ACCELERATED PUBLICATION

Ras activation in response to lysophosphatidic acid requires a permissive input from the epidermal growth factor receptor

Ignacio RUBIO¹, Knut RENNERT, Ute WITTIG and Reinhard WETZKER

Institute of Molecular Cell Biology, Medical Faculty, Friedrich-Schiller-University, Drackendorfer Strasse 1, 07747 Jena, Germany

The topology of the signalling pathway linking the G-protein-coupled receptor agonist lysophosphatidic acid (LPA) to extracellular-signal-regulated kinase activation remains undeciphered. In the present study, we report that analysis of LPA signals at the level of Ras-GTP formation and Ras nucleotide exchange discriminates true mediatory signals from permissive activities that do not participate in signal relay. Hence, whereas pertussis toxin (PTX) treatment impairs stimulation of nucleotide exchange, epidermal growth factor receptor (EGFR) inhibition does

not compromise LPA-induced acceleration of nucleotide exchange, but instead attenuates basal nucleotide turnover on Ras. Our data indicate that LPA activation of Ras proceeds via PTX-sensitive $G_{i/o}$ -proteins and requires a permissive input from basal EGFR activity.

Key words: epidermal growth factor receptor (EGFR), lysophosphatidic acid (LPA), nucleotide exchange, pertussis (PTX) toxin, Ras, transactivation.

INTRODUCTION

According to a firmly established model, occupancy of receptor tyrosine kinases (RTKs) initiates tyrosine phosphorylation of the RTK and of adapter proteins, including Shc, leading to the recruitment of the Grb-2/Sos guanine nucleotide-exchange factor (GEF) complex and activation of Ras as a result of elevated nucleotide exchange. In contrast, mechanisms underlying Ras activation in response to G-protein-coupled receptor (GPCR) agonists remain more poorly characterized. The consensus view is that GPCR agonists, acting via pertussis toxin (PTX)-sensitive heterotrimeric G-proteins of the $G_{i/o}$ type, prominently amongst them lysophosphatidic acid (LPA), engage a tyrosine kinase to activate the same Shc/Grb-2/Sos pathway that is also utilized by RTKs [1–9]. Accordingly, the interest of numerous investigators has focused on unveiling the identity of the tyrosine kinase(s) that mediate tyrosine phosphorylation of Shc in response to GPCR engagement.

Various studies document that LPA and other GPCR agonists induce transactivation of the epidermal growth factor (EGF) receptor (EGFR) in fibroblasts via the proteolytic release of heparin-binding EGF-like growth factor (HB-EGF) [3,5,10,11]. There is compelling evidence illustrating that the inhibition of EGFR-dependent signalling interrupts a variety of LPA-elicited responses, including tyrosine phosphorylation of Shc and Gab-1 adapter proteins [3,5,10,12,13], Ras activation [14], extracellular-signal-regulated kinase (ERK) activation [3,5,10,12,15–17], ERK-dependent gene expression [17] and LPA-dependent DNA synthesis [3,17]. These reports identify the EGFR RTK as a potential mediator in LPA signalling to Shc/Grb-2/Sos.

On the other hand, several studies based, in part, on the use of PTX have yielded puzzling observations concerning the involvement of EGFR and/or Shc in LPA signalling to Ras/ERK. PTX impairs Ras and ERK activation by LPA by blocking G-proteins of the $G_{i/o}$ subclass. As this represents a most receptor proximal

