12}/G_{13} in GPCR-induced Rho activation, we restricted coupling of receptors to these two G-protein families by pretreating cells with pertussis toxin (PTX), which uncouples receptors from G_i -type G-proteins. Fig. 2C shows that PTX treatment completely ADP-ribosylated α -subunits of G_i in $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient cells under the used experimental conditions. As expected, none of the stimuli was able to induce Ca^{2+} transients in $G_{\alpha_q}/G_{\alpha_{11}}$ -deficient cells whereas Ca^{2+} transients were induced in wild-type and $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient cells (Fig. 2A and data not shown). In pertussis toxin-treated $G_{\alpha_q}/G_{\alpha_{11}}$ -deficient cells, in which coupling of receptors is restricted to G_{12}/G_{13} , thrombin, and LPA resulted in an activation of RhoA (Fig. 2B). This confirms that G_{12}/G_{13} are able to mediate receptor-dependent RhoA activation independently of any signaling via G_q/G_{11} . Bradykinin, which induced Rho activation in PTX-treated wild-type cells had no effect on Rho activity in $G_{\alpha_q}/G_{\alpha_{11}}$ -deficient cells, suggesting that the bradykinin B_2 receptor is not coupled to G_{12}/G_{13} , but may induce Rho activation through G_q/G_{11} (Fig. 2B). To test the

