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Protein Kinase D Interacts with Golgi via Its Cysteine-Rich Domain

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Protein kinase D (PKD)/protein kinase Cμ is a serine/threonine protein kinase that has been localized in the cytosol and in several intracellular compartments including Golgi, mitochondria and plasma membrane. Using real time imaging of fluorescent protein (GFP)-tagged PKD, we have found that the accumulation of PKD in the Golgi compartment, following a temperature shift from 37 to 20°C, was mediated by the cysteine-rich domain (CRD) of PKD. The CRD of PKD also mediates its interaction with the plasma membrane, further supporting the conclusion that the CRD of PKD may act as a subcellular localization signal.

Key Words: **PKD; Golgi; subcellular localization; Swiss 3T3.**

Protein kinase D (PKD)/protein kinase Cμ is a serine/threonine protein kinase with structural, enzymological and regulatory properties different from other PKC family members (1, 2). The most salient features of PKD structure include the presence of a catalytic domain distantly related to Ca^{2+}-regulated kinases, a pleckstrin homology (PH) domain that regulates its enzymatic activity and a highly hydrophobic stretch of amino acids in its N-terminal region (3–5). The N-terminal region also contains a cysteine-rich domain (CRD) comprising a tandem repeat of cysteine-rich zinc-finger like motifs, termed cys1 and cys2, which confers high affinity binding to phorbol esters and plays a negative role in the regulation of catalytic activity of PKD (1, 2, 6–8). PKD can be activated in intact cells by pharmacological agents, including biologically active phorbol esters and cell permeant diacylglycerol (DAG) as well as by physiological stimuli including G-protein-coupled receptor (GPCR) agonists, growth factors and antigen-receptor engagement (9–17). In all cases, PKD activation is mediated by a PKC-dependent signal transduction pathway that involves the phosphorylation of Ser744 and Ser748 within the activation loop of PKD catalytic domain (9, 15, 16, 18).

PKD, which has been localized in the cytosol, Golgi, plasma membrane and mitochondria (12, 19–24), is implicated in the regulation of a variety of cellular functions including the organization of the Golgi compartment and its vesicular transport (20–22). Targeting of signaling proteins to different cellular compartments is a fundamental process in the regulation of their activity (25–27). Consequently, the identification of the localization signals mediating the targeting of these signaling proteins is a critical step to understand their regulation. Although recent studies demonstrated that the translocation of PKD to the plasma membrane is mediated by its CRD (12, 23, 24), the domain mediating its interaction with Golgi was not previously characterized.

In order to identify the domain of PKD responsible for its interaction with the Golgi compartment we examined the spatial distribution of PKD in Swiss 3T3 mouse fibroblasts using real time visualization of fluorescent tagged-PKD. We have found that the accumulation of PKD in the Golgi compartment, following a temperature shift from 37 to 20°C, is completely prevented by the deletion of the cys1, cys2 or the whole CRD. Our results imply that the interaction between PKD and the Golgi compartment is mediated by the CRD of PKD and that it does not require PKD's catalytic activity.

MATERIALS AND METHODS

cDNA constructs. Vectors encoding chimeric fusion proteins between green (GFP) or red (RFP) fluorescent protein and PKD wild-

