Articles

RACK1, a Protein Kinase C Anchoring Protein, Coordinates the Binding of Activated Protein Kinase C and Select Pleckstrin Homology Domains in Vitro†

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ABSTRACT: The pleckstrin homology (PH) domain, identified in numerous signaling proteins including the β-adrenergic receptor kinase (βARK), was found to bind to various phospholipids as well as the β subunit of heterotrimeric G proteins (Gβ) [Touhara, K., et al. (1994) *J. Biol. Chem. 269*, 10217−10220]. Several PH domain-containing proteins are also substrates of protein kinase C (PKC). Because RACK1, an anchoring protein for activated PKC, is homologous to Gβ (both contain seven repeats of the WD-40 motif), we determined (i) whether a direct interaction between various PH domains and RACK1 occurs and (ii) the effect of PKC on this interaction. We found that recombinant PH domains of several proteins exhibited differential binding to RACK1. Activated PKC and the PH domain of β-spectrin or dynamin-1 concomitantly bound to RACK1. Although PH domains bind acidic phospholipids, the interaction between various PH domains and RACK1 was not dependent on the phospholipid activators of PKC, phosphatidylserine and 1,2-diacylglycerol. Binding of these PH domains to RACK1 was also not affected by either inositol 1,4,5-triphosphate (IP_3) or phosphatidylinositol 4,5-bisphosphate (PIP_2). Our in vitro data suggest that RACK1 binds selective PH domains, and that PKC regulates this interaction. We propose that, in vivo, RACK1 may colocalize the kinase with its PH domain-containing substrates.

The platelet protein kinase C (PKC)[1] substrate pleckstrin contains sequences in the C and N termini which are homologous to each other over a region spanning approximately 120 amino acids (*1, 2*). Homologues of this domain (pleckstrin homology, or PH domain) are found in over 120 proteins including serine/threonine kinases, tyrosine kinases, phospholipases, GTPase activating proteins, GTPases, cytoskeletal proteins, and guanine nucleotide releasing factors (*2*−*5*). The PH domain can be divided into six subdomains (*4*). Although the homology between PH domains is limited, NMR studies of the PH domains from pleckstrin (*6*), β-spectrin (*7*), and the β-adrenergic receptor kinase (*8*) and crystal structures of the PH domains of human dynamin (*9*) and phospholipase Cδ_1 (*10*) suggest that PH domains fold to a common tertiary structure. It has been suggested that PH domains are involved in the anchoring of selective proteins to membranes by binding directly to lipids (reviewed in ref *11*) and by direct protein−protein interactions (*12*−*18*). In addition, the phosphotyrosine binding (PTB) domain possesses a tertiary structure similar to that of the PH domain and mediates protein−protein interactions via ligand phosphotyrosine residues (*11*).

A direct protein−protein interaction between the $\beta\gamma$ subunits of the heterotrimeric G protein (G$\beta\gamma$) and the PH domain of the β-adrenergic receptor kinase (βARK) was demonstrated (*12*). Following ligand binding to the β-adrenergic receptor, βARK translocated to the plasma membrane where it binds to G$\beta\gamma$ (*20, 21*). The G$\beta\gamma$ interacting site on βARK was mapped to the PH domain and sequences in close proximity to it (*13, 21*). Glutathione-*S*-transferase (GST) fusion proteins containing PH domains from other proteins also bound G$\beta\gamma$ (*12*), and the binding of G$\beta\gamma$ to Gα (the third subunit of the heterotrimeric G protein) and the PH domains was found to be mutually exclusive (*12*). Wang et al. showed that the Gβ subunit, but not the γ subunit, was responsible for this interaction with the PH domains of

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[1] Abbreviations: PH domain, pleckstrin homology domain; PKC, protein kinase C; RACK, receptor for activated C-kinase; PS, phosphatidylserine; DAG, 1,2-diacylglycerol; IP_3, inositol 1,4,5-triphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PL, phospholipid; GST, glutathione-*S*-transferase; MBP, maltose binding protein; GRF, guanine nucleotide releasing factor; OSBP, oxysterol binding protein; βARK, β-adrenergic receptor kinase; PLCγ, phospholipase Cγ; IRS-1, insulin receptor substrate-1; RAC, related to A- and C-kinase.

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β-spectrin and βARK (*13*).

Gβ contains seven repeats referred to as WD-40 domains (*22*). The WD-40 domain is found in many signaling proteins and may mediate protein−protein interactions (*23*). The crystal structure of Gβ (*24*) suggests that WD-repeat proteins, containing multiple WD-40 domains, are comprised of a core "propeller" structure of β sheets and loops. These loops present variable surface residues for protein−protein interactions and may enact conformational changes induced by these interactions (*23*). These predictions, together with the findings of Wang et al. (*13*), Touhara et al. (*12*, *25*), and Koch et al. (*21*), suggest that PH domains of select proteins and the WD-40 domains of Gβ interact directly with each other. To determine whether binding to PH domains is a common feature of WD-40-containing proteins, we determined the ability of another WD-40 protein, RACK1, to bind the PH domains of different proteins.

RACK1 (GenBank Accession Number U03390) has been cloned as a receptor for activated C-kinase (PKC) but is not itself a PKC substrate (*26*). According to our working hypothesis, PKC translocates upon activation from the cytosol to the cell particulate fraction (*27*) where it binds to RACKs (*28*). This translocation is analogous to that of βARK, which, following activation, translocates to the plasma membrane where it binds to G$\beta\gamma$ proteins (*20*, *29*). In vitro, RACK1 binds PKC only in the presence of PKC activators, and this binding enhances the catalytic activity of PKC (*26*). Similar to Gβ, RACK1 consists of seven WD-40 repeats (*26*), and is 69% identical and 81% homologus to Gβ (GenBank Accession Number U44850) [Basic BLASTP, v1.4.9MP search (*30*)].

