Articles

p65 Fragments, Homologous to the C2 Region of Protein Kinase C, Bind to the Intracellular Receptors for Protein Kinase C†

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ABSTRACT: Receptors for activated protein kinase C (RACKs) have been isolated from the particulate cell fraction of heart and brain. We previously demonstrated that binding of protein kinase C (PKC) to RACKs requires PKC activators and is via a site on PKC that is distinct from the substrate binding site. Here, we examine the possibility that the C2 region in the regulatory domain of PKC is involved in binding of PKC to RACKs. The synaptic vesicle-specific p65 protein contains two regions homologous to the C2 region of PKC. We found that three p65 fragments, containing either one or two of these PKC C2 homologous regions, bound to highly purified RACKs. Binding of the p65 fragments and PKC to RACKs was mutually exclusive; preincubation of RACKs with the p65 fragments inhibited PKC binding, and preincubation of RACKs with PKC inhibited binding of the p65 fragments. Preincubation of the p65 fragments with a peptide resembling the PKC binding site on RACKs also inhibited p65 binding to RACKs, suggesting that PKC and p65 bind to the same or nearby regions on RACKs. Since the only homologous region between PKC and the p65 fragments is the C2 region, these results suggest that the C2 region on PKC contains at least part of the RACK binding site.

Protein kinase C (PKC)[1] isozymes are a family of cytosolic enzymes (Nishizuka, 1988) that translocate to the particulate fraction on activation (Kraft & Anderson, 1983). Recent studies by Mochly-Rosen et al. suggest that activated PKC binds to specific proteins, termed *r*eceptors for *a*ctivated *C-k*inase or RACKs, in the particulate fraction (Mochly-Rosen et al., 1991a). At least two major RACKs have been identified in the Triton-insoluble material from the particulate fraction of neonatal heart that bind the activated α-, β-, and γPKC isozymes (Mochly-Rosen et al., 1991a). PKC binding to these RACKs involves direct protein–protein interaction (Mochly-Rosen et al., 1991b). The binding is dependent on the PKC activators phosphatidylserine (PS), diacylglycerol (DG), and calcium, and is to a site different from the substrate binding site on PKC (Mochly-Rosen et al., 1991a).

The RACK binding site on PKC has not been identified. Since, in vitro, at least three PKC isozymes bind the same RACKs (Mochly-Rosen et al., 1991a), it is likely that the conserved sequences between these PKC isozymes are involved in PKC binding to RACKs. Comparison of the primary structures of these PKC isozymes reveals four conserved regions: C1, C2, C3, and C4 (Nishizuka, 1986; Parker et al., 1989). The C1 and C2 conserved regions are in the regulatory domain of PKC and are involved in binding of PKC activators (Nishizuka, 1986; Parker et al., 1989; Burns & Bell, 1991). The C3 and C4 conserved regions are in the catalytic domain of PKC; a PKC fragment containing the C3 and C4 regions has kinase activity that is independent of PKC activators (Nishizuka, 1986; Mochly-Rosen & Koshland, 1987). In vivo, the catalytic fragment does not associate with the particulate fraction (Tapley & Murray, 1984). These data suggest that the RACK binding site is in the regulatory domain of PKC, possibly in the C1 and/or C2 regions.

Recently, the sequences of the p65 synaptic vesicle-specific protein from rat (Perin et al., 1990), drosophila and human (Perin et al., 1991a), and the marine ray *Discopyge ommata* (Wendland et al., 1991) have been obtained. Two homologues of the C2 region of PKC are present in p65 (Perin et al., 1990; Wendland et al., 1991; see Figure 1). These two C2 homologous repeats, termed A and B, are, respectively, 46% and 50% homologous to the C2 region of bovine αPKC (amino acids 147–262; Perin et al., 1990). The C2 homologous regions in p65 are highly conserved among different species (78% invariant residues in human, rat, and drosophila; Perin et al., 1990, 1991a,b; Wendland et al., 1991). On the basis of these observations and the suggestion that p65 binds lipids in vitro, Perin et al. (1990, 1991b) proposed that this synaptic vesicle-specific protein may mediate the docking and/or fusion of synaptic vesicles at the active zone in the nerve terminus, leading to synaptic vesicle exocytosis. It has also been

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[1] Abbreviations: DG, diacylglycerol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PKC, protein kinase C; RACKs, receptors for activated C-kinase; PS, phosphatidylserine.

