Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 492–496, January 1995
Biochemistry

An autoregulatory region in protein kinase C: The pseudoanchoring site

(pseudo-RACK1/agonist/WD40 motif/*Xenopus* oocyte/translocation)

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Communicated by Edwin G. Krebs, University of Washington, Seattle, WA, October 5, 1994

ABSTRACT **We have previously identified receptors for activated C kinase (RACKs) as components of protein kinase C (PKC) signaling. RACK1, a recently cloned 36-kDa RACK, has short sequences of homology to PKC. A possible explanation for the homologous sequences between the ligand (PKC) and its intracellular receptor (RACK1) may be that, similar to the pseudosubstrate autoregulatory sequence on PKC, there is also a pseudo-RACK1 binding site on the enzyme. If this is the case, peptides with these sequences (derived from either RACK1 or PKC) are expected to affect PKC binding to RACK1 *in vitro* and PKC-mediated functions *in vivo*. Here, we show that the PKC-derived peptide (pseudo-RACK1 peptide), but not its RACK1 homologue, modulated PKC function both *in vitro* and *in vivo*. Our data suggest that the pseudo-RACK1 peptide binds and activates PKC in the absence of PKC activators and thereby acts as an agonist of PKC function *in vivo*. Therefore, the pseudo-RACK1 sequence in PKC appears to be another autoregulatory site; when PKC is in an inactive conformation, the pseudo-RACK1 site interacts with the RACK-binding site. Activation of PKC exposes the RACK-binding site, enabling the association of the enzyme with its anchoring RACK. Similar pseudoanchoring sites may regulate the function of other protein kinases.**

Protein kinase C (PKC) isozymes translocate on activation from one cellular compartment to another (1–6). We have previously suggested that receptors for activated C kinase (RACKs) are required for the translocation and subsequent function of the enzyme (7). We have recently obtained a cDNA clone encoding a protein, RACK1, that meets the criteria previously established for RACKs (8). RACK1 is a homologue of the guanine nucleotide-binding protein (G protein) β subunit; it contains seven WD40 repeat elements. RACK1 binds PKC in a dose-dependent and saturable manner. Binding is specific for PKC, since other kinases do not interact with RACK1. RACK1 is not phosphorylated by PKC nor does it cause inhibition of histone III phosphorylation by PKC, and therefore it is neither a substrate nor a PKC inhibitor. Rather, RACK1 increases histone III phosphorylation, indicating its ability to stabilize the active conformation of PKC.

Activation of many serine/threonine protein kinases such as cAMP kinase, calcium/calmodulin-dependent protein kinase II, and PKC is associated with conformational changes from inactive to catalytically active forms (9, 10). The inactive conformation is stabilized by an autoinhibitory sequence that masks the catalytic site from exposure. This sequence was identified because of its homology to the consensus sequence surrounding a phospho-acceptor amino acid in the substrates of these kinases (11). Since alanine substitutes for the phospho-acceptor amino acid (threonine or serine), it was termed a pseudosubstrate sequence (9). In PKC, this pseudosubstrate sequence is composed of 13 highly conserved amino acids in the N-terminal C1 domain of the protein (11). This basic pseudosubstrate sequence is thought to bind, in the inactive state, to an acidic conserved sequence within the catalytic domain between amino acids 530 and 583 (12).

Similar to the homology between sequences in PKC and the consensus sequence of phosphorylation in its substrates, short sequences of homology between PKC and RACK1 were also noted (8). We hypothesized that the RACK1 homologue sequences in PKC may serve as autoregulatory regions between the ligand (PKC) and its intracellular receptor (RACK1) (8). To test this hypothesis, two homologous peptides derived from PKC and RACK1 were synthesized. The PKC-derived peptide SVEIWD (pseudo-RACK1) is located in the middle of the C2 region within the regulatory domain of β-PKC. The RACK1-derived peptide SIKIWD is located in the sixth WD40 repeat but shares homology with sequences in three other repeats. If one of these homologue sequences (or both) is functionally important, it should bind to either PKC or to RACK1, alter PKC binding to RACK1, and affect PKC-mediated function *in vivo*. In this paper, we provide data supporting an autoregulatory role for the pseudo-RACK1 sequence in PKC.

