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RESEARCH COMMUNICATION

Platelet-derived growth factor activation of mitogen-activated protein kinase depends on the sequential activation of phosphatidylcholine-specific phospholipase C, protein kinase C-ζ and Raf-1

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The mechanism of Raf-1 activation by platelet-derived growth factor (PDGF) is poorly defined. We previously reported that, in Rat-1 fibroblasts, PDGF activates a phosphatidylcholine-specific phospholipase C (PC-PLC) and that the product, diacylglycerol, somehow activates protein kinase C-ζ (PKC-ζ). Both PC-PLC and PKC-ζ activities were required for PDGF activation of mitogen-activated protein kinase (MAPK). Now we report that MAPK activation by exogenous PC-PLC depends on Raf-1 activation. PKC-ζ co-immunoprecipitates with, phoshorylates and activates Raf-1, suggesting that in the PDGF- and PC-PLC-activated MAPK pathway, PKC-ζ operates in a signalling complex as a direct activator of Raf-1.

INTRODUCTION

Platelet-derived growth factor (PDGF) activation of the mitogen-activated protein kinase (MAPK or ERK) pathway is known to involve the sequential activation of Ras, Raf-1 and MAPK kinase (MEK) [1]. Activated Ras recruits Raf-1 to the plasma membrane where it becomes activated by poorly defined mechanisms, including phosphorylations [2,3]. Kinases thus far shown to phosphorylate and activate Raf-1 are Src [4,5] and phorbol ester-responsive protein kinase C (PKC) isotypes α, β and ϵ [6–10]. Remarkably, PKC-mediated activation of the Raf-1–MAPK pathway can occur independently of Ras [8–13].

We previously reported that, in Rat-1 fibroblasts, PDGF elicits phosphatidylcholine (PC) hydrolysis by phospholipases D (PLD) and -C (PLC) [14]. Based on inhibitor (D609) studies, phosphatidylcholine-specific-PLC (PC-PLC), but not PLD, was implicated in MAPK activation by PDGF [14]. We furthermore demonstrated that exogenous PC-PLC (from *Bacillus cereus*) mimicked endogenous PC-PLC in generating diacylglycerol (DG), and mimicked PDGF in activating PKC-ζ and MAPK, but without Ras activation [13]. Finally, we showed that activation of PKC-ζ, rather than classical or 'new' PKC isotypes, was required for MAPK activation by PDGF and PC-PLC in this cell system [13]. However, the target of PKC-ζ in the activation of the MAPK pathway remained to be identified.

The present study aims at elucidating how PDGF activation of PC-PLC and PKC-ζ couples to the Raf-1–MAPK pathway. We report that, in Rat-1 cells, PC-derived DG by itself leads to activation of Raf-1. Using a dominant-negative construct or 8-bromo-cAMP (8-Br-cAMP) to inhibit Raf-1, we show that Raf-1 activation, like PKC-ζ activation, is required for PDGF- and PC-PLC-induced MAPK activation. We present evidence that PKC-ζ directly interacts with Raf-1 in a signalling complex in which it phosphorylates and activates Raf-1. The results suggest that, in the PDGF-stimulated MAPK pathway, the sequential activation of PC-PLC and PKC-ζ is directly linked to Raf-1 activation.

EXPERIMENTAL

Materials, plasmids, antibodies and cell culture

[^{32}P]P_i, [γ-^{32}P]ATP, [^{3}H]thymidine and the enhanced chemiluminescence (ECL) system were from Amersham. Epidermal growth factor (EGF) was from Collaborative Research. Human recombinant PDGF-B/B, PC-PLC (from *B. cereus*), 8-Br-cAMP, dithiothreitol and leupeptin were from Boehringer Mannheim. PMSF and myelin basic protein were from Sigma. Aprotinin was from Fluka and orthovanadate from BDH. Ro-31-8220 (compound 3 [15]) was from Roche Research Centre (Welwyn Garden City, Herts., U.K.). Glutathione–Sepharose CL-4B and Protein A–Sepharose CL-4B were from Pharmacia. Dulbecco's modified Eagle's medium (DMEM) was from Gibco and P_i-free minimal essential medium from Flow.

pEXV-ERK2 containing a C-terminal epitope-tag from c-*myc* [16], pmtSM-PKC-ζ [17], pmtSM-PKC-ζ(K281W) [13] and pmtM-NΔRaf [16] have been described previously. Haemagglutinin (HA)-tagged Raf-1 was generated by subcloning human Raf-1 [16] in an pmtSM-HA expression vector.