Because RACK1 is a Gβ homologue, we reasoned that RACK1 may also bind specific PH-containing proteins. Additionally, PKC has been reported to bind to the PH domains of the Tec family of protein kinases, including Btk (*17*, *31*), as a means of regulating PKC phosphorylation of these proteins, and several PH domain-containing proteins are substrates of PKC (*1*, *2*, *39*). Finally, the regulatory domain of ζPKC has also been shown to associate with the PH domain of RAC-protein kinase (*32*, *33*). Here, we present evidence that the PH domains of select proteins (expressed as fusion proteins with glutathione-*S*-transferase, GST) bind to RACK1. We also report that PKC concomitantly binds to RACK1 and the PH domains of dynamin-1 or β-spectrin in vitro. Last, we suggest a model for the association between PKC, RACKs, and PKC substrates containing the PH domain.

EXPERIMENTAL PROCEDURES

Recombinant Proteins. The PH domains of β-adrenergic receptor kinase (βARK), guanine nucleotide releasing factor (GRF), oxysterol binding protein (OSBP), phospholipase Cγ (PLCγ), β-spectrin, insulin receptor substrate-1 (IRS-1), RAC kinase β, and dynamin-1 were expressed as fusion proteins with glutathione-*S*-transferase as described previously (*12*). Purified, recombinant, full-length human dynamin-1 was a gift from Dr. Sandra Schmid. Recombinant RACK1 was expressed as a fusion protein to maltose binding protein (MBP) as described in ref *34*.

PKC Purification. A PKC preparation containing the α, βI, βII, γ, δ, and ϵ isozymes (approximately 4−8 μg/mL total PKC; 2−8 units of total activity/μg relative to histone III-S) was partially purified from rat brain according to methods previously described (*35*).

Binding Assays. The binding of GST−PH proteins to RACK1 in the absence or presence of PKC and/or PKC-activating phospholipids (60 μg/mL phosphatidylserine, 2 μg/mL diacylglycerol) and calcium (1 mM) was analyzed by a column assay as described (*34*). Briefly, RACK1−MBP was immobilized on an amylose−agarose resin column such that RACK1 was always in excess of other analyte proteins (PH domains and PKC) in the binding assay, unless otherwise noted. After washing with 20 column volumes of column wash buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 12 mM β-mercaptoethanol), the various purified GST−PH fusions were incubated with or without PKC (see preparation, above) and/or PKC activators. Incubations were carried out in a final volume of 1 mL of column overlay buffer [50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1% bovine serum albumin, 5 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 20 μg/mL aprotinin, 10 μM phenylmethylsulfonyl fluoride, 0.1% poly(ethylene glycol), and 12 mM β-mercaptoethanol] for 30 min at 25 °C with gentle agitation of the amylose resin immobilization slurry. After washing (with column wash buffer) of unbound material, the MBP fusion complex was eluted with 10 mM maltose and the resultant material subjected to SDS−PAGE and Western blot analysis.

GST−PH fusions were similarly immobilized on glutathione−Sepharose resin (Pharmacia) for analysis of PKC binding to these fusion proteins. Assays were carried out as for the MBP fusion proteins with identical reagents, except for the following: the immobilizing resin comprised 50 μL bead volume; all washes and incubations were performed in Eppendorf tubes; washes were performed by spinning these tubes at approximately 1000*g* for 15 s; proteins were eluted with the addition of 50 μL of Laemmli sample buffer. RACK1 was recombinantly expressed (see above) as an MBP fusion protein, purified on 0.5 mL amalose−agarose columns, and eluted with 10 mM maltose. The purified MBP−RACK1 fusion was cleaved with 24 h digestion with factor Xa (New England Biolabs; 10 μg of factor Xa/mg of protein) at 4 °C.

Western Blot Analysis. Column eluates were suspended in Laemmli sample buffer, boiled for 10 min, and loaded onto 10% SDS−PAGE gels. Proteins were transferred to nitrocellulose membranes and Western analyzed with anti-GST monoclonal antibodies (1 μg/mL; Santa Cruz Biotechologies), anti-βPKC monoclonal antibodies (1:1000; Seikagaku America, Inc.), or anti-RACK1 monoclonal antibodies (1:1000; Transduction Labs, Inc.). Primary antibodies were detected using the appropriate horseradish peroxidase-conjugated secondary antibodies (1:1000; Amersham Life Science) and detected by a chemiluminescent reaction utilizing luminol (Sigma) as a substrate. Quantitation of binding was performed by densitometric analysis on either a Microscan 1000 gel analyzer (Galai Inc., Israel) or an Arcus II scanner (Agfa) and NIH image (v1.57) analysis software with similar results. The accuracy of this method within the measured sample range was confirmed by quantitation of the same Western blots by chemifluorescence (Molecular Dynamics, Sunnyvale, CA) detection using a Molecular Dynamics STORM 850 fluorescence scanner and Molecular

