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# A 20-Amino Acid Module of Protein Kinase C $\epsilon$ Involved in Translocation and Selective Targeting at Cell-Cell Contacts\*<sup>§</sup>

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Barthélémy Diouf<sup>†1,2</sup>, Alejandra Collazos<sup>†1,3</sup>, Gilles Labesse<sup>§</sup>, Françoise Macari<sup>‡</sup>, Armelle Choquet<sup>‡</sup>, Philippe Clair<sup>¶</sup>, Cécile Gauthier-Rouvière<sup>||</sup>, Nathalie C. Guérineau<sup>\*\*</sup>, Philippe Jay<sup>‡</sup>, Frédéric Hollande<sup>†4</sup>, and Dominique Joubert<sup>†5</sup>

From the <sup>†</sup>CNRS UMR5203, INSERM, U661, University of Montpellier I and II, and the Department of Cellular and Molecular Oncology, Institut de Génomique Fonctionnelle, 141, rue de la Cardonille, F-34094 Montpellier Cedex 5, France, the <sup>\*\*</sup>Department of Endocrinology, Institut de Génomique Fonctionnelle, 141, rue de la Cardonille, F-34094 Montpellier Cedex 5, France, <sup>§</sup>Centre de Biologie Structurale, 29, rue de Navacelles, CNRS UMR5048, INSERM, U554, University of Montpellier I, F-34094 Montpellier Cedex 5, France, <sup>¶</sup>Plateforme qPCR Haut Débit, Université Montpellier II, 34095 Montpellier, France, and <sup>||</sup>Centre de Recherche de Biochimie Macromoléculaire-CNRS Formation de Recherche 2593, 1919, route de Mende, 34293 Montpellier Cedex 5, France

In the pituitary gland, activated protein kinase C (PKC) isoforms accumulate either selectively at the cell-cell contact ( $\alpha$  and  $\epsilon$ ) or at the entire plasma membrane ( $\beta$ 1 and  $\delta$ ). The molecular mechanisms underlying these various subcellular locations are not known. Here, we demonstrate the existence within PKC $\epsilon$  of a cell-cell contact targeting sequence (3CTS) that, upon stimulation, is capable of targeting PKC $\delta$ , chimerin- $\alpha$ 1, and the PKC $\epsilon$  C1 domain to the cell-cell contact. We show that this selective targeting of PKC $\epsilon$  is lost upon overexpression of 3CTS fused to a (R-Ahx-R)<sub>4</sub> (where Ahx is 6-aminohexanoic acid) vectorization peptide, reflecting a dominant-negative effect of the overexpressed 3CTS on targeting selectivity. 3CTS contains a putative amphipathic  $\alpha$ -helix, a 14-3-3-binding site, and the Glu-374 amino acid, involved in targeting selectivity. We show that the integrity of the  $\alpha$ -helix is important for translocation but that 14-3-3 is not involved in targeting selectivity. However, PKC $\epsilon$  translocation is increased when PKC $\epsilon$ /14-3-3 interaction is abolished, suggesting that phorbol 12-myristate 13-acetate activation may initiate two sets of PKC $\epsilon$  functions, those depending on 14-3-3 and those depending on translocation to cell-cell contacts. Thus, 3CTS is involved in the modulation of translocation via its 14-3-3-binding site, in cytoplasmic sequestration via the  $\alpha$ -helix, and in selective PKC $\epsilon$  targeting at the cell-cell contact via Glu-374.

Activation of cytoplasmic kinases often induces their targeting to various subcellular locations where they phosphorylate their substrates and exert their biological functions. Representative examples of proteins for which targeting involves complex

and various molecular mechanisms are provided by the protein kinase C (PKC)<sup>6</sup> family, which comprises 10 known isoforms, displaying ubiquitous, tissue- or cell type-specific expression and playing crucial roles in signal transduction (1, 2). Depending on the cell type and the stimulus, various inactive cytoplasmic PKC isoforms may, upon activation, associate with the plasma, Golgi, or nuclear membranes (3–5). Even within a given cell type, a particular isoform can be targeted and accumulated at various subcellular locations (6, 7), and these processes involve direct interaction with phospholipids or other proteins (8, 9).

In pituitary GH3B6 cells, PKC isoforms accumulate at different subcellular locations upon phorbol 12-myristate 13-acetate (PMA) stimulation or thyrotropin-releasing hormone (TRH) receptor activation (10, 11). Activated PKC $\alpha$  and  $\epsilon$  accumulate selectively at cell-cell contacts, whereas PKC $\beta$ 1 and  $\delta$  are detected along the entire plasma membrane. The selective partitioning of specific PKC isoforms at cell-cell contacts is not restricted to the GH3B6 cell line. It was also observed in blastocysts (12), in the pituitary gland (11), at heterotypic contacts between fibroblasts and epithelial cells (13), at the interface between macrophages and IgG-coated beads (14), and at the immunological synapse (15–17). Although the molecular mechanism underlying this partitioning remains largely unknown, an interesting clue was provided by the discovery in human pituitary and thyroid tumors of a natural PKC $\alpha$  D294G mutant (18, 19), which is devoid of cell-cell contact targeting selectivity (20). A similar loss of selectivity is found when an E374G mutation is introduced in PKC $\epsilon$  (11), indicating that the Asp-294 and Glu-374 amino acids located within the V3 region of PKC $\alpha$  and  $\epsilon$ , respectively, are essential for proper targeting after activation. Interestingly, the PKC $\alpha$  D294G mutant was also shown to be a loss-of-function mutant (21). However, because replacing Phe by Glu in the corresponding position does not induce the targeting of PKC $\delta$  to the cell-cell contact, it is likely that other amino acids are required for cell-cell contact targeting selectivity.

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental "Experimental Procedures" and Figs. 1–4.

<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> Supported by a grant from the Association pour la Recherche contre le Cancer. Present address: Dept. of Pharmaceutical Sciences, St. Jude Children's Research Hospital, 262 Danny Thomas Pl., Memphis, TN 38105.

<sup>3</sup> Present address: Protein Phosphorylation Laboratory, London Research Inst., Cancer Research UK, London WC2A 3PX, UK.

<sup>4</sup> To whom correspondence may be addressed. Tel.: 33-467-668-144; Fax: 33-467-542-432; E-mail: frederic.hollande@univ-montpl.fr.

<sup>5</sup> To whom correspondence may be addressed. Tel.: 33-467-142-818; Fax: 33-467-542-432; E-mail: dominique.joubert@igf.cnrs.fr.

<sup>6</sup> The abbreviations used are: PKC, protein kinase C; aa, amino acid(s); Ahx, 6-aminohexanoic acid; GFP, green fluorescent protein; HPLC, high pressure liquid chromatography; PMA, phorbol 12-myristate 13-acetate; RhoB, rhodamine B; TRH, thyrotropin-releasing hormone; aa, amino acid.