Monoclonal anti-Raf-1 (R19120) was obtained from Transduction Labs. Polyclonal anti-PKC-ζ, anti-Raf-1 (SP-63) and anti-MAPK (ERK2) were raised against C-terminal peptides of these proteins. Monoclonal antibody 9E10 is directed against a C-terminal epitope of c-*myc*. Monoclonal antibody 12CA5 is directed against an epitope of HA. Peroxidase-conjugated antibodies, normal mouse serum and rabbit-(anti-mouse) serum were from DAKO (Gastrup, Denmark).

Rat-1 and COS7-M6 cells were grown in 5 cm plastic dishes in DMEM containing 7.5% (v/v) fetal calf serum (Life Technologies Inc.) to 80% confluency, and serum-starved for 24 h before stimulation.

Protein kinase assays

Measurement of MAPK (ERK2) phosphorylation and activity

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DG, diacylglycerol; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; GST, glutathione S-transferase; HA, haemagglutinin; LB, lysis buffer; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NP40, Nonidet-P40; NΔRaf, regulatory domain of Raf-1; PC-PLC, phosphatidylcholine-specific phospholipase C; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLD, phospholipase D; 8-Br-cAMP, 8-bromo-cAMP.

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was performed exactly as described previously [13]. Briefly, an electrophoretic mobility shift, visualized by Western blotting, is indicative of MAPK phosphorylation and activation [18]. To analyse kinase activity, cells were transfected with pEXV-ERK2 (myc-tagged) with or without pmtM-NΔRaf [16] and stimulated 2 days later. MAPK was immunoprecipitated with anti-myc antibody 9E10 and subjected to an *in vitro* kinase assay using myelin basic protein (250 μg/ml) as a substrate.

To determine Raf-1 kinase activity, cells were lysed with non-denaturating lysis buffer (LB): 10 mM Tris/HCl, pH 7.4/ 150 mM NaCl/1 mM EDTA/1 mM EGTA/0.2 mM Na_3VO_4/ 0.2 mM PMSF/1 % (v/v) Triton X-100/0.5 % (v/v) Nonidet P40 (NP40)/1 μg/ml aprotinin/1 μg/ml leupeptin. Lysates were clarified by centrifugation (15000 ***g*** for 10 min) and precleared twice for 1 h with 3 μl of normal mouse serum and protein A–Sepharose. Raf-1 was immunoprecipitated, washed three times in LB, twice in 10 mM Pipes, pH 7.0/100 mM NaCl, and once in kinase buffer (20 mM Pipes, pH 7.0/10 mM $MnCl_2$/ 1 μg/ml aprotinin). Kinase reactions were performed for 30 min at 30 °C in 50 μl of this buffer with 10 μCi of [γ-^{32}P]ATP, with or without 1 μg of the natural substrate MEK1 (Santa Cruz Biotechnology) or fusion protein glutathione S-transferase (GST)–MEK1 (generated from hamster MEK1 cDNA, provided by J. Pouysségur, Nice, France). Reactions were terminated by addition of SDS-sample buffer and and analysed on 10 % SDS/PAGE gels.

For hyperphosphorylation of Raf-1 *in vivo*, cells were labelled with 250 μCi of [^{32}P]P_i in phosphate-free medium for 4 h. After stimulation, cells were lysed in RIPA buffer [50 mM Tris/HCl, pH 8.0/150 mM NaCl/0.2 mM Na_3VO_4/0.2 mM PMSF/1 % Nonidet P40 (NP40)/0.5 % deoxycholate/0.1 % (w/v) SDS/ 1 μg/ml aprotinin/1 μg/ml leupeptin]. Lysates were clarified by centrifugation and precleared with 2 μl of normal rabbit serum and Protein A–Sepharose. [^{32}P]Raf-1 was immunoprecipitated with SP-63 antibody and Protein A–Sepharose and resolved by 10 % SDS/PAGE.