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suggested that the C2 region homologue found in phospholipase Cγ, GTPase activating protein (GAP), PKC, and cytosolic phospholipase A_2 encodes a calcium-dependent phospholipid binding motif that may mediate association of these proteins with the plasma membrane (Clark et al., 1991).

We have previously shown that PKC binding to RACKs involves a protein to protein interaction (Mochly-Rosen et al., 1991a,b). Translocation and binding to intracellular structures are a feature common to PKC and p65. We, therefore, hypothesized that the common C2 region of these unrelated proteins may mediate translocation and binding of PKC and p65 to their receptors. In the following, we demonstrate that a p65 fragment, containing the C2 homologue, bound to RACKs and inhibited PKC binding to RACKs. Moreover, PKC inhibited the binding of the p65 fragments to RACKs. These results suggest that the homologous C2 domains of p65 and PKC contain a site required for binding to RACKs.

EXPERIMENTAL PROCEDURES

p65 Fragments. The recombinant p65 fragments were produced as glutathione *S*-transferase (GST) fusion proteins with the addition of a consensus thrombin cleavage site between the GST and the recombinant fragment as described (Guan & Dixon, 1991; Miller et al., unpublished results). Following purification of the fusion protein on a glutathione–agarose affinity matrix, the p65 fragments were separated from the GST protein by cleavage with human thrombin (Guan & Dixon, 1991; Miller et al., unpublished results). The p65 AB fragment consists of amino acids 79–421, the p65 AB_s fragment contains amino acids 97–421, and the B fragment contains amino acids 248–421 (Figure 1). All the fragments contain an additional 13 amino acid linker between the thrombin cleavage site and the start of the recombinant protein.

Overlay Assay. (A) PKC Binding. Triton-insoluble protein fractions from neonatal rat hearts were subjected to SDS–PAGE and blotted onto nitrocellulose, and the blot was incubated with block buffer [50 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl, 3.0% bovine serum albumin, and 0.1% poly(ethylene glycol)] for at least 1 h as described elsewhere (Mochly-Rosen et al., 1991a). Nitrocellulose strips were preincubated for 30 min in overlay buffer [50 mM Tris-HCl, pH 7.5, with 0.1% bovine serum albumin, 0.2 M NaCl, 0.1% poly(ethylene glycol), 12 mM 2-mercaptoethanol, 10 μg/mL soybean trypsin inhibitor, and 10 μg/mL leupeptin] in the presence or absence of PS (50 μg/mL), DG (0.8 μg/mL), and calcium (1 mM), with or without the p65 fragments at the indicated concentrations. Unbound material was then removed by incubating the strips with fresh overlay buffer for 5 min, repeated 3 times. Binding of PKC (0.3 unit of partially purified rat brain PKC; ~200 units/mg) to RACKs was then determined in overlay buffer, using either PKC-mediated phosphorylation (Figure 3) or anti-βPKC monoclonal antibody diluted 1:1000 (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) followed by anti-mouse antisera (1:1000) and ^{125}I-labeled protein A (Figure 2), with both methods giving similar results (Mochly-Rosen et al., 1991a). Where indicated, highly purified RACKs purified from neonatal rat heart or adult rat brain were used (B. L. Smith and D. Mochly-Rosen, unpublished results).

(B) p65 Binding. Blotted nitrocellulose strips were also incubated with p65 at the indicated concentration after 0.5-h preincubation in the presence or absence of PKC activators. Where indicated, p65 AB_s was preincubated with peptide I in the presence of PKC activators. p65 binding was assayed using an anti-p65 monoclonal antibody (Matthew et al., 1981) at 1:1000 dilution followed by anti-mouse antisera (1:1000) and ^{125}I-labeled protein A. Where indicated, the nitrocellulose strips were incubated with PKC (0.3 unit) in the presence of PKC activators for 0.5 h prior to the addition of p65. Unbound material was removed, and p65 binding was determined as above.