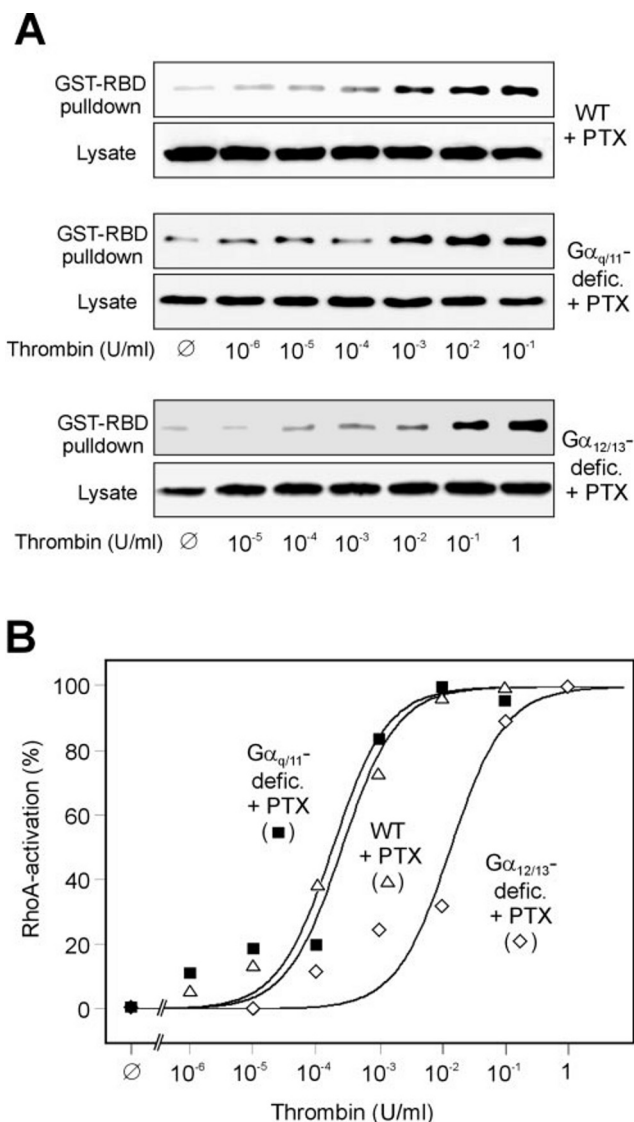
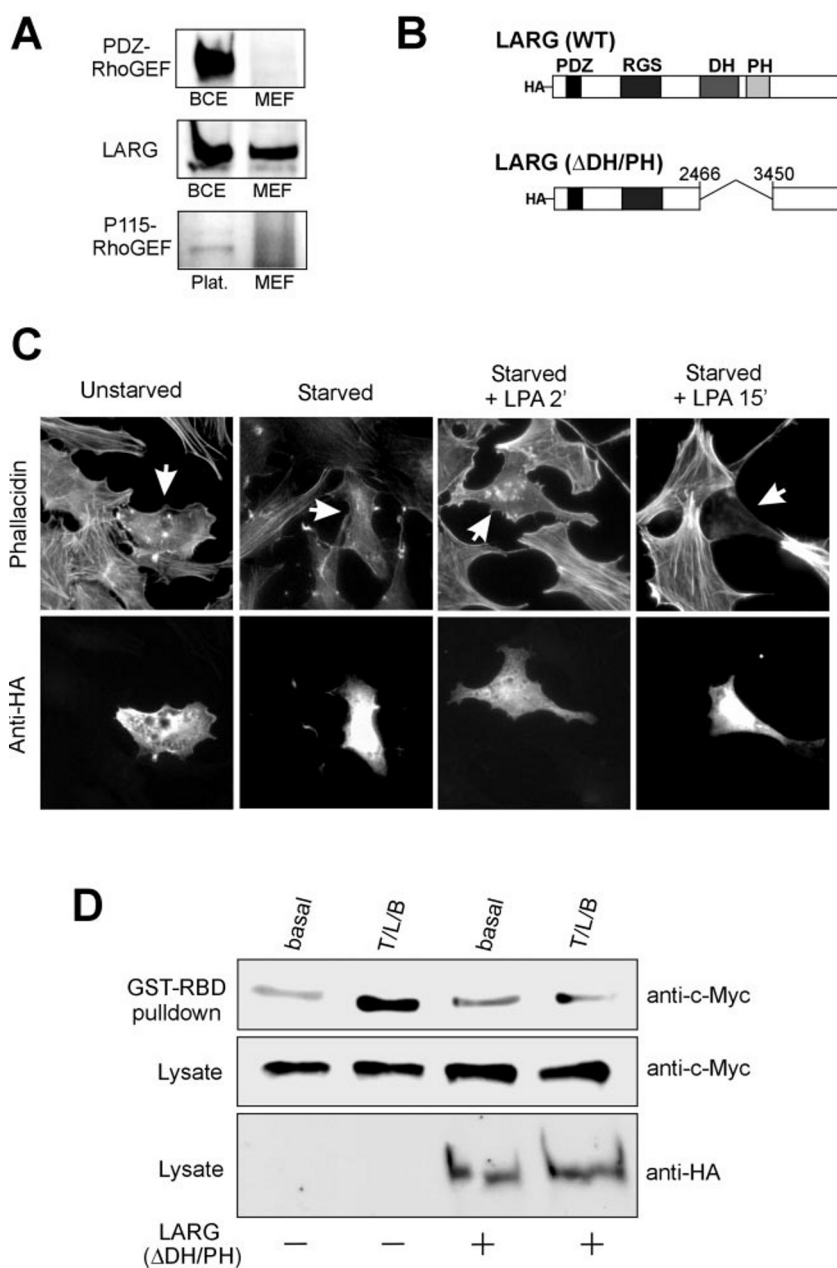


FIG. 4. Concentration-response relationships for thrombin-induced RhoA activation in PTX-treated wild-type, $G_{\alpha_q}/G_{\alpha_{11}}$ - and $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient cells. A, serum-starved and PTX-treated MEFs were stimulated with increasing concentrations of thrombin. Activated RhoA levels are determined in a Rho-pull-down assay as described under "Materials and Methods." B, concentration-response curves. The intensities of RhoA bands in the Western blots from two separate experiments were determined by densitometry (FluoS-Max, BioRad) and related to the maximum stimulation level. WT, wild-type MEFs; $G_{\alpha_{q/11}}$ -defic., $G_{\alpha_{q/11}}$ -deficient MEFs; $G_{\alpha_{12/13}}$ -defic., $G_{\alpha_{12/13}}$ -deficient MEFs.

potential ability of G_q/G_{11} to mediate receptor-dependent Rho activation more directly, we tested the effect of thrombin, LPA, and bradykinin on RhoA activation in PTX-pretreated $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient cells, in which coupling of receptors is restricted to G_q/G_{11} . All three receptor agonists were still able to activate RhoA through G_q/G_{11} (Fig. 2B).