To determine phosphorylation of Raf-1 by PKC-ζ, COS cells grown in 8.5 cm dishes were transfected with GST-NΔRaf (the N-terminal regulatory domain of Raf-1 fused C-terminally to GST), GST alone, PKC-ζ or empty pmtSM vector (10 μg of total DNA). After 2 days, GST- and GST-NΔRaf-transfected cells were lysed in LB (see above). Lysates were precleared with Sepharose CL-4B. GST and GST-NΔRaf proteins were subsequently isolated through binding to reduced glutathione–Sepharose CL-4B. Beads were washed with LB and with kinase buffer (see above). Cytosols from empty vector- (pmtSM) and PKC-ζ-transfected cells [17] were precleared with Sepharose CL-4B and with GST coupled to glutathione–Sepharose beads, sequentially. Next, 100 μl portions of these cytosols were incubated with GST-NΔRaf beads for 1 h at 4 °C. Beads were washed five times with kinase buffer and phosphorylation of GST-NΔRaf was accomplished with 5 μCi of [γ-^{32}P]ATP in 50 μl of kinase buffer for 20 min at 37 °C. The reaction was terminated by the addition of SDS-sample buffer and analysed by 10 % SDS/PAGE. Radioactivity on the gels was quantified by a PhosphorImager.

RESULTS AND DISCUSSION

We reported previously that PDGF elicits PC hydrolysis by PLD and PC-PLC, the latter of which appeared to be essential for PDGF-induced MAPK activation [14]. Treatment of Rat-1 cells with exogenous PC-PLC (from *B. cereus*) mimicked PDGF in generating DG and in activating MAPK [13]. Unexpectedly, this MAPK activation occurred independently of phorbol ester-sensitive PKC isotypes but, instead, via PKC-ζ activation [13]. The question now is how does PKC-ζ couple to the Raf-1–MEK–MAPK pathway? While Ras activation was not

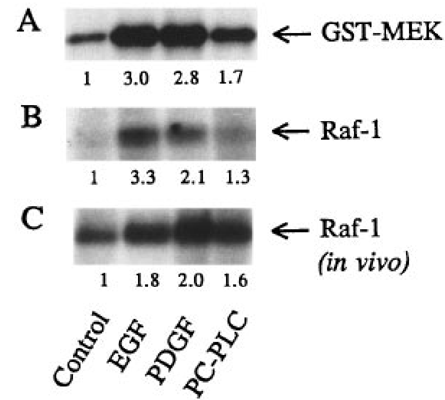


Figure 1 Activation of Raf-1

Rat-1 cells were stimulated for 10 min with EGF (50 ng/ml), PDGF (25 ng/ml), *B. cereus* PC-PLC (1.0 units/ml) or DMEM (control). Cells were lysed and Raf-1 was immunoprecipitated with monoclonal antibody (**A** and **B**) or polyclonal SP-63 (**C**). Raf-1 immunoprecipitations were subjected to an *in vitro* kinase assay in the presence (**A**) or absence (**B**) of 1 μg of GST-MEK1 per assay. (**B**) Raf-1 autophosphorylation *in vitro*. (**C**) Hyperphosphorylation of Raf-1 *in vivo*. Radiolabelled Raf-1 or GST-MEK1 bands (indicated) after SDS/PAGE were quantified by a PhosphorImager relative to control samples (factors given). Results are representative of three experiments with similar findings.

Table 1 Inhibition of MAPK (ERK2) activation by a dominant-negative mutant (NΔRaf) of Raf-1

Rat-1 cells were transfected with c-myc-tagged ERK2 with or without NΔRaf. Cells were stimulated for 10 min and subsequently lysed. ERK2 immunoprecipitates were subjected to an *in vitro* kinase assay with myelin basic protein as a substrate. Data are means ± S.E.M. of two experiments performed in duplicate. The control value represents 77 ± 12 c.p.m. (100%)

Stimulus	[^{32}P]Myelin basic protein (% of control)	
	Control cells	NΔRaf cells
DMEM (control)	100 ± 16	81 ± 22
EGF (50 ng/ml)	445 ± 22	230 ± 28
PDGF (25 ng/ml)	443 ± 41	184 ± 9
B. cereus PC-PLC (1.0 umnits/ml)	261 ± 36	123 ± 19