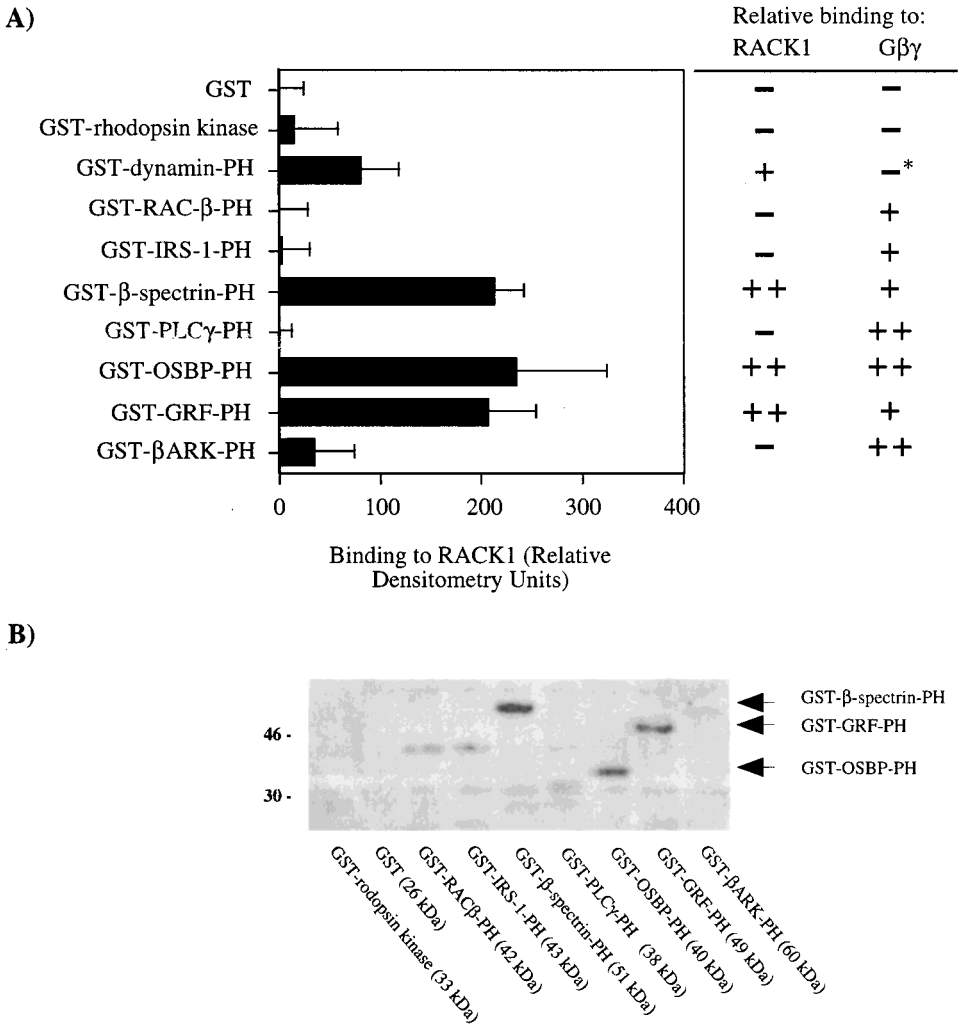

FIGURE 1: Binding of GST−PH domains to RACK1. The binding of various PH domains was determined by immobilizing RACK1−MBP on an amylose column and incubating with 100 nM GST−PH fusion proteins. After extensive washing, bound material was extracted with SDS−PAGE sample buffer and subjected to Western blot analysis. Bound GST−PH fusion proteins were detected with anti-GST antisera. GST and a region from the C-terminus of rhodopsin kinase lacking a PH domain were used as negative controls. (A) Left Panel: Densitometry of binding of various GST fusion proteins relative to GST alone. Results are mean ± SEM of 4 independent experiments. Right Panel: Comparison of the binding (arbitrary units) of GST−PH constructs to RACK1 vs G$\beta\gamma$ (*12*). (B) Western blot representative of results from one such binding experiment. *Kazushige Touhara, unpublished observations (Figure 1; p 21).

Dynamics ImageQuant v1.2 software. Numeric values were determined by either (i) verifying that densitometry of four internal standards of the detected protein run on the same blots as the eluates in select experiments fit to a linear equation with $R^2 > 0.970$ or (ii) using an exponential fit within NIH Image to calibrate these standards.

RESULTS

PH Domain Binding to RACK1. The binding of different recombinant PH domains to RACK1 was tested in vitro. As shown in Figure 1, the PH domains of dynamin-1, β-spectrin, oxysterol binding protein (OSBP), and Ras-guanine nucleotide releasing factor (GRF) bound to RACK1. However, none of these PH domains bound to an MBP−amylose column (data not shown), suggesting that the interactions observed were specific to the RACK1 moiety. Interestingly, the PH domains differed in their ability to bind to RACK1 and G$\beta\gamma$ (Figure 1A). Using the same experimental conditions, we found that the PH domain of OSBP bound well to both RACK1 and G$\beta\gamma$ (*12*) (Figure 1A). However, the PH domain of PLCγ did not bind to RACK1, whereas it bound to G$\beta\gamma$ (*12*). Conversely, the GST−PH domains of GRF and β-spectrin bound well to RACK1 (Figure 1A) but not as strongly to G$\beta\gamma$ (*12*). Whereas the binding of Ras-guanine nucleotide releasing factor (Ras-GRF−PH) to G$\beta\gamma$ required sequences outside the PH domain, the RACK1-binding site on Ras-GRF−PH was mapped entirely within the PH domain per se (data not shown). Therefore, these data suggest differential binding of PH domains by the WD-40 domain-containing proteins RACK1 and G$\beta\gamma$.

