Regulated Binding of the Protein Kinase C Substrate GAP-43 to the V0/C2 Region of Protein Kinase C-δ*

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The interaction between protein kinase C-δ and its neuronal substrate, GAP-43, was studied. Two forms of protein kinase C-δ were isolated from COS cells and characterized by differences in gel mobility, GAP-43 binding, and specific GAP-43 and histone kinase activities. A slow migrating, low specific activity form of protein kinase C-δ bound directly to immobilized GAP-43. Binding was abolished in the presence of EGTA, suggesting Ca2+ dependence of the interaction. The free catalytic domain of protein kinase C-δ did not bind GAP-43, suggesting the existence of a binding site in the regulatory domain. Glutathione S-transferase-protein kinase C-δ regulatory domain fusion proteins were generated and tested for binding to GAP-43. The V0/C2-like amino-terminal domain was defined as the GAP-43-binding site. GAP-43 binding to this region is inhibited by EGTA, suggesting Ca2+ dependence of the interaction. The free catalytic domain of protein kinase C-δ did not bind GAP-43, suggesting the existence of a binding site in the regulatory domain.

The interaction between protein kinase C-δ and GAP-43 was studied in intact cells by coexpression of the two proteins in human embryonic kidney cells followed by immunoprecipitation. Complex formation occurred only after treatment of the cells with the Ca2+ ionophore ionomycin, indicating that elevation of intracellular Ca2+ is required for interaction in vivo. It is concluded that protein kinase C-δ interacts with GAP-43 through the V0/C2-like domain, outside the catalytic site, and that this interaction is modulated by intracellular Ca2+.

The protein kinase C (PKC) family is a ubiquitous and abundant kinase family involved in the transduction of extracellular signals in a large number of different tissues (reviewed in Refs. 1–4). PKC isotypes are activated by Ca2+1, phospholipids, and diacylglycerol (or its pharmacomimetic phorbol ester), which bind at conserved (C) regions in the regulatory domain. Three PKC subfamilies are distinguished on the basis of the variability in their regulatory domains and consequential differences in cofactor dependence. The regulatory domains of PKC-α, -β, and -γ contain a C1 and a C2 region, rendering them responsive to diacylglycerol/phorbol ester, which bind at C1, as well as to Ca2+2, which binds at C2. The regulatory domains of PKC-δ, -ε, -η, and -θ contain C1, but lack C2, resulting in Ca2+ independence. A variable extension (V0) is present N-terminal of the C1 region. PKC-ζ and -ι/λ resemble the isotypes in the latter subfamily; however, their C1 region is different and will not bind phorbol ester or diacylglycerol (1–7).

Activation of PKC leads to phosphorylation of substrate proteins and ultimately to a biological response. The growth-associated protein GAP-43 (also known as neuromodulin, p57, B-50, F1, pp46, and γ5) is one of the better characterized cellular PKC substrates known (8, 9). It has an almost exclusively neuronal localization and is present at high levels during development or during neuronal regeneration processes (10, 11). In early stages of development, it is not confined to any particular part of the neuron; however, upon maturation, its expression becomes restricted to the axon, where it remains detectable in the axon shaft as well as in the axon terminal (12, 13).

GAP-43 is a very acidic, “rod-shaped” protein with a predicted molecular mass of 23 kDa (9). It contains a single PKC phosphorylation site at Ser-41 and is further modified by palmitoylation at two adjacent Cys residues, Cys-2 and Cys-3 (9).

