14-3-3 Isotypes facilitate coupling of protein kinase C- ζ to Raf-1: negative regulation by 14-3-3 phosphorylation

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14-3-3 Proteins may function as adapters or scaffold in signal-transduction pathways. We found previously that protein kinase C- ζ (PKC- ζ) can phosphorylate and activate Raf-1 in a signalling complex [van Dijk, Hilkmann and van Blitterswijk (1997) *Biochem. J.* **325**, 303–307]. We report now that PKC- ζ –Raf-1 interaction is mediated by 14-3-3 proteins *in vitro* and *in vivo*. Co-immunoprecipitation experiments in COS cells revealed that complex formation between PKC- ζ and Raf-1 is mediated strongly by the 14-3-3 β and - θ isotypes, but not by 14-3-3 ζ . Far-Western blotting revealed that 14-3-3 binds PKC- ζ directly at its regulatory domain, where a S186A mutation in a putative 14-3-3-binding domain strongly reduced the binding and the complex formation with 14-3-3 β and Raf-1. Treatment of PKC- ζ with lambda protein phosphatase also reduced its binding to 14-3-3 β *in vitro*. Preincubation of an immobilized Raf-1 construct with 14-3-3 β facilitated PKC- ζ binding. Together, the results suggest that 14-3-3 binds both PKC- ζ (at phospho-Ser-186) and Raf-1 in

a ternary complex. Complex formation was much stronger with a kinase-inactive PKC- ζ mutant than with wild-type PKC- ζ , supporting the idea that kinase activity leads to complex dissociation. 14-3-3 β and - θ were substrates for PKC- ζ , whereas 14-3-3 ζ was not. Phosphorylation of 14-3-3 β by PKC- ζ negatively regulated their physical association. 14-3-3 β with its putative PKC- ζ phosphorylation sites mutated enhanced co-precipitation between PKC- ζ and Raf-1, suggesting that phosphorylation of 14-3-3 by PKC- ζ weakens the complex *in vivo*. We conclude that 14-3-3 facilitates coupling of PKC- ζ to Raf-1 in an isotype-specific and phosphorylation-dependent manner. We suggest that 14-3-3 is a transient mediator of Raf-1 phosphorylation and activation by PKC- ζ .

Key words: mitogen-activated protein kinase, platelet-derived-growth-factor receptor, scaffold/adaptor protein, signalling complex, signal transduction.

INTRODUCTION

Activation of the mitogen-activated protein kinase (MAPK or extracellular-signal-regulated protein kinase, ERK) pathway is a central event in the response of cells to mitogenic stimuli. In this pathway, Raf-1 is recruited to the plasma membrane by activated Ras, and is there phosphorylated and activated by protein tyrosine kinases and by protein kinase C (PKC) [1–3]. Members of all three subclasses of PKC (conventional, novel and atypical) are capable of activating the MAPK pathway. However, which particular PKC isotype is acting at a given level in the Raf-1-MAPK cascade depends on the cell type. For example, in 3T3 fibroblasts, both conventional and novel PKCs can activate Raf-1 [4–6], whereas atypical PKC- ζ apparently bypasses Raf-1 to activate MEK-1 directly [7] or indirectly [6]. However, in Rat-1 fibroblasts stimulated by platelet-derived growth factor (PDGF), the situation is different [3,8]. In this system, PKC- ζ is an essential direct activator of Raf-1, whereas the MAPK pathway is hardly activated by phorbol ester-sensitive PKCs. Also, down-regulation of these PKCs by prolonged exposure to phorbol ester did not influence MAPK activation by PDGF or exogenous phospholipase C. We were curious to learn what cellular factor(s) might govern this subtype specificity of PKC in different cell types. There could be a role of scaffold/adaptor proteins, known to convey specificity in the interaction between two consecutive protein kinases in a signalling pathway [9]. For this and other reasons (see below), we tested 14-3-3 proteins as candidate scaffold/adapters between PKC and Raf-1.

Members of the 14-3-3 protein family form a group of highly conserved acidic proteins of about 30 kDa that exist as homo- or heterodimers in all eukaryotic cells. They bind to certain phosphoserine motifs in (proto)oncogene products and other proteins participating in signal-transduction pathways [10–13]. In this way, they may sequester and regulate (inactivate) proteins, such as BAD in apoptotic signalling [14,15] and Cdc25 in nuclear export [16,17]. Heterodimeric 14-3-3 may also interconnect two different proteins (e.g. signalling kinases), thus acting as an adapter/scaffold protein in signal-transduction complexes [18]. For example, it has been suggested that 14-3-3 can physically couple Raf-1 to Bcr [19] and to A20 [20]. However, the functional significance of this coupling is unclear. Genetic studies in *Drosophila* have indicated, though, that 14-3-3 proteins are essential for Raf-1-mediated signalling [21,22]. In yeast, 14-3-3 proteins are essential for Ras/MAPK-cascade signalling during pseudohyphal development [23].

Whereas 14-3-3 binding to Raf-1 is well established, existence of a physical interaction of 14-3-3 with PKCs is less clear [11]. A functional association has only been demonstrated, in T-cells, for 14-3-3 τ to PKC- θ [24] and to PKC- μ [13]. In the present study, we therefore addressed the likely hypothesis that one or more isotypes of 14-3-3 might function as a scaffold/adaptor, facilitating the formation of a complex between PKC- ζ to Raf-1.

To date, at least seven mammalian 14-3-3 isoforms have been described [25,26]. For the present study, five of these (14-3-3 β , - γ , - η , - θ and - ζ [26–28]) were subcloned and overexpressed

Abbreviations used: GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PDGF, platelet-derived growth factor; DTT, dithiothreitol; λ -PPase, lambda protein phosphatase; VSV, vesicular stomatitis virus.

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individually in COS cells together with PKC- ζ and Raf-1. We studied the interactions between these molecules by co-immunoprecipitation and overlay assays. We show that several but not all 14-3-3 isotypes bind to PKC- ζ , and particularly that 14-3-3 β and - θ amplify complex formation between PKC- ζ and Raf-1. We furthermore present evidence that this complex dissociates upon PKC- ζ -mediated phosphorylation of 14-3-3, suggesting that it is a functional and transient event in PKC- ζ -mediated signal transduction towards Raf-1.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium was from ICN-Flow and foetal calf serum was from BioWhittaker. [γ - 32 P]ATP and the enhanced chemiluminescence system (ECL[®]) were from Amersham. Leupeptin and aprotinin were from Fluka, and PMSF was from Sigma. ATP was from Boehringer. Acrylamide and *N,N'*-methylendiacylamide were from Merck (Darmstadt, Germany). Glutathione–Sephadex and Protein A–Sephadex (CL-4B) were from Pharmacia.

