Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3997–4000, May 1991
Biochemistry

Identification of intracellular receptor proteins for activated protein kinase C

(heart/annexin/lipocortin/translocation)

DARIA MOCHLY-ROSEN*†‡, HANITA KHANER†, AND JAMIE LOPEZ†

*Departments of Neurology and Pharmacology, and †The Ernest Gallo Clinic and Research Center, University of California at San Francisco, San Francisco General Hospital, San Francisco, CA 94110

Communicated by Daniel E. Koshland, Jr., January 31, 1991

ABSTRACT **Protein kinase C (PKC) translocates from the cytosol to the particulate fraction on activation. This activation-induced translocation of PKC is thought to reflect PKC binding to the membrane lipids. However, immunological and biochemical data suggest that PKC may bind to proteins in the cytoskeletal elements in the particulate fraction and in the nuclei. Here we describe evidence for the presence of intracellular receptor proteins that bind activated PKC. Several proteins from the detergent-insoluble material of the particulate fraction bound PKC in the presence of phosphatidylserine and calcium; binding was further increased with the addition of diacylglycerol. Binding of PKC to two of these proteins was concentration-dependent, saturable, and specific, suggesting that these binding proteins are receptors for activated C-kinase, termed here "RACKs." PKC binds to RACKs via a site on PKC distinct from the substrate binding site. We suggest that binding to RACKs may play a role in activation-induced translocation of PKC.**

Activation-induced translocation of protein kinase C (PKC) to the particulate fraction is thought to reflect PKC binding to membrane lipids (1). However, trypsin treatment of the particulate fraction reduces binding of exogenously added PKC (2). Furthermore, immunofluorescence studies have demonstrated that endogenous PKC binds to cytoskeletal elements associated with the particulate fraction (3–8), and binding of exogenous PKC to cytoskeletal elements is dependent on phosphatidylserine (PtdSer) and calcium (9) or on PtdSer, diacylglycerol, and calcium (8). Binding of PKC to perinuclear or intranuclear structures has also been reported (8, 10–15). Taken together, these data suggest that activated PKC may bind to receptor proteins located at various intracellular sites. We have called these proteins "RACKs" for putative receptors for activated C kinase and predicted that RACKs should have the following characteristics: (*i*) RACKs should be proteins present in the particulate fraction; (*ii*) PKC binding to RACKs should be dependent on PtdSer, diacylglycerol [or phorbol 12-myristate 13-acetate (PMA)], and calcium; and (*iii*) binding to RACKs should be saturable and specific. Here, we describe at least two proteins in the detergent-insoluble fraction of neonatal rat heart that fulfill these criteria.

MATERIALS AND METHODS

Preparation of Detergent-Insoluble Protein Fraction. Neonatal rat hearts were homogenized in 20 mM Tris·HCl (pH 7.5) containing 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 10 μM phenylmethylsulfonyl fluoride, and 20 μg of leupeptin and 20 μg of soybean trypsin inhibitor per ml. The homogenate was then centrifuged at 100,000 × *g* for 1 hr, and the pellet (particulate fraction) was extracted with 1% Triton X-100 for 30 min. The Triton-insoluble protein fraction was obtained by centrifugation for 1 hr at 100,000 × *g*. The pellet was resuspended in 20 mM Tris·HCl (pH 7.5) containing 1 mM EGTA, 1 mM EDTA, 12 mM 2-mercaptoethanol, and 50% (vol/vol) glycerol and stored at −20°C. Where indicated, the particulate fraction was treated with trypsin (1:50 protein ratio) for 10 min at room temperature, digestion was stopped by adding soybean trypsin inhibitor (50:1 protein ratio), and the incubation mixture was centrifuged at 100,000 × *g* for 1 hr. The pellet was then extracted with Triton X-100 as described above.

Assays for PKC Binding. *Centrifugation assay.* The Triton-insoluble protein fraction (500 μg) was incubated in the presence or absence of a mixture of rat brain PKC isozymes [1.5 μM; 150 units/mg; purified and characterized as described elsewhere (8, 16)], sonicated PtdSer liposomes (20 μg/ml), calcium (1 mM), and PMA (0.1 μM) for 15 min; 10 mM EGTA was then added, and the reaction mixture was centrifuged at 15,000 × *g*. The washed pellet was subjected to SDS/PAGE, and bound PKC was determined by immunoblot analysis (17) with anti-PKC polyclonal serum 2977 (a gift from A. Flint and D. E. Koshland, Jr., University of California, Berkeley). This rabbit antiserum was prepared against the catalytic domain of the PKC β isozyme.