interruption, PTX is inferred to block downstream events on the assumed linear pathway to Ras. With regard to the possible mediatory role of Shc in LPA signalling to Ras, one laboratory has documented PTX-sensitive Shc phosphorylation by LPA in fibroblasts [2,18,19], whereas others observed no such effect of PTX [20]. At the level of EGFR transactivation, available experimental evidence is also inconclusive. PTX blocks LPA-induced EGFR tyrosine phosphorylation in some cell types, indicating that $G_{i/o}$ -proteins and EGFR lie on the same pathway linking the LPA receptor to Ras/ERK [21,22]. In other cellular backgrounds, PTX does not impinge, or has only moderate effects, on LPA-induced EGFR transactivation [11,12,14]. Yet in other cell types, LPA induces PTX-sensitive activation of the Ras/ERK pathway in the absence of EGFR transactivation [5,24,25] and/or independently of EGFR activity [23,24], arguing against the participation of EGFR in LPA signalling to Ras. In conclusion, although a requirement for EGFR in LPA activation of the ERK pathway is firmly documented in various cellular systems, several lines of experimental evidence are not readily reconciled with a role of the EGFR as a constituent of PTX-sensitive pathway(s) targeting Ras.

To shed light on this issue, we have investigated the role of EGFR in LPA signalling at the level of Ras activation. The experiments described in the present paper exclude the EGFR as a mediator of LPA signalling to Ras in COS-7 cells and reveal that basal EGFR activity is permissive for LPA-induced accumulation of Ras-GTP.

EXPERIMENTAL

Materials

PTX, LPA and streptolysin O were purchased from Sigma. PD153035, AG1478 and AG1296 were from Calbiochem.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular-signal-regulated kinase; GEF, guanine nucleotide-exchange factor; GPCR, G-protein-coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; LPA, lysophosphatidic acid; PDGFR, platelet-derived growth factor receptor; PTX, pertussis toxin; RBD, Ras-binding domain of c-Raf1; RTK, receptor tyrosine kinase; S1P, sphingosine 1-phosphate.

¹ To whom correspondence should be addressed (e-mail b5igru@rz.uni-jena.de).

Diphtheria toxin mutant protein CRM197 was from List Biologicals (Campbell, CA, U.S.A.). [α - 32 P]GTP (3000 Ci/mmol) was from NEN Life Science Products (Brussels, Belgium). PMA was from ICN Biomedicals (Eschwege, Germany). Glutathione–Sepharose and Protein G–Sepharose (Gamma-Bind) were from Amersham Biosciences. EGF and sphingosine 1-phosphate (S1P) were gifts from Dr F. Böhmer (Institute of Molecular Cell Biology, Friedrich-Schiller-University) and Dr R. Heller (Institute of Molecular Cell Biology, Friedrich-Schiller-University) respectively.

Antibodies

Anti-pan-Ras (Ab-4) was from Oncogene Science (Cambridge, MA, U.S.A.). Y13-259 rat monoclonal anti-Ras antibodies were purified from hybridoma cells (A. T. C. C., Manassas, VA, U.S.A.). 4G-10 monoclonal anti-phosphotyrosine, and polyclonal anti-Shc and anti-EGFR antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Monoclonal anti-Shc was from Transduction Laboratories (Lexington, KY, U.S.A.).

Cell culture

COS-7 cells (A. T. C. C.) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) foetal calf serum at 37 °C in a 5 % CO₂ incubator. For all experiments cells were serum-starved for 24 h in DMEM.

Permeabilization, nucleotide-exchange measurement and analysis of Ras-bound nucleotides

Nucleotide-exchange measurement in permeabilized COS-7 cells was performed exactly as described in [28]. The procedure involves cell permeabilization in the presence of [α - 32 P]GTP, followed by Ras immunoprecipitation at various time points [26,27]. Final Ras immunoprecipitates were subjected to Cerenkov counting. After counting, Ras-bound nucleotides were eluted and analysed by TLC [26,27] or HPLC [28]. In all panels, zero-time as recorded is defined by quenching of the first assay point 5 min after streptolysin O addition, and 1 or 5 min after administration of growth factors. For inhibitor-treated cells, all solutions were supplemented with the relevant drugs during the permeabilization protocol.

Ras-binding domain of c-Raf1 (RBD) pull-down assays for analysis of Ras-GTP levels

Serum-starved COS-7 cells seeded in six-well plates were treated/stimulated as appropriate and Ras-GTP was pulled down from cell extracts with the RBD. Samples were processed exactly as described [33].