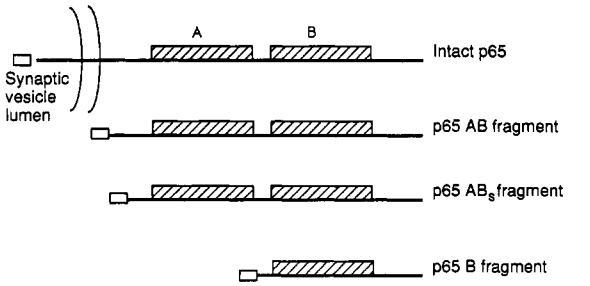

FIGURE 1: p65 fragments. The orientation of the p65 protein in a synaptic vesicle is schematically depicted. The three p65 fragments used in this study are also shown. The hatched boxes are the two C2 homologous regions, A and B. The empty box is a sequence of 13 amino acids, GSPGISGGGGGIQ, which was used as a linker between the thrombin site and the start of the recombinant protein (see Experimental Procedures).

Data Analysis. Quantitation of PKC or p65 binding was carried out by analyzing autoradiograms using a MicroScan 1000 gel analyzer (Technology Resources Inc., Nashville, TN) as described (Mochly-Rosen et al., 1991a). Each experiment was carried out at least 3 times, yielding similar results.

RESULTS

On activation, PKC binds to plasma membranes (Kraft & Anderson, 1983) and cytoskeletal elements (Jaken et al., 1989; Mochly-Rosen et al., 1990). Synaptic vesicles also bind to plasma membranes and cytoskeletal elements (Kelly, 1988; Trimble et al., 1991). Since binding to these structures appears to be a function common to both p65 and PKC, it may be mediated by the C2 region common to both proteins. To test this hypothesis, we used soluble rat p65 fragments expressed in *Escherichia coli*. These fragments contained the following PKC C2 homologous regions: repeat B, amino acids 248–421 (p65 B fragment; Figure 1); repeats A and B, amino acids 79–421 (p65 AB fragment; Figure 1); or a shorter fragment containing repeats A and B, amino acids 97–421 (p65 AB_s fragment; Figure 1).

PKC binds to 30-, 33-, and 36-kDa RACKs in the particulate fraction of cardiac myocytes or brain (Figure 2, lane 2; Mochly-Rosen et al., 1991a). If this binding were due to the interaction of RACKs with the C2 domain on PKC, then a p65 fragment with C2 homology might also bind to RACKs. Using a monoclonal antibody specific to p65, we assayed the binding of the p65 AB fragment to RACKs using an overlay assay. This fragment bound to polypeptides of 30, 33, and 36 kDa (Figure 2, lane 4); the bound fragment could not be removed by an overnight incubation in 1 M NaCl. The binding of the p65 B fragment could not be demonstrated directly, since the anti-p65 monoclonal antibody used in this study does not cross-react with this fragment.

PKC binding to RACKs is dependent on phosphatidylserine and calcium and is further increased in the presence of diacylglycerol (Figure 2, lanes 2 vs 1; Mochly-Rosen et al., 1991a). The binding of the p65 AB fragment to the 30-, 33-, and 36-kDa proteins had a similar dependence on PKC activators (Figure 2, lanes 4 vs 3). Minimal binding of the p65 AB fragment to these polypeptides was observed in the absence of phospholipids and calcium (Figure 2, lane 3) and

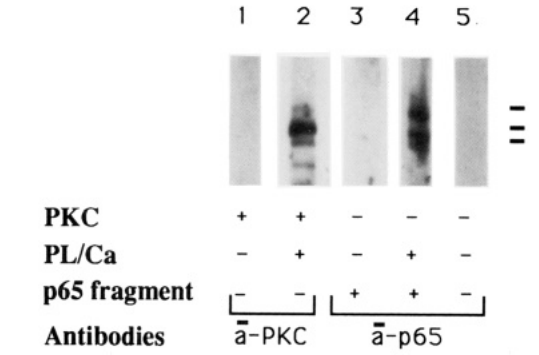

FIGURE 2: p65 AB fragment binds to RACKs. Binding of PKC (lanes 1 and 2) and the p65 AB fragment (lanes 3–5) was determined in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of PS, DG (PL), and calcium (Ca) using anti-PKC monoclonal antibodies (ā-PKC, lanes 1 and 2) and anti-p65 monoclonal antibodies (ā-p65, lanes 3–5). No p65 immunoreactivity was observed unless the p65 fragment was added to the assay (lane 5). The 36-, 33-, and 30-kDa RACKs from neonatal heart are indicated on the right, from top to bottom.