Overlay assay. Triton-insoluble protein fraction (100–250 μg per lane) was subjected to SDS/PAGE and blotted onto nitrocellulose. PKC binding was determined by the method of Wolf and Sahyoun (9) with some modifications. The nitrocellulose strips were incubated for 30 min to 16 hr in the presence or absence of PtdSer (5–20 μg/ml), diacylglycerol (0.8 μg/ml), calcium (1 mM), and the indicated amount of a mixture of rat brain PKC isozymes (1.5 μM; 150 units/mg; see refs. 8 and 16) in 50 mM Tris·HCl (pH 7.5) containing 12 mM 2-mercaptoethanol, 0.2 M NaCl, 0.1% bovine serum albumin, 1% polyethylene glycol, and 10 μg of soybean trypsin inhibitor and 10 μg of leupeptin per ml. Unbound material was removed by five subsequent 5-min incubations in the above buffer without bovine serum albumin and protease inhibitors. Where indicated, PKC activators were incubated with the nitrocellulose strips, and unbound material was removed prior to the addition of PKC. Polypeptides that bound PKC were detected by two methods: PKC-dependent phosphorylation or use of anti-PKC antibodies. PKC-dependent phosphorylation was carried out by incubating the nitrocellulose strips with 20 mM Tris·HCl (pH 7.5) containing [^{32}P]ATP (20 μM; 0.5–1.0 × 10^6 cpm/nmol) and 20 mM $MgCl_2$. After 10–30 min, the reaction mixture was

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Abbreviations: PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PtdSer, phosphatidylserine; RACK, receptors for activated C kinase; PMA, phorbol 12-myristate 13-acetate.

‡To whom reprint requests should be addressed at: The Gallo Center, Building 1 Room 101, San Francisco General Hospital, San Francisco, CA 94110.

removed, and the nitrocellulose strips were washed three times in wash buffer and twice in wash buffer containing 0.8 mM NaCl, followed by autoradiography for 1–20 hr at −70°C with an intensifier screen.

PKC binding to polypeptides on the nitrocellulose strips was also determined by using a monoclonal antibody for PKC β isozyme diluted 1:500 (Seikagaku Kogyo, Tokyo) and ^{125}I-labeled protein A, followed by autoradiography. Similar results were obtained with the polyclonal antiserum 2977 or with an anti-PKC α isozyme monoclonal antibody (data not shown).

Where indicated, the catalytic subunit of cAMP-dependent protein kinase was substituted for PKC (10 units/ml). Highly purified rat brain PKC (>1500 units/mg) or purified PKC α or β isozyme (expressed in insect cells with the baculovirus expression system; >1000 units/mg) gave the same results in the overlay assay as the partially purified PKC used in this study (data not shown).

The effect of occupying the substrate binding site of PKC on the overlay assay was determined by incubating the nitrocellulose strips with PKC in the presence of substrate peptide {based on the pseudo-substrate sequence of PKC amino acids 19–36 (18) with alanine at position 25 replaced by serine—i.e., [Ser^{25}]PKC-(19–36)} or in the presence of pseudo-substrate PKC fragments PKC-(19–36) or PKC-(19–31).

Annexins. Annexins I-VII, a gift from J. Ernst and R. A. Blackwood, were purified to homogeneity as described (19). Annexins I and V were purified from *Escherichia coli* that were expressing the respective human cDNA clones; annexins II, III, IV, and VII were purified from human lung, and annexin VI was purified from bovine lung.

Overlay Assay for [^{14}C]PtdSer Binding. Annexins (2 μg per lane) or RACK preparations (50 μg per lane) were electrophoresed on SDS/PAGE and blotted onto nitrocellulose as in the overlay assay. PtdSer binding was determined by incubating the blots with sonicated [^{14}C]PtdSer liposomes (20 μg/ml; 0.3 × 10^6 cpm/ml) in the presence of 1 mM calcium. Unbound PtdSer was removed as in the overlay assay, and the nitrocellulose sheet was subjected to autoradiography for 3–8 days at −70°C, in the presence of intensifier screens. Autoradiograms were scanned three to six times with the MicroScan 1000 gel analyzer (version 4.0; Technology Resources, Nashville, TN). The differences in values obtained on repetitive determinations of the same autoradiogram exposure or of different exposures were between 1% and 5%.