In the present work, we sought to deepen our understanding of the requirements for efficient targeting to the cell-cell contact by focusing our analysis on the sequence surrounding position Asp-294 of PKC $\alpha$  and Glu-374 of PKC $\epsilon$ . On the basis of isoform sequence comparison, we identified a 20-aa stretch in the V3 region of PKC $\epsilon$  that includes Glu-374 and contains one of the two 14-3-3-binding sites of PKC $\epsilon$  and a putative amphipathic  $\alpha$ -helix. This 20-aa module fulfills the criteria of a cell-cell contact targeting sequence, and we therefore propose to name this sequence 3CTS.

## EXPERIMENTAL PROCEDURES

**Materials**—Plasmids containing the full-length PKC $\beta$ 1, - $\epsilon$ , and - $\delta$  cDNAs were provided generously by P. J. Parker (Protein Phosphorylation Laboratory, London Research Institute, Cancer Research UK, London, WC2A 3PX, UK). The plasmid containing the full-length PKC $\gamma$  was generously provided by Dr. N. Saito (Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, Kobe, Japan). Monoclonal anti-pan14-3-3 was purchased from Santa Cruz Biotechnology, and polyclonal anti-PKC $\epsilon$  used in Western blotting was from Euromedex. Mouse monoclonal anti-PKC $\epsilon$  used for immunoprecipitation experiments was purchased from Pharmingen. Mouse monoclonal anti-green fluorescent protein (GFP) was purchased from Roche Applied Science. Anti-pSer antibody was purchased from Zymed Laboratories Inc.

**Construction of Plasmids, Site-directed Mutagenesis, RNA Extraction and Reverse Transcription-PCR, Cell Culture, Transfection, and Western Blotting**—Experimental procedures and primer sequences are described in the [supplementary "Experimental Procedures"](#).

**Vectorization of 3CTS and the Scramble Peptide**—Two peptides were synthesized using standard automated solid-phase *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)/*tert*-butyl chemistry and purified on a reverse phase HPLC C18 column to 98% purity. Their molecular weight was checked by mass spectrometry. The vector peptide H-Ahx-R-Ahx-RR-Ahx-RR-Ahx-RR-Ahx-R-NH<sub>2</sub> was coupled to the C terminus of 3CTS or a scramble peptide, with both peptides including 3 additional aa of the PKC $\epsilon$  sequence (to maintain a distance between Glu-374 and the vector peptide) and a cysteine to allow the chemical reaction for the linkage with the peptide vector. Rhodamine B (RhoB) was added to the N terminus of both constructs, yielding a RhoB-LKELENNIRKALSFDNRGEEHRA-C-vector peptide (for 3CTS) or a RhoB-GEHDFLKERLKNIREAESNRLAN-C-vector peptide complex (scramble peptide). Coupling of the vector peptide with the other peptides was achieved in a one-pot two-step reaction using the heterobifunctional linker *N*-succinimidyl 3-maleimidopropionate (Fluka 63179) in dry *N,N*-dimethylformamide, in the presence of *N,N*-diisopropylethylamine. The N-terminal function of the vector peptide was first reacted, resulting in maleimidopropionyl-GPS1168. Addition of either 3CTS or the control peptide resulted in the formation of a thioether linkage between the thiol of their cysteine and the maleimide moiety. The progression of each step of the coupling process was monitored by analytic reverse phase C18 HPLC. The resulting coupling products were purified to 98% on a reverse phase C18 HPLC column, aliquoted, and lyophilized.

**Immunoprecipitation**—For immunoprecipitation, anti-PKC $\epsilon$ , anti-GFP, or anti-IgG was cross-linked on protein G Plus-agarose beads. Four  $\mu$ g of each antibody was cross-linked to 50  $\mu$ l of protein G Plus-agarose beads with dimethyl pimelimidate. GH3B6 cells were washed twice with phosphate-buffered saline, harvested, resuspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM  $\beta$ -glycerophosphate) containing a mixture of protease inhibitors, and incubated for 45 min at 4 °C. Cell lysates were prepared from cells incubated or not with PMA and centrifuged for 10 min at 13,000 rpm. Supernatants were collected. Aliquots of supernatants containing 500  $\mu$ g of proteins were precleared with 50  $\mu$ l of protein G Plus-agarose beads for 30 min at 4 °C. Precleared supernatants were then incubated with 50  $\mu$ l of anti-PKC $\epsilon$ -, anti-GFP-, or anti-IgG-coupled beads overnight at 4 °C. Beads were then washed three times with lysis buffer and two times with 50 mM Tris, pH 7.5, 150 mM NaCl. Bound material was eluted with Laemmli buffer for SDS-PAGE.

**Real-time Fluorescence Microscopy**—Real time recording was performed as described (22). At the time of observation, the culture medium was replaced by a prewarmed buffer, at 37 °C, composed of 140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM Hepes, 6 mM glucose, pH 7.4. Images were acquired continuously, with intervals between frames of 0.533 s.

**Modeling**—Fold compatibility for the full-length and truncated sequences of the human PKC $\epsilon$  sequence was searched using the meta-server @TOME (23). Domain organization was refined using fold recognition results. Automatic modeling of recognized domains was performed with SCWRL (24) and MODELLER (25), and the validity of the resulting models was evaluated with PROSA (26) and Verify3D (27). Fold recognition was resumed for the variable regions. Improved three-dimensional models were built using MODELLER 7.0 with the loop optimization procedure.

