Protein kinase C activation by acidic proteins including 14-3-3

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14-3-3 proteins may function as adapter or scaffold proteins in signal transduction pathways. We reported previously that several 14-3-3 isotypes bind to protein kinase C (PKC)-ζ and facilitate coupling of PKC-ζ to Raf-1 [van der Hoeven, van der Wal, Ruurs, van Dijk and van Blitterswijk (2000) Biochem. J. **345**, 297–306], an event that boosts the mitogen-activated protein kinase (ERK) pathway in Rat-1 fibroblasts. The present work investigated whether bound 14-3-3 would affect PKC-ζ activity. Using recombinant 14-3-3 proteins and purified PKC-ζ in a convenient, newly developed *in vitro* kinase assay, we found that 14-3-3 proteins stimulated PKC-ζ activity in a dose-dependent fashion up to approx. 2.5-fold. Activation of PKC-ζ by 14-3-3 isotypes was unrelated to their mutual affinity, estimated by co-immunoprecipitation from COS cell lysates. Accordingly, PKC-ζ with a defective (point-mutated) 14-3-3-binding site, showed the same 14-3-3-stimulated activity as wild-type PKC-ζ. As 14-13-3 proteins are acidic, we tested several other acidic proteins, which turned out to stimulate PKC-ζ activity in a similar fashion, whereas neutral or basic proteins did not. These effects were not restricted to the atypical PKC-ζ, but were also found for classical PKC. Together, the results suggest that the stimulation of PKC activity by 14-3-3 proteins is non-specific and solely due to the acidic nature of these proteins, quite similar to that known for acidic lipids.

INTRODUCTION

Members of the 14-3-3 family of proteins are emerging as adapter or scaffold proteins that bind to and may interconnect a variety of proteins involved in signal transduction, cell cycle regulation and apoptosis [1]. Among these proteins is protein kinase C (PKC), a major serine/threonine kinase family involved in cell growth and differentiation. 14-3-3 isotypes have been reported to bind to mammalian PKC-γ, PKC-ϵ [2], PKC-θ [3], PKC-ζ [4] and PKC-μ [5], as well as to a *Dictyostelium* PKC [6]. We reported previously that several 14-3-3 isotypes bind to PKC-ζ and facilitate coupling of PKC-ζ to Raf-1 in a phosphorylation-dependent manner [4]. We found that this heterotrimeric complex dissociates upon PKC-ζ-mediated phosphorylation of 14-3-3, suggesting that complexation is a functional and transient event in PKC-ζ-mediated signal transduction towards Raf-1 and mitogen-activated protein kinase ('ERK'), as occurs in Rat-1 fibroblasts [4,7]. We were wondering whether, in this complex, 14-3-3 would have an effect on PKC-ζ activity.

Several studies have suggested that 14-3-3 proteins can modulate PKC activity [3,5,6,8–10], but the data are conflicting and the conclusions confusing. Initially, a group of kinase C inhibitor proteins ('KCIP') from brain were identified as 14-3-3 proteins [8,9]. Their inhibitory action on PKC was partially overcome by phorbol ester or diacylglycerol, suggesting that the cysteine-rich 'zinc finger' domains in PKC are involved in the interaction with 14-3-3 [10]. In contrast, other investigators reported a stimulatory effect (approx. 1.7-fold) of 14-3-3 on PKC activity [11–13]. All these studies were performed on PKC isolates without the definition of PKC isotypes. Differential activation of various PKC isoenzymes by 14-3-3ζ was reported by Acs et al. [14]; the classical PKCs were activated approx. 2-fold, while PKC-ϵ showed 5-fold activation in the absence of phospholipids.

Several explanations have been put forward to reconcile these conflicting data: the effect of 14-3-3 on PKC could depend on different 14-3-3 isoforms [10] or PKC subtypes [14,15] involved, or on the PKC substrate used [16]. Others proposed inadequate renaturation of the 14-3-3 preparations [17], or contamination of 14-3-3 preparations with an inhibitory protein [12]. None of these explanations, however, satisfactorily account for the discrepancies.