MATERIALS AND METHODS

Materials. The PKC-derived peptide (pseudo-RACK1 peptide; SVEIWD), [A^3]pseudo-RACK1 peptide (SVAIWD), [A^5]pseudo-RACK1 peptide (SVEIAD), pseudo-RACK1 scrambled peptide (WEVDIS), the RACK1-derived peptide (SIKIWD), and the control peptide (LKGKIL) were synthesized at the Protein and Nucleic Acid Facility at Stanford University. The purity and structural integrity of the peptides were confirmed by HPLC and amino acid analysis. PKC from rat brain was partially purified as described (13). Histone type III was purchased from Sigma, and phosphatidylserine (PS) and diacylglycerol (DG) were purchased from Avanti.

Preparation of RACK1. The recombinant RACK1 was produced as a fusion protein with the maltose-binding protein using pMAL-c2 (New England Biolabs) as an expression vector. An 8-aa FLAG (IBI) epitope sequence (DTKD-DDDK) was attached to the N terminus of RACK1 by the PCR technique using pRACK1 (8). The fusion protein was then purified on an amylose affinity column (New England Biolabs). The 36-kDa RACK1 was recovered after removal of the maltose-binding protein by incubating with factor Xa protease (5 μg/ml; New England Biolabs) for 48 hr at 4°C.

Overlay Assay. RACK1 was subjected to SDS/PAGE and blotted onto nitrocellulose as described elsewhere (7, 8). Strips of the nitrocellulose sheet (0.5–1 μg of RACK1 per strip) were preincubated in overlay buffer [50 mM Tris·HCl (pH 7.5) containing 0.1% (wt/vol) bovine serum albumin, leupeptin at

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Abbreviations: PKC, protein kinase C; RACK, receptor for activated C kinase; PS, phosphatidylserine; DG, diacylglycerol; GVBD, germinal vesicle breakdown; G protein, guanine nucleotide-binding protein.
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5 μg/ml, soybean trypsin inhibitor at 10 μg/ml, 0.1% PEG, 0.2 M NaCl, 0.1 mM $CaCl_2$, and 12 mM 2-mercaptoethanol] with the indicated peptides for 30 min at room temperature. Partially purified rat brain PKC (containing a mixture of PKC isozymes; 0.3 unit; 200 units/mg) was added together with PKC activators (PS at 50 μg/ml, DG at 0.8 μg/ml, and 1 mM calcium), and the mixture was further incubated for 30 min at room temperature. Unbound material was removed, and the strips were washed three times for 5 min with overlay wash buffer [50 mM Tris·HCl (pH 7.5) containing 0.1% PEG, 0.2 M NaCl, 0.1 mM $CaCl_2$, and 12 mM 2-mercaptoethanol]. PKC binding to RACK1 was detected by anti-β-PKC monoclonal antibodies (Seikagaku America, Rockville, MD; 1:1000). The strips were then incubated with anti-mouse horseradish peroxidase-linked antibodies diluted 1:1000 (Amersham) followed by detection of chemiluminescence (Amersham). A Western blot with anti-FLAG antibodies (1:10,000; Kodak) for RACK1 detection was carried out as a control.

PKC and RACK1 Binding to the Peptides. Increasing concentrations of the peptides were blotted onto nitrocellulose paper in a slot-blot apparatus (Schleicher & Schuell). Unbound material was removed, and the nitrocellulose was incubated for 2 hr in overlay block buffer (20 mM Tris·HCl, pH 7.5/3% bovine serum albumin/0.1% PEG/0.2 M NaCl). PKC or RACK1 (≈5 nM) was added in overlay buffer in the presence and absence of PKC activators (PS at 50 μg/ml, DG at 0.8 μg/ml, and 1 mM calcium), and the blot was incubated for 30 min at room temperature. Binding of PKC isozymes was determined as described above with anti-β-PKC or anti-δ-PKC and anti-ε-PKC polyclonal antibodies (1:300; GIBCO/BRL). Anti-FLAG monoclonal antibodies (1:10,000) were used for the detection of RACK1 binding to the peptides.