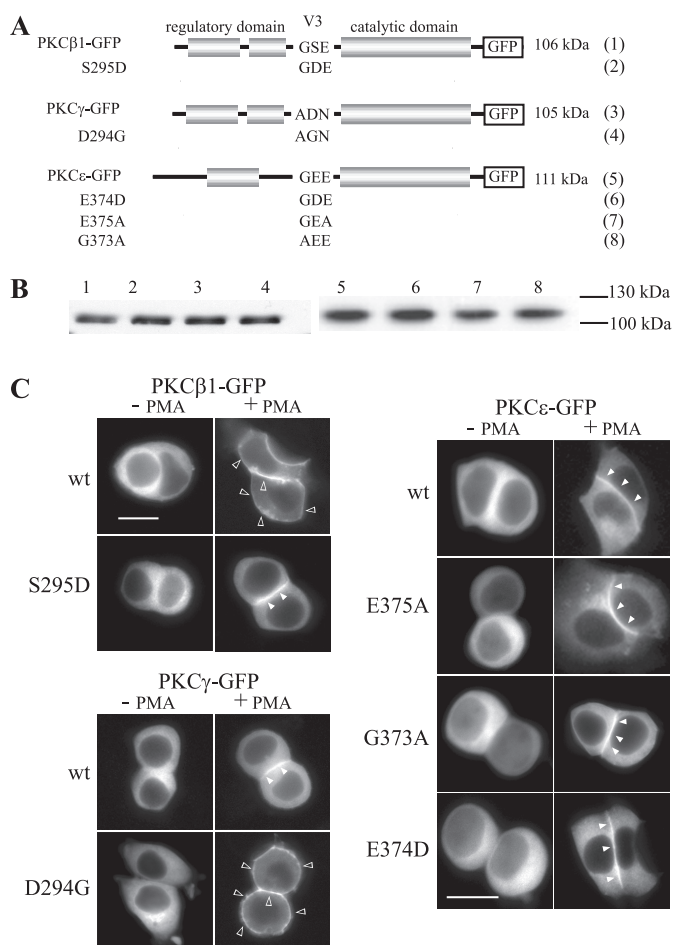
## RESULTS

### Different V3 Domain Sequences and Localizations of PKC $\alpha$ , - $\epsilon$ , - $\delta$ , - $\beta$ 1, and - $\gamma$

Previous studies showed that, in the V3 region of PKC $\alpha$  and PKC $\epsilon$ , respectively, the GDE or GEE motif is essential but not sufficient for selective cell-cell contact targeting. Accordingly, replacing GFE by GEE in the PKC $\delta$  V3 domain does not result in a selective cell-cell contact targeting of a PKC $\delta$ -GFP fusion protein (11). To improve our understanding of the molecular mechanisms underlying PKC targeting to the cell-cell contact, we compared the V3 sequences of PKC $\alpha$ ,  $\epsilon$ , and  $\delta$  (11) with those of PKC $\beta$ 1 and - $\gamma$  (Fig. 1A) and analyzed PKC $\beta$ 1 and - $\gamma$  localization when fused to GFP.

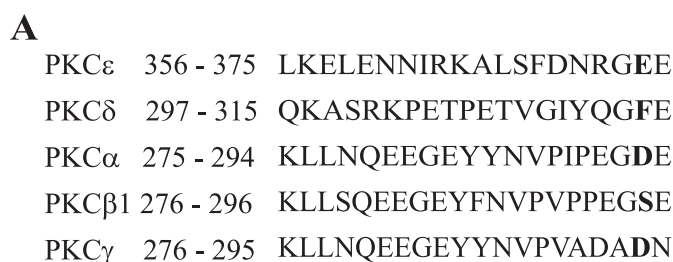
In TRH-stimulated GH3B6 cells, PKC $\beta$ 1-GFP translocated to the entire plasma membrane, including the cell-cell contact region (Fig. 1C). Changing the GSE of PKC $\beta$ 1 into the GDE of PKC $\alpha$  induced its selective localization at cell-cell contacts (Fig. 1C). We then analyzed the behavior of PKC $\gamma$  (Fig. 1A), an isoform that, when activated, selectively

## PKC $\epsilon$ Cell-Cell Contact Targeting



**FIGURE 1. Targeting of PKC $\beta$ 1,  $\gamma$ , and  $\epsilon$  and their different mutants in GH3B6 cells.** Various point mutations were performed in PKC $\beta$ 1 (GSE mutated into GDE), PKC $\gamma$  (ADN mutated into AGN) and PKC $\epsilon$  (GEE mutated into GEA, AEE, or GDE). GH3B6 cells were transiently transfected with the various GFP-tagged constructs (A). All constructs were translated at their expected size (B). Gels were loaded with 15  $\mu$ g of proteins. Two days after transfection, cells were treated or not with 100 nM PMA for 30 min, and observations were performed with conventional microscopy with an Axiophot 2.0 from Zeiss (C). The scale bars represent 5  $\mu$ m. Note that targeting is considered to be selective for the cell-cell contact (e.g. wild-type (wt) PKC $\epsilon$ ) when there is no accumulation anywhere else along the cell membrane. The seemingly higher staining detected at the cell-cell contact for PKC isoforms that also translocate along the whole membrane (e.g. wild-type PKC $\beta$ 1) is because these isoforms are present along both apposed cell membrane constituting the contact. A plot profile is shown in supplemental Fig. 1B to describe a selective versus a nonselective targeting to the cell-cell contact, as we already reported (10).

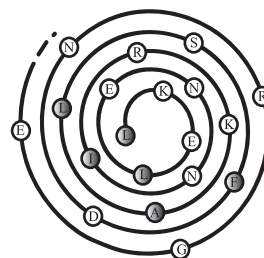
localizes at cell-cell contacts but that is not naturally expressed in GH3B6 cells. Like PKC $\alpha$ , it possesses an Asp in position 294, but, unlike PKC $\alpha$ , the motif reads ADN instead of GDE. Here, we show that the targeting selectivity of PKC $\gamma$ -GFP was lost when ADN was changed to AGN (Fig. 1C), suggesting that only the central Asp or Glu within the ADN, GDE, or GEE motif is essential for selectivity. Accordingly, activated PKC $\epsilon$  still localized at cell-cell contacts after changing the GEE motif into AEE (G373A) or GEA (E375A), and the Glu-374 of PKC $\epsilon$  can be replaced by an Asp (as in PKC $\alpha$ ) without affecting the selective targeting to cell-cell contacts (Fig. 1C). The genuine nature of PKC $\epsilon$  translocation at the actual cell-cell contact upon activation was then confirmed by costaining with p120-catenin, a marker of adher-