The best and most straightforward way to resolve the issue, if and how 14-3-3 modulates PKC-ζ activity, is to use a well-defined *in vitro* kinase assay system with pure proteins of defined isotype. For this purpose, we used three bacterially expressed and purified 14-3-3 isotypes (β, ζ and θ), PKC-ζ purified from recombinant baculovirus-infected insect Sf9 cells, and a new, sensitive *in vitro* kinase assay with biotinylated ϵ-peptide (derived from PKC-ϵ) as a substrate. Using this assay system we demonstrated that 14-3-3 stimulates PKC-ζ (and classical PKC) activity. However, we also show that other negatively charged proteins stimulate PKC activity as well, indicative of little specificity. Furthermore, we show that at least part of the controversy over whether 14-3-3 stimulates or inhibits PKC is not real but relates to the definition of PKC activity.

MATERIALS AND METHODS

Materials and cells

Dulbecco's modified Eagle's medium was from ICN-Flow and foetal calf serum from BioWhittaker. ATP was from Boehringer. Acrylamide and *N*,*N*′-methylendiacrylamide were from Merck. [γ-^{32}P]ATP (3000 Ci/mmol) and the enhanced chemiluminescence system were from Amersham. Classical PKC (mixture of rat brain PKC-α, PKC-β and PKC-γ isoforms) was from Alexis Corporation. L-α-Phosphatidylserine, cytochrome *c* (horse heart)

Abbreviation used: PKC, protein kinase C.

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and apo-transferrin (human) were from Sigma. Myoglobin (horse skeletal muscle) was from Calbiochem and lactoglobulin from Serva.

COS7-M6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum and containing penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM L-glutamine. Transfection of the cells (using the DEAE-Dextran method) with PKC-ζ [wild-type or S186A ($Ser^{186} \rightarrow Ala$) mutant] and 14-3-3 isotypes were described in our previous paper [4].

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

PKC-ζ was partially purified from Sf9 insect cells infected with PKC-ζ recombinant baculovirus [18], kindly provided by Dr. D. Fabbro (Novartis, Basel, Switzerland). Purification was based on the method of Dimitrijevic et al. [19]. 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 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 0.3% β-mercaptoethanol, sonicated and centrifuged at 100000 ***g*** for 30 min. Supernatant (5 ml; 25 mg of protein) was loaded on a Mono Q column and eluted in a linear gradient of 0–1 M NaCl in lysis buffer with 0.02% Triton X-100 (10 ml, flow rate 0.5 ml/min). Fractions between 0.7–0.9 M NaCl were diluted 6-fold in elution buffer without NaCl, loaded on to a Mono S column and eluted with the same gradient. Fractions between 0.65–1 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, 1 mM dithiothreitol. The final PKC-ζ preparation (50 μg/ml; purity shown in Figure 1) was frozen in aliquots and stored at -70 °C until use.

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

Synthesis of biotinylated ϵ-peptide substrate

The epsilon substrate peptide (ERMRPRKRQGSVRRRV) was synthesized by standard Fmoc chemistry on a Millipore 9050 *plus* Pepsynthesizer with reagents supplied by the manufacturer. ϵ-Peptide was biotinylated (on its free N-terminus) at room temperature for 3 h in 200 mM sodium phosphate buffer, pH 8, by the addition of 35 mg of sulphosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce) to the 200 mg of peptide still attached to the resin (arginines and lysines were protected). After biotinylation, protective groups were removed and the peptide was released from the resin. Biotinylated and non-biotinylated peptides were separated by HPLC. The structural integrity of the peptide was confirmed by amino acid analysis.

Antibodies, immunoprecipitation and immunoblotting

Antibodies against PKC-ζ were raised in rabbits and were directed against an oligopeptide (GFEYINPLLLSAEESVC) 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 to the N-terminus of 14-3-3β (KSELVQKAKLAEQAERYDDC). 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). Monoclonal antibodies against c-Myc were from the hybridoma clone 9E10. Conditions for COS cell lysis, immunoprecipitation and immunoblotting were as described in our previous paper [4].

