Current Biology, Vol. 15, 1867–1873, October 25, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.09.050

P-Rex1 Regulates Neutrophil Function

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Summary

Rac GTPases regulate cytoskeletal structure, gene expression, and reactive oxygen species (ROS) production [1, 2]. Rac2-deficient neutrophils cannot chemotax, produce ROS, or degranulate upon G protein-coupled receptor (GPCR) activation [3–10]. Deficiency in PI3Kγ, an upstream regulator of Rac, causes a similar phenotype [11–13]. P-Rex1, a guanine-nucleotide exchange factor (GEF) for Rac [14], is believed to link GPCRs and PI3Kγ to Rac-dependent neutrophil responses. We have investigated the functional importance of P-Rex1 by generating a P-Rex1$^{-/-}$ mouse. P-Rex1$^{-/-}$ mice are viable and healthy, with apparently normal leukocyte development, but with mild neutrophilia. In neutrophils from P-Rex1$^{-/-}$ mice, GPCR-dependent Rac2 activation is impaired, whereas Rac1 activation is less compromised. GPCR-dependent ROS formation is absent in lipopolysaccharide (LPS)-primed P-Rex1$^{-/-}$ neutrophils, but less affected in unprimed or TNFα-primed cells. Recruitment of P-Rex1$^{-/-}$ neutrophils to inflammatory sites is impaired. Surprisingly, chemotaxis of isolated neutrophils is only slightly reduced, with a mild defect in cell speed, but normal polarization and directionality. Secretion of azurophil granules is unaffected. In conclusion, P-Rex1 is an important regulator of neutrophil function by mediating a subset of Rac-dependent neutrophil responses. However, P-Rex1 is not an essential regulator of neutrophil chemotaxis and degranulation.

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Results and Discussion

Generation of P-Rex1$^{-/-}$ Mice

P-Rex1 is the major PtdIns(3,4,5)P_3-sensitive Rac-GEF in neutrophils and directly activated by G$\beta\gamma$ subunits [14]. To investigate its functional importance, we have generated a P-Rex1$^{-/-}$ mouse. To delete P-Rex1, we replaced exon 5 with a neomycin resistance cassette and translational stop codon (see Figure S1 in the Supplemental Data available with this article online). Targeting was monitored by Southern blotting (Figure 1A). Homozygous P-Rex1$^{-/-}$ mice were derived by standard protocols into specific-opportunistic pathogen-free isolator conditions and were obtained with the expected Mendelian frequency. The P-Rex1 deletion was confirmed by northern (Figure 1B) and western blotting (Figure 1C).

Characterization of P-Rex1$^{-/-}$ Mice

P-Rex1$^{-/-}$ mice were viable, fertile, and apparently healthy, with normal life spans. Ten-week-old P-Rex1$^{-/-}$ mice weighed on average 14% less than P-Rex1$^{+/+}$ mice (Figure 1D) and remained smaller throughout adult life (not shown). The livers of P-Rex1$^{-/-}$ mice were disproportionately smaller than those of P-Rex1$^{+/+}$ mice (Figure 1E).

Peripheral blood leukocyte counts were slightly elevated in P-Rex1$^{-/-}$ mice, as a result of mild neutrophilia (61% more peripheral P-Rex1$^{-/-}$ than P-Rex1$^{+/+}$ neutrophils), whereas erythrocyte, lymphocyte, monocyte, and platelet numbers were normal (Figure 1F). Numbers and maturity of bone-marrow-derived P-Rex1$^{-/-}$ neutrophils, splenic B and T cells (including marginal zone B cells), and thymocytes (CD4$^-$/CD8$^-$, CD4$^+$/CD8$^+$, CD4$^+$/CD8$^-$, and CD4$^-$/CD8$^+$ cells) appeared normal (Figure 1G, and data not shown).

Rac Activation in Neutrophils from P-Rex1$^{-/-}$ Mice

We measured fMLP-stimulated Rac1 and Rac2 activation in purified bone-marrow-derived neutrophils (Figures 2A–2C). In P-Rex1$^{+/+}$ cells, activation was robust and rapid: 2% of Rac1 and 5% of Rac2 were activated after 5 s, similar to previously reported levels [3]. In P-Rex1$^{-/-}$ neutrophils, Rac1 peak activation was reduced by 25% (Figure 2B) and Rac2 activation by 50% (Figure 2C), suggesting that P-Rex1 acts through both Rac isoforms but mainly through Rac2. This is a first description of the substrate specificity of endogenous P-Rex1 and of a GEF being able to distinguish between Rac isoforms.