βPKC and PH Domains Binding to RACK1. Because RACK1 is a receptor for activated PKC (*26*), specifically βPKC (*34*), we determined the effects of activated βPKC on the binding of PH domains to RACK1. For this purpose, we focused on two examples: the PH domain of β-spectrin was employed because β-spectrin−PH binds well to RACK1 (Figure 1) and β-spectrin has been shown to localize with βPKC in T lymphocytes (*36*−*38*); and, while dynamin-1−PH exhibits a lower level of binding to RACK1 (Figure 1), dynamin-1 is a PKC substrate (*39*) with high binding affinity for PKC (*40*). In contrast, evidence in the literature for an interaction between PKC or RACK1 and either Ras-GRF or

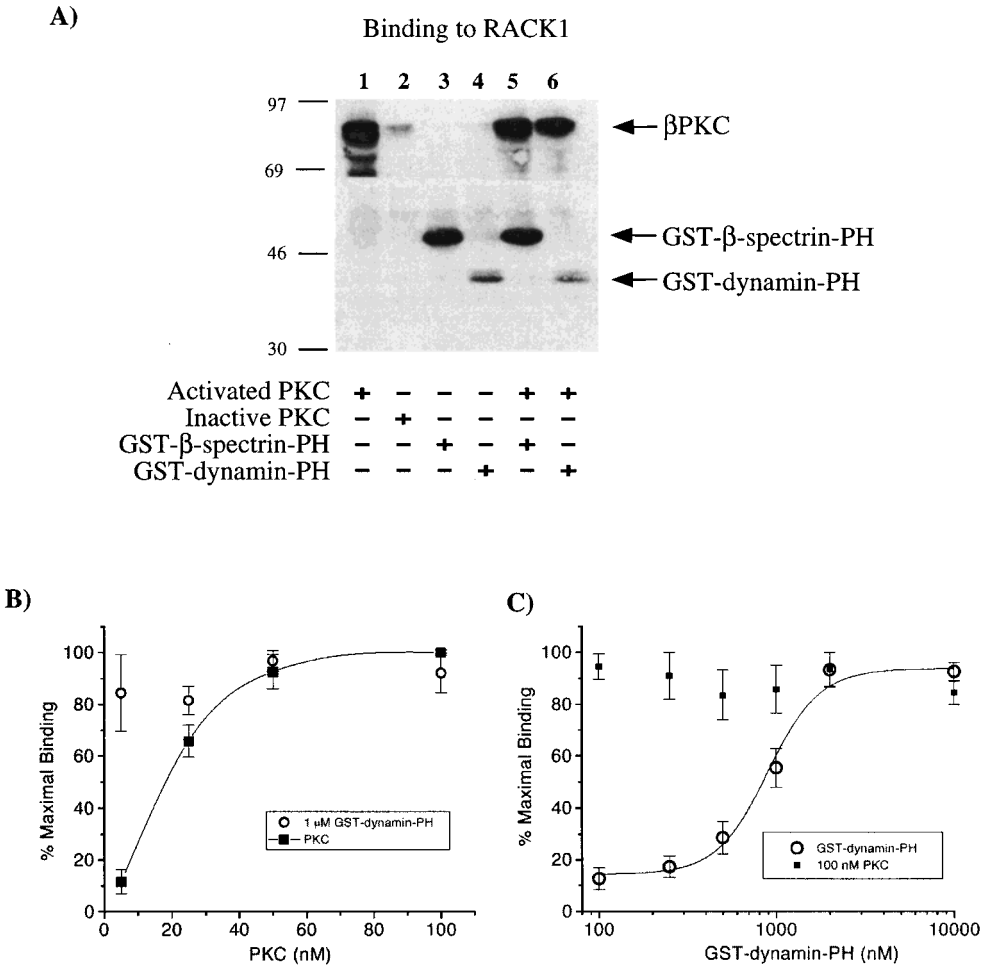

FIGURE 2: Activated βPKC and PH domains concomitantly bind to RACK1. (A) Equal amounts of MBP−RACK1 were immobilized on amylose columns and incubated with either GST−dynamin−PH (500 nM) or GST−β-spectrin−PH (100 nM) in the presence or absence of partially purified rat brain PKC ($\cong$50 nM). PKC was activated by 60 μg/mL phosphatidylserine (PS), 2 μg/mL 1,2-diacylglycerol (DAG), and 1 mM $CaCl_2$, or kept inactivated by 10 mM EGTA. Nitrocellulose membranes were cut horizontally (just above the 69 kDa MW marker), and the top portion was subjected to Western blotting with anti-βPKC monoclonal antibodies (1:300; Seikagaku America) to determine the extent of βPKC binding. The bottom portions of these blots were Western blotted with polyclonal anti-GST (IgG; Santa Cruz Biotech.) to determine the extent of GST−PH domain fusion binding. The image is representative of 6 independent experiments. (B, C) MBP−RACK1 was immobilized as in (A), but in limiting quantities (<100 ng/sample). Increasing doses of either $CaCl_2$−PS−DAG-activated PKC (B; filled squares) or dynamin−PH (C; open circles) as indicated were then coincubated with 1 μM GST−dynamin−PH (in panel B) or 100 nM PKC (in panel C). The amounts of bound βPKC and GST−dynamin−PH were detected as in (A). Results shown are the mean ± SEM of densitometry data from 3 (B) and 5 (C) experiments. Binding was normalized to the maximal level of binding observed for both analyte proteins in each experiment.

OSBP (the PH domains of which bound strongly to RACK1; Figure 1) is minimal. Thus, these proteins were not examined further in the present study.

PKC was activated by incubation with phosphatidylserine (PS), diacylglycerol (DAG), and 1 mM calcium. In the presence of activated PKC, binding of β-spectrin−PH to RACK1 was not significantly attenuated (Figure 2A, lanes 3 vs 5). Similarly, activated PKC did not inhibit binding of dynamin-1−PH to RACK1 (Figure 2A, lanes 4 and 6); the binding of dynamin-1−PH to RACK1 in the presence of activated PKC was 126 ± 23% (mean ± SEM; $n = 6$) of the binding measured in the absence of activated PKC. Moreover, incubation of activated PKC with RACK1 prior to the addition of any PH domains (preincubation) did not significantly affect the binding of either dynamin-1−PH or β-spectrin−PH to RACK1 observed during coincubation (data not shown). When PKC and the PH domain of dynamin-1 were simultaneously incubated with limiting amounts of immobilized RACK1, saturating bound quantities of activated PKC did not affect the amount of dynamin-1−PH bound to RACK1 and vice-versa (Figure 2B,C). These data suggest that there are separate and independent binding sites for PH domains and PKC on RACK1.