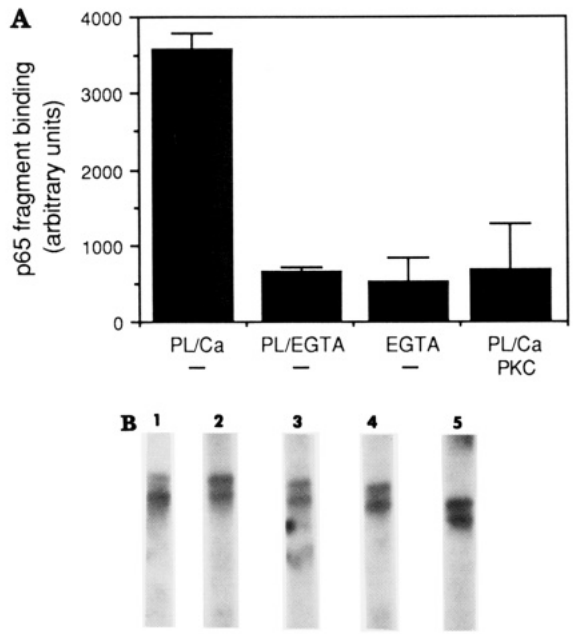

FIGURE 3: p65 AB and AB_s fragments bind to purified RACKs, and PKC inhibits this binding. (A) p65 AB fragment binding to highly purified RACKs from rat neonatal heart (30 and 33 kDa, ~1000 higher RACK activity as compared to the Triton-insoluble material used in Figures 2 and 4; B. L. Smith and D. Mochly-Rosen, unpublished results) was assayed after preincubation of the nitrocellulose-immobilized RACKs with PS, DG (PL), and calcium (Ca) in the presence or absence of 0.3 unit (~1.5 nM) of rat brain PKC. For comparison, binding of the p65 AB fragment to this RACK preparation in the presence of EGTA alone is also given. Results are an average of two determinations of the combined binding to the 30- and 33-kDa RACKs. (B) p65 AB_s fragment binding to RACKs is independent of PL and Ca. The binding of p65 AB_s fragment to purified adult rat brain RACKs in the presence (lanes 2 and 3) or absence (lanes 4 and 5) of PL and Ca is similar. For comparison, PKC binding in the presence of PL and Ca to RACKs from the same blot is also shown (lane 1).

in the presence of calcium alone (data not shown) or EGTA alone (Figure 3A).

A crude preparation of RACKs was used in the latter experiment. Thus, p65 might be binding to proteins other than RACKs, but with a similar molecular mass. We, therefore, determined whether p65 fragments could bind to a highly enriched preparation of 30- and 33-kDa RACKs (~1000-fold higher RACK specific activity than the crude preparation; B. L. Smith and D. Mochly-Rosen, unpublished