We measured the fMLP-dependent activation of two known neutrophil Rac targets, p38 MAPK and p42/p44 Erk [6, 7], as well as of the PI3K target PKB [11–13] (Figure S2). They were unaffected by the P-Rex1 deficiency, suggesting that partial Rac activation is suffi-

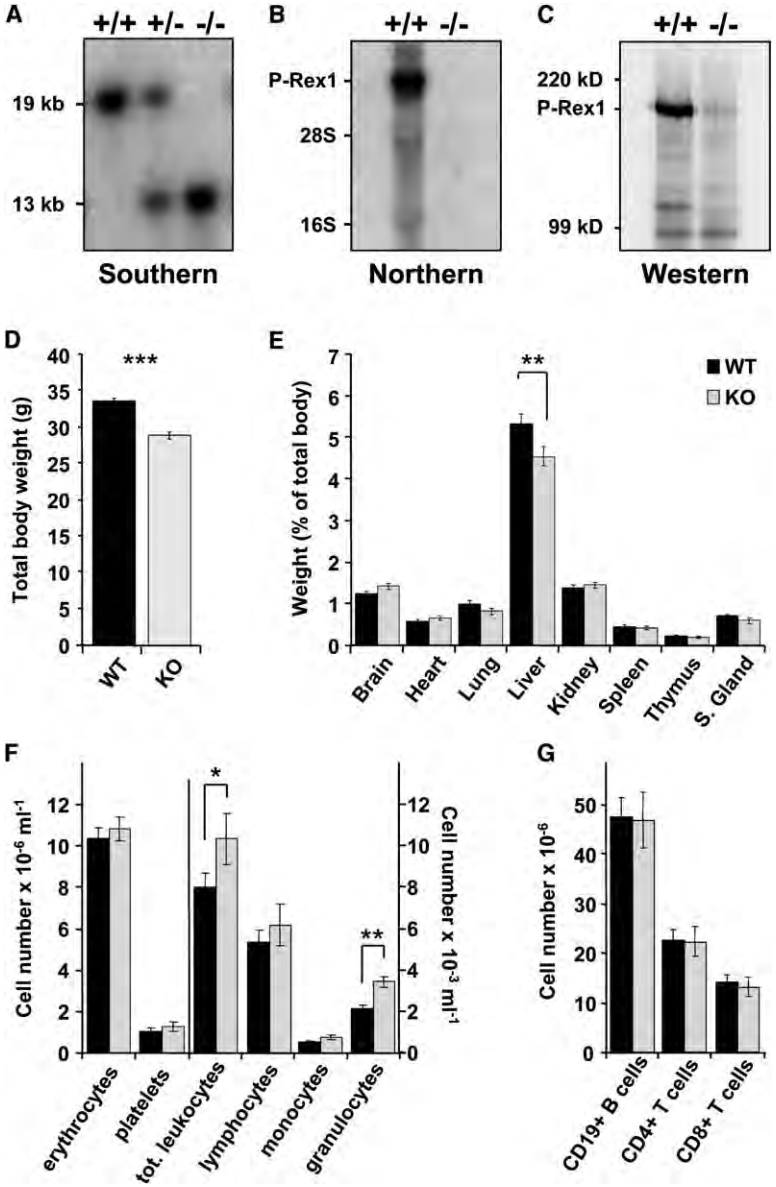

Figure 1. Generation and Characterization of P-Rex1$^{-/-}$ Mice

(A) Southern blot. A 3′ external probe (see Figure S1) was used with Xmn1-digested genomic DNA to screen ES cells and mice for wild-type and targeted P-Rex1 alleles.

(B) Northern blot. RNA from bone-marrow-derived neutrophils of P-Rex1$^{+/+}$ and P-Rex1$^{-/-}$ mice was blotted with a probe corresponding to exon 5. Equal loading was confirmed by ethidium bromide staining.

(C) Western blot. Total protein from 4 × 10^6 bone-marrow-derived neutrophils of P-Rex1$^{+/+}$ and P-Rex1$^{-/-}$ mice was blotted with monoclonal anti-P-Rex1 6F12 antibody.