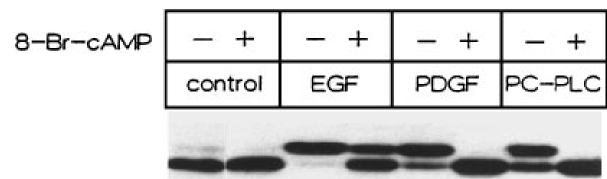
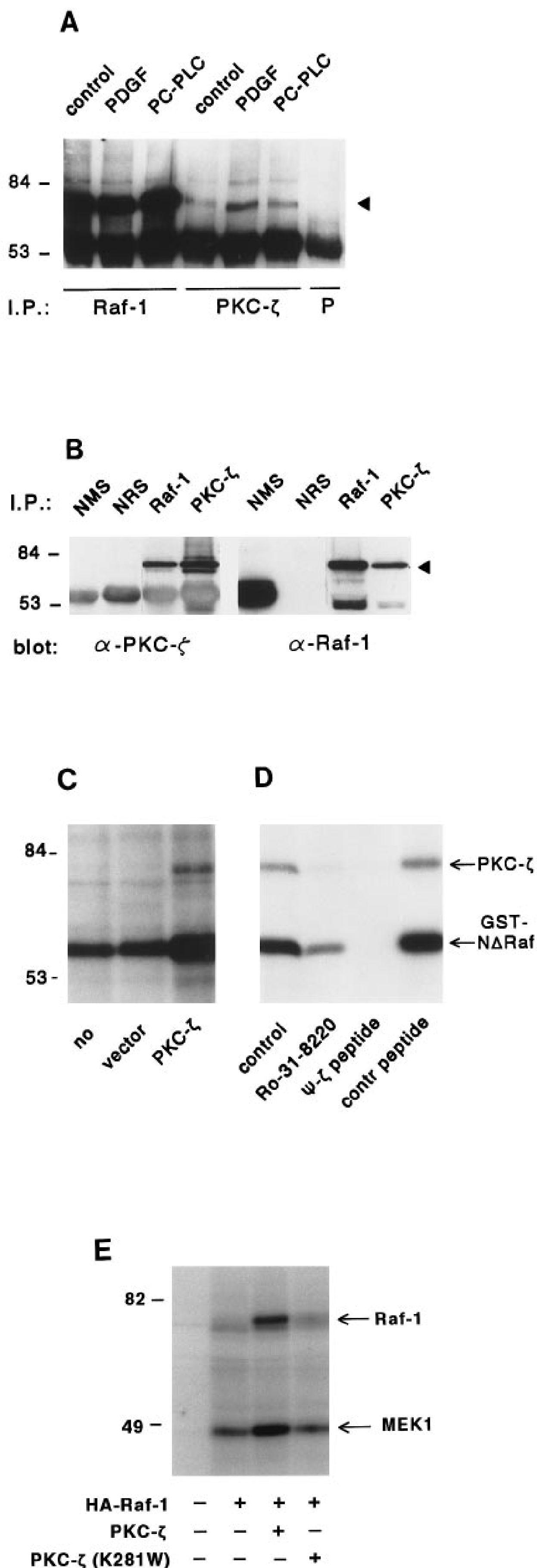


Figure 2 cAMP inhibits MAPK activation

Rat-1 cells were pretreated for 10 min with either DMEM (−) or 8-Br-cAMP (0.5 mM) (+) and stimulated for 10 min with EGF (50 ng/ml), PDGF (25 ng/ml), *B. cereus* PC-PLC (1.0 units/ml) or DMEM (control). Cell lysates were separated by SDS/PAGE and immunoblotted with anti-MAPK antibodies. Activation of MAPK is shown by the electrophoretic mobility shift [18].



required for PKC-ζ-mediated activation of MAPK by PC-PLC (via DG) [13], Raf-1 and MEK are two remaining candidate targets for the activated PKC-ζ. It has been suggested that PKC-ζ is capable of activating MEK directly [19], as Raf-1 does. However, the two known serine phosphorylation sites in MEK, essential for MEK activation (by Raf-1) and conserved among all known MEK family members [20], do not look at all like PKC phosphorylation sites [21,22]. Accordingly, in *in vitro* kinase assays (not shown) we confirmed results of others [23,24] that PKC-ζ phosphorylates MEK only very weakly (as compared with active Raf-1), and is, therefore, a poor activator of MEK. Also *in vivo* (transfected COS cells), PKC isotypes were incapable of acting as an MEK kinase [16]. In order to address this issue more precisely, the following experiments were aimed at demonstrating that PKC-ζ-dependent MAPK activation by PC-PLC and PDGF does not bypass Raf-1.