### B an amphipathic helix



alpha helix: 60%  
random coil: 40%



### C 14-3-3 binding sites



PKC $\epsilon$  343-RSKSAP-348  
364-RKALSFD-370

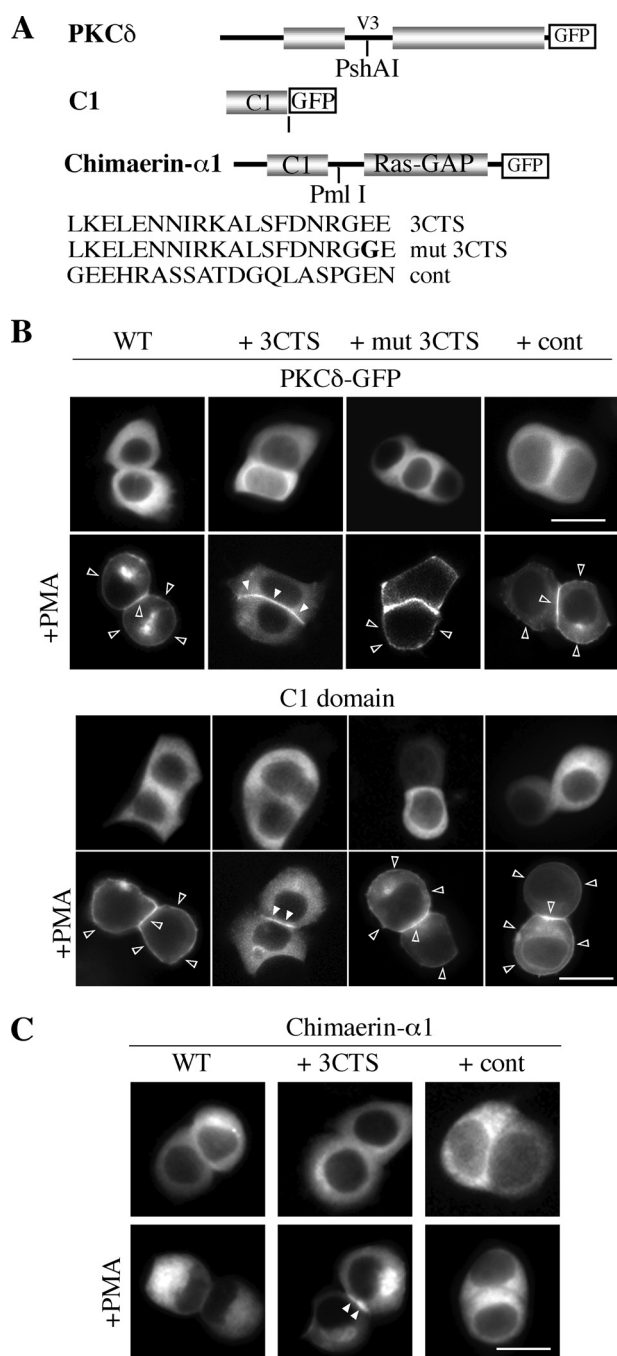


**FIGURE 2. Rationale for the selection of PKC $\epsilon$  cell-cell contact targeting sequence.** A, sequences of PKC $\epsilon$ ,  $\delta$ ,  $\alpha$ ,  $\beta$ 1, and  $\gamma$  upstream of the D(E) amino acid. B, amphipathic  $\alpha$ -helix of PKC $\epsilon$ , and as a comparison, its absence in PKC $\alpha$ . The amphipathic  $\alpha$ -helix of PKC $\epsilon$  is characterized by a hydrophobic residue every 3 or 4 amino acids and by the opposed distribution of the hydrophobic and charged amino acids in the helix. Dark circles represent hydrophobic residues. C, position of the 14-3-3-binding sites.

ens junctions (supplemental Fig. 1A). Indeed, both proteins colocalized at the cell-cell contacts.

### In Search of 3CTS, the Cell-Cell Contact Targeting Sequence

**Identification of a Candidate 20-Amino Acid Sequence in the PKC $\epsilon$  V3 Region**—Glu-374 and Asp-294 are necessary but not sufficient to target PKC $\epsilon$  and  $\alpha$  to cell-cell contacts (11), implying that neighboring amino acids could be important. We first compared the amino acid sequence close to the Glu-374 or to the corresponding amino acid of various PKC isoforms (Fig. 2A) and detected putative structural motives very close to the Glu-374 of PKC $\epsilon$ : a putative amphipathic  $\alpha$ -helix (aa 358–369) (see the Network Protein Sequence Analysis server) (Fig. 2B) and a binding site for the 14-3-3 protein (aa 364–370) (Fig. 2C) (see the ELM server). PKC $\epsilon$  possesses another 14-3-3-binding site, in position 343–348 (Fig. 2C), in agreement with the fact that 14-3-3 generally dimerizes to achieve efficient binding. The 356–375 sequence of PKC $\epsilon$  thus includes the amino acid Glu-374 essential for cell-cell contact targeting selectivity, a putative amphipathic  $\alpha$ -helix, and one of the two 14-3-3-binding sites present in PKC $\epsilon$  (28).



**FIGURE 3. 3CTS is a targeting sequence.** *A*, 3CTS bearing or not the substitution of Glu-374 by Gly (*mut 3CTS*) and a control sequence consisting of GEE plus the 17 amino acids downstream of the GEE (*cont*) were introduced in PKCδ-GFP, in C1-GFP, and in chimaerin-α1-GFP. The PshAI site gacagctgtc in PKCδ was in positions 951–960; the PmlI site cac/gtg in chimaerin-α1 was in positions 406–411. All proteins were expressed at their expected size (supplemental Fig. 3). Two days after transient transfection, GH3B6 cells were observed as in Fig. 1, in the presence or absence of PMA. *B*, the translocation of PKCδ-GFP (upper) and C1-GFP (lower) with 3CTS, *mut 3CTS*, or the control sequence is shown. *C*, the translocation of chimaerin-α1-GFP with 3CTS or the control sequence is shown.

**The PKCε 356–375 Sequence Is a 3CTS**—To determine whether the 356–375 sequence of PKCε contains the essential molecular determinants for selective cell-cell contact targeting, it was introduced into the V3 region of PKCδ, fused to the C1 region of PKCε, or inserted into chimaerin-α1, a non-PKC pro-

tein normally localized in the Golgi in the presence of PMA. These constructs were fused to GFP. Controls consisted of the 356–375 sequence containing the E374G mutation (*mut 3CTS*) and of the 17 aa downstream of the GEE motif (*cont*) (Fig. 3*A*). Although PKCδ-GFP translocated to the entire plasma membrane upon activation, including the cell-cell contact, the chimeric 3CTS-PKCδ-GFP selectively translocated to cell-cell contacts, suggesting that this 20-aa PKCε sequence probably contains the essential molecular determinants for cell-cell contact targeting (Fig. 3*B*). Indeed, when the inserted 20-aa sequence was mutated (E374G, +*mut 3CTS*) or replaced by the 17 aa found downstream of the GEE motif in wild-type PKCε (+*cont*), the resulting PKCδ chimeras translocated to the entire plasma membrane upon activation, similar to wild-type PKCδ. It is of note that wild-type PKCδ and PKCδ bearing the control 3CTS also accumulated in the perinuclear region. In addition, the C1-3CTS-GFP fusion protein was found to translocate and accumulate selectively at the cell-cell contact upon PMA activation, in contrast with the same fusion protein bearing the E374G mutation, the construct bearing the control sequence, or C1-GFP, which all accumulated indiscriminately along the entire cell membrane (Fig. 3*B*).

Finally, in the presence of PMA, chimaerin-α1 bearing the 20-aa sequence partially accumulated at cell-cell contacts, whereas the control sequence did not alter chimaerin-α1 localization (Fig. 3*C*). Thus, the 20-aa sequence does behave as a selective targeting sequence regardless of the protein it is fused to, and we consequently propose to call it 3CTS for cell-cell contact targeting sequence.