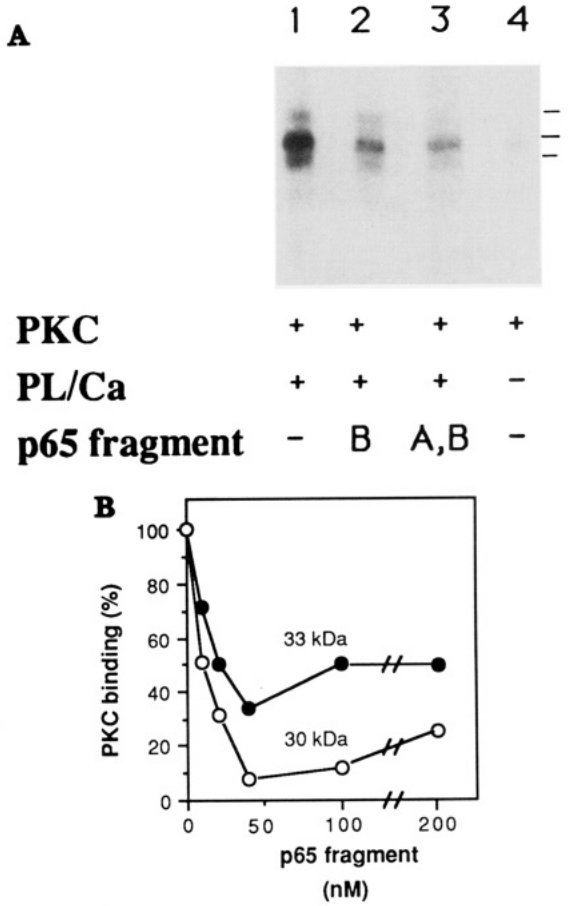

FIGURE 4: p65 B and AB fragments inhibit PKC binding to RACKs. (A) PKC binding to crude RACK preparations was determined without (lane 4) or with (lanes 1–3) preincubation with PS, DG (PL), and calcium (Ca) in the presence of p65 B fragment (lane 2) or p65 AB fragment (lane 3) or in their absence (lane 1). The 36-, 33-, and 30-kDa RACKs are indicated on the right, from top to bottom. (B) The p65 B fragment inhibits PKC binding to RACKs in a dose-dependent manner. PKC binding to RACKs after preincubation with PS, DG, and calcium in the presence of increasing amounts of the p65 B fragment was determined as in (A). Binding of PKC to the 30- and 33-kDa RACKs was quantified as described under Experimental Procedures.

results). The purified RACK preparation bound the p65 AB and p65 AB_s fragments (Figure 3A,B). These data suggest that the p65 fragments bound to the same RACKs as PKC.

Binding of the p65 AB fragment to the highly purified RACK preparation also occurred in the presence of phospholipids and calcium, but was minimal with EGTA alone or with phospholipids and EGTA (Figure 3A). In contrast, the binding of the shorter p65 AB_s fragment to both crude and pure RACKs was independent of phospholipids and calcium (Figure 3B and data not shown), indicating a direct protein–protein interaction between the p65 fragment and RACKs. Since binding of the p65 AB_s fragment to RACKs does not require phospholipids, binding of phosphatidylserine and calcium is likely to be dependent on or affected by amino acids 79–97, a sequence that lies outside the C2 homologous regions.

If p65 binds to RACKs, then PKC and p65 binding should be mutually exclusive; PKC should inhibit the binding of the p65 AB fragment to RACKs, and p65 should inhibit PKC binding to RACKs. Preincubation of the nitrocellulose strips with 0.3 unit (~1.5, nM) of PKC inhibited binding of the p65 AB fragment to purified RACKs (Figure 4A). Moreover, the binding of PKC to the 30- and 33-kDa RACKs was inhibited by both p65 B and p65 AB fragments (Figure 4A,

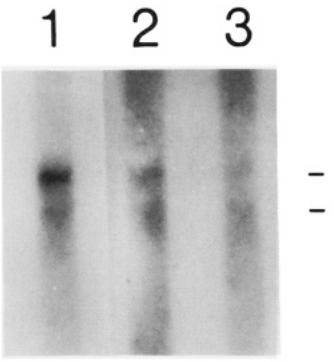

FIGURE 5: Peptide I inhibits p65 binding to purified RACKs. p65 AB_s fragment was preincubated in the absence (lane 1) or presence of 5 μM (lane 2) or 10 μM (lane 3) peptide I. The binding of the p65 fragment was then determined as in Figure 2 using purified RACKs. 30- and 33-kDa RACKs are indicated on the right.

lanes 2 and 3 vs lane 1) in a dose-dependent manner, with maximum inhibition obtained at ~37 nM B fragment (Figure 4B). Therefore, the binding of the C2-containing p65 fragments and PKC to RACKs is mutually exclusive.

We next determined whether the C2-containing fragments of p65 bind to the PKC binding site on RACKs. We have recently identified a peptide (peptide I), homologous to annexin I and a protein kinase C inhibitor, which appears to contain the PKC binding site on RACKs (Mochly-Rosen et al., 1991b). Peptide I inhibits PKC binding to RACKs with an IC_{50} of approximately 10 μg/mL. If p65 binds to RACKs at the same site as PKC, then peptide I should also inhibit the binding of p65 to RACKs. We found that peptide I inhibited p65 binding to purified RACKs in a dose-dependent manner and at a concentration similar to the concentration needed to inhibit PKC binding (Figure 5). Taken together, our data indicate that the p65 fragments and PKC bind to the same site on RACKs. Since the C2 region is homologous between these proteins, it is likely that the C2 region contains the RACK binding site on both these proteins.