(D) Body weights of P-Rex1$^{-/-}$ mice. The body weights of adult male P-Rex1$^{+/+}$ (black bars) and P-Rex1$^{-/-}$ mice (gray bars) are plotted. Data are means ± standard error of the mean (SEM) (n = 18 animals per group at 72 ± 10 days of age).

(E) Organ weights of P-Rex1$^{-/-}$ mice. The weights of selected organs of adult P-Rex1$^{+/+}$ (black bars) and P-Rex1$^{-/-}$ mice (gray bars) are plotted as % of total body weight. S. gland = salivary gland. Data are means ± SEM (n = 14), pooled from animals between 3 months and 1 yr of age and from both sexes, equally matched between groups.

(F) Peripheral blood cells. Peripheral blood from 3-month-old female P-Rex1$^{+/+}$ mice (black bars) and P-Rex1$^{-/-}$ mice (gray bars) was analyzed in a Vetabc animal blood cell counter. Data are means ± SEM (n = 7).

(G) Splenocytes. Cells isolated from the spleens of 3-month-old female P-Rex1$^{+/+}$ mice (black bars) and P-Rex1$^{-/-}$ mice (gray bars) were fluorescently labeled and analyzed by flow cytometry essentially as in [25]. Data are means ± SEM (n = 4). Statistical analysis is unpaired Student's t test throughout.

cient to stimulate these signaling pathways in neutrophils.

It is worthwhile discussing that P-Rex1 activates Rac2 preferentially over Rac1, because these play distinct roles in neutrophils. In humans, Rac2 is the predominant neutrophil isoform, whereas in mice, Rac2 and Rac1 are expressed equally [3]. Rac2-deficiency in humans (D57N mutation) causes severe recurrent bacterial infections, with neutrophils largely unable to chemotax to sites of infection, produce ROS, or secrete granule proteins [4, 5]. Similarly, Rac2$^{-/-}$ mice clear fungal infections badly, and Rac2$^{-/-}$ neutrophil adhesion, chemotaxis, ROS formation, and degranulation are impaired [6–8]. In contrast, Rac1$^{-/-}$ neutrophils produce ROS normally [15]. Furthermore, whereas Rac2$^{-/-}$ neutrophils cannot polarize or move per se [6, 9, 10], Rac1$^{-/-}$ neutrophils move, but with poor directionality [10, 16]. Hence, Rac2 is the main regulator of ROS formation and chemotaxis and also controls degranulation. Because P-Rex1 deficiency impacts more on Rac2 than Rac1 activation, one might expect defects in all these neutrophil responses.

ROS Formation in Neutrophils from P-Rex1$^{-/-}$ Mice

We tested GPCR-dependent ROS production. In unprimed fMLP- or C5a-stimulated P-Rex1$^{+/+}$ neutrophils, ROS formation was low, but it was even lower in P-Rex1$^{-/-}$ cells (up to 30%), and with slightly delayed onset (Figures 3A and 3B). Priming of P-Rex1$^{+/+}$ cells with LPS or TNFα prior to fMLP or C5a stimulation resulted in a 6–8-fold increase in ROS production (Figures 3C–3E). The LPS-primed fMLP- or C5a-stimulated response was almost completely absent in P-Rex1$^{-/-}$ neutrophils, i.e., roughly at unprimed levels (Figures 3C and 3D), whereas the TNFα-primed response was only reduced by 30% (Figure 3E, and data not shown).