This allows for membrane localization of the protein in the absence of an obvious hydrophobic domain. Phosphorylation of GAP-43 in vivo is significantly stimulated by phorbol ester and, in various systems, by membrane depolarization, nerve growth factor treatment, and conditions under which hippocampal slices show long-term potentiation (14–16). Manipulation of GAP-43 levels suggests a role for the protein in neurite outgrowth, possibly by modulating the adhesive properties of the growth cone (17, 18). Furthermore, a role for GAP-43 in transmitter release has been proposed based on observations that interference with GAP-43 affects the release of neurotransmitter (19–23) and that overexpression of GAP-43 changes the competence of pituitary cells to release hormone (24). Some of the biochemical properties of GAP-43, such as its capacity to bind calmodulin and actin or to affect GTP-binding proteins, may underlie its cellular functions (8). It has been shown that GAP-43 serves as a substrate for a number of PKC isotypes (25, 26) and that PKC-mediated phosphorylation of GAP-43 directly modulates its calmodulin binding capacity (8, 9).

It has been postulated that in addition to being effector molecules, PKC substrates also bind to the kinase directly, a property that may be important for intracellular targeting of PKC. Little is known concerning the manner in which PKC interacts with GAP-43, although the existence of a high affinity binding site outside the phosphoacceptor region has been proposed on the basis of enzyme kinetic studies (26). In this study, we have addressed this issue by investigating the molecular determinants in PKC involved in GAP-43 binding. We demonstrate that GAP-43 interacts directly with PKC-δ outside the catalytic domain at the V0/C2 region. This defines a protein interaction domain on PKC-δ and suggests new ways to evaluate the functional relevance of the PKC/GAP-43 system.

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† The abbreviations used are: PKC, protein kinase C; C1 and C2 regions, conserved regions 1 and 2, respectively; GST, glutathione S-transferase; hisGAP-43, histidine-tagged GAP-43; PAGE, polyacrylamide gel electrophoresis.

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EXPERIMENTAL PROCEDURES

Materials—A monoclonal antibody to GAP-43 was obtained from Affinity Laboratories. The PKC-δ rabbit polyclonal antiserum was raised against a C-terminal 10-mer peptide (27). An affinity-purified polyclonal antibody to glutathione S-transferase (GST) was obtained from Pat Warne and Julian Downward (Imperial Cancer Research Fund, London). The calmodulin polyclonal antibody, produced by Dr. A. Merril (Cancer Research Fund, London), was used at 1:1000 dilution in 1% (w/v) bovine serum albumin (BSA) in PBS (50 mM sodium phosphate, pH 7.5, 150 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂) (QIAGEN Inc.; 0.5-ml bed volume, washed in 50 mM sodium phosphate, pH 7.5, 150 mM NaCl, 30 mM β-mercaptoethanol, 50 μg/ml phenylmethylsulfonyl fluoride, 1% (v/v) Triton X-100). The resulting cell suspension was homogenized in a Dounce homogenizer by 20 strokes, washed three times in ice-cold buffer A (137 mM NaCl, 3 mM KCl, 10 mM sodium phosphate, pH 7.2) and scraped in harvesting buffer (20 mM Tris-Cl, pH 7.5, 10 mM EDTA, 5 mM EDTA, 0.3% (v/v) β-mercaptoethanol, 250 μg/ml leupeptin, 10 mM benzamidine, 50 μg/ml phenylmethylsulfonyl fluoride, 1% (v/v) Triton X-100). The resulting cell suspension was homogenized in a Dounce homogenizer by 20 strokes, incubated for 15 min at 4 °C, diluted to 1% (v/v) in buffer B (20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 10 mM benzamidine, 0.3% (v/v) β-mercaptoethanol, 0.02% (v/v) Triton X-100), and centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was loaded onto a MonoQ column (Pharmacia Biotech Inc.) equilibrated with buffer B. Fractions (0.5 ml) were collected at a flow rate of 0.2 ml/min with a 25 min isocratic wash followed by a gradient of NaCl in buffer B (0–0.5 M in 20 min). After collection, an equal volume of glycerol was added to each fraction, and fractions were stored at –20 °C.