Next we assessed whether G_q/G_{11} -mediated RhoA activation requires a functional phospholipase C- β or increases in intracellular free $[Ca^{2+}]$. Receptor-mediated Rho activation in PTX-treated $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient cells was not inhibited by the phospholipase C- β inhibitor U73122 or the Ca^{2+} -chelator BAPTA, although both agents completely blocked receptor-mediated induction of Ca^{2+} transients in the same cells (Fig. 3, A and B). These data indicate that GPCRs can mediate RhoA activation via G_q/G_{11} in a phospholipase C- β -independent manner.

FIG. 5. Involvement of LARG in RhoA activation in G_{α₁₂}/G_{α₁₃}-deficient MEFs. *A*, expression of RhoGEF proteins in G_{α₁₂}/G_{α₁₃}-deficient cells. Shown is the Western blot analysis using polyclonal antibodies against LARG, PDZ-RhoGEF, and p115-RhoGEF on total mouse brain cholate extracts (BCE; for PDZ-RhoGEF and LARG) or platelet extracts (*Plat.*; for p115RhoGEF) as well as on lysates of G_{α₁₂}/G_{α₁₃}-deficient MEFs (*MEF*). *B*, model of the HA-tagged ΔDH/PH-LARG mutant. *C*, PTX-treated G_{α₁₂}/G_{α₁₃}-deficient MEFs transiently transfected with HA-tagged ΔDH/PH-LARG were either kept in 10% fetal calf serum (unstarved) or were maintained in 0.5% fatty acid-free bovine serum albumin overnight (starved). After a short incubation in the absence or presence of LPA (1 μM), cells were fixed and stained with BODIPY-phalloidin and anti-HA antibodies to visualize F-actin or the epitope tagged ΔDH/PH-LARG, respectively. *D*, effect of ΔDH/PH-LARG on RhoA activation in G_{α₁₂}/G_{α₁₃}-deficient MEFs. Cells were transfected with c-Myc-tagged RhoA, either without (*two left lanes*) or with the HA-tagged ΔDH/PH mutant of LARG (*two right lanes*). After serum starvation and pertussis toxin treatment thrombin (1 unit/ml), LPA (5 μM), and bradykinin (10 μM) were added together (T/L/B) to the cells, and activated RhoA was precipitated as described. Precipitates (GST-RBD pulldown) and a defined part of the lysates were immunoblotted with monoclonal c-Myc antibody (anti-c-Myc) to detect activated and total RhoA in transfected cells. The expression of the HA-tagged ΔDH/PH mutant of LARG was controlled by an immunoblot of lysates using HA-monoclonal-antibody (anti-HA). Data shown are representative of at least three independently performed experiments.



To further characterize G₁₂/G₁₃- and G_q/G₁₁-mediated RhoA activation through GPCRs, we determined concentration-response relationships for thrombin-induced RhoA activation in PTX-treated wild-type, G_{α_q}/G_{α₁₁}, and G_{α₁₂}/G_{α₁₃}-deficient cells (Fig. 4, A and B). Thrombin-induced RhoA activation in PTX-treated wild-type cells and in G_{α_q}/G_{α₁₁}-deficient cells with half-maximal and maximal concentrations of 10⁻⁴–10⁻³ and 10⁻² units/ml, respectively. However, when G-protein coupling was restricted to G_q/G₁₁ in PTX-treated G_{α₁₂}/G_{α₁₃}-deficient cells, thrombin was considerably less potent, and maximal RhoA activation was observed only at thrombin concentrations of 1 unit/ml. While both G₁₂/G₁₃ and G_q/G₁₁ can mediate RhoA activation, receptor-dependent activation through G_q/G₁₁ requires agonist concentrations about two orders of magnitude higher than activation through G₁₂/G₁₃.