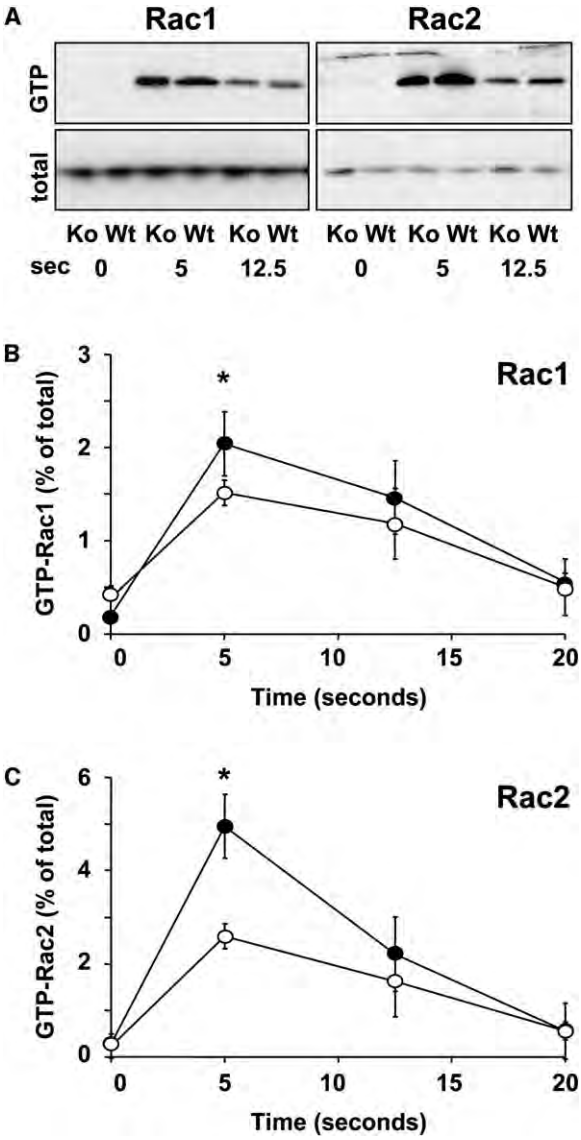

Figure 2. Rac Activation Is Impaired in P-Rex1$^{-/-}$ Neutrophils

(A) Western blots of Rac1 and Rac2. Purified bone-marrow-derived neutrophils [26] from P-Rex1$^{+/+}$ and P-Rex1$^{-/-}$ mice were stimulated for the indicated periods of time with 10 μM fMLP, and GTP bound Rac1 and Rac2 were isolated by PAK-Crib pull-down from total lysates [27]. GTP-Rac1 and Rac2 levels are compared to total Rac1 and Rac2 levels (2% of total lysate loaded) by western blotting. Data are from one experiment representative of five.

(B) fMLP-stimulated Rac1 activation. Anti-Rac1 western blots such as those shown in (A) were obtained in five experiments by stimulation of P-Rex1$^{+/+}$ (black circles) and P-Rex1$^{-/-}$ neutrophils (white circles) with 10 μM fMLP. Blots from all experiments were densitometrically scanned and analyzed with Image J software. Data were pooled and expressed as means ± SEM.

(C) fMLP-stimulated Rac2 activation. Data were obtained as in (B), except from anti-Rac2 western blots, and are means ± SEM of five experiments.

Hence, P-Rex1 regulates GPCR-dependent ROS formation both in unprimed and primed neutrophils, but its biggest impact is on LPS-primed GPCR-dependent ROS formation.

The finding that P-Rex1 regulates ROS formation corroborates data obtained previously through antisense treatment of NB4 and HL60 cells [14] and is consistent with the notion that P-Rex1 preferentially activates Rac2 over Rac1. The fact that P-Rex1 distinguishes between LPS and TNFα priming suggests that the LPS-priming pathway specifically, rather than a primed state in general, depends on P-Rex1. LPS signals through TLR4 via various adaptor proteins, IRAKs, Btk, and members of the MAPK family [17]. We do not know whether any of these LPS-signaling mediators are involved in regulating P-Rex1.

We tested ROS formation in P-Rex1$^{-/-}$ neutrophils stimulated via GPCR-independent pathways. IgG-opsonized zymosan, which engages mainly FcγRIIa, induced normal ROS formation (Figure 3F), implying that P-Rex1 plays little role in FcR-mediated signaling. ROS formation was also normal in PMA-stimulated cells, suggesting that P-Rex1 is not involved in protein kinase C-dependent activation of the NADPH oxidase (Figure 3G).