***Xenopus* Oocyte Assay.** Microinjection of *Xenopus* oocytes was carried out by Mike Wu and Associates (Berkeley, CA) as described (14). Oocytes were injected with 50 nl of the indicated peptide or injection vehicle (20 mM NaCl) 1 hr before insulin treatment (8.25 μg/ml). Insulin-induced oocyte maturation was then determined by monitoring the appearance of a white spot in the animal pole of the oocyte, indicative of germinal vesicle breakdown (GVBD) and maturation. Ten to 15 oocytes were included in each treatment, and oocytes were scored for up to 24 hr after insulin treatment. Where indicated, peptide-induced oocyte maturation was determined in the absence of insulin stimulation.

Histone III Phosphorylation by PKC. The peptides (10 μM) were first incubated with PKC (≈10 nM) for 15 min in overlay buffer as described above. Histone type III (41 μg/ml) was then added to the mixture with or without PS (50 μg/ml), DG (0.8 μg/ml), and calcium (1 mM). Histone phosphorylation by PKC was obtained by adding 20 mM Tris·HCl at pH 7.5 containing $MgCl_2$ (20 mM), 2-mercaptoethanol (12 mM), ATP (20 μM), and [γ-^{32}P]ATP (5 μCi/ml; 1 Ci = 37 GBq). The mixture was incubated for 15 min at room temperature, and the reaction stopped by addition of sample buffer. The samples were then boiled for 10 min and loaded onto an SDS/10% PAGE minigel. The gel was fixed with 50% methanol and 10% acetic acid for 1 hr, and the autophosphorylation of PKC was determined by autoradiography.

Sequence Alignment. Homologous sequences between rat PKC isozymes and RACK1 were identified using the MACVECTOR 4.1.4 program (Kodak) by aligning the RACK1 amino acid sequence to the Entrez data bank (hash value = 2, scoring matrix PAM 250 or PAM 250S). Sequences of PKC isozymes were taken from refs. 15 and 16.

RESULTS AND DISCUSSION

When the amino acid sequence of RACK1 was compared to the Entrez protein data bank, some regions of homology with α-, β-, γ-, δ-, ε-, ζ-, θ-, and η-PKC isozymes were found (8). Although these homologies to PKC isozymes were restricted to short stretches of amino acids, this finding was intriguing. If the homologous sequences between PKC and RACK1 are functionally important, peptides derived from these sequences should bind to either PKC or RACK1, alter PKC binding to RACK1, and affect PKC-mediated function *in vivo*.

For the α-, β-, and γ-PKC isozymes, the RACK1-like sequences are located within the C2 region in the regulatory domain previously found to contain at least part of the RACK-binding site on PKC (17). These sequences have a conserved tryptophan and are not present in another WD40 motif-containing protein, the β subunit of the G protein (18). The corresponding sequences in RACK1 are clustered around the end of the WD40 motif and aligned with regions in four out of the seven WD40 repeats in RACK1. The other (C2-less) PKC isozymes also have RACK1-like sequences in the regulatory domain, most of which are different from these found in the C2-containing isozymes.

Since RACK1 binds β-PKC better than it binds other isozymes (8), we focused here on the sequence homology between RACK1 and β-PKC. Two homologous peptides derived from PKC and RACK1 were synthesized: the PKC-derived sequence, SVEIWD (pseudo-RACK1), located in the middle of the C2 region within the regulatory β-PKC, and the RACK1-derived sequence, SIKIWD, from the sixth WD40 repeat, homologous to sequences in three other repeats.

Incubation with the pseudo-RACK1 peptide inhibited β-PKC binding to RACK1, *in vitro* (Fig. 1), in a dose-dependent manner (IC_{50} ≈1μM; data not shown). The RACK1-derived peptide and the control peptide did not affect β-PKC binding to RACK1 (Fig. 1). These results suggest that the pseudo-RACK1 sequence may be important in regulating the interaction between β-PKC and RACK1.