Generation of Recombinant Histidine-tagged GAP-43 (hisGAP-43)—DNA representing the human GAP-43 coding domain (28) was generated by polymerase chain reaction using a human fetal brain library as template. The DNA was cloned in BamHI-XhoI sites of pRSETb (Invitrogen) using restriction sites engineered in the primers, and the resulting construct was transformed into XL-1 Blue cells. Full-length hisGAP-43 protein production, 400 ml of LB medium was inoculated (1:20) with an overnight culture of XL-1 Blue cells transformed with the appropriate pGEX-KG-PKC-δ construct and incubated with exogenous recombinant hisGAP-43 using the calcium phosphate precipitation method (29). After 3 days, the cells were treated for 15 min with 1.3 mM ionomycin. Subsequently, the cells were resuspended in buffer A containing 1% Triton X-100, 100 μg/ml leupeptin, 100 μg/ml aprotinin, and 10 mM benzamidine and centrifuged at 10,000 × g for 20 min at 4 °C. To the supernatant were added 50 μl of PKC-δ antiserum PP084 and 100 μl of protein A-agarose (50% bead volume in buffer A). The mixture was incubated for 16 h at 4 °C, after which the protein A beads were collected by centrifugation and washed three times with buffer A. PKC-δ was eluted from the protein A-antibody complex by incubation for 1 h with 20 μg of peptide antigen (27) in 65 μl of buffer A. The eluate was analyzed by SDS-PAGE and Western blotting.

RESULTS

Phosphorylation of GAP-43 by PKC-δ—It has been reported that GAP-43 is phosphorylated by a number of PKC isoforms (25, 26). The phosphorylation reaction may involve a complex interaction between GAP-43 and PKC since the kinetics of phosphorylation of GAP-43 polypeptide and phosphorylation site oligopeptide differ (26). We noticed differences among PKC-δ preparations in their activity toward GAP-43, but not peptide substrates, also indicating complexity of interaction (data not shown). To investigate this in more detail, PKC-δ was overexpressed in COS cells and partially purified by MonoQ ion-exchange chromatography. MonoQ fractions were analyzed for kinase activity using exogenous recombinant hisGAP-43 or histone III-S as substrate. GAP-43 kinase activity eluted in three main peaks (peaks 1–3), two of which were dependent on cofactor, with the third being largely cofactor-independent (Fig. 1A). Histone kinase activity in these fractions showed an additional cofactor-dependent activity (peak 4) eluting before peak 3 (Fig. 1B).

Fig. 1C shows a Western blot of the individual fractions probed with an antibody recognizing the PKC-δ C terminus. Full-length forms of PKC-δ coeluted with peaks 1 and 2, whereas a PKC-δ breakdown product of ~45 kDa (representing the catalytic domain) eluted in peak 3. Peak 1 contained a fast migrating form of PKC-δ, and peak 2 contained a slower migrating form; fraction 25, between these peaks, contained both.
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Neither of the forms reacted with an antibody recognizing phosphotyrosine. Western blotting also revealed that peak 4 shows that peak 2 PKC-δ bound directly to the Ni²⁺-NTA-agarose beads (Fig. 2B). Furthermore, no binding of the PKC-δ catalytic domain (peak 3) to GAP-43 occurred (Fig. 2C).

PKC-δ is known for its Ca²⁺-independent catalytic activity. Indeed, both PKC-δ forms phosphorylated histone III-S in a Ca²⁺-independent manner, whereas PKC-α (peak 3) showed Ca²⁺ dependence under the same conditions (Fig. 3A). Surpris-