Chemotaxis of Neutrophils from P-Rex1$^{-/-}$ Mice

P-Rex1 has often been proposed to link GPCRs and PI3Kγ to Rac in neutrophil chemotaxis, without there being experimental evidence. We measured chemotaxis of P-Rex1$^{-/-}$ neutrophils in transwell and Dunn chamber assays [3, 5, 6, 10]. In the transwell assay, fMLP- or C5a-induced chemotaxis was surprisingly little affected by the P-Rex1-deficiency: fMLP-stimulated migration was variable but on average normal, and C5a-dependent migration was reduced by 30% (Figure 4A). We tested wide dose ranges (0.1–30 nM C5a), timeframes (15 to 90 min), and LPS priming of fMLP- or C5a-stimulated chemotaxis without finding bigger defects (Figure S3A and data not shown). In Dunn chamber assays, P-Rex1$^{-/-}$ and P-Rex1$^{+/+}$ neutrophils polarized well and moved with good directionality toward the chemoattractant fMLP (Rayleigh test for unimodal clustering, p value << 0.0001, for both P-Rex1$^{-/-}$ and P-Rex1$^{+/+}$ cells; see Figures 4B and 4C, Figure S3B, and Movies S1 and S2). The persistence of migration was also equal between P-Rex1$^{-/-}$ and P-Rex1$^{+/+}$ cells (mean migratory index of 0.465 ± 0.0162 for P-Rex1$^{-/-}$ and 0.471 ± 0.0161 for P-Rex1$^{+/+}$ cells). In contrast, the average speed of P-Rex1$^{-/-}$ cells was slightly but significantly lower than that of P-Rex1$^{+/+}$ cells (by 10%) (Figure 4D), which might explain why fewer P-Rex1$^{-/-}$ neutrophils migrated long distances (Figure 4B). We also measured chemokinesis, under uniform fMLP conditions. Again, cell speed was reduced in P-Rex1$^{-/-}$ neutrophils (by 28%), resulting in fewer cells traveling far (Figure 4D and Figure S3C). Similarly, fMLP- or C5a-induced actin polymerization was slightly reduced over a range of doses at 10 and 30 s, but again the defect was small (Figure 4E and Figure S3D). Hence, although P-Rex1-deficiency causes a defect in neutrophil motility, it is surprisingly mild, suggesting that P-Rex1 is not an essential regulator of neutrophil chemotaxis.

Interestingly, a recent report has shown that P-Rex1 is expressed in brain as well as neutrophils, and P-Rex1-RNAi treatment of PC12 cells results in a striking defect in NGF-induced motility [18]. Hence, intriguingly, in neuronal cells that move slowly and only during development in vivo, the role of P-Rex1 in chemotaxis