To further demonstrate the concomitant interaction between the PH domain of either β-spectrin or dynamin-1, RACK1, and activated βPKC, the converse experiment was carried out. That is, GST−PH domains of β-spectrin or dynamin were immobilized on glutathione−Sepharose, and, in the presence and absence of RACK1, the binding of activated βPKC to these fusion proteins was determined. As seen in Figure 3, activated (but not inactive) βPKC bound to the PH domains of both β-spectrin (1 μM) and dynamin (1 μM). Furthermore, RACK1 (1.5 μM) bound to this complex without diminishing the amount of associated activated βPKC (Figure 3, lanes 4 and 8 vs 3 and 7, respectively). These data further suggest that a concomitant trimolecular interaction occurs between PKC, RACK1, and the PH domains of dynamin-1 and β-spectrin.

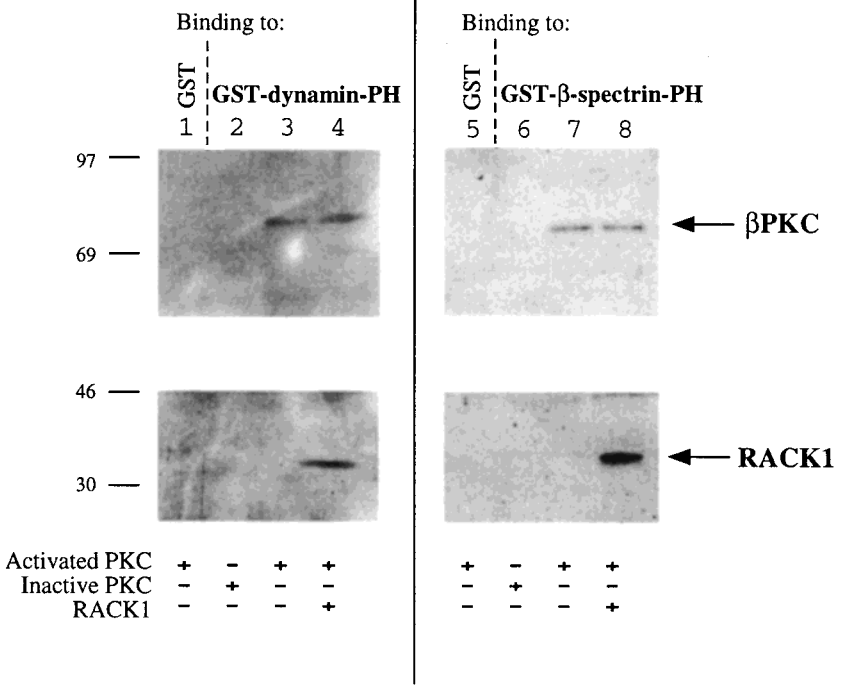

FIGURE 3: Activated βPKC directly binds to the PH domains of β-spectrin and dynamin-1; RACK1 associates with this complex. GST (lanes 1 and 5) or GST−PH domain fusion proteins (as indicated; approximately 1 μM at incubation volume) were incubated with glutathione−Sepharose beads in the presence of rat brain PKC ($\cong$50 nM) rendered inactive (10 mM EGTA; lanes 2 and 6) or active (60 μg/mL PS, 2 μg/mL DAG, 1 mM $CaCl_2$; all other lanes) and purified recombinant RACK1 (1.5 μM; lanes 4 and 8). Lammeli sample buffer eluates were then subjected to Western blot analysis as in Figure 2. RACK1 was detected with anti-RACK1 monoclonal antibodies (1:1000; Transduction Laboratories). Blots are representative of 6 independent experiments.

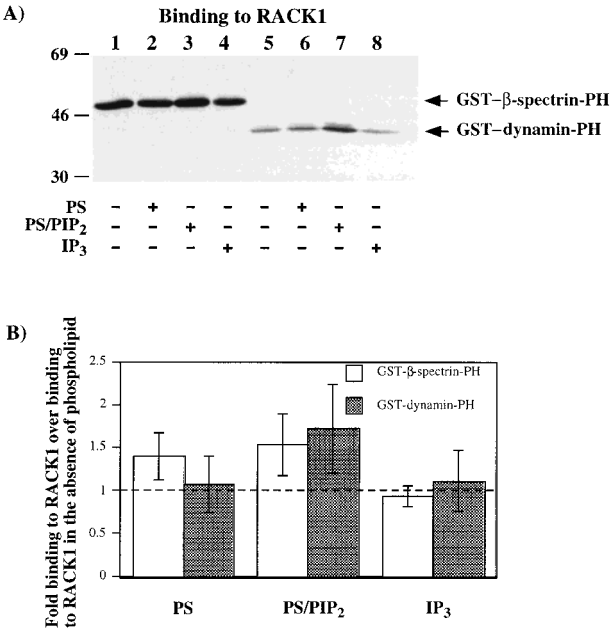

FIGURE 4: Phospholipids do not affect PH domain binding to RACK1. (A) Binding of the PH domains of β-spectrin (100 nM) and dynamin-1 (500 nM) to RACK1 was determined in the presence of PS (40 μg/mL, lanes 2 and 6), PS/PIP_2 (1:1 molar ratio, 40 μg/mL total lipid, lanes 3 and 7), or IP_3 (40 μg/mL, lanes 4 and 8). (B) Averaged data of experiments represented in (A); binding of GST−β-spectrin−PH (white bars) or GST−dynamin−PH (gray bars) to RACK1 in the presence of the indicated components. Error bars represent SEM of 4 (GST−β-spectrin−PH) and 5 (GST−dynamin−PH) independent experiments. Binding assays were performed as described in Figure 1.