MAPK activation by PC-PLC depends on Raf-1 activation

Figure 1 shows that, in Rat-1 cells, exogenous (bacterial) PC-PLC mimicks growth factors (PDGF, EGF) in activating Raf-1, as demonstrated by the phosphorylation of its natural substrate MEK1 (used as GST-MEK1) (Figure 1A) and by Raf-1 autophosphorylation *in vitro* (Figure 1B) and hyperphosphorylation *in vivo* (Figure 1C), measured in immunoprecipitates. Dent and Sturgill [25], who also found activation of Raf-1 by exogenous PC-PLC (in NIH 3T3 cells), did not elucidate the mechanism, but showed that the product of PC hydrolysis, DG, failed to activate Raf-1 directly.

That activation of MAPK by PC-PLC and PDGF, like that by EGF, indeed depends on Raf-1, was demonstrated by two different approaches: first, by transfecting a dominant-negative construct of Raf-1 (NΔRaf) in the cells. We successfully used this method to inhibit Raf-1 activity in transfected COS cells [16]. Table 1 shows that, in Rat-1 cells, NΔRaf blocks MAPK activation by EGF, PDGF and PC-PLC. The second approach to demonstrate involvement of Raf-1 is its inhibition by cAMP through direct phosphorylation by protein kinase A. cAMP has been shown to inhibit both Ras-dependent [26–28] as well as Ras-independent Raf-1 activation [29]. Treatment of Rat-1 cells with 8-Br-cAMP (0.5 mM) abrogated MAPK activation induced by PC-PLC and PDGF completely and that by EGF partially (Figure 2). 8-Br-cAMP did not inhibit other early signalling

Figure 3 PKC-ζ forms a complex with Raf-1 (A, B), phosphorylates Raf-1 at its regulatory domain (NΔRaf) (C, D) and activates Raf-1 (E)

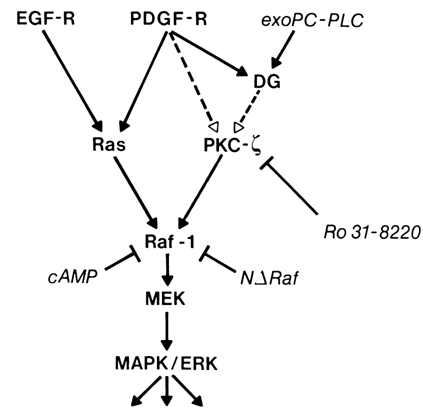
(**A**) Raf-1 co-immunoprecipitates with PKC-ζ in lysates from unstimulated (control) and stimulated (by PDGF or PC-PLC) Rat-1 cells. The position of Raf-1 is indicated (arrowhead) on the immunoblot. P, preclear with normal rabbit serum. (**B**) Raf-1 and PKC-ζ co-expressed in COS cells are immunoprecipitated with their respective antibodies, as indicated (I.P.), after preclearing with normal mouse serum (NMS) or normal rabbit serum (NRS). The precipitates were subjected to SDS/PAGE (minigel) and immunoblotted with polyclonal anti-PKC-ζ or monoclonal anti-Raf-1, as indicated (blot). The position of Raf-1 and PKC-ζ is indicated by the arrowhead. (**C**) *In vitro* phosphorylation of GST-NΔRaf, expressed in COS cells and isolated via binding to glutathione beads, by bound PKC-ζ. GST-NΔRaf beads were incubated with cytosol from COS cells in which PKC-ζ was overexpressed (indicated), or with control cytosol (vector-transfected) or in the absence of cytosol (no). Beads were washed, subsequently subjected to phosphorylation with [γ-^{32}P]ATP, and analysed by SDS/PAGE. Note that bound PKC-ζ itself (indicated by an arrow; identified by Western blotting, not shown) becomes autophosphorylated. (**D**) PKC-ζ-mediated *in vitro* phosphorylation of GST-NΔRaf and PKC-ζ autophosphorylation (indicated by arrows), executed as described for (**C**), is inhibited by Ro 31-8220 (3 μM) and pseudo-ζ peptide (ψ-ζ peptide SIYRRGARRWRKL) (40 μM), but not by an irrelevant control peptide (contr peptide GQLIDSMANSFVGTR) (40 μM). (**E**) Raf-1 activation by PKC-ζ. HA-tagged Raf-1 is transfected in COS cells with (+) or without (−) wild-type PKC-ζ or kinase-dead PKC-ζ(K281W), as indicated. Raf-1 was immunoprecipitated with 12CA5 antibody and subjected to an *in vitro* kinase reaction with [γ-^{32}P]ATP in the presence of 1 μg of MEK1. Phosphorylated Raf-1 and MEK1 (indicated by arrows) were separated by SDS/PAGE.