FIG. 1. MonoQ chromatography of PKC-δ extracted from COS cells. COS cells were transfected with a PKC-δ expression construct and, after 3 days, extracted in 1% Triton X-100 buffer. The extract was cleared, and the supernatant was subjected to MonoQ chromatography as described under “Experimental Procedures.” A, 5 μl of each fraction was analyzed for GAP-43 kinase activity in the presence of 0.5 mM CaCl₂ and 2 mM MgCl₂ in the presence or absence of phosphatidyserine and phorbol ester as described (30). Phosphorylated proteins were separated by 12.5% SDS-PAGE, and the phosphate incorporation in GAP-43 was measured by Cerenkov counting of the GAP-43 protein band. Peaks 1–3 are indicated. B, 5 μl of each fraction was assayed for histone kinase activity in the presence of 0.5 mM CaCl₂ and 1 mM MgCl₂ and in either the presence (○) or absence (□) of phosphatidyserine and phorbol ester as described for A. A kinase activity in the presence of 0.5 mM CaCl₂ and 1 mM MgCl₂ in the presence or absence of phosphatidyserine and phorbol ester as described for A. Peaks 1–4 are indicated. C, 1 μl of each fraction was separated by 8% SDS-PAGE. The proteins were transferred to nitrocellulose, and the filters were incubated for 16 h at 4 °C in PKC-δ antiserum PP084 diluted 1:10,000 in buffer A supplemented with 1% fat-free milk powder and 0.05% Tween 20. The open arrowheads indicate the two forms of full-length PKC-δ. The positions of molecular mass markers (in kDa) are given on the right. PKC-δ-cat, catalytic fragment of PKC-δ. D, short exposure of the autoradiograph shown in C.
GAP-43 binding to PKC-δ (peak 2) was found to be reduced in the presence of EGTA, suggesting Ca\(^{2+}\) dependence of this interaction (Fig. 3B). In contrast to the Ca\(^{2+}\)-independent phosphorylation of histone by peak 2 PKC-δ, phosphorylation of GAP-43 increased upon EGTA addition (Fig. 3C). This was not the case for peak 1 PKC-δ, which showed Ca\(^{2+}\)-dependent phosphorylation of histone as well as GAP-43 (Fig. 3, A and C).

These data indicate that GAP-43 and PKC-δ interact directly with each other in a Ca\(^{2+}\)-dependent fashion at a site outside the catalytic domain. Furthermore, PKC-δ is functionally heterogeneous since only the later eluting, slow migrating form (peak 2) bound to GAP-43. (It should be noted that since GAP-43 is not expressed in COS cells, the lack of binding to peak 1 is not due to a pre-existing GAP-43/PKC-δ complex.)

Ca\(^{2+}\)-dependent Binding of GAP-43 to the V0/C2 Domain of PKC-δ—In view of the fact that full-length PKC-δ binds to GAP-43, but the catalytic domain of PKC-δ did not, we investigated where the interaction occurs in the regulatory domain. PKC-δ regulatory domain fragments of different length were produced as recombinant GST fusion proteins. Fig. 4 shows the relative yield of the various fragments after extraction of the proteins and purification using glutathione-Sepharose 4B. Of four regulatory domain fragments, GST-PKC-δ-(1–121) showed the highest levels of expression and the highest yield. PKC-δ-(1–121) represents the region of PKC-δ that previously has been identified as V0 (1, 2) and that shares structural homology with the C2 region of the classical PKC isotypes (33). GST-PKC-δ-(1–298) and GST-PKC-δ-(1–165) showed, in addition to proteins of the predicted size, a breakdown product of ~40 kDa. GST-PKC-δ-(1–121) and GST-PKC-δ-(1–61) were homogeneous proteins of the predicted molecular mass.

To establish whether the regulatory domain of PKC-δ was able to bind GAP-43, the various GST-PKC-δ fusion proteins were assayed for binding to hisGAP-43 as described above. Fig. 5A shows that both GST-PKC-δ-(1–298) and GST-PKC-δ-(1–121) co-immobilized with hisGAP-43 on Ni\(^{2+}\)-NTA beads. GST-PKC-δ-(1–61) and GST did not associate with hisGAP-43. In the absence of hisGAP-43, GST-PKC-δ-(1–298) and GST-PKC-δ-(1–121) were not present in the eluate, indicating that they do not bind directly to the beads. Fig. 5B shows the concentration dependence of the binding of GST-PKC-δ-(1–121) to hisGAP-43. At 0.5 μM GAP-43, half-maximum binding of GST-PKC-δ-(1–121) was estimated to occur at 50 nM.