Antibodies

Antibodies against PKC- ζ were raised in rabbits and were directed against an oligopeptide (GFEEYNPLLSAEESVC) based on the C-terminal region of rat PKC- ζ . Anti-14-3-3 (pan) antibodies were raised in rabbits and were directed against an oligopeptide corresponding to amino acids 3–21 mapping at the N-terminus of 14-3-3 β (KSELVQKAKLAQEARYDDC). This antibody detects all 14-3-3 isoforms used in this study with equal sensitivity as judged by immunoblotting in combination with Ponceau S staining (results not shown). The peptides were coupled via a cysteine residue (added to the C- or N-terminus) to keyhole limpet haemocyanin via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Specific polyclonal antibodies against 14-3-3 β , - γ and - ϵ were provided kindly by Dr. A. Aitken (N. I. M. R., London, U.K.). Anti-14-3-3 θ and - ζ rabbit polyclonal antibodies were from Santa Cruz Biotechnology. Anti-(c-Raf-1) monoclonal antibody (catalogue no. R-19120) was from Transduction Labs. Monoclonal antibodies against c-Myc (clone 9E10), influenza haemagglutinin epitope (HA, clone 12CA5) or VSV (vesicular stomatitis virus) glycoprotein (clone P5D4) were obtained from the growth medium of the hybridomas producing these antibodies. Peroxidase-conjugated rabbit anti-mouse secondary antibodies were from Dako (Glostrup, Denmark).

cDNA constructs

pmtM is a pMT2-derived cell expression vector that contains a multiple cloning region. pmtSM is derived from pmtM and contains three upstream stop codons in all three reading frames [29]. Rat pmtSM-PKC- ζ [30], pmtM-Raf-1, pmtSM-HA-Raf-1 and pmtM-GST- Δ Raf [8,29] have been described previously. Myc-tagged PKC- ζ was generated by subcloning of rat PKC- ζ into a pmtSM-myc vector. pmtSM-mycPKC- ζ (S186A) was constructed using the Quickchange[™] site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) with pmtSM-mycPKC- ζ as template. Murine PKC- ζ (K281W) [31] was subcloned into a pmtSM-myc vector. pmtSM-catPKC- ζ was constructed through ligation of the catalytic domain of rat PKC- ζ (cut at an internal *Hind*III site as a *Hind*III–*Xba*I fragment) with a *Sal*I–*Hind*III PCR fragment into pmtSM. pmtSM-regPKC- ζ -VSV was constructed through ligation of the regulatory domain of rat PKC- ζ (cut at an internal *Alw*N1 site as a *Sal*I–*Alw*N1 fragment) into

pmtSM-VSVtag. Cloning of the cDNAs of rat 14-3-3 subtypes β , γ , η , θ and ζ has been described previously [26–28]; they were subcloned as *Eco*RI fragments from their vector (pUC118 for 14-3-3 β , - γ and η ; pBluescript for 14-3-3 θ and - ζ) into pmtSM. (Note that the rat 14-3-3 θ isoform differs from the 14-3-3 τ (T-cell) isoform by only one amino acid (Glu-147 instead of Asp [26]). 14-3-3 β mutants S130A and T141A were constructed using the Quickchange[™] site-directed mutagenesis kit with pGEX14-3-3 β as a template. The double mutant S130A/T141A was generated with the mutant S130A as template. The mutated 14-3-3 β cDNAs were subcloned into the pmtSM vector.

Cell culture and transfection

COS7-M6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % foetal calf serum and containing penicillin (100 units/ml), streptomycin (100 μ g/ml) and 2 mM L-glutamine. For transfection, 5×10^5 cells were seeded into 5-cm dishes and 6 μ g of plasmid DNA per plate was used for transfection following the DEAE–Dextran method. Cells were not serum-starved. Cells were used for experiments 48 h after transfection.

Cell lysis and immunoprecipitation

COS cells were lysed in 1 ml of ice-cold buffer containing 50 mM Hepes, pH 7.4, 0.2 % (w/v) Triton X-100, 1 mM EGTA, 1 mM Na_3VO_4 , 100 mM NaCl, 10 mM NaF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 0.2 mM PMSF. Cell lysates were kept on ice for 30 min, scraped and centrifuged for 15 min at 15000 *g* (Eppendorf microfuge). Protein expression was checked using 50 μ l of supernatant, and the remainder was used for immunoprecipitation. Cell lysates were precleared three times with Protein A–Sephadex beads conjugated to normal rabbit serum or rabbit anti-mouse serum. Immunoprecipitation was performed using 2 μ l of serum of polyclonal anti-PKC- ζ , 2 μ g of monoclonal anti-Raf-1 or 100 μ l of medium containing 9E10 or 12CA5 monoclonal antibodies. Precipitates and control beads were washed at least five times in lysis buffer, subjected to SDS/PAGE, blotted on to nitrocellulose membranes, probed with the appropriate antibody and subjected to ECL[®]. The amounts of PKC- ζ and Raf-1 immunoprecipitated with their respective antibodies closely reflected the amounts present in the cell lysates, as determined by Western blotting.

Purification of 14-3-3 proteins and of PKC- ζ

14-3-3 Proteins were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins using pGEX vector (Pharmacia) and purified by glutathione–Sephadex affinity chromatography. Free 14-3-3 proteins were obtained by cleavage with factor X, and were further purified by incubation with glutathione–Sephadex beads. Purified 14-3-3 was dialysed against 20 mM Tris/HCl (pH 7.5)/200 mM NaCl, concentrated (Centricon, mass cut-off 10 kDa) to approx. 0.4 μ g/ml and stored in aliquots at -70°C until use.