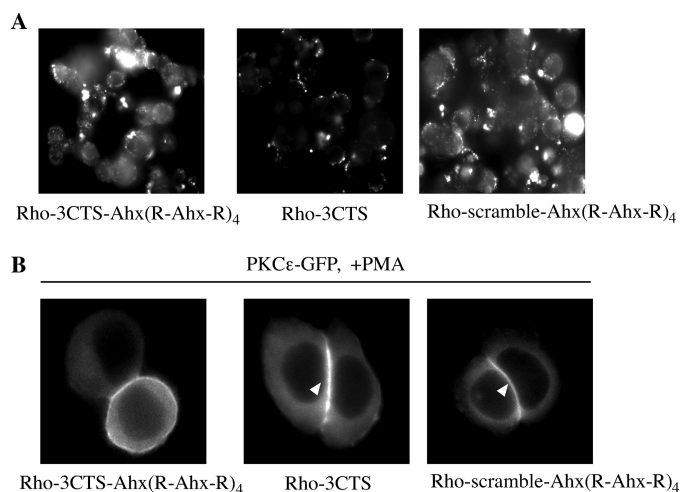
It is of note that, in agreement with data showing that the V3 region does not contain any phosphatidylserine-binding site, 3CTS is not by itself capable of accumulating at cell-cell contacts (29, 30). When fused to GFP alone, the resulting construct is only able to accumulate transiently and very weakly at cell-cell contacts (supplemental Fig. 2*D*).

**Dominant-negative Effect of 3CTS Overexpression**—To assess the potential role of 3CTS in the regulation of targeting selectivity, we analyzed whether exogenous 3CTS could disrupt the PKCε translocation process. To deliver intracellularly the 3CTS sequence, RhoB-3CTS was chemically linked to the peptide vector sequence (R-Ahx-R)<sub>4</sub> already used for the vectorization of oligonucleotides (31). Cells were incubated in the presence of 2.5 μM RhoB-3CTS-(R-Ahx-R)<sub>4</sub> for 30 min. A 3CTS scramble peptide also linked to (R-Ahx-R)<sub>4</sub> was used as a control. As shown in Fig. 4*A*, 3CTS entered the cells only when fused to (R-Ahx-R)<sub>4</sub>, and incubating cells with RhoB-3CTS-(R-Ahx-R)<sub>4</sub> abolished targeting selectivity but not translocation (Fig. 4*B*), meaning that 3CTS is indeed essential for specifying the location of activated PKCε in pituitary cells. The control sequence did not affect targeting selectivity.

**PKCε and 3CTS Interact with 14-3-3 in Pituitary Cells**

14-3-3 adaptor proteins are conserved polypeptides that mediate the cellular effects of many protein kinases through their ability to bind specific serine- or threonine-phosphorylated peptide motifs (32). Recently, phosphorylation sites within PKCε, which control its association with 14-3-3, have been identified (28, 33). It was demonstrated that association of

## PKC $\epsilon$ Cell-Cell Contact Targeting



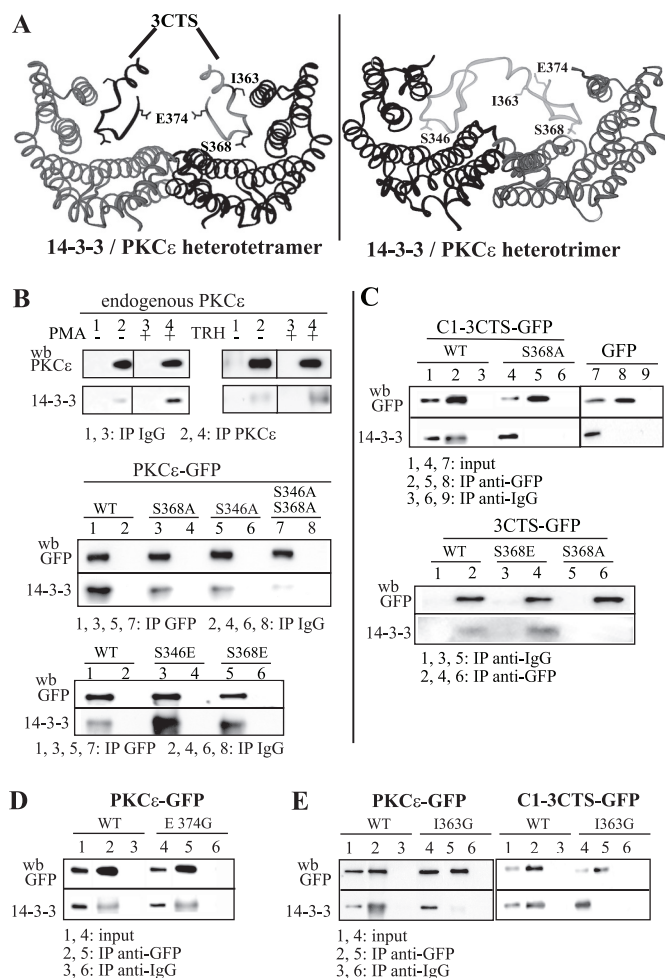
**FIGURE 4. Peptide vectorization of 3CTS abolishes selectivity of translocation.** RhoB-3CTS and RhoB-scramble 3CTS were chemically linked to the peptide vector sequence Ahx(R-Ahx-R)<sub>4</sub>. Cells were incubated for 30 min in the presence of either peptide (2.5  $\mu$ M) followed by a 15-min incubation with 100 nM PMA. **A**, RhoB fluorescence was observed intracellularly only when the peptides were coupled to the vectorization peptide (*left* and *right*). **B**, in the presence of RhoB-3CTS alone, fluorescence was observed only at the plasma membrane. In these conditions, selectivity was abolished when cells were incubated in the presence of RhoB-3CTS-Ahx(R-Ahx-R)<sub>4</sub> (*left*).

PKC $\epsilon$  with 14-3-3 is essential for the completion of cytokinesis. To determine whether the 14-3-3-binding site located within 3CTS is involved in the translocation of PKC $\epsilon$ , we first built an *in silico* model of the interaction between 3CTS and 14-3-3 and then tested it by characterizing functionally the interaction between PKC $\epsilon$  or 3CTS and 14-3-3.

**Modeling the Interaction between 3CTS and 14-3-3**—The model was derived from the crystal structure Protein Data Bank code 1ib1 of the 14-3-3 $\zeta$ -serotonin *N*-acetyltransferase complex (34). This structure contains tetramers of 14-3-3-acetyltransferase with one-to-one interactions involving mainly the phosphopeptide and its recognition site. This suggests that PKC could, similarly, bind as soon as one of its 14-3-3-binding motifs is phosphorylated. Alternatively, the presence of two putative 14-3-3-binding sites on the same polypeptide implies that one monomeric PKC could interact with two 14-3-3 polypeptides as part of a heterotrimer. A model of both types of complexes can thus be generated.