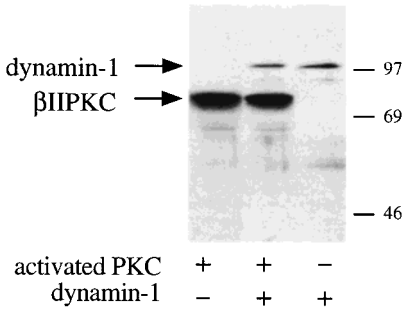

FIGURE 5: Activated βIIPKC and full-length dynamin-1 interact with RACK1. Equal amounts of MBP−RACK1 were immobilized as in Figure 2 and incubated with either 1 μg/mL dynamin-1 and/or 250 ng/mL βIIPKC (PanVera, Inc.) activated as in Figure 2. Western blotting was accomplished by simultaneously probing with anti-dynamin-1 (1:1000; Transduction Labs) and anti-βIIPKC (1 μg/mL; Santa Cruz Biotechnologies) antibodies. The image is representative of 3 independent experiments.

Effect of Phospholipids on PH Domain Binding to RACK1. The PH domains of multiple proteins, including dynamin-1, have been shown to bind phosphatidylinositol 4,5-bisphosphate (PIP_2) (*41*, *42*). Also, the binding of G$\beta\gamma$ to βARK is mediated, at least in part, by PIP_2 (*25*, *43*), and inositol 1,4,5-triphosphate (IP_3) binds to β-spectrin (*44*, *45*). In our assay, IP_3 (40 μg/mL) had no significant ($p > 0.5$) effect on the interaction between either dynamin-1−PH or β-spectrin−PH and RACK1 (Figure 4A,B). PS together with PIP_2 (1:1 molar ratio, 40 μg/mL total concentration) marginally increased the binding of dynamin-1−PH and β-spectrin−PH to RACK1, but this effect was not significant ($0.1 < p < 0.2$; $n = 4$ with β-spectrin-PH, $n = 5$ with dynamin-1-

PH; Figure 4B). Therefore, the observed binding of the PH domains of dynamin-1 and β-spectrin to RACK1 is not dependent on these acidic phospholipids and thus appears to be mediated by direct protein−protein interactions.

βIIPKC and Full-Length Dynamin-1 Binding to RACK1. To evaluate the significance of the interaction between isolated PH domains, RACK1, and activated PKC, we determined whether a PH domain-containing protein could interact concomitantly with PKC and RACK1. Using dynamin-1 as an example (Figure 5), either phospholipid and calcium-activated recombinant βIIPKC (PanVera, Inc.) or dynamin-1 binds independently to RACK1 in assays similar to those used in Figures 1 and 2. When these two proteins were coincubated with RACK1, neither component displaced significant amounts of the other, indicating that both βIIPKC and full-length dynamin-1 concomitantly bind to RACK1 in this assay.

DISCUSSION

Multiple PH domain-containing proteins are PKC substrates (*2*, *3*, *40*). In this study, a role for select PH domains in binding βPKC and its anchoring protein, RACK1, has been suggested. Since RACK1 was initially identified as a binding protein for activated PKC, it was interesting to find that binding of activated PKC to RACK1 did not inhibit the binding of either dynamin-1−PH or β-spectrin−PH to RACK1 (Figure 2). Our data suggest that a trimolecular complex between PKC, RACK1, and a PH domain-containing protein is formed in which all three proteins contain binding sites for the other two partners.

Using the same GST−PH protein constructs, Touhara et al. have also demonstrated differential binding to G$\beta\gamma$ (*12*). Interestingly, the binding specificity of the various PH domains to G$\beta\gamma$ as compared with RACK1 differed (Figure 1). The differences in binding patterns between RACK1 and G$\beta\gamma$ could be due to the contribution of the Gγ subunit toward binding. That is, Gγ may selectively contribute to the interactions observed between Gβ and other PH domains. It is also possible that differential specificity of certain PH domains for RACK1 vs Gβ exists. Our observations reported here with RACK1 present evidence for a third WD-40-containing protein [along with Gβ (*13*) and the protein product of the *lis-1* gene (*46*)] which associates with PH domains. Based on these independent findings, the PH domain of specific proteins appears to bind a select group of WD-40-containing proteins.

Our finding that a molar excess of various phospholipids does not affect the interaction between β-spectrin−PH or dynamin-1−PH and RACK1 indicates that these associations in the presence of activated PKC (Figures 2 and 3) reflect direct protein−protein interactions between the respective PH domains and RACK1. However, we currently cannot exclude the possibility that protein−lipid interactions do not contribute to the association between PH domains and βPKC in the trimolecular complex as well.

It is worth noting that the interactions between select PH domains, RACK1, and activated PKC are unlike those which occur between Gα, G$\beta\gamma$, and PH-containing proteins. Whereas Gα inhibits the binding of PH-containing proteins to the G$\beta\gamma$ dimer (*12*), activated βPKC, RACK1, and dynamin-1−PH or β-spectrin−PH interact concomitantly. Thus, it appears that PH domain binding and PKC binding to RACK1 occur at independent sites on RACK1 and are not allosterically controlled.