DISCUSSION

In this study, we show that fragments of the synaptic vesicle-specific protein p65, containing regions homologous to the C2 region of PKC, bound directly to 30-, 33-, and 36-kDa RACKs from heart and brain (Figures 2 and 3). The p65 B and AB fragments inhibited binding of PKC to RACKs (Figure 4), and PKC inhibited the binding of the p65 AB fragment to RACKs (Figure 3A), indicating that their binding is mutually exclusive. In addition, peptide I inhibited both PKC binding (Mochly-Rosen et al., 1991a) and p65 binding to RACKs (Figure 5), suggesting that p65 and PKC bind to the same region on RACKs. Since these two proteins share a sequence homology at the C2 region, our data suggest that this region contains at least part of the RACK binding site.

p65 is a synaptic vesicle-specific protein with the C2 repeat domain extending into the cytosol [Figure 1 and see Perin et al. (1990) and Wendland et al. (1991)]. Since binding of synaptic vesicles to the active zone at the nerve terminal is specific, a docking protein or receptor in the active zone membrane has been proposed (Kelly, 1988; Trimble et al., 1991). Furthermore, synaptic vesicles bind the cytoskeleton at nerve terminals (Kelly, 1988; Trimble et al., 1991). Here, we show that three p65 fragments, which contain one or two repeats of the C2 homologue, bound to RACKs. It is possible that the binding of p65 to RACKs or RACK homologues mediates synaptic vesicle binding to the active zone and/or cytoskeletal structures at the nerve terminal. It is interesting to note that the C2 region is the most highly conserved region between the p65 proteins from different species (78% homology at the C2 region vs 57% for the full-length drosophila and rat proteins; Perin et al., 1990, 1991a,b; Wendland et al., 1991), emphasizing the functional importance of this region.

Although our data suggest that p65 binds to RACKs (Figures 2 and 3), it is not clear whether RACKs bind p65 in vivo. Whereas 0.3 unit of PKC (~1.5 nM) blocked 95% of the p65 binding to RACKs (Figure 3), ~15 nM p65 fragment (Figure 4B) was required for 50% inhibition of PKC binding to RACKs. In addition, whereas the 33-kDa RACK had the highest PKC binding activity [Figure 2, lane 2; see also Mochly-Rosen et al. (1991a)], p65 appears to bind equally well to the 30-, 33-, and 36-kDa RACKs (Figure 2, lane 4). The differences in binding to RACKs between PKC and the p65 fragments may be due to differences in the C2 region of PKC and p65. There may be a p65-specific receptor that is similar but not identical to RACKs. In addition, p65 and/or PKC may be dependent on additional factors for optimal binding. Finally, the C2 region may contain only part of the RACK binding site on PKC and p65; other regions may also interact with RACKs.

Three additional proteins have PKC C2 homologues: phospholipase Cγ (Stahl et al., 1988; Baker, 1989), GTPase activating protein (GAP; Vogel et al., 1988), and cytosolic phospholipase A_2 ($cPLA_2$; Clark et al., 1991). Similar to PKC and p65, these cytosolic enzymes translocate to the plasma membrane in response to extracellular stimuli [Clark et al., 1991; reviewed in Koch et al. (1991)]. Recently, a 16-kDa fragment of $cPLA_2$, containing the C2 homologue, was found to mediate binding of $cPLA_2$ to the plasma membrane on calcium elevation (Clark et al., 1991). These data support our hypothesis that the C2 domain is important for the docking of translocating proteins to the plasma membrane. Clark et al. suggested that the C2 region in all the proteins containing the C2 homologue is responsible for their association with the lipids in the plasma membrane (Clark et al., 1991). However, since they used a cell membrane preparation and not pure lipid preparations, they could not exclude the possibility that the association of the C2-containing proteins with the plasma membrane is through binding to RACKs or RACK-like proteins. Our findings with the p65 AB_s fragment, demonstrating that this fragment binds to RACKs in the absence of lipids and calcium (Figure 3B), further indicate that the C2 domain contains a protein recognition site which is sufficient to mediate binding to RACKs in the absence of lipids.

In summary, our results demonstrate that PKC and fragments of p65 containing a region homologous to the C2 region of PKC bind to RACKs at the same or a nearby site. These results suggest that the C2 region contains at least part of the RACK binding site on PKC. In addition, similar to the SH2 region of several translocating proteins (Moran et al., 1990; Anderson et al., 1990), this region may mediate the translocation of other C2-containing proteins such as p65 and phospholipase Cγ to intracellular structures. It should now be possible to design peptides to inhibit the translocation of specific proteins based on differences in their C2 sequences. These peptides will be useful probes to elucidate the role of translocation in the function of these proteins.

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