In vitro kinase assay for PKC

We developed a new, sensitive *in vitro* kinase assay for PKC, especially for PKC-ζ. This quick and highly reproducible assay utilizes a biotinylated-peptide substrate (Biotin-spacer-ERMRPRKRQGSVRRRV) derived from the pseudo-substrate region of PKC-ϵ in combination with immobilized streptavidin. This ϵ-peptide is a very good substrate for PKC-ζ [20,21]. Kinase activity was assayed in a volume of 50 μl in 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM Na_2VO_4, 50 μM [γ-^{32}P]ATP (unlabelled ATP added to yield a specific raidoactivity of approx. 500 c.p.m./pmol ATP), 25 μM biotinylated ϵ-peptide, 25 ng of PKC-ζ (or other PKC) and 50 μg/ml phosphatidylserine as indicated. The assay was performed at 37 °C for 30 min and was started by the addition of a 1:1 mixture of a 10× stock solution of ATP and biotinylated ϵ-peptide. After 25 min 20 μl of immobilized streptavidin (50% slurry; Pierce) was added and incubated with shaking for 5 min. The reaction was stopped by the addition of 3 ml of ice-cold buffer containing 25 mM EDTA, 1 M NaCl, 25 mM Hepes, pH 7.4. The reaction mixture was immediately filtered over Whatman GF/A glass-fibre filters mounted on a Millipore filtration manifold (filter diameter 2.5 cm; pre-incubated in 50 mM ATP to reduce non-specific ATP binding). Filters were washed at least ten times with 10 ml of 1 M NaCl and two times with water and then counted by liquid scintillation counting. Non-specific radioactivity bound to filters (background) was routinely monitored by leaving out kinase or by adding 10 mM biotin to the assay as indicated. The advantage of using the biotinylated peptide substrate, rather than the non-biotinylated substrate in a filter-binding assay, is the specific isolation (via bead filtration) of phosphorylated ϵ-peptide only, since, for example, autophosphorylated protein kinase or other proteins would not be retained by this method. Although ϵ-peptide is a preferred substrate for PKC-ζ, other protein kinases in COS cell lysates might in principle also be capable of phosphorylating the peptide. However, the major contribution of PKC-ζ could be assessed by its selective inhibitor Ro 31-8220 [21].

Specific PKC-ζ activity was calculated as follows: First, the specific radioactivity (in c.p.m./nmol) of [γ-^{32}P]ATP was calculated. Then the specific kinase activity (in nmol P_i/min per mg of protein) was calculated using the following equation: (c.p.m. − background)/(specific radioactivity × min × mg of protein).

RESULTS AND DISCUSSION

We measured the effect of purified 14-3-3 proteins on the activity of purified recombinant PKC-ζ (purity shown in Figure 1). For this purpose, we developed a new sensitive *in vitro* kinase assay, using a biotinylated ϵ-peptide (derived from the PKC-ϵ pseudo-substrate) as a substrate (see the Materials and methods section). In this assay, we tested three recombinant 14-3-3 isotypes, which show a differential interaction/affinity with PKC-

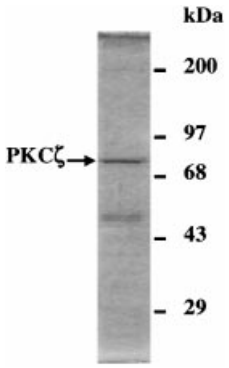

Figure 1 Purity of PKC-ζ preparation

PKC-ζ was purified as described in the Materials and methods section. The final PD-10 column fraction was subjected to SDS/PAGE and Coomassie Brilliant Blue staining. The major PKC-ζ band is indicated. Note the absence of 14-3-3 proteins at 30 kDa.