Immunoprecipitations

Serum-starved COS-7 cells seeded in 10 cm dishes were treated as appropriate with inhibitors/agonists and lysed in 1.5 ml of ice-cold lysis solution (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % Nonidet P40, protease inhibitors and phosphatase inhibitors). Cell material was scraped off and lysates were cleared by centrifugation at 20 000 *g* for 20 min. Supernatants were incubated for 1 h with 3 μ g of immunoprecipitating antibody for Shc (polyclonal) or EGFR. Immunocomplexes were collected on Protein G–Sepharose by a 2 h incubation with constant rotation. Precipitates were washed three times with lysis solution,

and analysed by Western blotting with anti-phosphotyrosine, monoclonal anti-Shc and anti-EGFR antibodies.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. Two-sample comparisons were performed with Student's *t* test. A *P* value < 0.05 was considered significant.

RESULTS

LPA activation of Ras involves EGFR and PTX-sensitive G-proteins

We used COS-7 cells in the present study because both EGFR inhibition and PTX treatment compromise ERK activation in response to LPA in these cells [5,12,20,29]. In line with those findings, LPA-induced formation of Ras-GTP was abolished in cells exposed to AG1478 or PD153035 (200 nM), two selective EGFR inhibitors, whereas the platelet-derived growth factor receptor (PDGFR)-selective drug AG1296 (1 μ M) had no effect (Figure 1A, and results not shown). In contrast, AG1478 only marginally impinged on Ras and ERK activation induced by the phorbol ester PMA (Figure 1A, and results not shown) [30,31]. To substantiate the inhibitor results, we used the diphtheria toxin mutant CRM197, which interrupts LPA-induced EGFR transactivation and Shc tyrosine phosphorylation in COS-7 cells by sequestering HB-EGF [10,31]. Although not as potent as AG1478, CRM197 markedly reduced LPA-induced Ras-GTP accumulation by > 50 % (Figure 1A). Taken together, these experiments evidenced a requirement for the EGFR in LPA activation of Ras.

PTX reportedly impairs LPA-induced ERK activation in COS-7 cells [20,29]. As shown in Figure 1(B), inhibition was also evident at the level of Ras, in line with previous observations in other cell types [6,7,14]. Ras-GTP formation in response to PMA or EGF was unaffected by PTX (Figure 1B, and results not shown), confirming specific action of the toxin. Since COS-7 cells do not express G_o-proteins [32], these data indicate that G_i-proteins are involved in Ras activation by LPA in this cell type.

The inhibition exerted by PTX is situated most proximal to the LPA receptor and should thus compromise downstream events on a presumptive linear pathway to Ras. We therefore tested whether or not PTX treatment impaired activation of EGFR and EGFR-dependent tyrosine phosphorylation of Shc in response to LPA. In agreement with Daub et al. [12], LPA-induced tyrosine phosphorylation of EGFR was only partially attenuated in PTX-treated cells (10–15 % inhibition under the experimental conditions used) (Figure 1C). At the level of tyrosine phosphorylation of Shc, no effect of PTX was apparent. Shc/EGFR co-immunoprecipitation upon LPA administration was also not compromised in PTX-treated cells (Figure 1C). These results indicate that the majority of EGFR-dependent LPA signalling does not lie downstream of PTX-sensitive G-proteins. Since PTX inhibited LPA-induced Ras-GTP formation by over 80 % (Figure 1B), the simplest interpretation is that LPA activation of Ras requires independent inputs from EGFR and PTX-sensitive G-proteins and that these two activities reside on separate pathways downstream of the LPA receptor.