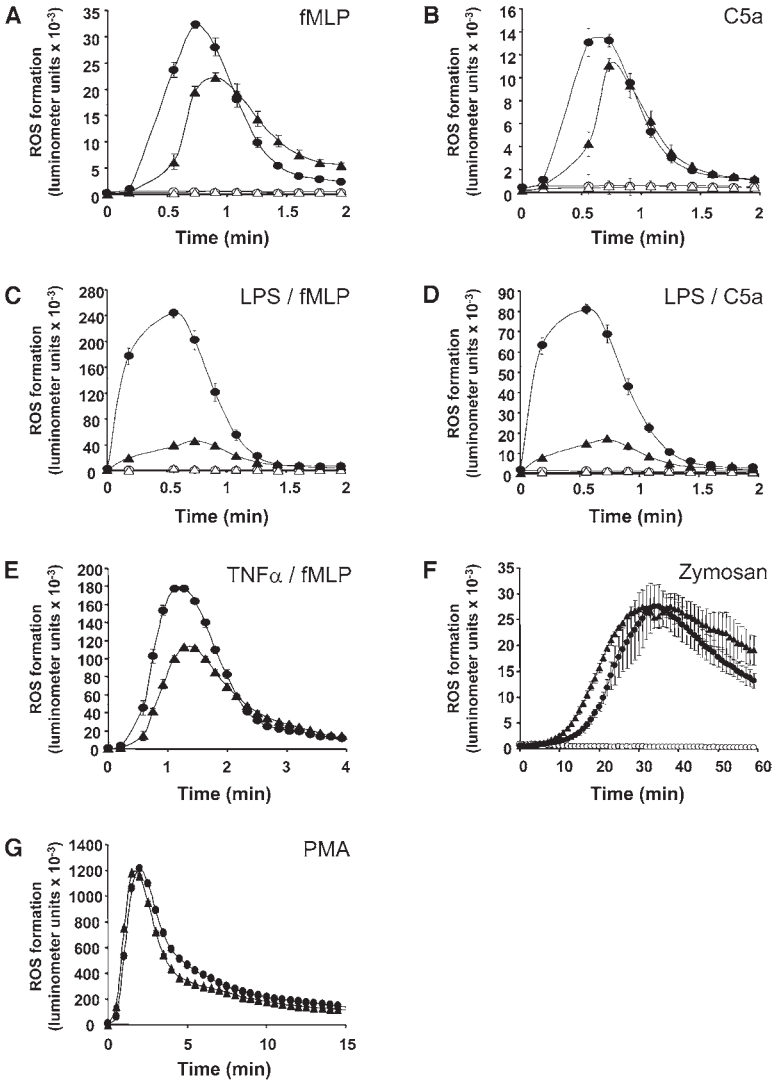

Figure 3. ROS Formation Is Impaired in P-Rex1$^{-/-}$ Neutrophils

(A–G) Bone-marrow-derived neutrophils from P-Rex1$^{+/+}$ (black circles) and P-Rex1$^{-/-}$ mice (black triangles) were treated with either (A) 3 μM fMLP; (B) 25 nM C5a; (C) 1 μg/ml LPS, 1 hr, then 3 μM fMLP; (D) 1 μg/ml LPS, 1 hr, then 25 nM C5a; (E) 500 units/ml mouse TNFα, 30 min, then 3 μM fMLP; (F) 500 nM PMA; or (G) zymosan opsonized with rabbit anti-zymosan antibody (5 particles per neutrophil); and ROS formation was measured in the presence of horseradish peroxidase and luminol in a temperature-controlled luminometer at 37°C as described [26]. Stimuli in (A–E) were delivered through automated injection ports. Data are means ± standard deviation (n = 3) from one experiment representative of three to six. Open symbols are mock-stimulated controls that, in (C) and (D), included priming with LPS.

seems greater than in "professionally" chemotaxing neutrophils.

Recruitment of P-Rex1$^{-/-}$ Neutrophils to Sites of Inflammation

Neutrophil recruitment to sites of infection is crucial for host defense against bacteria and fungi. This involves neutrophil chemotaxis but also their ability to leave the blood stream and reach the infected site. We measured neutrophil recruitment to sites of inflammation in P-Rex1$^{-/-}$ animals in an aseptic-peritonitis model [6, 11], with thioglycollate as the inflammatory agent. Leukocytes present in the peritoneum of untreated animals were mainly macrophages but no neutrophils (Figure 4F). Thioglycollate elicited a robust recruitment of neutrophils to the peritoneum of P-Rex1$^{+/+}$ animals within 4–5 hr. This was reduced by 50% in P-Rex1$^{-/-}$ animals (Figure 4F). Hence, P-Rex1 controls neutrophil recruitment to sites of inflammation in intact animals.

Degranulation of Azurophil Granules from Neutrophils of P-Rex1$^{-/-}$ Mice

An important Rac2-dependent neutrophil response is degranulation of azurophil myeloperoxidase (MPO)-containing granules [4, 8]. We measured MPO secretion in cytochalasin B-pretreated fMLP-stimulated neutrophils. It was unaffected by the P-Rex1-deficiency (Figure S4). The amount of MPO was equal in P-Rex1$^{-/-}$ and P-Rex1$^{+/+}$ cells (data not shown), and the level of degranulation was comparable to previous reports (fMLP-stimulated MPO secretion in Figure S4 corresponds to 35%) [8]. Without finding a defect, we measured fMLP- and C5a-stimulated degranulation with or without cytochalasin B pretreatment and LPS or TNFα priming, over timeframes of 30 s to 90 min and over wide dose ranges, or upon Ig-opsonized zymosan-stimulation (data not shown). Hence, P-Rex1 does not regulate Rac2 pathways leading to azurophil granule release.

The Role of P-Rex1 in Neutrophil Responses

This paper shows that P-Rex1 regulates Rac2 activation and ROS formation in neutrophils stimulated via GPCRs, as well as neutrophil recruitment to inflammatory sites. It also demonstrates a surprising role for P-Rex1 in LPS priming of GPCR-dependent ROS formation. Together, our results suggest that P-Rex1 is an