ζ in a COS cell overexpression system. Figure 2 shows that 14-3-3β and 14-3-3θ co-immunoprecipitate with PKC-ζ from COS cell lysates, while 14-3-3ζ does not. Despite the differences in affinity to PKC-ζ the effects of these 14-3-3 isotypes on PKC-ζ activity are virtually the same. Figure 3(A) shows that in the absence of phospholipids 14-3-3β, 14-3-3θ and 14-3-3ζ stimulate PKC-ζ activity 2.2–2.6-fold. This is in agreement with the results of others using other PKCs [11–14]. Acs et al. [14] found no stimulation of PKC-ζ activity by 14-3-3ζ, but this can be explained by the fact that they used 0.5 mM $CaCl_2$ in their assay which is known to inhibit PKC-ζ activity [22].

The above results indicate that 14-3-3 isotypes stimulate PKC-ζ activity independent of their mutual affinity. To confirm this further, we used a PKC-ζ mutant, PKC-ζ(S186A), that is unable to bind 14-3-3 at the putative phosphoserine motif RHDMpS^{186}VMP in its regulatory domain, and is thus defective in 14-3-3-mediated complex formation with Raf-1 [7]. We overexpressed this mutant as well as the wild-type PKC-ζ in COS cells and measured their kinase activity in cell lysates in the presence or absence of 14-3-3β. Figure 4 shows that the two overexpressed PKC-ζ constructs show a 4-fold increase in kinase activity in cell lysates, compared with empty-vector-transfected control cells. This enhanced activity was fully blocked by Ro 31-8220 (5 μM) (results not shown), confirming that this was PKC-

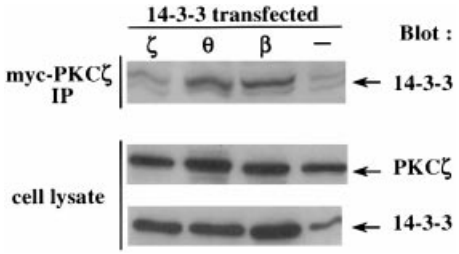

Figure 2 Differential association of 14-3-3 isotypes to PKC-ζ in immunoprecipitates

COS cells were transfected with pmtSM plasmids encoding PKC-ζ (Myc-tagged) together with 14-3-3 isotypes β, θ or ζ, as indicated. Cells were lysed 48 h later and PKC-ζ was immunoprecipitated (IP) with 9E10 antibody. IPs and cell lysates (expression controls) were subjected to SDS/PAGE and immunoblotted with anti-14-3-3 (pan) or anti-PKC-ζ serum.

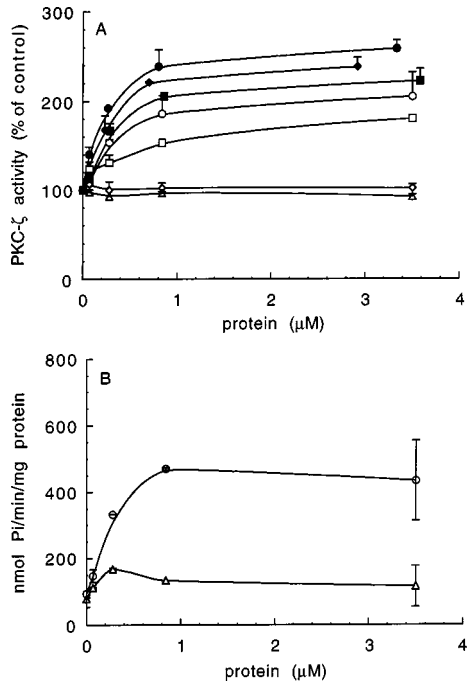

Figure 3 Effect of 14-3-3 isotypes and control proteins on the activity of PKC-ζ (A) or classical PKC (B) *in vitro*

(**A**) Effect of 14-3-3β (pI 4.54; ●), 14-3-3ζ (pI 4.50; ◆), 14-3-3θ (pI 4.46; ■), lactoglobulin (pI 5.1–5.3; ○), apo-transferrin (pI 5.56; □), myoglobin (pI 7.0–7.4; ◇) and cytochrome *c* (pI 10.1; △) on the activity of PKC-ζ. The pI values of the 14-3-3 isotypes were calculated from NCBI-GenBank sequences, using http://www.expasy.ch/. (**B**) Effect of lactoglobulin (○) and cytochrome *c* (△) on the activity of classical PKC (mix of PKC-α, PKC-β and PKC-γ). Data are expressed as percentage of control ± S.D.; 100% values approximately equal the incorporation of 5 nmol of P_i/min per mg of protein.