The first one, composed of two identical phosphosites, would be predicted to fit perfectly with the crystal structures revealed for various 14-3-3 complexes (Fig. 5A). The interactions with the 3CTS motif may extend to its helical region, including Ile-363. In this configuration, the model predicted that the 14-3-3 and 3CTS interface may span 10 residues of the 3CTS central region (from Ile-363 to Arg-372), whereas the N terminus of the helix and the motif GEE at the C terminus would not be expected to interact with 14-3-3. When unbound, and except for the 5 last C-terminal residues (including Glu-374), 3CTS was predicted to adopt a helical conformation. However, upon binding to 14-3-3, partial unfolding of the helix is expected to allow a perfect fit into the 14-3-3 groove.

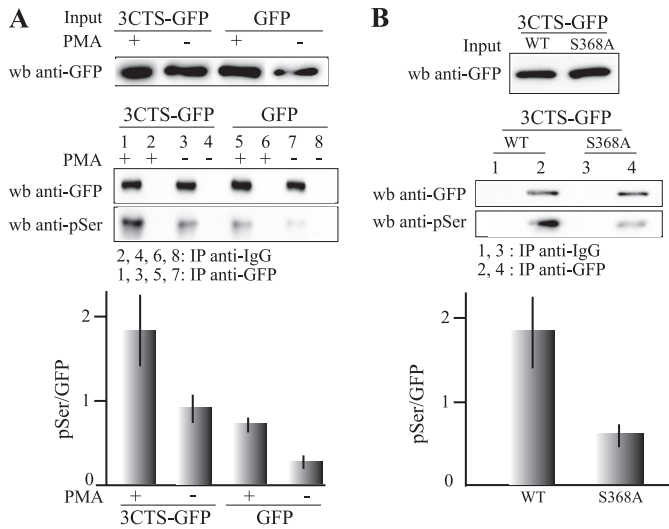
In the alternative complex, both phosphoserines 346 and 368 would be interacting with the 14-3-3-binding pocket. The predicted helical segment of the central region of 3CTS



**FIGURE 5. PKC $\epsilon$  interaction with 14-3-3.** **A**, molecular models of two complexes of a 14-3-3 protein dimer with two 3CTS (*left*) or with a sequence encompassing Ser-346 and Ser-368 (*right*) from PKC $\epsilon$ . The models are derived from the crystal structure Protein Data Bank code 1ib1 of the 14-3-3 $\zeta$ -serotonin *N*-acetyltransferase complex (34) using MODELLER 7.0. *Left*, two identical phosphosites would be predicted to fit perfectly with the crystal structures revealed for various 14-3-3 complexes. The interactions with the 3CTS motif may extend to its helical region including Ile-363. *Right*, both phosphoserines 346 and 368 would be interacting with the 14-3-3-binding pocket. The predicted helical segment of the central region of 3CTS would bridge the two phosphosites. **B**, endogenous PKC $\epsilon$  and PKC $\epsilon$ -GFP interact with 14-3-3 upon PMA or TRH stimulation. *Middle* and *bottom*, effect of the S368A and S346A and of the S346E and S368E mutations on PKC $\epsilon$ -GFP interaction with 14-3-3. **C**, C1-3CTS-GFP bearing or not a S368A mutation and 3CTS-GFP bearing or not a S368E or S368A mutation interact with 14-3-3. **D** and **E**, incidence of the E374G mutation (**D**) and of the I363G mutation (**E**) on the interaction of PKC $\epsilon$ -GFP or C1-3CTS-GFP with 14-3-3. Experiments shown in **B–E** have been performed in the presence of 100 nM PMA.

would bridge the two phosphosites. The resulting heterotrimeric model is represented in Fig. 5A. The precise orientation of the amphipathic helix cannot be clearly determined using this model, and additional interactions of PKC $\epsilon$  with 14-3-3 could thus involve the hydrophobic region of the amphipathic helix, particularly via its Ile-363. The functionality and significance of this PKC $\epsilon$ /14-3-3 interaction are assessed below.

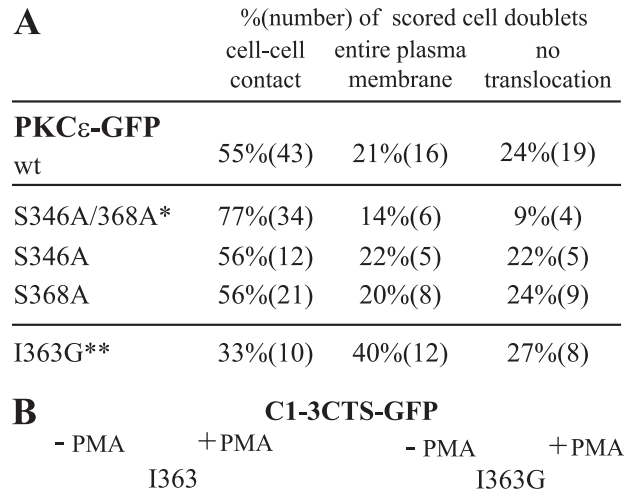
In addition, the model predicted that the remaining helical segment, which contains the hydrophobic Ile-363 residue, should not prevent direct interaction of Ile-363 with 14-3-3 at the edge of the binding groove. This model further implied



**FIGURE 6. Phosphorylation-dependent interaction of PKCε and 3CTS with 14-3-3 after PMA treatment.** Extracts (500 μg of proteins) generated from 3CTS-GFP-transfected GH3B6 cells treated or not with 100 nM PMA were used to immunoprecipitate endogenous 3CTS-GFP. 3CTS-GFP bore (B) or not (A) a S368A mutation. Immunoblots were probed with an anti-pSer antibody. 3CTS phosphorylation was increased in the presence of PMA (A) and greatly decreased after substitution of Ser-368 by an alanine (B). Quantification, performed on three independent experiments, is shown at the bottom of each panel. Bars represent S.E. wb, Western blot; WT, wild-type; IP, immunoprecipitation.

that substituting an Ala, Phe or Gly for Ile-363 should affect protein/protein interaction either directly (I363A, I363F) or indirectly (I363G) by destabilizing the helical conformation. The precise role of this helical segment is analyzed further below.