events [28], including PKC-ζ activation (results not shown). In contrast, under the same conditions, 8-Br-cAMP was a potent inducer of MAPK activation in Swiss 3T3 cells (result not shown), indicating that it is not deleterious to cells and that its effect depends on the cell type.

We conclude that MAPK activation in Rat-1 cells by exogenous PC-PLC, as with PDGF, proceeds not only via PKC-ζ [13] but also via Raf-1 activation.

Raf-1 activation independent of Ras

Unlike the growth factors PDGF and EGF [1,2,26,27], PC-PLC activates Raf-1 in the absence of Ras activation, as we reported previously [13]. In this regard we confirm similar findings by Cai et al. [30] in a different cell system. However, PC-derived DG does not seem to activate Raf-1 directly [25] and, in our Rat-1 cells, it also activates PKC-ζ by an unknown mechanism that is essential for subsequent MAPK activation [13]. Since DG- and PKC-ζ-mediated MAPK activation depends on Raf-1 activation (see above), which, in turn, requires phosphorylation by a kinase (see the Introduction), it was likely that PKC-ζ could be a direct activator of Raf-1 through phosphorylation. Conceivably, plasma-membrane-bound PKC-ζ [31] may function to some extent like Ras-GTP in recruiting Raf-1 to the membrane. The next experiments were aimed at demonstrating that active PKC-ζ does indeed bind to Raf-1, and that Raf-1 is a direct substrate of PKC-ζ activity.



Scheme 1 Summary of signalling pathways based on our present and previous studies

In Rat-1 cells, PDGF-receptor (PDGF-R)-activated PC-PLC [14] and exogenous (exo)PC-PLC generate DG. Although DG activates PKCα, δ and ϵ (not shown here), this does not lead to MAPK (ERK) activation [13]. However, DG also somehow activates PKC-ζ [13] and Raf-1, which proved to be essential for PDGF- and exoPC-PLC-induced MAPK activation, since Ro 31-8220 [13,14], dominant-negative mutants of PKC-ζ [13] and Raf-1 (NΔRaf) as well as elevated cAMP (protein kinase A activation), were inhibitory (indicated). In contrast with growth factors, exoPC-PLC does not activate Ras but, unexpectedly, activates PKC-ζ [13]. PKC-ζ forms a complex with, and directly phosphorylates and activates, Raf-1, thus mediating signals from PDGF-R and PC-PLC towards MAPK. Broken arrows denote mechanistically undefined signalling pathways.

PKC-ζ binds to, phosphorylates and activates Raf-1

Figure 3(A) shows that Raf-1 co-immunoprecipitates with PKC-ζ in lysates from unstimulated Rat-1 cells and, even more clearly so, from PDGF- and PC-PLC-stimulated cells. Apparently, only very little of the available Raf-1 is complexed with PKC-ζ. Together with the relatively high constitutive kinase activity in immunoprecipitated PKC-ζ [13], this caused difficulties in obtaining meaningful results from further phosphorylation experiments in Rat-1 cells. To circumvent this problem, we overexpressed the two kinases by cDNA transfection in COS cells. Figure 3(B) shows that, in lysates of these cells, Raf-1 co-immunoprecipitated with PKC-ζ and vice versa. These results in both cell systems indicate that the two kinases physically interact in a complex that is already present in unstimulated cells.