LPA stimulates nucleotide exchange on Ras

What is the nature of the EGFR and G_i-protein inputs in LPA signalling to Ras? LPA-elicited tyrosine phosphorylation of Shc

Figure 1 Ras-GTP accumulation in response to LPA requires independent inputs from EGFR and PTX-sensitive G-proteins

(A) Serum-starved COS-7 cells were exposed to AG1478 (250 nM, 10 min), CRM197 (5 µg/ml, 45 min) or the respective vehicles and challenged with 10 µM LPA or 1 µM PMA (TPA) for the indicated lengths of time. Active Ras-GTP was pulled down from cell extracts and visualized as described in the Experimental section. (B) Same as in (A), except COS-7 cells were serum-starved for 16 h in the presence of 250 ng/ml PTX or vehicle before administration of TPA or LPA. (C) Serum-starved COS-7 cells were treated with PTX or vehicle as in (B), followed by stimulation with 10 µM LPA for the indicated time periods. EGFR and Shc were immunoprecipitated (IP) from the corresponding cell extracts and immunoprecipitates were analysed by Western blotting (WB) as indicated on the left of the panels. Molecular-mass markers (in kDa) are given on the right side of the panels. The tyrosine-phosphorylated band around 170 kDa associated with Shc immunoprecipitates was identified as the EGFR using specific EGFR antibodies (results not shown). pY, phosphotyrosine. All experiments were performed at least three times with similar results.

is unaffected by PTX treatment in our experimental system, but depends on the activity of the EGFR [10,12] (results not shown). Because extensive experimental evidence links Shc tyrosine phosphorylation to the activation of Sos, it seems likely that EGFR/Shc-dependent Sos recruitment underlies LPA-induced activation of Ras. According to this scenario, LPA-induced Ras activation should ultimately result from Sos-dependent stimulation of nucleotide exchange, but this has not yet been rigorously addressed.

To collect evidence in favour of, or against, this model, we investigated whether or not LPA stimulates nucleotide exchange on Ras. For the analysis of nucleotide exchange on cellular Ras, COS-7 cells were permeabilized with streptolysin O in the presence of radiolabelled GTP, and the amount of radioactivity associated with Ras immunoprecipitates was evaluated after increasing periods of time. As shown previously for other cell types [26,27,33], the assay monitored the reversible binding of radiolabelled guanine nucleotides to endogenous COS-7 Ras proteins specifically (results not shown). To test if the assay detected growth-factor-dependent changes in nucleotide exchange, we first employed EGF as a well-established Sos activator. In line with previous findings [26,27,34–36], exposure of permeabilized cells to EGF elevated the rate of nucleotide uptake by Ras (Figure 2A), consistent with the notion that EGF engages Ras-GEF systems, most probably Grb-2/Sos, to speed up nucleotide exchange on Ras. Acceleration of nucleotide uptake was accompanied by accumulation of Ras-GTP (Figure 2B) (see below), which demonstrated that EGF-dependent signal transduction remained operational in permeabilized cells.

To answer the question whether LPA stimulates GEFs to activate Ras, we administered LPA to permeabilized [α - 32 P]GTP-loaded COS-7 cells and measured the nucleotide-uptake rate as described above. LPA induced a clear acceleration of nucleotide uptake (Figure 2C) concomitant with Ras-GTP accumulation

[$13.6 \pm 2.1\%$ GTP/(GDP + GTP) compared with $30.7 \pm 3.3\%$ in cells treated with 5 ng/ml EGF and $6.3 \pm 0.7\%$ in unstimulated cells; all values calculated at 6 min as recorded in Figure 2] (Figure 2D). The related lysophospholipid S1P did not affect nucleotide turnover on Ras (Figure 2C), nor did it promote Ras-GTP accumulation in Raf-RBD pull-down experiments (results not shown), consistent with published data arguing for the absence of the relevant S1P receptors in COS-7 cells [37]. We conclude from these data that LPA activation of Ras proceeds via stimulation of nucleotide exchange.