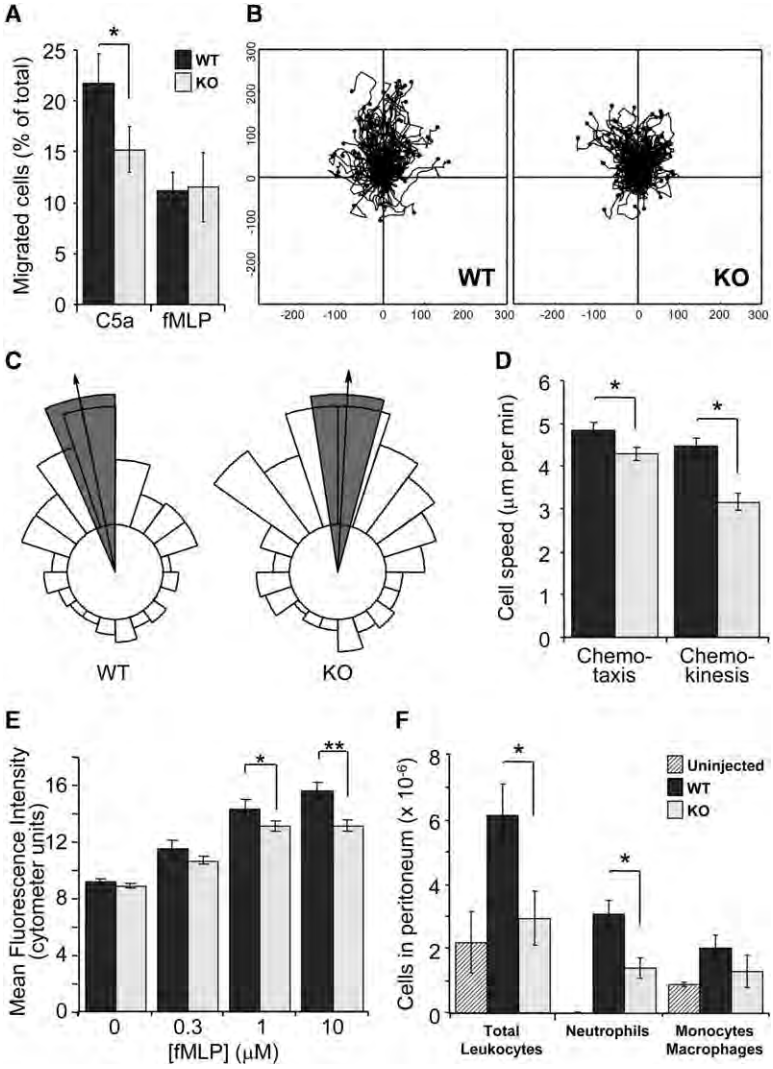

Figure 4. Chemotaxis of P-Rex1$^{-/-}$ Neutrophils Is Mildly Impaired

(A) Transwell assay: Bone-marrow-derived neutrophils from P-Rex1$^{+/+}$ (black bars) and P-Rex1$^{-/-}$ (gray bars) mice were stimulated with 1 μM fMLP, 3 nM C5a, or vehicle for 30 min at 37°C in a 24-well format transwell assay (Millipore, Millicell-PC, 3 μm pores). Cells that had migrated through the filter were counted with a Coulter counter. Unstimulated migration through the filter (1.43 ± 0.17% of P-Rex1$^{+/+}$ and 2.17 ± 0.40 of P-Rex1$^{-/-}$ cells) was subtracted. Data are means ± SEM of 12 experiments with C5a and 8 with fMLP.

(B) Cell-track diagrams of migrating cells in a Dunn chamber chemotaxis assay: Bone-marrow-derived neutrophils from P-Rex1$^{+/+}$ and P-Rex1$^{-/-}$ mice were assayed in a Dunn chamber [28], in a 0–300 nM fMLP gradient for 30 min at 37°C. The diagrams show the combined traces from four independent experiments. Units are μm. The source of fMLP is at the center top of the diagram.

(C) Horizon plots of cell tracks shown in (B): As a measure of directionality, the position of each cell at its origin and at the 20 μm horizon was used to calculate its angle of movement relative to the orientation of the gradient, with Mathematica software (highest concentration of fMLP at center top of diagram). Wedges show the proportion of vectors within an 18° segment. Arrows shows the mean angle of movement (11° for P-Rex1$^{+/+}$ and 3° for P-Rex1$^{-/-}$ cells, relative to the orientation of the gradient). Gray wedges show the 95% confidence limit for cell trajectories (23° for P-Rex1$^{+/+}$ and 15° for P-Rex1$^{-/-}$ cells, relative to the orientation of the gradient).

(D) Average speed in chemotaxis and chemokinesis: The average speeds (means ± SEM) of the all the P-Rex1$^{+/+}$ (black bars) and P-Rex1$^{-/-}$ neutrophils (gray bars) in (B) (0–300 nM fMLP gradient), and those in Figure S3C (uniform 150–150 nM fMLP gradient), were calculated with Mathematica software to give values for speed of chemotaxis and chemokinesis, respectively.

(E) Actin polymerization: Bone-marrow-derived neutrophils from P-Rex1$^{+/+}$ (black bars) and P-Rex1$^{-/-}$ mice (gray bars) were stimulated for 30 s with the indicated concentrations of fMLP, fixed, and permeabilized, stained for polimerized actin with TRITC-phalloidin and their fluorescence levels analyzed by FACS. Results are means ± SEM from three independent experiments.