PKC- ζ was purified partially from Sf9 insect cells infected with PKC- ζ recombinant baculovirus [32], kindly provided by Dr. D. Fabbro (Novartis, Basel, Switzerland). Purification was based on the method of Dimitrijevic et al. [33]. Briefly, a 72-h infected 250-ml spinner culture of Sf9 cells was pelleted and lysed in 25 ml of buffer containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 % (v/v) Triton X-100, 50 μ M PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 0.3 % (v/v) β -mercaptoethanol, sonicated and centrifuged at 100000 *g* for 30 min. Supernatant (5 ml, 25 mg of protein) was loaded on to a MonoQ column and eluted in a

linear gradient of 0–1 M NaCl in lysis buffer with 0.02% (v/v) Triton X-100 (10 ml, 0.5 ml/min). Fractions between 0.7 and 0.9 M NaCl were diluted 6-fold in elution buffer without NaCl and loaded on to a MonoS column and eluted with the same concentration gradient. Fractions between 0.65 and 1 M were pooled, loaded on to a PD-10 column (Pharmacia) and eluted with 3.5 ml of 20 mM Tris/HCl (pH 7.5), 1 mM EGTA, 10% (v/v) glycerol, 0.1 mM PMSF, 0.02% Triton X-100 and 1 mM dithiothreitol (DTT). The PKC- ζ preparation (50 μ g/ml) was frozen in aliquots and stored at -70°C until use.

Overlay assay (far-Western Blotting)

Cell lysates from COS cells overexpressing PKC- ζ constructs were subjected to SDS/PAGE and blotted on PVDF membranes. Proteins were denatured by submerging the PVDF membranes in 7 M guanidine/HCl, 50 mM DTT, 2 mM EDTA and 50 mM Tris/HCl (pH 8.3) for 1 h and renatured in 20 mM Tris/HCl (pH 7.4), 140 mM NaCl, 4 mM DTT, 1 mM MgCl_2 , 10 μ M ZnCl_2 , 0.1% BSA and 0.1% Nonidet P-40 for at least 4 h at 4°C . Blots were blocked in 4% dried skimmed milk and incubated overnight with 14-3-3 θ (5 μ g/ml), washed and probed with anti-14-3-3 (pan) serum. Subsequently, the same PVDF membrane was stripped and probed with antibodies against the PKC- ζ constructs to check their expression.

Phosphorylation of 14-3-3 by PKC- ζ

Purified recombinant PKC- ζ (0.5 μ g, 10 μ l) and 1 μ g of purified recombinant 14-3-3 β , - θ or - ζ (or 14-3-3 β mutants) were incubated in 50 μ l (final volume) of 20 mM Hepes (pH 7.4), 10 mM MgCl_2 , 1 mM EGTA and 5 μ Ci [γ - ^{32}P]ATP for 30 min at 37°C . The reaction was stopped by the addition of 50 μ l of $2\times$ SDS sample buffer. Samples were subjected to SDS/PAGE (10% gel) and autoradiography.

Treatment of PKC- ζ with lambda protein phosphatase (λ -PPase)

Purified PKC- ζ (1 μ g, 20 μ l) was incubated for 30 min with 40 units of λ -PPase (New England Biolabs, > 95% homogeneous protein devoid of detectable protease activity) at 30°C in 40 μ l of 20 mM Hepes (pH 7.4), 2 mM MnCl_2 , 5 mM DTT, 1 mM PMSF, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin. The reaction was stopped by the addition of 100 μ l of binding buffer (see below) containing Na_3VO_4 (1 mM final concentration) and NaF (10 mM final concentration), and binding to GST-14-3-3 was performed as described below.

PKC- ζ binding to 14-3-3

In vitro binding of purified PKC- ζ to 14-3-3 β was carried out by incubating 0.5 μ g of PKC- ζ and 25 μ g of GST-14-3-3 β (or 14-3-3 β bound to GST- Δ Raf; see Figure 3B below), precoupled to glutathione-Sepharose in 100 μ l of binding buffer (20 mM Hepes, pH 7.4/100 mM NaCl/1 mM EGTA/10 mM MgCl_2) for 30–45 min at 37°C . To test the effects of pseudo-PKC- ζ substrate peptide [31], 0.5 μ g of PKC- ζ was preincubated with 200 μ M pseudo-PKC- ζ peptide in $2\times$ binding buffer (50 μ l total volume) for 15 min at 37°C . After preincubation, glutathione-Sepharose-linked GST-14-3-3 β and ATP (100 μ M + 10 μ Ci [γ - ^{32}P]ATP) were added in 50 μ l of $2\times$ binding buffer. After 30–45 min of incubation, the beads were washed four times in 1 ml of binding buffer and subjected to SDS/PAGE (12% gel) and immunoblotting with anti-PKC- ζ polyclonal antibody (1:1000). Blots were also subjected to autoradiography to check 14-3-3 phosphorylation.

The effect of ATP on the binding of 14-3-3 to PKC- ζ was tested *in vitro* by immunoprecipitating mycPKC- ζ (wild-type or kinase-dead, using 9E10 anti-Myc in combination with rabbit anti-mouse pre-coupled Protein A-Sepharose beads) from COS cells overexpressing the kinase constructs in equal amounts. Precipitates were washed in binding buffer, and incubated for 60 min with 1 μ g of 14-3-3 β (recombinant) in the presence or absence of 100 μ M ATP. After incubation, immunoprecipitates were washed and subjected to SDS/PAGE (12% gel). Since 14-3-3 migrates to about the same position in the gel as the light chains of immunoglobulins, electrophoresed gels were incubated for 30 min in 10% glutardialdehyde in PBS at room temperature, washed three times for 10 minutes in PBS and subjected to Western blotting. Using this protocol, there was no more interference of secondary antibodies with immunoglobulin light chains, and a clear result was obtained.

RESULTS

We have demonstrated previously that, in Rat-1 fibroblasts and in transfected COS cells, atypical PKC- ζ forms a signalling complex with Raf-1, in which it can phosphorylate Raf-1 at its regulatory domain, leading to Raf-1 activation [8]. Since some 14-3-3 isotypes are known to bind to Raf-1 and to affect its kinase activity, we surmised that 14-3-3 might be a scaffold/adaptor protein in this PKC- ζ -Raf-1 complex. To address this concept, we performed the following experiments in a COS-cell overexpression system.

Physical association of 14-3-3 isotypes with PKC- ζ and Raf-1 *in vivo*

Five different 14-3-3 isotypes (β , γ , η , θ and ζ) were overexpressed individually in COS cells, together with PKC- ζ or Raf-1, and the physical association of these isotypes with the two kinases was determined by co-immunoprecipitation. Figure 1 (top panel, right-hand side) shows that all 14-3-3 isotypes co-precipitate with Raf-1 to a variable extent. Doublet bands could be due to a different degree of 14-3-3 phosphorylation (see Figure 6 below) and due to co-precipitation of endogenous 14-3-3 isotypes, expression of which in COS cells is shown in Figure 1 (bottom panel). Figure 1 (top panel, left-hand side) shows that 14-3-3 β , - γ , - η and - θ co-precipitate with PKC- ζ . In some experiments, 14-3-3 γ and - η co-precipitated to a lesser extent than 14-3-3 β and - θ . PKC- ζ , in contrast with Raf-1, hardly co-precipitates 14-3-3 ζ .