*TRH or PMA Induces an Association of PKCε and 3CTS with 14-3-3 and a Phosphorylation of Ser-368 within 3CTS*—Fig. 5B shows that the amount of endogenous PKCε coimmunoprecipitating with 14-3-3 increased drastically in the presence of PMA or TRH, suggesting that a PMA- or TRH-stimulated kinase activity is involved in modulating the direct or indirect PKCε/14-3-3 interaction. To decipher the molecular details of this interaction selectively, we then used GFP-tagged PKCε, C1-3CTS, and 3CTS constructs, which were all found to coimmunoprecipitate with 14-3-3, unlike GFP itself (Fig. 5, A and B, and supplemental Fig. 3). These interactions were increased in the presence of PMA (data not shown), which was therefore used for all subsequent coimmunoprecipitation experiments, unless otherwise specified. Because serine or threonine phosphorylation of the prospective partner is a prerequisite for 14-3-3 binding, the fact that the PKCε/14-3-3 interaction is increased in the presence of PMA could mean that serine phosphorylation is increased within the 3CTS under these conditions. Fig. 6A shows that 3CTS-GFP serine phosphorylation was strongly increased in the presence of PMA and that introducing the S368A substitution decreased serine phosphorylation to GFP background phosphorylation levels (Fig. 6B), in agreement with what was shown by Durgan *et al.* (33). Substitution of Ser-368 by Ala also resulted in a diminished PKCε/14-3-3 interaction (Fig. 5B). The Ser-346 of the second 14-3-3-binding site of PKCε was also involved in this interaction because abolition of the interaction was observed only when



**FIGURE 7. Incidence on translocation of S346A and/or S368A or I363G mutation.** A, quantification of PKCε translocation in TRH-stimulated cells was performed from real-time recordings after transfection with PKCε-GFP bearing or not the S368A or the S346A mutation or both and bearing or not the I363G mutation. Results are expressed as percentage (and absolute numbers) of cells translocating or not to the specified location. \*,  $p = 0.04$  and \*\*,  $p = 0.07$  against wild-type (wt) PKCε-GFP ( $\chi^2$  test). B, translocation of C1-3CTS-GFP is abolished in the presence of the I363G mutation.

both sites were mutated. Mimicking constitutive phosphorylation with S368E within PKCε resulted in an increased amount of 14-3-3 coimmunoprecipitating with PKCε (Fig. 5B). Similar results were obtained with the S346E substitution, arguing in favor of the 14-3-3-binding sites of 3CTS being involved in the PKCε/14-3-3 interaction. These results were confirmed with a 3CTS-GFP construct (Fig. 5C).

Fig. 5D also shows that E374G mutation does not alter PKCε/14-3-3 interaction, suggesting that selectivity of translocation, which is abolished by the E374G mutant, does not involve PKCε interaction with 14-3-3. However, to get further insights on the relationship between 14-3-3 and PKCε translocation, we analyzed the effect of introducing the Ser-368 and Ser-346 mutation in PKCε-GFP on the translocation of this enzyme.

*Preventing PKCε/14-3-3 Binding Increases the Occurrence of PKCε Translocation*—Disrupting the PKCε/14-3-3 interaction increases the number of cells where PKCε translocation is observed. Indeed, the percentage of cells where PKCε S346A/S368A-GFP was targeted exclusively to cell-cell contacts increased significantly compared with wild-type PKCε-GFP (77% versus 55%;  $p = 0.04$ ) (Fig. 7A). No change was observed when using the single mutants, in agreement with the fact that these mutants are still capable of interacting with 14-3-3, although less efficiently than the native enzyme. Thus, PKCε interaction with 14-3-3 appears to control PKCε translocation negatively, although both are induced by PMA or TRH. Furthermore, the fact that selectivity of targeting is maintained when PKCε/14-3-3 interaction is abolished suggests that 14-3-3 is not involved in the control of cell-cell contact targeting selectivity.

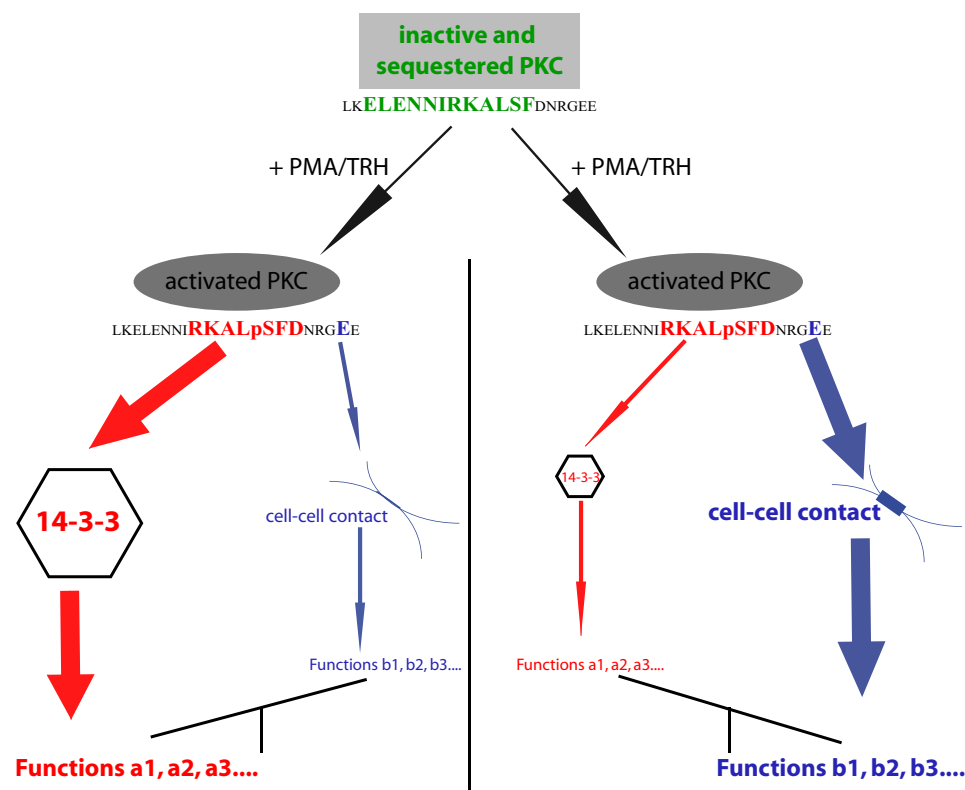


FIGURE 8. Integrated model for the coordinated involvement of the amphipathic  $\alpha$ -helix, the 14-3-3 binding site, and the Glu-374 amino acid of 3CTS in the control of PKC $\epsilon$  translocation and function. The  $\alpha$ -helix is involved very early in the translocation process because its alteration inhibits translocation. Upon PMA or TRH activation, the amount of PKC $\epsilon$  that localizes at the cell-cell contact might depend on the local amount of 14-3-3 available to bind PKC $\epsilon$ . The higher the amount of 14-3-3, the lower the amount of PKC $\epsilon$  translocated to cell-cell contacts will be. This implies that two sets of functions might be regulated by PMA/TRH because both PKC $\epsilon$  translocation to cell-cell contact and phosphorylations at Ser-368 and Ser-346, which are necessary for PKC $\epsilon$ /14-3-3 interaction, are PMA/TRH-dependent.