(F) Aseptic peritonitis model [6, 13]: P-Rex1$^{+/+}$ (black bars) and P-Rex1$^{-/-}$ mice (gray bars) were injected intraperitoneally with 0.25 ml of 3% thioglycollate. After 4.5 hr, leukocytes in the peritoneal exudates were counted and assessed for proportions of neutrophils, monocytes, and macrophages by microscopy and flow cytometry. Data are means ± SEM of three experiments performed with at least four injected animals per group plus one uninjected P-Rex1$^{+/+}$ control animal (hatched bars). Statistical analysis is unpaired Student's t test.

important mediator of neutrophil-dependent host defense. However, they also show that P-Rex1 is not an essential regulator of all Rac2-dependent neutrophil responses downstream of GPCRs. Notably, chemotaxis is only mildly affected by the P-Rex1 deficiency, and degranulation is unaffected. These results suggest two scenarios, possibly in combination: The levels of active Rac2 required for ROS formation might be higher than for chemotaxis or degranulation, particularly in LPS-primed cells, so the partial inactivation caused by P-Rex1 deficiency could suffice to impair the former but not the latter cellular responses. Alternatively, P-Rex1 could scaffold the pool of Rac2 needed for ROS formation, but not those for chemotaxis and degranulation, assuming such specialized pools exists. In any event, other Rac-GEFs than P-Rex1 must regulate the pathways governing neutrophil chemotaxis and degranulation.

About a dozen Rac-GEFs have tissue distributions that would allow them to regulate neutrophil function, but only the Vav family, best known for its role in B and T cell development and signaling, has so far been assayed for this. Vav1$^{-/-}$ mice have a similar neutrophil phenotype to that of P-Rex1$^{-/-}$ mice, with a defect in fMLP-induced ROS formation and a minor impairment in fMLP-induced chemotaxis, although Rac activation and thioglycollate-induced neutrophil recruitment to the peritoneum are normal [19]. Even deletion of Vav1 and Vav3, which together make up 99% of total neutrophil Vav, does not impair neutrophil chemotaxis and peritoneal recruitment, whereas integrin-dependent functions such as cell spreading and complement-

mediated phagocytosis are defective [20]. Hence, there might be some functional overlap between the Vav family and P-Rex1 in neutrophils, and possible redundancy needs to be investigated.

The Dock and Tiam families of Rac-GEFs also signal downstream of GPCRs and are therefore candidate regulators for those GPCR-dependent neutrophil responses that are not governed by P-Rex1 or the Vav family [21, 22]. Irrespective of their identity, the notion that several Rac-GEFs must govern neutrophil responses downstream of GPCRs gives us valuable insights into the complexity of the signaling pathways involved, without even considering that GPCRs are only one of several routes to Rac activation, and that other GTPases like CDC42 and RhoA and their GEFs also play crucial roles in regulating neutrophil responses [2, 23, 24].

Conclusions

P-Rex1 governs GPCR-dependent Rac2 activation and ROS formation in neutrophils, as well as neutrophil recruitment to inflammatory sites, and it plays a modulatory role in neutrophil chemotaxis, but not in degranulation. This defines P-Rex1 as an important regulator of neutrophil responses.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four Supplemental Figures, and two Supplemental Movies and are available with this article online at http://www.current-biology.com/cgi/content/full/15/20/1867/DC1/.

Acknowledgments

We thank the Babraham Institute's Gene Targeting Facility, Small Animal Breeding Unit, and Flow Cytometry Facility for their meticulous work; Sarah Donald and Mark Barber from the Welch lab for help with genotyping and assays; Sylvia Thelen (IRB, Bellinzona) for help with developing the 6F12 antibody; Gary Bokoch (Scripps) for the gift of anti-Rac2 antibody; Anthony Green's lab (Dept. Haematology, Cambridge University) for use of their animal cell counter; and Martin Turner for critically reading the manuscript. A.M.C. was the recipient of a Wellcome Intermediate Fellowship. K.H. received a PhD case studentship from the Medical Research Council and Novartis. H.C.E.W. holds a Career Development Award from the Medical Research Council.

Received: July 8, 2005
Revised: August 31, 2005
Accepted: September 6, 2005
Published: October 25, 2005

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