Proposed Model for a Multiprotein Interaction with RACK1 as a Scaffolding Protein. The anchoring of PH domain proteins by RACK1 may be independent of the role of RACK1 in anchoring activated PKC. However, according to the results obtained in Figures 2, 3, and 5, we favor the following model for interaction of these proteins in vivo (Figure 6): RACK1 serves as an anchoring protein both for activated βPKC and for selective PH domain proteins, some of which are PKC substrates [e.g., pleckstrin (*1*, *2*) and dynamin-1 (*39*)]. Prior to PKC activation (Figure 6A), RACK1 is bound to a PH domain-containing protein. This interaction is likely to be dynamic and possibly regulated by other signaling systems. Following activation (Figure 6B), PKC translocates and binds to its anchoring protein, RACK1. Since RACK1 is not a PKC substrate (*26*), the interaction between these two molecules should not be governed by enzyme kinetics. Other cellular factors may prevent this trimolecular interaction in vivo. However, based on the data gathered here, an enticing hypothesis is that the simultaneous binding of PKC and a PH domain-containing protein to RACK1 may provide a means to bring PKC in close proximity with its substrate(s), thus enabling substrate phosphorylation with higher efficiency (Figure 6).

Proper localization and activation of βARK is mediated by simultaneous binding of both lipid (PIP_2) and G$\beta\gamma$ subunits to the PH domain of βARK (*43*, *47*). In addition, membrane targeting of the PH domain of Sos has been suggested to involve specific ligands distinct from PIP_2 (*48*), and others have modeled the PH domain as a dual lipid/protein binding motif (*49*). Similarly, our model (Figure 6) suggests that the role of phospholipids is important for the trimolecular interaction as they are required for PKC activation and localization of PH domain-containing proteins to the membrane. However, we also provide evidence that lipids are not sufficient for governing the specificity of the interaction between RACK1 and PH domains. Rather, our in vitro data suggest that the trimolecular complex of activated PKC, a PH domain-containing protein, and RACK1 is in part mediated via direct protein−protein interactions.

Because many of the PH domain-containing proteins are also involved in signal transduction, the colocalization of activated PKC and these signaling proteins may provide a means for coordinated signaling. Such multivalent organizers, or scaffolding proteins, have been previously described. For example, the anchoring protein for cAMP-dependent protein kinase, AKAP79, binds PKC as well as calcineurin (*50*). In the *Drosophila* eye, the *inaD* gene product organizes a multiprotein complex that includes phospholipase C-β, PKC, and a PKC substrate: the calcium channel encoded by the *trp* gene (*51*). Similarly, RACK1 may act as a scaffold protein to organize signaling molecules such as dynamin-1 with a protein kinase, activated PKC.

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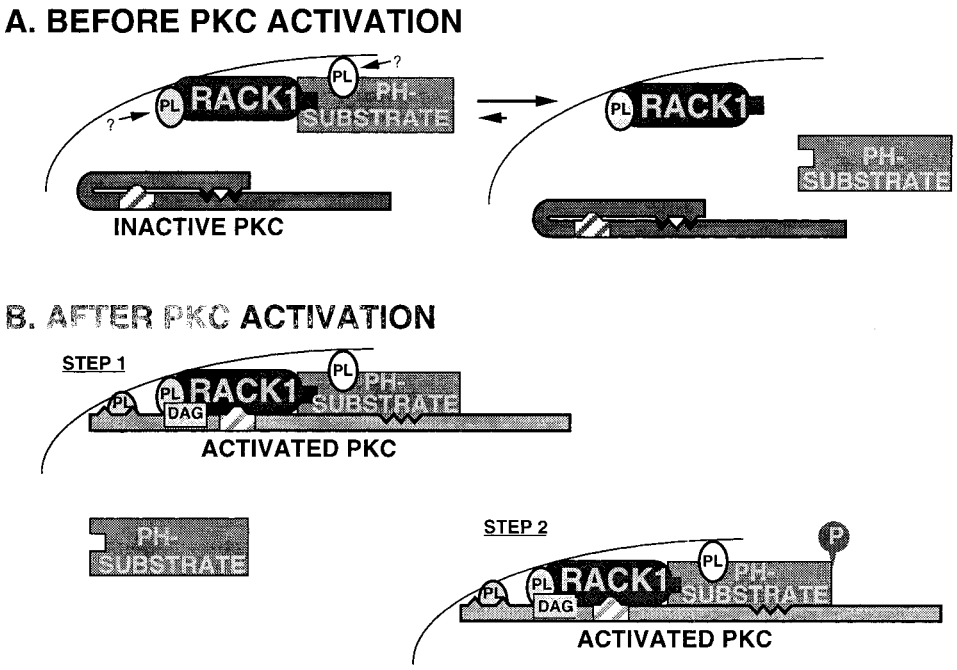

FIGURE 6: Model of the interaction between PKC, RACK1, and specific PH domain proteins. This hypothetical model depicts PKC, a PH domain-containing protein (as a PKC substrate), and RACK1 associated in a trimolecular complex following PKC activation. (A) Before PKC activation, a PH domain-containing protein (PH-substrate) associates with RACK1, possibly in a reversible manner. (B) Following activation, PKC binds RACK1 which in turn may increase the affinity of RACK1 for the PH domain-containing protein. Substrate phosphorylation (step 2) is thus controlled by PKC binding to both RACK1 and the PH domain-containing protein. Putative roles for both phospholipid (PL) anchoring and direct protein−protein interactions are shown.