ζ activity [21]. Figure 4, furthermore, shows that stimulation of activity by 14-3-3β is the same (1.3-fold) for wild-type PKC-ζ and PKC-ζ(S186A), indicating that this stimulation is unrelated to the binding of 14-3-3 to PKC-ζ. The extent of stimulation when using COS cell lysates is reproducible but lower than that with purified compounds (Figure 3A). This is because PKC-ζ in these lysates has relatively high constitutive activity, as we have shown and discussed before [21]. Additional activation by any stimulus, or by 14-3-3 (in the present study), is therefore moderate [21].

Our conclusion that 14-3-3 stimulates PKC-ζ activity independent of their mutual binding may also be valid for classical PKCs (see below) but not for any PKC isotype. PKC-μ, a subtype that differs from other PKC members in structure and activation mechanisms, has been shown to bind 14-3-3τ via a Cbl-like serine-containing consensus motif [5]. This binding results in inactivation of PKC-μ [5]. Apparently, in this case and possibly also for 14-3-3τ binding to PKC-θ [3], the mode of interaction differs from PKC-ζ, so that kinase activity is down-regulated.

Since 14-3-3 proteins are acidic, having a pI of about 4.5 (see legend of Figure 3A), we tested the effect of several unrelated proteins with different isoelectric points. We found that acidic non-14-3-3 proteins stimulate PKC-ζ activity almost equally well, while neutral or basic proteins had no effect (Figure 3A). A similar stimulatory effect of acidic proteins is seen on the activity of classical PKCs (Figure 3B). We conclude that the moderate activation of PKC-ζ (and probably also classical PKCs) by 14-

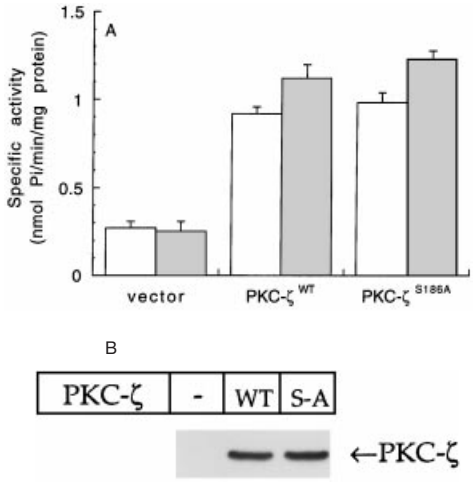

Figure 4 Effect of purified 14-3-3β on PKC-ζ activity in lysates of COS cells transfected with empty vector, wild-type PKC-ζ or PKC-ζ(S186A) mutant

(**A**) No 14-3-3β added (open bars); 14-3-3β added to 2.75 μM (filled bars). The *in vitro* kinase assay was performed as described in the Materials and methods section, with the following minor modifications: assay volume 25 μl, 100 μM ATP, 50 μM ϵ-peptide substrate, 100 ng of protein (cell lysate). The specific filter binding of [γ^{32}P]ATP was estimated by including 10 mM biotin in the assay. Data are means $\pm$ S.D. of triplicates and are representative of three experiments that had similar results. (**B**) Immunoblots for PKC-ζ in COS cell lysates (expression controls). Cells were transfected with 3 μg of pmt2SM-mycPKC-ζ^{WT} (WT; plus 3 μg of empty vector) and 1 μg of pmt2SM mycPKC-$\zeta^{(S186A)}$ (S-A; plus 5 μg of empty vector). Transfected cells were lysed in 250 μl of buffer and 5 μl of total cell lysate was subjected to SDS/PAGE and immunoblotted with anti-PKC-ζ antibody.