Furthermore, we reasoned that if PKC-ζ is a direct activator of Raf-1, it is likely to act on the regulatory domain of Raf-1 by phosphorylation. We prepared a GST-fusion construct of this N-terminal regulatory, kinase-dead fragment (GST-NΔRaf) of Raf-1 in the pmtSM expression vector, and overexpressed it in COS cells. The fusion protein was then isolated from the cytosol by glutathione–Sepharose beads and was found to bind PKC-ζ, most prominently from the cytosol of COS cells in which PKC-ζ was overexpressed (immunoblotting results not shown). PKC-ζ remained bound after extensive washing and, being constitutively active [13], subsequently phosphorylated GST-NΔRaf in an *in vitro* kinase assay (Figures 3C and 3D). The substantial background phosphorylation of NΔRaf by untransfected or vector-transfected cell lysates most likely results from endogenous PKC-ζ activity. Bound PKC-ζ is visible as an (auto)-phosphorylated band on the gel at the predicted size of 78 kDa (Figures 3C and 3D). The NΔRaf moiety was the actual phosphorylation target of PKC-ζ, since control GST coupled to the beads was not phosphorylated (results not shown). Figure 3(D) shows that this NΔRaf phosphorylation (as well as the associated PKC-ζ autophosphorylation) was blocked by two highly selective PKC-ζ inhibitors, i.e. Ro 31-8220 [13,17] and the pseudo-substrate peptide of PKC-ζ [13], and was not affected by a control peptide, indicating that the phosphorylation was indeed due to PKC-ζ. Finally, Figure 3(E) shows an *in vitro* kinase assay on Raf-1 immunoprecipitates, demonstrating that Raf-1 overexpressed in COS cells is activated by co-expressed wild-type PKC-ζ (being enzymically active also in unstimulated cells [13,19]), but not by the kinase-inactive PKC-ζ (K281W) mutant. Figure 3(E) (lane 3 from the left) shows that Raf-1 becomes phosphorylated and, in turn, phosphorylates its substrate MEK1.

Concluding remarks

From the above we conclude that PKC-ζ, which is activated in PDGF- and PC-PLC-stimulated Rat-1 cells, physically couples to Raf-1 in a complex *in vivo*. The COS cell data have shown, furthermore, that PKC-ζ can phosphorylate Raf-1 directly at its regulatory domain, leading to Raf-1 activation. Taken together, the results support the concept that PKC-ζ is a direct upstream activator of the Raf-1–MAPK pathway *in vivo* (Scheme 1). In a different cell system, Moscat and co-workers [19,23] have shown that PKC-ζ can associate with and activate MEK and MAPK. However, they did not exclude the possibility that the interaction between PKC-ζ and MEK was indirect, through Raf-1. Bjørkøy et al. [32] showed in NIH 3T3 cells stably transfected with the *B. cereus* PC-PLC gene, that the transformed phenotype of these cells could be reversed with dominant-negative versions of either Raf-1 or PKC-ζ, suggesting that DG activation of both kinases is required for transformation. These authors suggest a bifurcation in the signalling pathway downstream of PC-PLC, whereas our results suggest that PKC-ζ acts upstream of Raf-1. We draw this conclusion from four observations, briefly summarized as follows (Scheme 1): (i) elevation of cAMP levels and (ii) expression of the dominant-negative NΔRaf construct inhibits the MAPK cascade at the level of Raf-1, without interfering with PKC-ζ activation; (iii) Ro 31-8220 inhibits PDGF- and PC-

PLC-induced MAPK signalling at the level of PKC-ζ, but not Raf-1 or MEK1 [13]; and (iv) PKC-ζ poorly phosphorylates MEK1, while it is capable of binding to and phosphorylating the regulatory domain of Raf-1, thereby activating Raf-1.

Although PC-PLC (via DG) can activate PKC-ζ and Raf-1 independently of Ras [13], it cannot be excluded that PDGF-induced PKC-ζ activation is Ras dependent. In fact, it has been shown that PDGF-induced MAPK activation depends on activated Ras [33] and that direct Ras-PKC-ζ interaction/activation is possible [34]. If the latter occurs in PDGF-stimulated Rat-1 cells, the dominant-negative Raf-1 construct (NΔRaf) does not compete with PKC-ζ for interaction with Ras [16]. In the case of PC-PLC stimulation, where Ras is not activated, the truncated NΔRaf construct may compete with full-length endogenous Raf-1 for binding to and phosphorylation by PKC-ζ, as shown in Figures 3(C) and 3(D). In PDGF-stimulated cells, the possibility remains that activated Ras facilitates the signalling from PKC-ζ to Raf-1 by recruiting both kinases to the plasma membrane. How, precisely, PC-PLC-generated DG activates the PKC-ζ–Raf-1 complex remains a challenge for future studies.

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