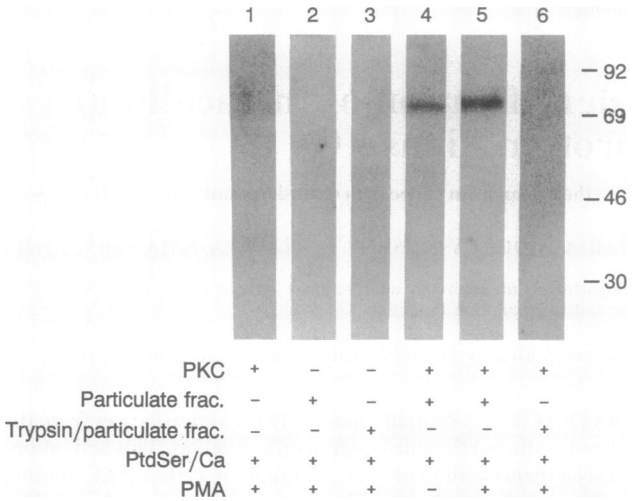

FIG. 1. PKC binds to proteins in the Triton-insoluble protein particulate fraction (centrifugation assay). PKC binding to the Triton-insoluble protein fraction (frac.) in the presence of PtdSer liposomes and calcium with (lane 5) or without (lane 4) PMA was compared with binding to PtdSer liposomes in the presence of PMA and calcium and in the absence of the Triton-insoluble proteins (lane 1). No PKC was detected in the Triton-insoluble fraction (lanes 2 and 3). The effect on PKC binding of trypsin treatment of the particulate fraction prior to the preparation of the Triton-insoluble protein fraction was also determined (lane 6).

RESULTS AND DISCUSSION

On activation, PKC translocates to cytoskeletal elements associated with the particulate fraction (3–9). Since cytoskeletal elements are present in the Triton-insoluble material of the particulate fraction, we determined whether proteins that bind PKC are also present in this fraction. The Triton-insoluble fraction was incubated with PKC in the presence or absence of calcium, diacylglycerol, and PtdSer. After 15 min,