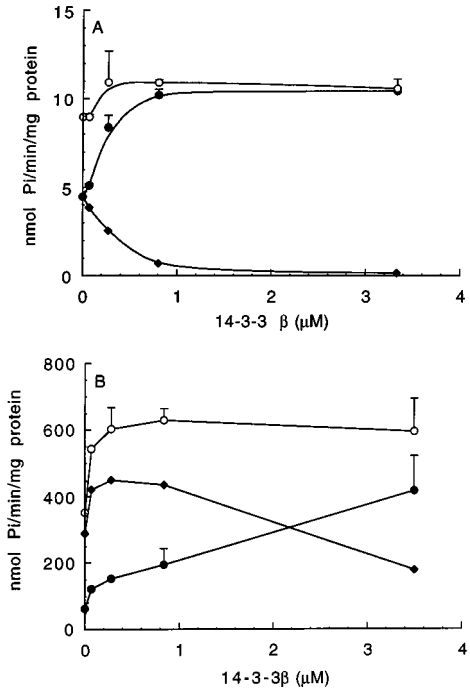

Figure 5 Effect of 14-3-3β on the activity of PKC in the presence of phosphatidylserine

The effect of 14-3-3β on the activity of PKC-ζ (**A**) or classical PKC (**B**) was measured in the presence (○) or absence (●) of phosphatidylserine. Alternative expression as phosphatidylserine-activated kinase is also shown (◆). The effect of lactoglobulin in the absence and presence of phosphatidylserine shows a similar effect (results not shown).

3-3 is solely caused by the acidic nature of the 14-3-3 protein. It thus appears that PKC activity is not only elevated by acidic lipids, such as phosphatidylserine [22–24], phosphatidic acid [22], *cis*-unsaturated fatty acids [22,25] and polyphosphoinositides [23,24], but also by acidic proteins in a rather non-specific way.

Finally, we wish to comment on the PKC inhibitory action of 14-3-3, reported by Aitken and co-workers [8–10]. In their studies, they mostly used (classical) PKC isolates from sheep brain and corrected for the background activity from other kinases (including the catalytic fragment of PKC) by defining PKC activity as phosphatidylserine-stimulated kinase activity. This definition of PKC activity is legitimate or even necessary when using partially purified PKC. In our study, however, such a correction is unnecessary since all kinase activity stems only from pure, unfragmented PKC-ζ. Legitimate or not, the correction for phosphatidylserine-independent kinase activity has important implications for the final statement on whether PKC is activated or inhibited. Figure 5(A) shows what happens when we measure the effect of acidic proteins, 14-3-3 and lactoglobulin on PKC-ζ activity in the absence and presence of phosphatidylserine. When subtracting the kinase activity in the absence of phosphatidylserine from that in the presence of phosphatidylserine, the effect of 14-3-3 on PKC-ζ appears to be inhibitory. The same holds for lactoglobulin [results not shown; note that lactoglobulin has similar effects on classical PKCs (Figure 3B)]. The dose-dependency of this apparent inhibition is very similar to that reported by Robinson et al. [10], who used recombinant 14-3-3τ. This means that the reported inhibitory effect of 14-3-3 on PKC activity may just be due to the correction for phosphatidylserine-independent kinase activity and is not a real inhibition in terms of a constraint on kinase activity. Without this correction, the effect of 14-3-3 on PKC-ζ *per se* would be stimulatory, as shown. Thus, the apparent discrepancy between our data (PKC stimulation by 14-3-3) and previous reports by Aitken's group (PKC inhibition) can be explained by the definition of PKC activity as phosphatidylserine-stimulated protein kinase activity.

In conclusion, 14-3-3, by virtue of its acidic nature, promotes PKC activity in the same way as acidic lipids do in a rather non-specific manner. As an adapter protein, however, 14-3-3 isotypes act more selectively and may help activating a protein kinase by connecting it to another (upstream) activating kinase in a signalling complex [1,4].

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