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REFERENCES

1. Mayer, B. J., Ren, R., Clark, K. L., and Baltimore, D. (1993) *Cell 73*, 629−630.
2. Haslam, R., Koide, H., and Hemmings, B. (1993) *Nature 363*, 309−310.
3. Ingley, E., and Hemmings, B. (1994) *J. Cell. Biochem. 56*, 436−443.
4. Musacchio, A., Gibson, T., Rice, P., Thompson, J., and Saraste, M. (1993) *Trends Biomed. Sci. 18*, 343−348.
5. Shaw, G. (1996) *Bioessays 18*, 35−46.
6. Macias, M. J., Musacchio, A., Ponstingl, H., Nilges, M., Saraste, M., and Oshkinat, H. (1994) *Nature 369*, 675−677.
7. Yoon, H. S., Hajduk, P. J., Petros, A. M., Olejniczak, E. T., Meadows, R. P., and Fesik, S. W. (1994) *Nature 369*, 672−675.
8. Fushman, D., Najmabadi-Haske, T., Cahill, S., Zheng, J., LeVine, H., III, and Cowburn, D. (1998) *J. Biol. Chem. 273*, 2835−2843.
9. Ferguson, K., Lemmon, M., Schlessinger, J., and Sigler, P. (1994) *Cell 79*, 199−209.
10. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) *Cell 83*, 1037−1046.
11. Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996) *Cell 85*, 621−624.
12. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) *J. Biol. Chem. 269*, 10217−10220.
13. Wang, D., Shaw, R., Winkelmann, J., and Shaw, G. (1994) *Biochem. Biophys. Res. Commun. 203*, 29−35.
14. Tsukada, S., Simon, M. I., Witte, O. N., and Katz, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11256−11260.
15. Mahadevan, D., et al. (1995) *Biochemistry 34*, 9111−9117.
16. Konishi, H., Kuroda, S., Tanaka, M., Matsuzaki, H., Ono, Y., Kameyama, K., Haga, T., and Kikkawa, U. (1995) *Biochem. Biophys. Res. Commun. 216*, 526−534.
17. Yao, L., Kawakami, Y., and Kawakami, T. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9175−9179.
18. Burks, D. J., Wang, J., Towery, H., Ishibashi, O., Lowe, D., Riedel, H., and White, M. F. (1998) *J. Biol. Chem. 273*, 31061−31067.
19. Wang, T., Pentyala, S., Rebecchi, M. J., and Scarlata, S. (1999) *Biochemistry 38*, 1517−1524.
20. Pitcher, J., et al. (1992) *Science 257*, 1264−1267.
21. Koch, W. J., Inglese, J., Stone, W. C., and Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 8256−8260.
22. Fong, H. K., Amatruda, T. T., Birren, B. W., and Simon, M. I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3792−3796.
23. Neer, E. J., Schmidt, C. J., Nambudripad, R., and Smith, T. F. (1994) *Nature 371*, 297−300.
24. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell 83*, 1047−1058.
25. Touhara, K., Koch, W. J., Hawes, G. E., and Lefkowitz, R. J. (1995) *J. Biol. Chem. 270*, 17000−17005.
26. Ron, D., Chen, C.-H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 839−843.
27. Kraft, A. S., and Anderson, W. B. (1983) *Nature 301*, 621−623.
28. Mochly-Rosen, D. (1995) *Science 268*, 247−251.
29. Inglese, J., Koch, W. J., Caron, M. G., and Lefkowitz, R. J. (1992) *Nature 359*, 147−150.
30. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.
31. Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W. B., Kawakami, Y., and Kawakami, T. (1997) *J. Biol. Chem. 272*, 13033−13039.
32. Konishi, H., Kuroda, S., and Kikkawa, U. (1994) *Biochem. Biophys. Res. Commun. 205*, 1770−1775.
33. Konishi, H., Matsuzaki, H., Tanaka, M., Ono, Y., Tokunaga, C., Kuroda, S., and Kikkawa, U. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7639−7643.
34. Ron, D., Luo, J., and Mochly-Rosen, D. (1995) *J. Biol. Chem. 270*, 24180−24187.
35. Mochly-Rosen, D., and Koshland, D. E., Jr. (1987) *J. Biol. Chem. 262*, 2291−2297.
36. Gregorio, C. C., Kubo, R. T., Bankert, R. B., and Repasky, E. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4947−4951.

37. Gregorio, C. C., Verstovsek, G., Black, J. D., and Repasky, E. A. (1992) *FASEB J. 6*, A1981.
38. Gregorio, C. C., Repasky, E. A., Fowler, V. M., and Black, J. D. (1994) *J. Cell Biol. 125*, 345−358.
39. Robinson, P. J., Sontag, J.-M., Liu, J.-P., Fykse, E. M., Slaughter, C., McMahon, H., and Sudhof, T. C. (1993) *Nature 365*, 163−166.
40. Liu, J. P., Powell, K. A., Sudhof, T. C., and Robinson, P. J. (1994) *J. Biol. Chem. 269*, 21043−21050.
41. Salim, K., et al. (1996) *EMBO J. 15*, 6241−6250.
42. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) *Nature 371*, 168−170.
43. Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995) *J. Biol. Chem. 270*, 11707−11710.
44. Wang, D. S., and Shaw, G. (1995) *Biochem. Biophys. Res. Commun. 217*, 608−615.
45. Zhang, P., Talluri, S., Deng, H., Branton, D., and Wagner, G. (1995) *Structure 3*, 1185−1195.
46. Wang, D., Shaw, R., Hattori, M., Arai, H., Inoue, K., and Shaw, G. (1995) *Biochem. Biophys. Res. Commun. 209*, 622−629.
47. Touhara, K. (1997) *FEBS Lett. 417*, 243−248.
48. Chen, R.-H., Corbalan-Garcia, S., and Bar-Sagi, D. (1997) *EMBO J. 16*, 1351−1359.
49. Alberti, S. (1998) *Proteins: Struct., Funct., Genet. 31*, 1−9.
50. Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996) *Science 271*, 1589−1592.
51. Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M., and Zuker, C. S. (1997) *Nature 388*, 43−49.

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