LPA-induced stimulation of Ras nucleotide exchange is mediated by PTX-sensitive G-proteins and does not involve the EGFR

To investigate the possibility that EGFR-dependent engagement of Grb-2/Sos underlies LPA stimulation of nucleotide exchange, we treated cells with the EGFR-inhibitor AG1478 and measured nucleotide exchange induced by LPA. As shown in Figure 3(A), AG1478 treatment caused a reduction in basal nucleotide turnover on Ras. The effect of AG1478 was mimicked by CRM197, whereas the PDGFR blocker AG1296 caused no alteration of basal nucleotide uptake (results not shown). Remarkably, LPA stimulated nucleotide exchange on Ras in a background of EGFR inhibition, as illustrated by the increased nucleotide uptake in AG1478-treated and LPA-challenged cells compared with cells treated only with the EGFR inhibitor (Figure 3A). Because fold-stimulation of nucleotide uptake induced by LPA was not affected by AG1478 treatment (Figure 3C, left-hand panel), we conclude that AG1478-sensitive activities do not participate in the activation of Ras-GEFs by LPA.

As this excluded the EGFR as a mediator in LPA signalling to Ras-GEFs, we tested the effect of PTX in the same assay. As opposed to EGFR inhibition, PTX treatment did not down-regulate basal nucleotide turnover in serum-starved COS-7 cells,

Figure 2 LPA stimulates nucleotide exchange on Ras

(A) EGF stimulates nucleotide exchange on Ras. Serum-starved COS-7 cells were permeabilized in the presence of [α - 32 P]GTP and exposed to 10 ng/ml EGF for 1 min before pore formation and quenching of the first assay point (zero-time as recorded). The chart displays time-dependent association of radioactivity to Ras immunoprecipitates determined by Cerenkov counting. (B) TLC separation of nucleotides bound to the Ras immunoprecipitates processed in (A). (C) LPA stimulates nucleotide exchange on Ras. Serum-starved COS-7 cells were permeabilized in the presence of [α - 32 P]GTP and challenged with 10 μ M LPA or 2 μ M SIP for 5 min before zero-time as recorded. Samples were processed as described above in the legend. (D) TLC separation of nucleotides bound to Ras immunoprecipitates processed in (B). Differences in absolute c.p.m. values associated with Ras immunoprecipitates among individual experiments, e.g. between (A) and (C), reflect the use of different batches of [α - 32 P]GTP. All panels depict representative results of at least four independent experiments.

but markedly reduced LPA-evoked stimulation of nucleotide exchange (Figures 3B and 3C). Together with the observation that the $G\beta\gamma$ subunits of heterotrimeric G_i -proteins activate ERK via Ras in COS-7 cells and other fibroblasts [1,9,38–40], these data represent strong evidence that G_i -proteins mediate activation of Ras-GEFs by LPA.

DISCUSSION

Using an established permeabilization assay [26–28,33–36], we have demonstrated that LPA stimulates nucleotide exchange on Ras in COS-7 cells, strongly indicating that LPA stimulates Ras-GEFs to activate Ras. Remarkably, although EGFR inhibition and PTX application result in largely indistinguishable inhibition of LPA-induced net Ras-GTP accumulation, these treatments have radically different consequences at the level of nucleotide exchange. PTX impairs up-regulation of nucleotide exchange by LPA, indicating that G_i -proteins function as intermediates linking LPA receptors to Ras-GEFs. In contrast, fold-stimulation of nucleotide exchange evoked by LPA is not affected by EGFR inhibition, demonstrating that EGFR does not relay the signal from LPA to Ras-GEFs. This implies that transactivation of the EGFR must not necessarily occur downstream of PTX-sensitive G -proteins, consistent with previous observations [11,12,14,20] and the findings of the present study (Figure 1C). Furthermore,

Figure 3 Differential regulation of basal compared with LPA-induced nucleotide exchange by EGFR and PTX-sensitive G -proteins