The fact that RhoA activation *via* G_q/G₁₁ occurred independently of phospholipase C-β prompted us to consider the involvement of RGS domain-containing RhoGEF proteins, which have been suggested to directly link G₁₂/G₁₃ to Rho activation. Of the

three members of this subgroup of RhoGEF proteins, p115 RhoGEF, PDZ-RhoGEF, and LARG, we found only LARG to be expressed in MEFs (Fig. 5A). This is consistent with the much wider expression pattern of LARG compared with p115RhoGEF and PDZ-RhoGEF, which are mainly found in hematopoietic and neuronal cells, respectively (26, 37, 38). To test the potential role of LARG in G_q/G₁₁-mediated Rho activation, we constructed a mutant of LARG lacking the DH and PH domains required for its RhoGEF activity (Fig. 5B). Expression of (ΔDH/PH)-LARG in PTX-treated G_{α₁₂}/G_{α₁₃}-deficient cells efficiently blocked rapid actin stress fiber formation induced by LPA (Fig. 5C). Furthermore, expression of ΔDH/PH-LARG also dramatically reduced the cellular F-actin content in the presence of 10% fetal calf serum (Fig. 5C). Similarly, ΔDH/PH-LARG was found to block activation of Myc-tagged RhoA in response to thrombin and LPA in PTX-treated G_{α₁₂}/G_{α₁₃}-deficient cells (Fig. 5D). These data suggest that LARG is critically involved in the phospholipase C-β-independent RhoA activation *via* G_q/G₁₁.

DISCUSSION

Many GPCRs have been shown to be able to mediate the activation of the small GTPase RhoA (6, 15). Activation of RhoA via GPCRs is involved in a variety of functions like regulation of cell movement, cell shape, and cell growth. Receptors that stimulate RhoA activity couple to G_{12}/G_{13} , G_q/G_{11} , and G_i -type G-proteins. The fact that Rho activation through GPCRs is not affected by PTX and since receptors coupling only to G_i -type G-proteins are unable to stimulate Rho activity suggests that G_i is unlikely to be directly involved in RhoA activation via GPCRs (5, 6). It has been difficult to evaluate the relative roles of G_{12}/G_{13} and G_q/G_{11} in receptor-mediated Rho activation since there are currently no specific inhibitors of these G-protein subtypes available. Multiple evidence has accumulated that G_{12} and G_{13} can mediate the activation of RhoA, and the recently described RhoGEF proteins, p115-RhoGEF, LARG, and PDZ-RhoGEF have been shown to link G_{12}/G_{13} to RhoA activation (13–16). However, it is not clear whether G_q/G_{11} are indeed directly involved in the rapid receptor-mediated RhoA activation by extracellular signals. Studies in platelets lacking $G_{\alpha_q}/G_{\alpha_{11}}$ demonstrated that receptor-mediated RhoA activation does not necessarily depend on activation of G_q/G_{11} (39). Sagi *et al.* (24) reported that a constitutively active mutant of G_{α_q} was unable to induce RhoA activation. In contrast, several recent reports clearly show that transient transfection of constitutively active mutants of G_{α_q} in NIH3T3 cells or in HEK293T cells results in elevated levels of active RhoA (21–23). Since these studies rely on the effects of transfected constitutively active mutants of G_{α_q} family members, they do not necessarily prove a direct involvement of G_q proteins in RhoA activation exerted by extracellular signals via receptors. We therefore chose a genetic approach to study the activation of RhoA through endogenous receptors in PTX-treated MEFs lacking either $G_{\alpha_q}/G_{\alpha_{11}}$ or $G_{\alpha_{12}}/G_{\alpha_{13}}$. In these cells, receptor coupling is restricted to G_{12}/G_{13} and G_q/G_{11} , respectively. Our data confirm that RhoA activation is potently induced through G_{12}/G_{13} . More importantly however, they provide genetic evidence that G_q/G_{11} proteins can also mediate the rapid RhoA activation via endogenously expressed GPCRs.