Since the pseudo-RACK1 peptide inhibited β-PKC binding to RACK1, one or both proteins should bind directly to this peptide. Therefore, increasing amounts of pseudo-RACK1 peptide, RACK1-derived peptide, and the control peptide were immobilized on nitrocellulose, and β-PKC and RACK1 binding was determined. RACK1 did not bind to any of the three tested peptides (data not shown). However, β-PKC bound to the pseudo-RACK1 peptide, but not to the RACK1-derived peptide, in the absence or presence of PKC activators (Fig. 2*A* and data not shown). Therefore, the pseudo-RACK1 peptide interacts selectively with β-PKC, presumably by binding to part of the RACK-binding site on PKC.

Homologous pseudo-RACK1 sequences are present in other PKC isozymes (e.g., δ and ε; ref. 8). We therefore determined whether these PKC isozymes interact with the pseudo-RACK1 peptide. Both δ- and ε-PKC isozymes bound to the pseudo-RACK1 peptide in the absence of PKC activa-

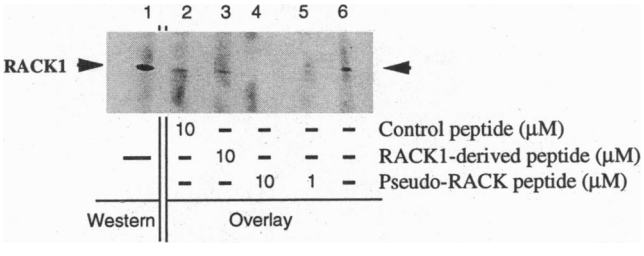

FIG. 1. Inhibition of β-PKC binding to RACK1 by the pseudo-RACK1 peptide. RACK1 blotted on nitrocellulose was coincubated with peptides, PKC, PS, DG, and calcium. Binding of PKC was then determined using anti-β-PKC antibodies as described in *Materials and Methods*, in the presence of 10 μM control peptide (LKGKIL) (lane 2), 10 μM RACK1-derived peptide (lane 3), 10 μM pseudo-RACK1 peptide (lane 4), or 1 μM pseudo-RACK1 peptide (lane 5) or in the absence of any peptides (lane 6). Lane 1 is a Western blot of RACK1 detected with anti-FLAG antibodies. Results are representatives of three independent experiments.

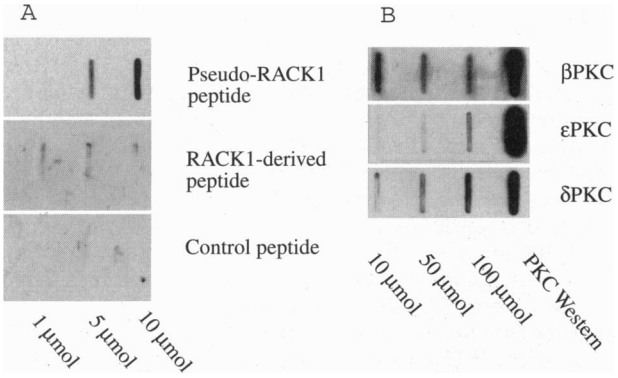

FIG. 2. (*A*) β-PKC binds to the pseudo-RACK1 peptide but not to the RACK1-derived peptide. Increasing concentrations of the pseudo-RACK1 peptide, the RACK1-derived peptide, and a control peptide were immobilized on nitrocellulose using a slot-blot apparatus, and the immobilized peptides were overlaid with PKC. Bound PKC was detected using anti-β-PKC antibodies. Recombinant RACK1 (≈0.5 μg/ml) did not bind to any of these peptides (not shown). Results are representative of three independent experiments. (*B*) β-PKC, δ-PKC, and ε-PKC bind to the pseudo-RACK1 peptide. Increasing concentrations of the pseudo-RACK1 peptide were immobilized on nitrocellulose as described in *A*. Rat brain PKC preparation (≈5 nM) was then added to the nitrocellulose, and the binding of the different PKC isozymes was detected using anti-β-PKC, anti-δ-PKC, and anti-ε-PKC antibodies. The relative amounts of β-, δ-, and ε-PKC isozymes were determined as in a Western blot by applying 20 μl of the PKC preparation to the nitrocellulose (right slots in each strip). Results are representative of three independent experiments.

tors (Fig. 2*B*). However, at all peptide concentrations, β-PKC bound considerably better than δ- or ε-PKC (Fig. 2*B*). Therefore, the pseudo-RACK1 peptide appears to have a greater specificity for the isozyme from which it was derived.