The binding of GST-PKC-δ-(1–121) to hisGAP-43 was not affected by high concentrations of NaCl (Fig. 6A). Similarly, 2 mM CaCl\(_2\) did not change binding, but it was completely lost in the presence of 1 mM EGTA (Fig. 6A). The interaction between PKC-δ-(1–121) and hisGAP-43 may therefore be supported by trace calcium ions present in the incubation buffers. Analysis of the Ca\(^{2+}\) dependence of GAP-43 binding to PKC-δ-(1–121) in an EGTA-buffered system showed binding to occur at Ca\(^{2+}\) concentrations higher than 10\(^{-5}\) M (Fig. 6B). At a concentration of 10\(^{-7}\) M or lower, binding was dramatically reduced. The binding of hisGAP-43 to the Ni\(^{2+}\)-NTA-agarose beads was not affected by these Ca\(^{2+}\) concentrations (data not shown).

Characterization of the PKC-δ-binding Region on GAP-43—To refine the binding of PKC-δ-(1–121) to GAP-43, we generated a C-terminal truncation mutant of GAP-43 (hisGAP-43-(1–146)) and analyzed its capacity to bind the various GST-PKC-δ regulatory domain constructs. In all aspects, hisGAP-43-(1–146) behaved in the same way as full-length hisGAP-43 both in terms of the binding preferences and concentration dependences of binding (Fig. 7). Thus, the binding of PKC-δ to GAP-43 occurs in the N-terminal part of the protein between residues 1 and 146.

Since GAP-43 is a calmodulin-binding protein, we investigated whether the binding of GST-PKC-δ-(1–121) would preclude calmodulin binding to GAP-43. Calmodulin was identi-
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Fied on SDS gel by silver stain (Fig. 8B) based on its migration at the appropriate size (17 kDa) and by Western blotting using a calmodulin antibody (Fig. 8C). In line with existing literature, calmodulin bound to hisGAP-43 (immobilized on Ni²⁺-NTA-agarose beads) in the absence, but not in the presence, of CaCl₂ (Fig. 8, B and C). GST-PKC-δ-(1–121) binding to hisGAP-43 occurred whether or not calmodulin was associated, and similarly, calmodulin binding was unaffected when increasing amounts of GST-PKC-δ-(1–121) were bound to hisGAP-43. Lanes 1–6 refer to 1:2 serial dilutions of the fusion protein subjected to the binding assay. For details, see A.

**Fig. 5.** Binding of GST-PKC-δ regulatory domain fusion proteins to hisGAP-43. A, equal amounts of GST-PKC-δ regulatory domain fusion proteins were analyzed for binding to immobilized hisGAP-43 as described under “Experimental Procedures.” Proteins were separated by 12.5% SDS-PAGE and visualized by silver staining. The left panel shows the total amount of each fusion protein subjected to the GAP-43 binding assay; the middle panel shows the imidazole eluate of assays performed in the presence of hisGAP-43; and the right panel shows that of assays in the absence of hisGAP-43. The arrows indicate the retained fusion proteins, and the arrowhead shows hisGAP-43. Lane MW shows the positions of molecular mass markers (in kDa). B, shown is the concentration dependence of GST-PKC-δ-(1–121) binding to hisGAP-43. Lanes 1–6 refer to 1:2 serial dilutions of the fusion protein subjected to the binding assay. For details, see A.