(A) Serum-starved COS-7 cells were exposed to 250 nM AG1478 for 10 min or left untreated, followed by 10 μ M LPA or 5% (w/v) BSA vehicle administration for 5 min before permeabilization in the presence of [α - 32 P]GTP. Samples were processed as described in the legend to Figure 2. Shown here is one out of three independent experiments that yielded similar results. (B) COS-7 cells were serum-starved overnight in the absence or presence of 250 ng/ml PTX, followed by administration of 10 μ M LPA or 5% (w/v) BSA vehicle for 5 min before permeabilization in the presence of [α - 32 P]GTP. Samples were processed as described in the legend to Figure 2. Two additional experiments produced virtually identical results. (C) Quantification of data presented in (A) and (B). The left-hand panel illustrates effect of AG1478 and PTX on LPA-induced stimulation of nucleotide exchange. The amount of GDP + GTP bound to Ras 6 min after chasing the first assay point was calculated as the fold-increase of radioactivity associated with Ras over the relevant control value in untreated or inhibitor-treated cells without agonist stimulation. The right-hand panel illustrates the effect of AG1478 and PTX on the basal rate of nucleotide uptake in serum-starved cells. The amount of GDP + GTP bound to Ras was evaluated at 6 min as recorded and plotted as percentage of the value from untreated cells (100%). * $P < 0.05$ compared with untreated. Results are means $\pm$ S.E.M. of at least three independent experiments.

previous data do support the idea that the EGFR and PTX-sensitive G -proteins fulfil different functions in LPA signalling to the Ras/ERK pathway. For example, in Rat-1 fibroblasts and smooth muscle cells, PTX exerts a complete block on LPA-induced Ras and/or ERK activation [6,7,16,18,20,38,41], whereas EGFR inhibition results in only partial attenuation of ERK activation

[3,16,41]. In Vero cells, PTX treatment and EGFR inhibition indistinctively abrogate Ras activation in response to LPA [14], whereas PTX does not compromise LPA-elicited EGFR transactivation [11,14]. Finally, in various cellular backgrounds, LPA elicits PTX-sensitive activation of the Ras/ERK pathway in the absence of EGFR activation [5,23–25]. All these findings support the notion that EGFR takes over a function different to that of $G_{i/o}$ -proteins, and by doing so contributes to full activation of Ras by LPA in some cellular backgrounds.

The conclusions drawn above for the EGFR extend to Shc and the role of its tyrosine phosphorylation since, in COS-7 cells, LPA-elicited tyrosine phosphorylation of Shc is fully dependent on the EGFR [10,12] (results not shown). Hence the present study confirms previous findings [20] and illustrates that LPA-induced tyrosine phosphorylation of Shc is neither sufficient nor required for the activation of Ras-GEF systems by LPA. This notion is not unprecedented as, for example, endothelin-1 reportedly fails to activate Ras in Rat-1 cells [6,42], despite its ability to induce tyrosine phosphorylation of EGFR and Shc to a similar extent as LPA [3].

The present study demonstrates that EGFR inhibition severely compromises Ras-GTP formation in response to LPA, despite the fact that EGFR does not mediate activation of Ras-GEFs by LPA. The permeabilization experiments provide a plausible explanation for this apparent paradox, since they illustrate a marked decline in basal nucleotide turnover on Ras as a result of EGFR inhibition. This observation indicates that basal EGFR activity drives constitutively operating Ras-GEF systems in serum-arrested COS-7 cells. Taking all the data together, the most straightforward interpretation is that the decline in basal nucleotide exchange upon application of EGFR inhibitors effectively raises the threshold for the LPA-induced stimulation of nucleotide exchange, which proceeds independently of the EGFR. Along this line of reasoning we hypothesize that 'futile Ras-GDP/GTP cycling' as driven by basal EGFR activity could provide an amplification system for secondary Ras-activatory inputs elicited by agents such as LPA, but possibly not by others, such as phorbol ester.

In conclusion, our data reveal a permissive function of basal EGFR activity and confirm a mediatory role of G_i -proteins in LPA activation of Ras. This scenario rationalizes previous contradictory observations regarding the role of EGFR and PTX-sensitive G-proteins in LPA signalling. Beyond that, the present paper stresses the importance of discriminating between permissive and true mediatory activities when studying the topology of signalling pathways.

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