Complex formation of Raf-1 with PKC- ζ is reinforced selectively by 14-3-3 β and - θ

We next tested if 14-3-3 couples both PKC- ζ and Raf-1 in one and the same complex. To this end, Raf-1, PKC- ζ and five different 14-3-3 subtypes were co-transfected in COS cells and immunoprecipitations with anti-Raf-1 and anti-PKC- ζ were performed. Figure 2 shows that Raf-1 co-precipitates with PKC- ζ , and vice versa, most efficiently in the presence of 14-3-3 β , - θ and, to a lesser extent, - γ isotypes. 14-3-3 θ and - γ are also expressed endogenously in COS cells (Figure 1B), which explains the low but clearly detectable PKC- ζ -Raf-1 co-immunoprecipitation in the absence of co-transfected 14-3-3. 14-3-3 β is only weakly expressed endogenously in COS cells (detectable at a longer exposure time; Figure 1B, *). Overexpressed 14-3-3 ζ , which failed to bind PKC- ζ (Figure 1, top panel), understandably also does not mediate PKC- ζ -Raf-1 co-immunoprecipitation (Figure 2). 14-3-3 η also fails to stimulate complex formation between the kinases. The reason for this is less clear but might be

Figure 1 *In vivo* association of 14-3-3 isotypes with PKC- ζ and Raf-1

(Top panel) COS cells were transfected with pmtSM plasmids encoding PKC- ζ (Myc-tagged), or Raf-1 together with various 14-3-3 isotypes. After 48 h cells were lysed and immunoprecipitates of PKC- ζ and Raf-1 (indicated) were made. Immunoprecipitates (I. P., upper panel) and cell lysates (lower panels, for expression control) were subjected to SDS/PAGE (15% gel) and immunoblotted with the respective anti-kinase antibodies or with anti-14-3-3 (pan), as indicated by arrows. Note that, in several lanes of the Raf-1 immunoprecipitates, the antibody detects two 14-3-3 bands, possibly due to different degrees of phosphorylation and/or the co-precipitation of endogenous 14-3-3 isotype(s) (e.g. γ or θ ; see bottom panel). (Bottom panel) Expression of endogenous 14-3-3 isotypes in COS cells. Cell lysate was subjected to SDS/PAGE and Western blotting. Each lane was probed with specific polyclonal antibodies (used at 1:2000 dilution) against the indicated 14-3-3 isotypes. 14-3-3 β is only detected after prolonged exposure (*, 5 min instead of 1 min). Note: no specific antibodies against 14-3-3 η and σ were available, and 14-3-3 (pan) antibodies, used for immunoblotting in several experiments, do not detect 14-3-3 ϵ but do detect 14-3-3 γ .

Figure 2 Co-immunoprecipitation of Raf-1 with PKC- ζ and vice versa is enhanced by some but not all 14-3-3 isotypes

COS cells were transfected with PKC- ζ and Raf-1, together with 14-3-3 isotypes, as indicated. After 48 h of transfection, cells were lysed and the lysates were divided in two equal fractions to make immunoprecipitates of both PKC- ζ and Raf-1. Immunoprecipitates and cell lysates were subjected to SDS/PAGE (10% gel), and immunoblotted with antibodies against Raf-1, PKC- ζ or 14-3-3 (pan) as indicated by arrows.

due to variable (and sometimes very low) co-precipitation of this isotype with PKC- ζ , mentioned earlier. In general, however, the 14-3-3 subtype selectivity in mediating PKC- ζ -Raf-1 complex formation is in agreement with, and a superimposition of, their

co-immunoprecipitation with PKC- ζ and Raf-1 individually (Figure 1A).

14-3-3 binds PKC- ζ directly at a phosphoserine motif in its regulatory domain, thus forming a ternary complex with Raf-1

The above-described immunoprecipitations prove that PKC- ζ , 14-3-3 and Raf-1 participate in a complex, but not necessarily in direct association in a ternary complex. While the direct interaction of 14-3-3 with Raf-1 is well established [34–37], a direct interaction with PKC- ζ has not been reported previously. We therefore investigated whether the binding of 14-3-3 to PKC- ζ was direct or perhaps mediated by some bridging molecule. We investigated the interaction in an overlay (far-Western) assay with 14-3-3 θ , one of the isotypes that strongly mediates PKC- ζ -Raf-1 binding. Figure 3 (top panel) shows that binding of 14-3-3 θ to PKC- ζ is indeed direct and that it involves the regulatory domain of PKC- ζ . No binding to the catalytic domain was detected under these conditions. Similar results were obtained when the overlay was performed with 14-3-3 β (results not shown).

To provide further evidence that 14-3-3 actually participates in a ternary complex in direct interaction with PKC- ζ and Raf-1, we performed an *in vitro* reconstitution experiment. We argued that 14-3-3 should facilitate binding of PKC- ζ to Raf-1 when Raf-1 is preloaded with 14-3-3 *in vitro*. For this purpose, we used the regulatory domain of Raf-1 (named NΔRaf [29]), which binds non-phosphorylated 14-3-3 [38,39] and PKC- ζ [8], via 14-3-3 we presume. Figure 3 (bottom panel) shows that pre-incubation of GST-NΔRaf fusion protein with 14-3-3 β (in a COS-cell lysate) indeed facilitated PKC- ζ binding. This result together with the overlay data strongly suggest that 14-3-3 directly mediates and participates in ternary complex formation.

14-3-3 Proteins bind to other proteins via a phosphoserine motif [10–13]. In order to investigate whether the PKC- ζ -14-3-3 interaction is also mediated through phosphoserine, we treated purified recombinant PKC- ζ with purified recombinant λ -PPase.