**Fig. 6.** Binding of GST-PKC-δ-(1–121) to hisGAP-43 in the presence or absence of NaCl and CaCl₂. A, binding of GST-PKC-δ-(1–121) to hisGAP-43 was determined as described for Fig. 3 under standard conditions or in the presence of 1 M NaCl, 1 mM CaCl₂, or 1 mM EGTA. Proteins were separated by 12.5% SDS-PAGE and visualized by Coomassie Brilliant Blue staining (left panel) or silver staining (right panel). Lane MW shows the positions of molecular mass markers (in kDa). B, binding of GST-PKC-δ-(1–121) to hisGAP-43 was measured at increasing concentrations of free Ca²⁺ in the presence of 1 mM EGTA, pH 7.0. Proteins were separated by 12.5% SDS-PAGE and analyzed by Western blotting using an antibody against GST.

with the Ca²⁺ ionophore ionomycin to elevate intracellular Ca²⁺ levels. Fig. 9 shows GAP-43 to be present in PKC-δ immunoprecipitates from 293 cells cotransfected with GAP-43 and PKC-δ and treated with ionomycin. In the absence of ionomycin treatment, very little GAP-43 was detected. As a control, immunoprecipitations were carried out on cell lysates from cells that were transfected with GAP-43, but not PKC-δ. In these immunoprecipitates, background levels of GAP-43 were detected comparable to the level detected in the immunoprecipitates from cells not treated with ionomycin. We conclude that GAP-43 and PKC-δ form a complex in cells in response to elevation of intracellular Ca²⁺ as predicted on the basis of the in vitro binding studies.

**Fig. 7.** Binding of GST-PKC-δ-(1–121) to hisGAP-43-(1–146). Conditions were as described for Fig. 5B, except that hisGAP-43-(1–146) instead of GAP-43 was used to assess binding. Lane MW shows the positions of molecular mass markers (in kDa).

**DISCUSSION**

Evidence has been presented for the formation of a GAP-43-PKC-δ complex in intact cells and for the direct binding of
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GAP-43 at the regulatory domain of PKC-δ. Within the regulatory domain, the V0 region presents a minimal binding site. Little is known about the structure and function of this region, which is present in novel as well as atypical PKC isotypes. Recently, it was proposed that it may share structural homology with the C2 region in PKC-α, -β, and -γ (33). Our observation that GAP-43 binding occurs at the V0/C2 region of PKC-δ indicates that it may serve as a protein-protein interaction domain. Furthermore, the Ca^{2+} dependence of the interaction shows that circumstances may exist in which "Ca^{2+}-independent" PKCs, such as PKC-δ, may still respond to changes in Ca^{2+} levels.

The PKC-δ employed in this study was partially purified from COS cells and separated into two forms, characterized by differences in mobility on SDS gels. The slow migrating form of PKC-δ (peak 2) binds GAP-43, and binding of GAP-43 to this form was abolished by EGTA treatment, suggesting Ca^{2+} dependence of the interaction. No GAP-43 binding to the fast migrating form of PKC-δ (peak 1) was detected. The difference in migration of the two PKC-δ forms is likely to be a result of phosphorylation, as was previously shown for many other PKC isotypes (34, 35). Although tyrosine phosphorylation of PKC-δ is a well-established phenomenon (36–38), we could find no evidence that one of the forms in this study is tyrosine-phosphorylated.