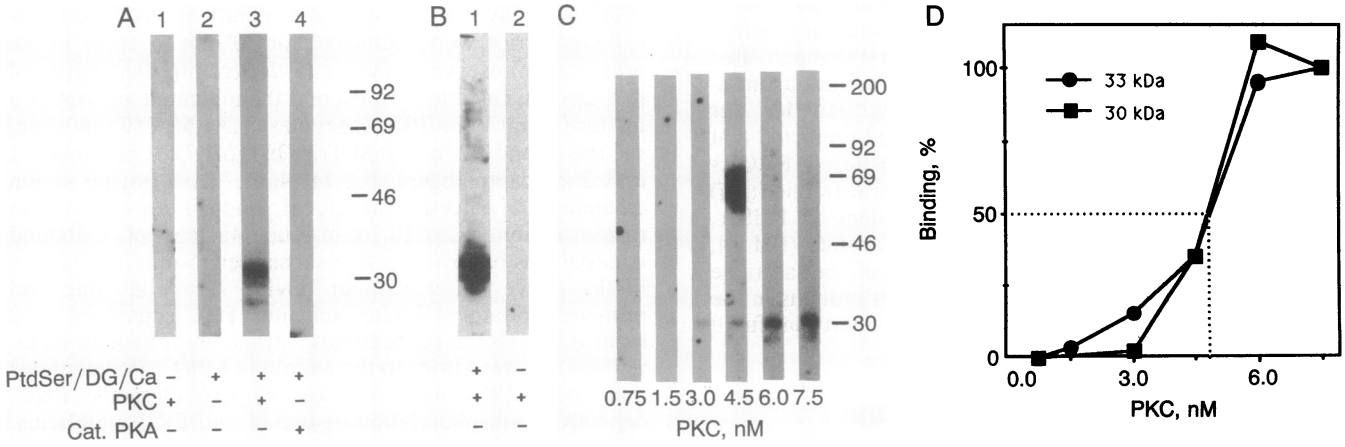

FIG. 2. PKC binding to RACKs requires PtdSer, diacylglycerol (DG), and calcium (overlay assay). (*A*) The Triton-insoluble protein fraction (100 μg per lane) was subjected to SDS/PAGE and blotted onto nitrocellulose. PKC binding was determined by incubating the blots with 5 nM PKC in the presence (lane 3) or absence (lane 1) of PtdSer, diacylglycerol, and calcium as described. Polypeptides that bound PKC were identified by PKC-dependent phosphorylation in the presence of [^{32}P]ATP, followed by autoradiography. Similar binding of PKC was obtained in the presence of PtdSer and calcium only (see also Fig. 3) but not in the absence of calcium (data not shown). The catalytic subunit of the cAMP-dependent protein kinase (Cat. PKA) did not bind polypeptides that bind PKC (lane 4). (*B*) PKC binding to polypeptides on the nitrocellulose strips was determined in the presence (lane 1) or absence (lane 2) of PtdSer, diacylglycerol, and calcium. Polypeptides that bound PKC were identified by using antibodies to the PKC β isozyme as described. (*C*) Concentration-dependent binding of PKC to two polypeptides was determined as in *A* by using 0.75–7.5 nM PKC in the presence of PtdSer, diacylglycerol, and calcium. (*D*) Densitometry of the autoradiogram in *C* was carried out by using a Scan 1000 image analyzer. Binding is given as the percentage of maximal binding. Sizes are shown in kDa.

the sample was centrifuged, and the amount of PKC in the pellet was determined by SDS/PAGE followed by an immunoblot with anti-PKC antibodies. Binding of PKC to the Triton-insoluble fraction occurred in the presence of PtdSer and calcium (Fig. 1, lane 4), was further increased by PMA (lane 5), but was almost nondetectable in the absence of PtdSer (data not shown). PKC binding was abolished when the particulate fraction was treated with trypsin prior to the Triton extraction (Fig. 1, lane 6 vs. lane 5). Loss of PKC binding after trypsin treatment was not due to proteolysis of PKC, since most of the unbound PKC was recovered as an 80-kDa protein in the supernatant (data not shown). These results suggest that PKC bound to the Triton-insoluble fraction and that the binding was via proteins.

The proteins that bound PKC in the Triton-insoluble fraction were identified by using an overlay method. The Triton-insoluble protein fraction was subjected to SDS/PAGE, blotted onto nitrocellulose paper, and the paper was incubated with PKC with or without PKC activators. Unbound PKC was removed, and the PKC-binding proteins were detected either by PKC-dependent phosphorylation (Fig. 2*A*) or by using anti-PKC antibodies (Fig. 2*B*), with both detection methods yielding similar results. Polypeptides of 30 ± 1 and 33 ± 1 kDa (n = 18 independent experiments) bound PKC in the presence of PtdSer, diacylglycerol, and calcium (Fig. 2*A*, lane 3; Fig. 2*B*, lane 1). In some experiments, additional polypeptides of 36 ± 1, 45 ± 1, and 61 ± 3 kDa (n = 13, 9, and 5, respectively) were also observed.

Binding of a ligand to its receptor should be specific, concentration-dependent, and saturable. Concentration-dependent binding of PKC to the 30- and 33-kDa polypeptides was observed, with half-maximal binding at ≈4 nM (≈0.3 unit/ml; Fig. 2 *C* and *D*). No cAMP-dependent protein kinase (PKA) binding to the 30- and 33-kDa polypeptides was observed either by PKA-dependent phosphorylation (Fig. 2*A*, lane 4) or by anti-PKA antibodies (data not shown). These results suggest that the 30- and 33-kDa polypeptides are RACKs. It remains to be determined whether the other PKC-binding polypeptides fulfill all the criteria for RACKs.

PKC could bind to RACKs via the PKC substrate binding site. If so, an excess of substrate peptide should inhibit PKC binding to RACKs. A substrate peptide [based on the pseudo-substrate sequence of PKC-(19–36) fragment; ref. 18] did not inhibit PKC binding to RACKs in either the overlay assay (Fig. 3, lane 2 vs. lane 1) or the centrifugation assay (data not shown). These data suggest that the PKC substrate site is not involved in PKC binding to RACKs.

PKC binding to RACKs requires PtdSer and calcium (Figs. 1 and 2). It is possible that PtdSer and calcium bind directly to RACKs. Therefore, we measured PKC binding to RACKs after preincubation of RACKs with PKC activators. Fig. 4*A* shows that after preincubation of RACKs with PtdSer and calcium with or without diacylglycerol, PKC binding occurred without further addition of PtdSer and calcium (lane 3 vs. lane 2 and data not shown). These results indicate that RACKs bound PtdSer and calcium in the preincubation step.