Figure 3 14-3-3 binds directly to the regulatory domains of PKC- ζ and Raf-1, thus forming a ternary complex

(Top panel) Overlay assay showing direct binding of 14-3-3 θ to PKC- ζ constructs. Lysates of COS cells overexpressing wild-type (wt) PKC- ζ , its regulatory (reg) or catalytic (cat) domains were subjected to SDS/PAGE (equal amounts of protein were loaded) and blotted on PVDF membrane. COS cells transfected with empty vector (pmtSM) were used as a control. The blot was probed with 5 μ g of recombinant 14-3-3 θ /ml, and binding was detected with anti-14-3-3 (pan) polyclonal antibody. The membrane was stripped and reprobed with anti-PKC- ζ (binds to wild-type PKC- ζ and PKC- ζ cat) and anti-VSV tag (P5D4) to detect the regulatory domain. (Please note that this overlay does not yield quantitative estimates of relative binding, because different primary and secondary antibodies were used to detect the expressed PKC- ζ constructs). Positions of these PKC- ζ constructs in the gel (arrows) and molecular-mass markers (in kDa) are indicated. (Bottom panel) 14-3-3 β mediates PKC- ζ binding to the regulatory domain of Raf-1 (NΔRaf). GST-NΔRaf fusion protein (NΔRaf) was expressed in COS cells, and precipitated from cell lysates with glutathione-Sepharose beads [8], washed and subsequently mixed with lysates of COS cells transfected with 14-3-3 β (+) or empty vector (—) as control. After a 60-min incubation, the beads were washed and then incubated with 0.5 μ g of purified recombinant PKC- ζ (see the Experimental section), washed and subjected to SDS/PAGE. Bound PKC- ζ was visualized by Western blotting.

Figure 4 (top panel) shows that such a treatment decreased its binding *in vitro* to GST-14-3-3 β , as found similarly for the 14-3-3-Raf-1 interaction [40]. The treatment did not abolish the binding completely, suggesting either the incomplete removal of phosphate or the presence of an additional, phosphatase-insensitive 14-3-3-binding site in PKC- ζ . Collectively, however, the results support the notion that, similar to other 14-3-3-binding proteins, the interaction of 14-3-3 with PKC- ζ occurs via a phosphoserine motif in a direct fashion.

The stringency of phosphoserine motifs for binding of 14-3-3 proteins is not as high as originally thought [10,11], and although PKC- ζ does not contain any of the published motifs [10–13], its amino acid sequence [41] reveals an **RHMD**S¹⁸⁶**VMP** sequence (with the essential residues in bold) in its regulatory domain,

Figure 4 Dephosphorylation or S186A mutation of PKC- ζ abrogates the binding of and heterotrimeric complex formation with 14-3-3 β

(Top panel) Phosphatase treatment of PKC- ζ leads to decreased 14-3-3 binding. Purified recombinant PKC- ζ was treated with λ -PPase (+ λ) or left untreated (— λ) and was incubated subsequently with GST-14-3-3 β or GST (control). Glutathione-Sepharose beads were added, and beads were washed and subjected to SDS/PAGE and Western blotting with anti-PKC- ζ . (Middle panel) PKC- ζ (S186A) mutant shows strongly reduced binding to (endogenous) 14-3-3. Lysates of COS cells transfected with wild-type PKC- ζ (WT) or PKC- ζ (S186A) constructs or empty vector were immunoprecipitated (I.P.) with anti-14-3-3 (pan) antibody. Equal amounts of cell-lysate protein were precipitated. Co-precipitated PKC- ζ was visualized by Western blotting. Expression controls are shown in the lower panels. (Lower panel) 14-3-3 β does not facilitate co-precipitation between Raf-1 and PKC- ζ (S186A). COS cells were transfected with wild-type PKC- ζ or PKC- ζ (S186A), together with 14-3-3 β and Raf-1 as indicated (+). Following transfection (48 h), cells were lysed, and lysates were divided into two equal fractions to make an immunoprecipitate of both PKC- ζ and Raf-1. Immunoprecipitates and cell lysates were subjected to SDS/PAGE (12% gel), and immunoblotted with antibodies against Raf-1, PKC- ζ or 14-3-3 (pan) as indicated by the arrows.

which differs from a described 'mode-2' motif for 14-3-3 binding [11] by only one extra amino acid residue between the (phospho)serine and the proline. To test whether this alternative motif in PKC- ζ might be involved in 14-3-3 binding, and thus in complex formation with Raf-1, we mutated the presumably essential Ser-186 in this motif into Ala. This mutant indeed

Figure 5 Comparison of wild-type with kinase-inactive PKC- ζ in complex formation with 14-3-3 β and Raf-1

COS cells were transfected with combinations of plasmids as indicated (+). Following transfection (48 h) cells were lysed, and lysates were divided into two equal fractions to make immunoprecipitates of both mycPKC- ζ (wild-type, wt, and kinase dead, kd) and Raf-1. Co-precipitation of Raf-1 or PKC- ζ was visualized after SDS/PAGE (12% gel) and immunoblotting with monoclonal antibodies against Raf-1 or mycPKC- ζ (9E10). To show how much kinase was actually precipitated, the blots were probed with the immunoprecipitating antibody. Expression controls (Western blots) for 14-3-3 β are shown in the lower panel. The same results were produced in four independent experiments.

shows greatly reduced co-immunoprecipitation with (endogenous) 14-3-3 compared with wild-type PKC- ζ (Figure 4, middle panel). Furthermore, Figure 4 (lower panel, top) shows that with PKC- ζ (S186A), in contrast with wild-type PKC- ζ , 14-3-3 β is no longer capable of enhancing the co-precipitation of Raf-1. Also, in the reverse experiment, 14-3-3 β does not enhance co-precipitation of PKC- ζ (S186A) with Raf-1 (Figure 4, lower panel). Together, these data suggest strongly that 14-3-3 can bind to PKC- ζ via a new phosphoserine motif RHMDSVMP. By doing so, it may act as an adapter or scaffold protein that facilitates the direct coupling of PKC- ζ to Raf-1.

Complex formation is stronger with kinase-inactive PKC- ζ

Since we have found previously that PKC- ζ activates Raf-1 directly through phosphorylation [8], and since it is now clear that 14-3-3 acts as a scaffold/adapter facilitating the complex formation between these protein kinases, the question arises as to what happens with 14-3-3 and the ternary complex when the kinase cascade is activated during signal transduction. It has been suggested that 14-3-3 maintains Raf-1 in an inactive conformation [2,22], and that activation of Raf-1 is accompanied by the partial release of 14-3-3 [38,42]. We therefore investigated the effect of PKC- ζ -mediated phosphorylation events on heterotrimeric complex formation or dissociation. PKC- ζ happens to be constitutively active in cells [7,31] and a stimulus-induced increase of overall PKC- ζ activity is moderate and probably

Figure 6 Phosphorylation of 14-3-3 isotypes by PKC- ζ

Kinase assay *in vitro* with purified recombinant PKC- ζ in the absence (lane 1) or presence of purified recombinant 14-3-3 β (lane 2), - θ (lane 3) or - ζ (lane 4) and [γ - 32 P]ATP. Kinase reactions were stopped with SDS sample buffer and subjected to SDS/PAGE (10% gel) and autoradiography. Positions of autophosphorylated PKC- ζ and phosphorylated 14-3-3 isotypes are indicated.

restricted to a small compartment in the cell [31]. In order to see a possible effect of phosphorylation most clearly, we therefore chose to compare wild-type PKC- ζ with the kinase-inactive mutant PKC- ζ (K281W) in transfected COS cells.