Under all circumstances, we observed a reciprocal correlation between the ability of PKC-δ to bind GAP-43 and its specific GAP-43 kinase activity. The high binding form shows low specific activity and vice versa. EGTA treatment of the binding form (abolishing GAP-43 binding) results in an increase in GAP-43 kinase activity. Under these conditions, histone kinase activity is not affected, indicating that EGTA impinges on a component in the reaction specifically related to GAP-43. Phosphorylation of GAP-43 by peak 1, the non-binding form, is not affected by EGTA, indicating that the effect of EGTA on GAP-43 phosphorylation is restricted to the binding form. The free catalytic domain of PKC-δ, which does not bind GAP-43, shows high activity. A unifying explanation for our observations is that GAP-43 binding may affect the specific GAP-43 kinase activity of PKC-δ, measured in the in vitro phosphorylation reaction. In addition to GAP-43 binding, other properties of PKC-δ play a role in the phosphorylation reaction. We observed that differences occur in histone kinase activity between the PKC-δ forms, although there is no complete quantitative agreement between the histone kinase activity of the two PKC-δ forms since histone is essentially a non-physiological substrate. Other determinants become relevant when a physiological substrate such as GAP-43 is used to assay kinase activity, as exemplified here by the non-catalytic interaction between the PKC-δ V0/C2 domain and GAP-43. Although the exact mechanism by which binding of GAP-43 to V0/C2 would contribute to specific activity is not formally demonstrated here, one possibility is that it affects the off-rate of phosphorylated product and hence the turnover of the phosphorylation reaction. Earlier work showing a low catalytic rate of phosphorylation of full-length GAP-43 versus GAP-43 phosphorylation site oligopeptide also implied rate-limiting interactions outside the direct site of catalysis (26), consistent with the above conclusion.

Binding of V0/C2 to GAP-43 is not prevented by binding of calmodulin to GAP-43. Therefore, calmodulin and V0/C2 have different binding sites on GAP-43. This binding site is not in the C-terminal part of the molecule since the binding characteristics of GAP-43(1–146) are identical to those of full-length GAP-43. The binding site may therefore lie between the calmodulin-binding site (residues 43–51) and residue 146 or in the extreme N terminus of GAP-43.

Binding of GAP-43 to the regulatory domain of PKC-δ indicates that this domain is not just a target region for cofactor, but in fact serves as a protein-protein interaction domain. As such, this observation falls within the more general pattern of data suggesting such a role for the regulatory domain (39, 40). For instance, several phospholipid-binding proteins have been shown to interact with the regulatory domain of PKC in a phospholipid-dependent way. This binding occurs, at least in part, at the pseudosubstrate site, which itself has phospholipid binding capacity (39, 41). Although GAP-43 has been shown to bind phospholipid (9), phospholipids were not present in the binding studies here. The binding of GAP-43 to PKC-δ at the V0/C2 region is reminiscent of the binding of RACK1 to PKC-β, which takes place at the C2 region (40, 42). However, in con-
Contrast to GAP-43, RACK1 is not a PKC substrate. Furthermore, PKC-δ does not need to be in an active conformation to bind to GAP-43. It was suggested that RACK1 binding to the C2 region is important for the subcellular redistribution of PKC-δ, but not of PKC-δ and -e, upon phorbol ester stimulation of cells (42). Such specificity is intriguing in light of our observations, in that the interaction between PKC-δ and GAP-43 at the V0/C2 region may be important for the subcellular localization of PKC-δ. The finding that, on coexpression of GAP-43 and PKC-δ, a complex of the two proteins can be immunoprecipitated indicates that interaction can occur physiologically. The demonstration that this complex formation is dependent on the pretreatment of cells with the Ca2+ ionophore ionomycin indicates that the interaction is regulated by Ca2+ (46). Our results show that Ca2+-dependent interactions can take place at a C2-like region that does not contain these four aspartate residues, indicating that the basis of the Ca2+ responses of the C2 regions may be more complex than initially thought. This can be concluded also from the recent molecular cloning of a large number of synaptotagmin isoforms, which revealed that at least two of them, while containing the same four aspartate residues in their C2A region as synaptotagmin I, did not show Ca2+-dependent syntaxin binding (43).

The data presented demonstrate that PKC-δ interacts with GAP-43 in a manner controlled by two regulatory devices. First, the binding is Ca2+-dependent both in vitro and in vivo. Second, fractionation of PKC-δ reveals the presence of binding and non-binding forms. Since this heterogeneity is stable to fractionation, it is likely to be a consequence of PKC-δ post-translational modifications. In vivo, the combined effects of these two regulatory devices will serve to determine whether or not PKC-δ and GAP-43 interact. Future efforts will need to address this interaction in situ to assess its physiological role.

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