Bazzi and Nelsestuen (20) suggested that a PtdSer bridge connects PKC and its protein substrate. Therefore, we determined whether such a PtdSer bridge is responsible for PKC binding to its receptor. If this were the case, any PtdSer- and calcium-binding proteins would bind PKC and serve as RACKs. Annexins are a family of homologous proteins that bind negatively charged phospholipids such as PtdSer in a calcium-dependent manner (21). Purified annexins were tested for their RACK activity by using the overlay assay (Fig. 4*B*). Only annexin I and, to a much lesser extent, annexins II and VII [known also as lipocortin I, calpectin I and synexin, respectively (22)] bound PKC in the presence of PtdSer and calcium (Fig. 4*B*). Annexins III, IV, V, and VI did not bind PKC under the same conditions (Fig. 4*B*).

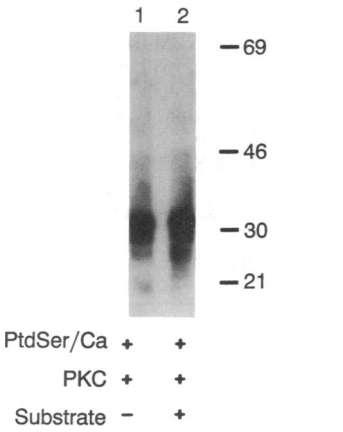

FIG. 3. Substrate peptide does not inhibit PKC binding to RACKs. Binding of PKC (5 nM) to RACKs was determined with PtdSer and calcium in the presence (lane 2) or absence (lane 1) of 30 μM substrate peptide. This concentration of peptide is about 150-fold greater than the K_m of the peptide for PKC in a kinase activity assay. Similar results were obtained when the substrate was present at an ≈100-fold excess or with the pseudo-substrate peptides PKC-(19–36) or PKC-(19–31) (data not shown). Sizes are shown in kDa.

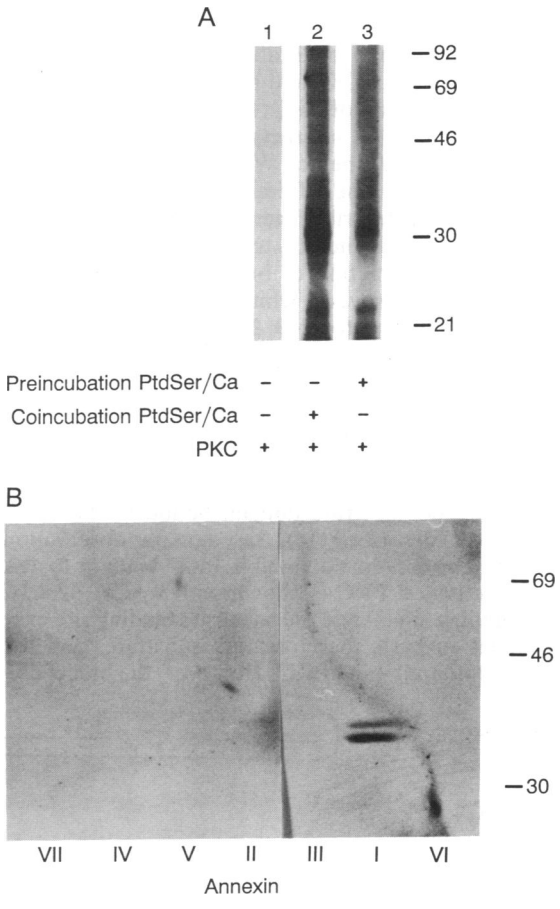

FIG. 4. RACKs bind PtdSer in the presence of calcium, but not all PtdSer-binding proteins are RACKs. (*A*) SDS/PAGE blots of RACKs were preincubated for 1 hr with PtdSer and calcium and washed to remove unbound activators prior to PKC addition; PKC binding to RACK was then determined as in Fig. 2*A*. For comparison, binding of PKC to RACKs when PtdSer and calcium were coincubated with PKC is also shown (lane 2). (*B*) Purified annexins I–VII (2 μg per lane) were analyzed for RACK activity as in Fig. 2*B* by using anti-PKC 2977 antiserum. Sizes are shown in kDa.