Figure 5 shows that kinase-dead PKC- ζ is also capable of complex formation with Raf-1 and, interestingly enough, does so much more effectively than wild-type PKC- ζ . This suggests that a PKC- ζ -mediated phosphorylation event negatively regulates formation or decreases the stability of the complex. In the following sections, we investigated whether PKC- ζ -mediated phosphorylation of 14-3-3 is instrumental in this negative regulation.

14-3-3 β and - θ are substrates of PKC- ζ

Aitken and co-workers [25,43,44] have already reported that some 14-3-3 isoforms can be phosphorylated by PKC, but the physiological relevance of this phosphorylation was not clear. As PKC- ζ was not included in these studies, we tested whether 14-3-3 is a substrate for PKC- ζ . This was done in a kinase assay *in vitro* using purified recombinant PKC- ζ and bacterially expressed, purified 14-3-3 isotypes. Figure 6 shows that 14-3-3 β and - θ , the best complex-forming isotypes (Figure 2), are readily phosphorylated by PKC- ζ . On SDS/PAGE, phosphorylated 14-

Figure 7 Reduced binding of 14-3-3 β to PKC- ζ under phosphorylation conditions

(Top panel) Comparison of association of (endogenous) 14-3-3 with wild-type (wt) and kinase-dead (kd) PKC- ζ *in vivo*. Lysates of COS cells transfected with the combinations of plasmids indicated (+) were equalized for protein contents and immunoprecipitations (I.P.) were performed with anti-14-3-3 (pan) antibody. Co-precipitated wtPKC- ζ or kdPKC- ζ (arrow) was visualized by SDS/PAGE and immunoblotting. Below is shown a control for equal expression (Western blot). (Middle panel) Binding *in vitro* of 14-3-3 β to wtPKC- ζ or kdPKC- ζ in the presence (+) or absence (−) of ATP. The PKC- ζ constructs (Myc-tagged) were expressed in COS cells. Cell lysates were immunoprecipitated with 9E10 monoclonal antibody coupled to Protein A–Sepharose beads. Equal amounts of mycPKC- ζ were incubated with purified 14-3-3 β under kinase conditions *in vitro* (+ ATP) or without ATP. Binding of 14-3-3 β to the beads was visualized after SDS/PAGE (12% gel) by immunoblotting with anti-14-3-3 (pan) serum. A similar result was obtained in a second independent experiment. (Bottom panel) Binding *in vitro* of PKC- ζ to immobilized 14-3-3 β under phosphorylation conditions, in the presence (+) or absence (−) of the pseudo-substrate peptide inhibitor (Ψ -substrate) of PKC- ζ . Glutathione–Sepharose-bound GST-14-3-3 β or GST (control) was incubated with purified PKC- ζ in the presence of ATP. Beads were washed, subjected to SDS/PAGE (12% gel) and analysed by Western blotting with anti-PKC- ζ serum.

3-3 β gives a single band, whereas 14-3-3 θ shows up as a doublet. In contrast, 14-3-3 ζ , which does not bind to PKC- ζ (Figure 1), is also no substrate for PKC- ζ .

Phosphorylation of 14-3-3 by PKC- ζ negatively regulates their association in the complex

Since 14-3-3 forms a ternary complex with PKC- ζ and Raf-1 (Figure 2) and since the complex is stronger with kinase-inactive PKC- ζ than with wild-type PKC- ζ (Figure 5), we addressed the likely hypothesis that PKC- ζ -mediated phosphorylation of 14-3-3 β or - θ (Figure 6) would negatively regulate ternary complex formation or favour dissociation of the complex. Indeed, support for this concept comes from three different experimental approaches, as follows.

First, we overexpressed wild-type PKC- ζ or kinase-dead PKC- ζ . Immunoprecipitates of endogenous 14-3-3 were then analysed for co-precipitated PKC- ζ by immunoblotting. Figure 7 (top panel) shows that 14-3-3 binds more strongly to kinase-dead

than to wild-type PKC- ζ . This indicates that the observed stronger association of kinase-dead PKC- ζ with Raf-1 may be caused by a stronger interaction of PKC- ζ with 14-3-3. In the second type of experiment, we incubated immunoprecipitated Myc-tagged PKC- ζ (wild-type or kinase-dead) with purified recombinant 14-3-3 β in the presence of 100 μ M [γ - 32 P]ATP. Figure 7 (middle panel) shows that under these phosphorylation conditions *in vitro*, wild-type PKC- ζ bound less 14-3-3 β than in the absence of ATP or than kinase-dead PKC- ζ . We verified by autoradiography (results not shown) that under these conditions the wild-type PKC- ζ indeed phosphorylated 14-3-3 β , consistent with Figure 6. In the third alternative approach we incubated, under the same phosphorylation conditions *in vitro*, purified GST-14-3-3 β fusion protein together with purified PKC- ζ in the presence or absence of the pseudo-PKC- ζ -substrate inhibitor peptide. The Western blot in Figure 7 (bottom panel) shows that binding was stronger in the presence of the inhibitor peptide. Also in this experiment phosphorylation of GST-14-3-3 β (and its inhibition) was verified by autoradiography (results not shown).

From the results of these three experiments (Figure 7), we conclude that phosphorylation of 14-3-3 β by PKC- ζ (Figure 6) weakens their physical association. Since the ternary complex of PKC- ζ with 14-3-3 and Raf-1 was much stronger with kinase-dead PKC- ζ (Figure 5), the data suggest that phosphorylation of 14-3-3 by PKC- ζ leads to dissociation of the ternary complex in between PKC- ζ and 14-3-3.