β-PKC bound to the pseudo-RACK1 peptide in the absence of PKC activators (Fig. 2*A*). Therefore, it is possible that binding of this peptide to PKC exposes the catalytic site, thereby increasing its catalytic activity. We, therefore, determined the ability of the peptides to induce substrate (histone) phosphorylation in the absence of any PKC activators. Only the pseudo-RACK1 peptide induced histone phosphorylation by PKC (Fig. 3); the RACK1-derived peptide and the control peptide did not induce histone phosphorylation under the same conditions (Fig. 3). The pseudo-RACK1-induced phosphorylation was equal to histone phosphorylation in the presence of optimal concentrations of PKC activators. Furthermore, the pseudo-RACK1 peptide also induced PKC autophosphorylation (data not shown).

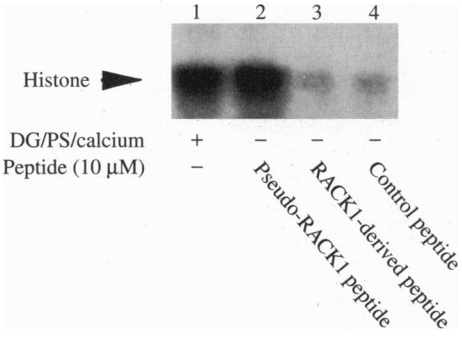

FIG. 3. The pseudo-RACK1 peptide, but not the RACK1-derived peptide, induces PKC-mediated histone phosphorylation in the absence of PKC activators. Histone type III was incubated with PKC and radiolabeled ATP in the presence of DG (0.8 μg/ml), PS (50 μg/ml), and calcium (1 mM; lane 1); pseudo-RACK1 peptide (10 μM; lane 2), RACK1-derived peptide (10 μM; lane 3), or a control peptide (10 μM; lane 4). Results are representative of two independent experiments.

Since the pseudo-RACK1 peptide activated PKC *in vitro*, we next determined whether this peptide activates PKC *in vivo*. We used the insulin-induced *Xenopus* oocyte maturation assay that is mediated in part by PKC (14). The pseudo-RACK1 peptide increased the rate of insulin-induced GVBD, which is indicative of *Xenopus* oocyte maturation (Fig. 4*A*); $t_{1/2}$ for oocytes injected with the pseudo-RACK1 peptide was 3 hr, compared with $t_{1/2}$ of ≈5 hr for control oocytes. The RACK1-derived peptide had no effect on insulin-induced *Xenopus* oocyte maturation (Fig. 4*A*). Therefore, pseudo-RACK1 peptide appeared to increase specifically the rate of insulin-induced maturation.

We then determined whether the pseudo-RACK1 peptide could induce GVBD in the absence of insulin stimulation. Microinjection of this peptide into oocytes caused 100% GVBD and maturation (Fig. 4*B*), with $t_{1/2}$ of 11 hr. If maturation is due to PKC activation by the pseudo-RACK1 peptide, maturation should be blocked by the PKC pseudosubstrate peptide, an inhibitor of PKC catalytic activity (11). Indeed, microinjection of PKC pseudosubstrate inhibitor (20 μM) together with pseudo-RACK1 (20 μM) completely inhibited the pseudo-RACK1-induced maturation (Fig. 4*B*).