Interestingly, we observed a clear difference in the potencies by which thrombin induced RhoA activation via G_{12}/G_{13} and G_q/G_{11} . Rho activation through G_q/G_{11} required agonist concentrations about two orders of magnitude higher than Rho activation via G_{12}/G_{13} . The observed potency difference for ligand-induced Rho activation could be due to different efficiencies by which the activated receptor couples to different G-protein subfamilies. For example, the thromboxane A_2 receptor, which couples to G_{12}/G_{13} and G_q/G_{11} has been shown to preferentially activate G_{12}/G_{13} in platelets (35). However, it is not known whether this is a general feature of G_q/G_{11} - and G_{12}/G_{13} -coupled receptors. Alternatively it is conceivable that RhoGEF proteins are less sensitive to regulation via $G_{\alpha_q}/G_{\alpha_{11}}$ than through $G_{\alpha_{12}}/G_{\alpha_{13}}$.

While it is well established that G_{12}/G_{13} -mediated RhoA activation involves RhoGEF proteins like PDZ-RhoGEF, LARG, and p115-RhoGEF, the mechanism of G_q/G_{11} -mediated Rho activation is less clear. We could not observe any effects of Ca^{2+} -chelators or of the phospholipase C- β inhibitor U73122 on G_q/G_{11} -mediated Rho activation (see Fig. 3A), indicating that G_q/G_{11} mediates Rho activation in a manner independent of β -isoforms of PLC. This is consistent with data showing that activation of protein kinase C (PKC) or elevation of intracellular Ca^{2+} is not able to promote RhoA activation and that inhibition of PKC does not interfere with Rho activation by an active mutant of G_{α_q} (23). If PLC is not involved in G_q/G_{11} -mediated RhoA activation, it is tempting to speculate that

G_q/G_{11} may activate RhoA by a mechanism analogous to G_{12}/G_{13} . Interestingly, recent data suggest that the RGS domain of LARG but not of p115 RhoGEF can interact with G_{α_q} when complexed with AlF_4^- to mimic the transition state of GTP hydrolysis (21). In contrast, the constitutively active mutant of G_{α_q} (Q229L) did not show a considerable interaction with the RGS domains or the full-length versions of LARG, PDZ-RhoGEF, or p115RhoGEF (23, 40, 41). However, mutants of PDZ-RhoGEF and p115RhoGEF which lack the N-terminal region including the RGS domain were able to interact with G_{α_q} (Q229L) (23). In functional experiments, it was shown that expression of full-length LARG but not of a LARG mutant, which lacks the PDZ and RGS domain, enhances RhoA activation by constitutively active G_{α_q} (21), suggesting that an interaction of the RGS domain with G_{α_q} is involved in RhoA activation. However, expression of the RGS domains of LARG and PDZ-RhoGEF did not interfere with G_{α_q} (Q229L)-induced serum response element (SRE) activation, an effect, which is mediated by Rho (23, 41). Thus, while there is some evidence that active G_{α_q} can interact with LARG and other RGS domain containing RhoGEF proteins, the exact mode of this interaction still remains elusive. In our study, we used a LARG mutant that lacks the RhoGEF domain (Δ DH/PH-LARG) to study the potential involvement of LARG in receptor-mediated, G_q/G_{11} -dependent RhoA activation. Expression of Δ DH/PH-LARG blocked receptor-mediated RhoA activation as well as actin stress fiber formation in PTX treated $G_{\alpha_{12}}/G_{\alpha_{13}}$ -deficient cells, suggesting that LARG is critically involved in G_q/G_{11} -mediated RhoA activation. Whether the mechanism of LARG-mediated Rho activation by G_q/G_{11} is identical to that by G_{12}/G_{13} remains to be elucidated.

In summary, based on a genetic model our data clearly demonstrate that both, G_{12}/G_{13} and G_q/G_{11} , can mediate the activation of RhoA via G-protein-coupled receptors. The G_q/G_{11} -mediated Rho activation occurs independently of β -isoforms of phospholipase C and involves the RhoGEF protein LARG.

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