14-3-3 β phosphorylation-site mutants are constitutive adapters

So far, our results have indicated that the adapter function of 14-3-3 depends on its phosphorylation status and that, therefore, 14-3-3 is likely to be a transient mediator of a phosphorylation signal from PKC- ζ to Raf-1 [8]. To verify this concept further, we made two point mutations, S130A and T141A, in putative PKC- ζ phosphorylation sites of 14-3-3 β . We reasoned that these mutants, being poorer substrates for PKC- ζ , would be kept in the ternary complex, even under phosphorylation conditions. We chose to mutate S130 and T141 in particular because these sites in the 14-3-3 β molecule are easily accessible to protein kinases [45], T141 is present in 14-3-3 β and a similar residue is present in 14-3-3 θ , but not in other 14-3-3 isoforms. S130 is unique to 14-3-3 β . Moreover, S130 is located in the annexin-like domain of 14-3-3 β (KGDYFRYLS¹³⁰EVASGDNK, consensus phosphorylation motif for PKC underlined), suggested to be involved in PKC binding [46,47].

Figure 8 shows that the 14-3-3 β mutants significantly enhance co-immunoprecipitation of PKC- ζ and Raf-1, and are, therefore, indeed much better mediators of PKC- ζ –Raf-1 complex formation than wild-type 14-3-3 β . The S130A mutant clearly is a stronger adapter than T141A, whereas the double mutant acts in a more or less additive fashion. We confirmed by kinase assays *in vitro* (similar to those shown in Figure 6) that the purified recombinant 14-3-3 β mutant proteins are indeed less phosphorylated by PKC- ζ than wild-type 14-3-3 β protein. The reduction in the phosphorylation of 14-3-3 β (S130A), 14-3-3 β (T141A) and the double mutant amounted to 26, 58 and 72 %, respectively. The results suggest that, whereas both S130 and T141 in 14-3-3 β are phosphorylated by PKC- ζ , the former phosphorylation site (unique to 14-3-3 β) is the most important one for the regulation of complex dissociation.

We conclude that the inability of PKC- ζ to phosphorylate 14-3-3 β at amino acid positions 130 and 141 promotes their association and ternary complex formation with Raf-1. The data support the concept that 14-3-3-mediated ternary complex formation is a transient event in signal transduction from PKC- ζ to

Figure 8 14-3-3 β mutated in its PKC phosphorylation sites amplifies co-immunoprecipitation of PKC- ζ with Raf-1

COS cells were co-transfected with PKC- ζ and Raf-1, together with wild-type (wt) 14-3-3 β , the point mutants 14-3-3 β (S130A) (S-A), 14-3-3 β (T141A) (T-A) or the double-mutant (S-A/T-A), as indicated. Cell lysates were equalized for protein content and were subjected to immunoprecipitation (I.P.) with antibodies against PKC- ζ or Raf-1, and the amount of co-precipitated Raf-1 or PKC- ζ (arrows), respectively, was visualized by SDS/PAGE and immunoblotting. Expression of the proteins in cell lysates was checked by Western blotting (lower panels).

Raf-1 [8] and is terminated by 14-3-3 phosphorylation. We propose that 14-3-3 mutants that lack essential PKC phosphorylation sites may function as constitutive adapters between PKC and the next protein kinase (Raf-1 in our system) in signal-transduction pathways.

DISCUSSION

With PKC- ζ we add a new member to the growing list of signalling molecules that bind to 14-3-3 [10–13,18]. More importantly, we found that 14-3-3, the β and θ isotypes in particular, can amplify coupling of PKC- ζ to Raf-1 in a ternary signalling complex. Herewith we extend our previous observations that, in Rat-1 and COS cells, PKC- ζ co-immunoprecipitates with Raf-1 and directly activates Raf-1 by phosphorylation [8]. We also found that formation of the heterotrimeric complex is dependent on phospho-amino acids, particularly phospho-Ser-186, in PKC- ζ and is negatively regulated by PKC- ζ -mediated phosphorylation of 14-3-3. Alternatively, this phosphorylation may lead to dissociation of pre-existing ternary complex. Importantly, this would imply a new concept of 14-3-3 operation in signal-transduction pathways. Besides acting as a scaffold protein facilitating the binding of two consecutive kinases, thus playing a functional role in signal transmission, 14-3-3 may also attenuate signalling when its phosphorylation leads to complex dissociation.

Whereas 14-3-3 binding to Raf-1 is well established [34–36], we demonstrated by overlay experiments that 14-3-3 binds directly to PKC- ζ at its regulatory domain. In this domain, the region RHMD^{S186}VMP differs from the described ‘mode-2’ motif for 14-3-3 binding [11] by only one extra residue between the (phospho)serine and the proline. We demonstrated that an S186A

point mutation at this site abrogated 14-3-3 binding as well as complex formation, strongly suggesting that this is the 14-3-3-binding site. This would add a new phosphoserine motif to the ones already described [10–13], determining the interaction with 14-3-3 adapters. Definitive proof that this amino acid sequence in PKC- ζ represents a genuine 14-3-3-binding motif should come from an independent study using phosphopeptides as competitive inhibitors. It thus appears that PKC- ζ is the first atypical PKC isotype, next to the classical and ‘new’ PKC isotypes [11,13,18], to contain a 14-3-3-binding site at its regulatory domain.

Analogous to our findings, Braselmann and McCormick [19] reported 14-3-3 β -mediated complex formation between Raf-1 and Bcr. Contrary to our data, however, 14-3-3 β overexpression failed to enhance co-immunoprecipitation between Raf-1 and Bcr. The authors explained this by the notion that 14-3-3 proteins are very abundant in cells, so that they would be not limiting for the formation of the complex. However, there is cell-type specificity in the expression of 14-3-3 isotypes [25] and there is some selectivity in 14-3-3 isotypes to mediate complex formation between different kinases, as we have found for different PKC isotypes and Raf-1 (results not shown). For example, we found that complex formation between PKC- ζ and Raf-1 is particularly strong with 14-3-3 β and θ , whereas 14-3-3 ζ and η do not facilitate this complex formation (Figure 2). Thus cell-type-specific expression and target selectivity of 14-3-3 isotypes could explain why, in different cell types, different PKC isotypes interact with and activate Raf-1 in a signalling complex via this type of adapter [4–6,8,48].

Although our data show clearly that 14-3-3 β and θ amplify complex formation of PKC- ζ with Raf-1 (Figures 2, 4 and 5), definite proof that this complex is absolutely dependent on these 14-3-3 isotypes should come from reconstitution experiments *in vitro* with purified proteins, showing that formation of the ternary complex is correlated with a certain stoichiometry of the individual components. The use of a dominant-negative 14-3-3 construct [37] may be helpful in this respect. Our present data do not necessarily imply that 14-3-3 acts as a bridging molecule that cross-links the two kinases. It may also act as a scaffold protein holding the kinases together and in touch with each other. In fact, our previous data showing that PKC- ζ phosphorylates Raf-1 directly [8] would be consistent with such a model.