The differences in the RACK activity between the various annexins were not due to differences in the amount of bound PtdSer in the overlay assay, as demonstrated by using [^{14}C]PtdSer and quantitation of the autoradiograms. Annexins II and VII had similar RACK activity, which was much lower than the activity of annexin I (Fig. 4*B*). Yet, annexin II bound 1.3-fold more PtdSer than did annexin I and 5.3-fold more PtdSer than did annexin VII. In addition, annexins V and VI (which had no RACK activity; Fig. 4*B*) bound amounts of PtdSer that were only ≈40% less than amount bound by the annexin with the highest RACK activity, annexin I (Fig. 4*B*). Thus, it appears that there was no correlation between the amount of bound PtdSer and RACK activity and that not all PtdSer-binding protein are RACKs.

Wolf and collaborators (9, 23) have described three proteins of 68, 110 and 115 kDa that bind PKC in the presence of PtdSer. However, it has not been determined whether the binding to these proteins is specific for PKC and saturable. Furthermore, the conclusion of their studies is that PtdSer is the primary ligand of these binding proteins and that PKC binding is mediated by a PtdSer bridge (23). If this were the case, any PtdSer-binding protein should have PKC binding activity. However, our data on annexins indicate that PtdSer binding is not sufficient for RACK activity. Furthermore, we have recently found that a peptide derived from annexin I binds PKC in the absence of PKC activators (unpublished observations). Taken together, our data suggest a direct protein–protein interaction between PKC and RACKs. Therefore, we conclude that PtdSer does not serve as a bridge between PKC and RACKs and propose that PtdSer may be required for activation of PKC, resulting in the exposure of the site(s) on PKC that interacts with RACK.

If the proteins described by Wolf and collaborators (9, 23) prove to be RACKs, the difference in their molecular mass from the RACKs described here may reflect variation between tissues. In fact, the 110- to 115-kDa proteins identified in the particulate fraction of brain and erythrocytes were either absent from several white blood cell types or were present only in their cytosol (9, 23). Alternatively, if RACKs are isozyme-specific, RACKs with different isozyme specificity may have been identified in these studies.

In summary, we describe PKC-binding proteins of 30 and 33 kDa that appear to be high-affinity receptors for activated PKC according to established criteria for receptors. A model is presented in Fig. 5 depicting inactive PKC as a folded rod with the pseudo-substrate domain bound to the substrate-binding site as described (18). Based on our observation that substrate peptides do not inhibit PKC binding to RACKs (Fig. 3), activated PKC is shown with RACK occupying a RACK binding site(s) and the substrate binding site exposed. This model suggests therefore that substrates and RACKs may concomitantly bind PKC. However, the model does not exclude the possibility that RACKs may also serve as substrates.

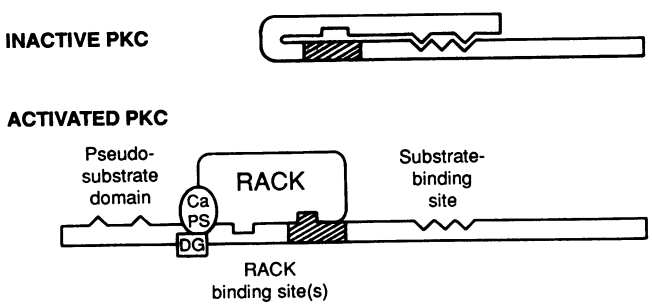

FIG. 5. Model of PKC with a RACK binding site. DG, diacylglycerol; PS, PtdSer.

PKC binding to RACKs may be subject to modulation because of modification(s) in or near the sites of interaction. This may provide a further level of regulation of PKC function. Therefore, it is important to identify the sequences in both PKC and RACKs that participate in this binding.

The concept of endogenous receptor proteins for protein kinases is not without precedent. On stimulation by platelet growth factor (PDGF), Raf-1 kinase translocates from the cytosol to the particulate fraction. The association with the particulate fraction appears to reflect binding of Raf-1 kinase to a protein in this fraction, the PDGF receptor (24). Recently, a 32-kDa receptor protein that binds the tyrosine kinase, p60$^{v\text{-}src}$, via a site different from the kinase substrate site has also been described (25). Therefore, it is possible that specific intracellular receptors for different protein kinases may be a general phenomenon.

We are grateful to Drs. A. S. Gordon, I. M. Goldstein, J. D. Ernst, and R. A. Blackwood for helpful discussions; to Drs. I. Diamond, R. O. Messing, and S. A. Krauss for reading the manuscript; and to Ms. C. J. Breaux for figure preparation. This work was supported in part by grants from the National Institutes of Health and the National Institute of Alcohol Abuse and Alcoholism.

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