### Functional Importance of the Amphipathic $\alpha$ -Helix in 3CTS

Taking advantage of an isoleucine located in a central position (Ile-363) on the hydrophobic face of the predicted  $\alpha$ -helix, three different aa substitutions were performed: one to destabilize the helical segment (I363G), one to affect the hydrophobicity (I363A) (which decreases the side chain size), and one to preserve membrane anchoring while disrupting the protein-protein interface (I363F) (supplemental Fig. 4). These substitutions were first introduced in the C1-3CTS-GFP construct. All aa substitutions were found to abolish C1-3CTS translocation (Fig. 7B and supplemental Fig. 4), suggesting that the 3CTS amphipathic  $\alpha$ -helix structure is an essential molecular determinant of the translocation process. When the I363G substitution was introduced in PKC $\epsilon$ -GFP, it also induced a decrease in translocation of the PKC $\epsilon$  I363G mutant, from 55 to 33% (Fig. 7A). This decrease was close to significance ( $p = 0.07$ ), suggesting that the  $\alpha$ -helix plays a role in the translocation process.

We then analyzed the effect of this I363G mutation on the interaction between 14-3-3 and PKC $\epsilon$ -GFP or C1-3CTS-GFP and found that both mutants did not interact with 14-3-3 (Fig. 5E). The likely explanation of this result is that the I363G mutation disrupted a process located upstream of the PKC $\epsilon$ /14-3-3 interaction. Indeed, if the amphipathic helix was merely facilitating the interaction between PKC $\epsilon$  and 14-3-3, this disruption

would have been expected to increase the translocation to cell-cell contacts, similar to what was seen with the double S368A/S346A mutant.

### DISCUSSION

Results of this work suggest that the 3CTS sequence within PKC $\epsilon$  controls both translocation and targeting selectivity of this enzyme to cell-cell contact. It controls translocation via the  $\alpha$ -helix because, when the helix is destabilized, translocation is inhibited. It controls selectivity because of the presence of the essential Glu-374 amino acid, and it controls the functions that PKC $\epsilon$  exerts via its association with 14-3-3 because one of the binding sites involved in PKC $\epsilon$  interaction with 14-3-3 is located within 3CTS.

The fact that GFP cannot accumulate efficiently at the cell-cell contact when fused to 3CTS indicates that 3CTS contains the molecular determinants needed for selective targeting but not for accumulation at the cell-cell contact. This agrees well with the fact that the predicted amphipathic  $\alpha$ -helix in 3CTS most probably does not mediate any strong inter-

action with phospholipids but is involved in protein/protein interaction. Indeed, the amphipathic  $\alpha$ -helix is involved upstream of the interaction of PKC $\epsilon$  with 14-3-3 because when it is destructured, PKC $\epsilon$  translocation is abolished. Also, our data show that overexpression of 3CTS abolishes selectivity of targeting. Because 3CTS cannot accumulate at cell-cell contacts, this argues in favor of selectivity being determined prior to accumulation at cell-cell contacts.

In the present work, we also provide substantial evidence demonstrating that PKC $\epsilon$  interacts with 14-3-3, as recently suggested by Saurin *et al.* (28) and by Durgan *et al.* (33), although in these two publications, the interaction of the endogenous proteins was not documented in contrast to our work. We also provide a model predicting the nature of this interaction via a 14-3-3-binding site located in 3CTS. Endogenous PKC $\epsilon$ /14-3-3 interaction was weak in basal conditions and markedly increased in the presence of PMA or TRH, suggesting that in GH3B6 cells also, phosphorylation of PKC $\epsilon$  by a PMA-dependent kinase is responsible for PKC $\epsilon$ /14-3-3 interaction. PKC $\alpha$  could participate in this process because its activation is necessary for PKC $\epsilon$  translocation (10) and because it was shown to be involved in PKC $\epsilon$  Ser-368 phosphorylation in the process of cytokinesis (28). The involvement of 3CTS in the PKC $\epsilon$ /14-3-3 interaction was further supported by the fact that C1-3CTS-GFP and 3CTS-GFP both coimmunoprecipitated with 14-3-3



and by showing that the Ser-to-Ala substitution in either of these two constructs abolished the interaction.

The data presented here show that 14-3-3 functions as an inhibitor of PKC $\epsilon$  translocation, in agreement with other previous examples found in the literature (35, 36). However, our results also point to a new paradigm; PMA (or TRH) may initiate two processes with seemingly opposed effects on two separate pools of PKC $\epsilon$ : it initiates PKC $\epsilon$  translocation, and at the same time, it initiates binding of PKC $\epsilon$  to 14-3-3, which inhibits translocation. The fact that 14-3-3 inhibits PKC $\epsilon$  translocation does not imply that it sequesters PKC $\epsilon$  to prevent its function. Instead, we propose that the interaction of PKC $\epsilon$  with 14-3-3 may initiate a function of PKC $\epsilon$  different from that exerted at the cell-cell contact, thus allowing the involvement of PKC $\epsilon$  in new aspects of cell biology, as shown by Saurin *et al.* (28) concerning cytokinesis. In this model, upon PMA stimulation, the amplitude of each PKC $\epsilon$  function could depend on the proportion interacting with 14-3-3 and that relocating to the cell membrane (Fig. 8). Interestingly, Par-1b membrane localization has been shown to be also negatively regulated when its binding to 14-3-3 is induced via phosphorylation of Ser-400 by protein kinase D activated by nPKC (37).

Thus, two molecular mechanisms involving 3CTS appear to be at work for PKC $\epsilon$ : one that allows translocation or not and the other that determines selectivity. The former involves the amphipathic  $\alpha$ -helix and 14-3-3; the latter occurs via Glu-374 (Fig. 8). This does not mean that other amino acids or regions of PKC $\epsilon$  are not involved in translocation/targeting, as already demonstrated (38, 39). Nevertheless, this work identifies for the first time a previously unsuspected function for the V3 domain of PKC $\epsilon$  in the translocation/targeting process and underlines the role of this region as a platform for protein/protein interactions.

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**Mechanisms of Signal Transduction:  
A 20-Amino Acid Module of Protein Kinase  
C? Involved in Translocation and Selective  
Targeting at Cell-Cell Contacts**

Barthélémy Diouf, Alejandra Collazos, Gilles Labesse, Françoise Macari, Armelle Choquet, Philippe Clair, Cécile Gauthier-Rouvière, Nathalie C. Guérineau, Philippe Jay, Frédéric Hollande and Dominique Joubert

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