Our results suggest that PKC- ζ -mediated phosphorylation of 14-3-3 leads to dissociation of the ternary complex between PKC- ζ and 14-3-3. This was concluded from four lines of evidence: (i) the ternary complex as well as the PKC- ζ –14-3-3 β complex is much stronger with kinase-inactive PKC- ζ , as shown by co-immunoprecipitation; (ii) 14-3-3 β (or θ , but not ζ) is a PKC- ζ substrate *in vitro*; (iii) binding of 14-3-3 β to PKC- ζ is weaker under phosphorylation conditions (+ATP) and stronger in the presence of an inhibitor (pseudo-substrate peptide) of the kinase; and (iv) 14-3-3 β mutants in which the ¹³⁰Ser and ¹⁴¹Thr phosphorylation sites for PKC- ζ are mutated into Ala showed increased ternary complex formation with PKC- ζ and Raf-1. It has been shown that 14-3-3 is a poor substrate for Raf-1 [42,49,50], and that 14-3-3 is constitutively associated with Raf-1, regardless of its activation state [34,38]. Indeed, we confirmed that, under phosphorylation conditions, when PKC- ζ dissociates from the complex, 14-3-3 remains associated with Raf-1 (results not shown).

Our results with the 14-3-3 mutants indirectly imply a new phosphorylation site, ¹³⁰Ser (and to a lesser extent ¹⁴¹Thr), in 14-3-3 β that regulates the association/dissociation of 14-3-3 β and PKC- ζ . The same mechanism may exist for 14-3-3 θ , as it forms a complex with PKC- ζ , it is an *in vitro* PKC- ζ substrate and it contains also a PKC phosphorylation site at a position similar to

¹⁴¹Thr in 14-3-3 β . The ¹³⁰Ser site, exclusively present in 14-3-3 β , is located in an annexin-like domain, thought to be involved directly in binding to PKC [46,47]. In interesting contrast, the 14-3-3 ζ isotype contains no phosphorylation site at this position, is no substrate for PKC- ζ (Figure 6), does not form a complex with PKC- ζ (Figure 1) and might compete with endogenous 14-3-3 in COS cells, thus impairing ternary complex formation (Figure 2), as if it were a dominant-negative 14-3-3 construct [37]. Spatially, the ¹³⁰Ser residue in 14-3-3 β is located near the C-terminus and near the edge of the groove-like tertiary structure of the protein, adjacent to two other phosphorylation sites, ¹⁸⁵Ser (in 14-3-3 β and - ζ isotypes) and ²³³Thr (in 14-3-3 ζ and - τ/θ , but not in - β) [45,51]. This region in 14-3-3 determines the binding to target proteins [52–54]. Phosphorylation of ²³³Thr negatively regulates 14-3-3 binding to the regulatory domain of Raf-1 [50]. Remarkably similar, our data suggest that phosphorylation of ¹³⁰Ser in 14-3-3 β negatively regulates the binding to PKC- ζ . Thus the specificity of 14-3-3 isotypes to determine the association with target proteins as well as their dissociation by phosphorylation is located in this relatively narrow region on the surface of the 14-3-3 protein. Although not explicitly proven here, our data imply that PKC- ζ -mediated phosphorylation of 14-3-3 is a likely event in the process of PDGF-induced, PKC- ζ -mediated Raf-1 activation [8]. We propose that in the ternary complex, PKC- ζ phosphorylates Raf-1 [8] and 14-3-3 sequentially. The first phosphorylation activates Raf-1, whereas 14-3-3 phosphorylation negatively regulates ternary complex formation (or favours dissociation) and can thus be tentatively considered to be an attenuation/termination signal of Raf-1 phosphorylation and activation by PKC- ζ .

In the ternary complex, each of the two 'wings' of the 14-3-3 dimer binds to the regulatory domain of either PKC- ζ or Raf-1 (Figures 3 and 4). In this particular 14-3-3-bound configuration, Raf-1 is inactive [22,39]. When in this complex, PKC- ζ phosphorylates and activates Raf-1 [8], the stoichiometry of 14-3-3 binding to Raf-1 seem to remain unchanged, as data of Rommel et al. [38] and ourselves (not shown) indicate. Yet, it is known that activation of Raf-1 is accompanied by displacement of 14-3-3 from the regulatory domain of Raf-1 to a second site (Ser-621) at the catalytic domain [2,38,39]. Since a kinase-defective construct of the catalytic domain of Raf-1 (K375E) is unable to bind 14-3-3 [38], it is reasonable to assume that 14-3-3 can only bind to the catalytic domain when Raf-1 is activated. Because of the unchanged 14-3-3–Raf-1 stoichiometry mentioned, it seems likely that upon PKC- ζ -mediated Raf-1 phosphorylation and activation, the position of 14-3-3 binding shifts from the regulatory to the catalytic domain of Raf-1. In this regard, our results support and extend the putative model proposed by Morrison and Cutler [2] for Ras-mediated Raf-1 activation. It can be envisioned that the 14-3-3 wing released by PKC- ζ (following 14-3-3 phosphorylation by PKC- ζ) is free to bind to the catalytic domain of the activated Raf-1, whereas in the same concerted reaction the other wing of the 14-3-3 dimer is then released from the Raf-1 regulatory domain, free to bind another target phosphoprotein, possibly a downstream substrate of Raf-1. Whereas 14-3-3 phosphorylation may be responsible directly for the release of 14-3-3 from the regulatory domain of Raf-1 [38], it remains to be determined whether PKC- ζ -mediated phosphorylation of the ¹³⁰Ser residue in 14-3-3 β is instrumental in this release.

Finally, the new concept that phosphorylation of the adapter/scaffold protein 14-3-3 leads to dissociation of signalling complexes may have broad implications. It not only sheds more light on the complexity of Raf-1 regulation [2], but may also be important for many other signalling processes where 14-3-3 is

involved, for example in apoptotic signal transduction initiated by dissociation of a BAD–14-3-3 complex [14,15]. Since the 14-3-3 β isotype associates with BAD [55], our 14-3-3(S130A) mutant may be of value because it may bind BAD and activate Raf-1 constitutively, and thus may promote cell survival/proliferation in certain systems.

After completion of the present work, a paper [56] appeared showing interaction of 14-3-3 γ with both Raf-1 and poorly defined PKC in vascular smooth-muscle cells. In this system, PDGF stimulated a delayed and transient phosphorylation of 14-3-3 γ , most likely by a PKC activity.

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