To further establish the sequence specificity of the pseudo-RACK1 peptide, we tested the ability of a peptide with a pseudo-RACK1 scrambled sequence and two peptides with alanine substitution in the third or the fifth positions of pseudo-RACK1 ([A³]pseudo-RACK1 and [A⁵]pseudo-RACK1) to bind β-PKC *in vitro*, and to induce GVBD *in vivo*. The scrambled peptide and [A⁵]pseudo-RACK1 did not bind β-PKC at amounts up to 100 μmol. Some binding of β-PKC to [A³]pseudo-RACK1 was observed when using 50–100 μmol of peptide (data not shown). However, in three independent experiments, none of these peptides induced maturation after their injection into oocytes. For example, in an experiment in which 50% of the oocytes reached GVBD at 5.5 hr after injection of the pseudo-RACK1 peptide (20 μM), none of the oocytes injected with either of these three control peptides (20 μM) reached GVBD even after 18 hr. Therefore, replacement of the acidic residue of glutamic acid at the third position of the pseudo-RACK1 sequence with alanine did not abolish the binding of β-PKC.

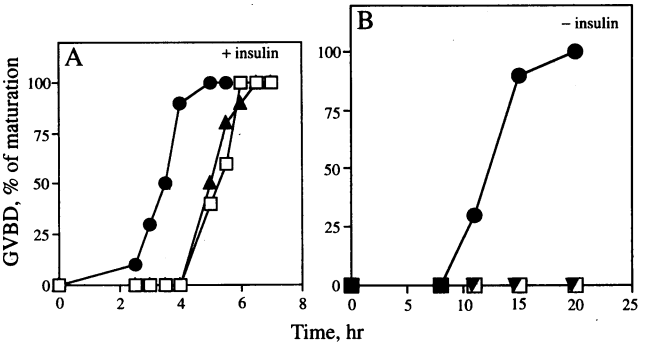

FIG. 4. (*A*) The pseudo-RACK1 peptide, but not the RACK1-derived peptide, increases the rate of insulin-induced *Xenopus* oocyte maturation. The time course of insulin-induced *Xenopus* oocyte maturation after microinjection of vehicle (20 mM NaCl; □), 20 μM RACK1-derived peptide (▲), or 20 μM pseudo-RACK1 peptide (●) is shown. Results are expressed as a percentage of oocytes reaching GVBD and are representative of three independent experiments. (*B*) Microinjection of the pseudo-RACK1 peptide induces *Xenopus* oocyte maturation in the absence of hormone stimulation. The time course of *Xenopus* oocyte maturation after microinjection of vehicle (20 mM NaCl; □), pseudo-RACK1 peptide alone (20 μM; ●), or pseudo-RACK1 peptide (20 μM) together with 20 μM PKC pseudosubstrate inhibitor peptide [β-PKC-(19–36)] (▼) is shown. The RACK1-derived peptide did not induce GVBD (data not shown). Results are expressed as the percentage of oocytes that reached GVBD and are representative of two to five independent experiments.

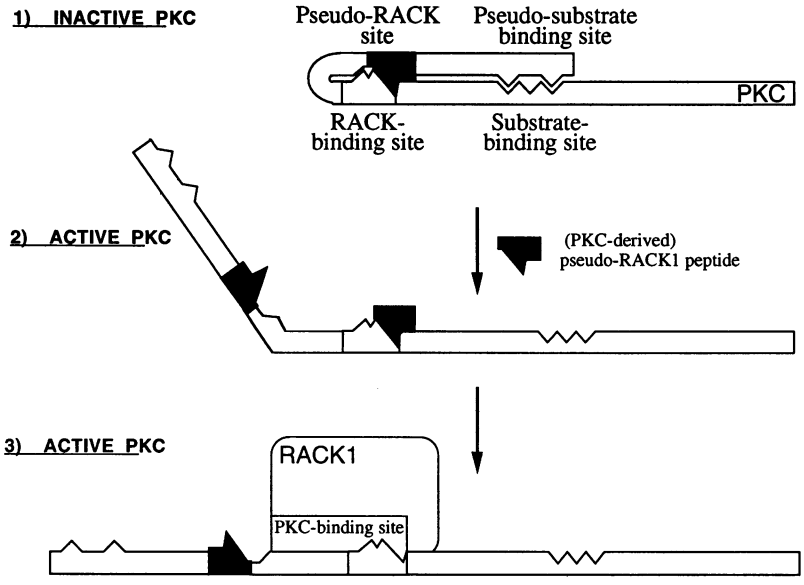

FIG. 5. Model of the pseudo-RACK1 site in the inactive and active forms of PKC. A model of PKC, depicting the pseudo-RACK1 site in an intramolecular association with the RACK-binding site when PKC is inactive and with the pseudo-RACK1 peptide or RACK1 bound to this site when PKC is active (see text for details) is shown.

However, this binding was not sufficient to fully activate the enzyme, and consequently oocyte maturation did not occur. Interestingly, substitution of the tryptophan residue at the fifth position of the pseudo-RACK1 sequence abolished both *in vitro* and *in vivo* activities of the pseudo-RACK1 peptide. Thus, the structural requirements for the pseudo-RACK1 sequence, SVEIWD, include a negatively charged residue at the third position and a tryptophan at the fifth position.

Since the RACK1-derived peptide had no *in vitro* or biological activities, this sequence may represent only part of the PKC-binding site on RACK1. Indeed, we have recently demonstrated that a peptide with a sequence derived from the middle of the same repeat of RACK1 binds β-PKC, inhibits β-PKC binding to RACK1, and affects its activity *in vivo* (8, 19). Further mapping of the PKC-binding site on RACK1 remains to be determined.

The data presented here suggest that the pseudo-RACK1 peptide, but not the RACK1-derived peptide, is an agonist of PKC *in vitro* and *in vivo*. Our data also suggest that the sequence, SVEIWD, located in the regulatory domain of βPKC, represents at least part of an additional autoregulatory region in PKC, the pseudo-RACK1 site (see model; Fig. 5). We suggest that similar to the interaction of the pseudosubstrate site with the substrate-binding site of PKC (11), the pseudo-RACK1 site interacts with the RACK-binding site in the inactive (closed) form of PKC (state 1). In the presence of the pseudo-RACK1 peptide, the equilibrium between the inactive and active conformations of the enzyme may be shifted. The active (open) form (state 2) may be stabilized by the binding of the pseudo-RACK1 peptide (shown in black) to part of the RACK-binding site in PKC, leaving the substrate-binding site available for catalytic activity. This activated PKC may then bind to anchored RACKs, either while the peptide remains associated or after the dissociation of the peptide (state 3), leading to full function of PKC in the absence of second messengers.

In this study, we focus on the role of the pseudo-RACK1 sequence in β-PKC. This sequence or similar sequences are present in other PKC isozymes. Since *in vitro*, both δ- and ε-PKC isozymes also bound to the peptide (albeit to a lesser extent), the pseudo-RACK1 peptide may not have absolute specificity for only one PKC isozyme. In this respect, it is interesting to note that the pseudosubstrate peptides of individual PKC isozymes, which differ somewhat in their sequence, are not isozyme-specific inhibitors (B. L. Smith and D.M.-R., unpublished data). However, the PKC pseudosubstrate peptides are specific inhibitors for PKC and only inhibit other protein kinases at much greater concentrations (11). Since the pseudo-RACK1 sequence was not found in other protein kinases, it is unlikely to affect kinases other than the PKC isozymes.

Finally, it is possible that there are similar autoregulatory sequences in other signal transduction enzymes that interact with specific proteins for their activity. For example, we have identified short sequences of homology between the β-adrenergic receptor kinase (20) and its anchoring receptor, the G protein β subunit (21), and between cAMP-dependent protein kinase (human RIIβ subunit; GenBank accession no. Z21958; J. S. Altala, N. Kalcheva, and B. Shafit-Zagardo, unpublished results) and the 150 A kinase-anchoring proteins (22, 23). It will be of interest to determine whether the homologous sequences in these and other signal transducing enzymes are *pseudoanchoring sites* and whether peptides derived from these sequences will modulate the biological activities of the respective enzymes *in vivo*.

This work was supported in part by grants from the National Institute of Health (RO1 HL-43380) and from The American Cancer Society (BE-158) to D.M.-R. We are grateful to Dr. Adrienne S. Gordon for helpful discussions.

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