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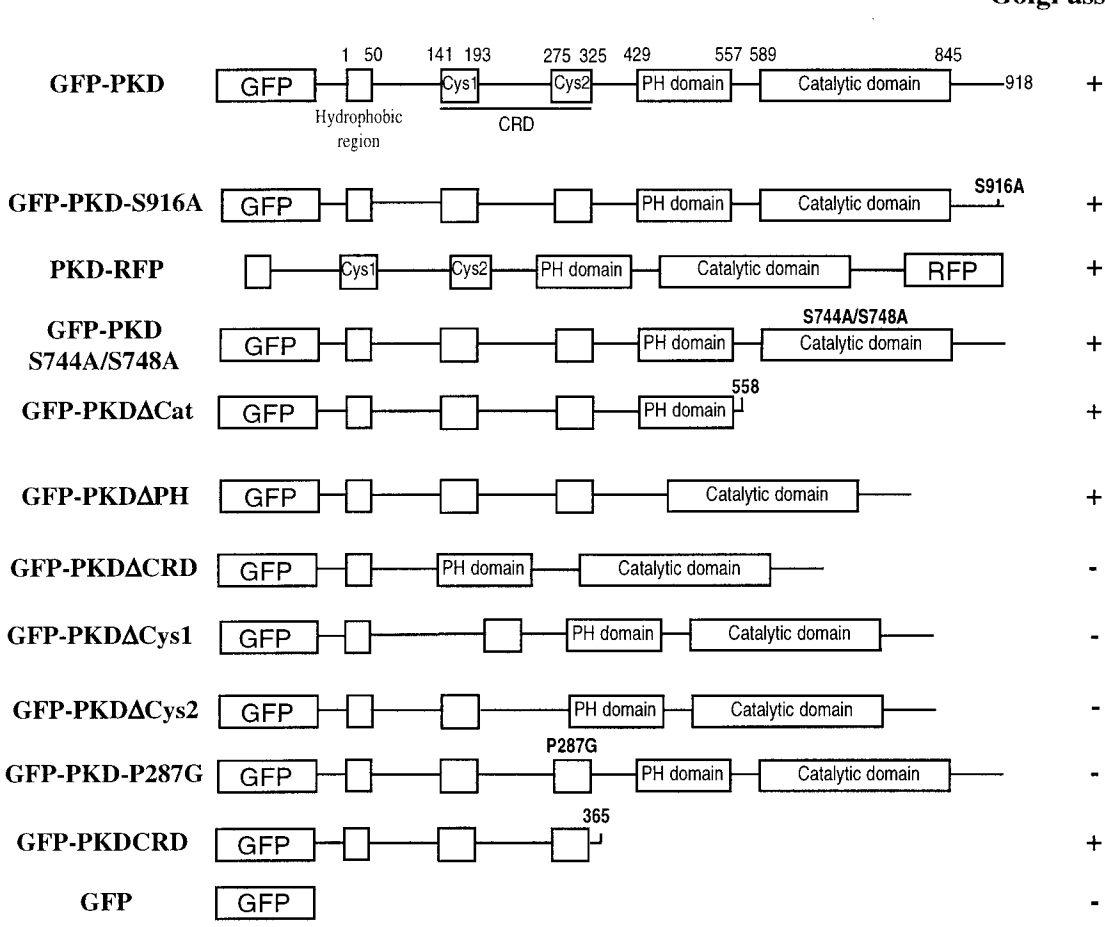

FIG. 1. Schematic representation of the expressed wt and mutant PKD fusion proteins and summary of their Golgi association properties. Swiss 3T3 cells were transfected with constructs encoding the wt and mutant PKD proteins and incubated at 37°C for 18 h. The cultures were then shifted from 37 to 20°C and after 20 min the association of the different fusion proteins with the Golgi compartment was analyzed by real time imaging. The results summarized are representative of at least 3 independent experiments. The employed plasmids were constructed as described under Materials and Methods.

type (wt) and mutants PKDΔPH, PKDΔCRD, PKDP287G, PKDS916A, PKDS744A/S748A, PKD-CRD, PKDΔCat were previously described (13, 23, 24). The pGFP-PKDΔCys1 and pGFP-PKDΔCys2 vectors were constructed by subcloning the *Eco*RI cDNA fragments isolated from pcDNA3-PKDΔCys1 and pcDNA3-PKDΔCys 2 (7) into the pEF-plink2-GFP_{C3} expression vector previously digested with *Eco*RI (12). All the constructs generated were confirmed by DNA sequence analysis and the products of expression of the pGFP-PKDΔCys1 and pGFP-PKDΔCys 2 analyzed by Western blot using antibodies against GFP and PKD/PKCμ (C20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A schematic representation of all these constructs is shown in Fig. 1.

Cell culture and transfections. Stock cultures of Swiss 3T3 were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO_2 and 90% air. For experimental purposes, cells were plated onto 15-mm No. 1 round glass coverslips (Warner Instrument Corp., Hamden, CT) inside 33-mm dishes at 7×10^4 cells/dish and transfected 18–20 h later for real time image analysis. Cells were transfected with 1 μg DNA/33 mm dish using LipofectAMINE PLUS (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's suggested conditions. Transfected cells were analyzed 18–20 h after transfection.

Real time cell imaging. In order to control the temperature during the experimental procedures, cells grown in the 15-mm glass coverslips were mounted in a RC-25 perfusion chamber (Warner Instrument Corp., Hamden, CT) and perfused with medium circulated through a TC-344B Chamber System Heater Controller (Warner Instrument Corp., Hamden, CT). The medium was supplemented with 10 mM Hepes, pH: 7.2. The microscope used was a Zeiss epifluorescent Axioskop with a Zeiss Achroplan 40×/0.75w water immersion objective (Carl Zeiss Inc., Jena, Germany). Images were captured as uncompressed 24-bit TIFF files with a SPOT cooled (−12°C) single CCD color digital camera (three pass method) driven by SPOT version 2.1 software (Diagnostic Instruments, Inc., Sterling Heights, MI). GFP fluorescence was observed with a HI Q filter set for FITC (Chroma Technology, Brattleboro, VT). Fifty cells were analyzed per experiment and each experiment was performed in duplicate. Unless otherwise indicated, the selected single cell displayed in the appropriate figures was representative of 90% of the population of positive cells.

RESULTS AND DISCUSSION

We recently demonstrated that after GPCR agonist stimulation, PKD is transiently translocated to the

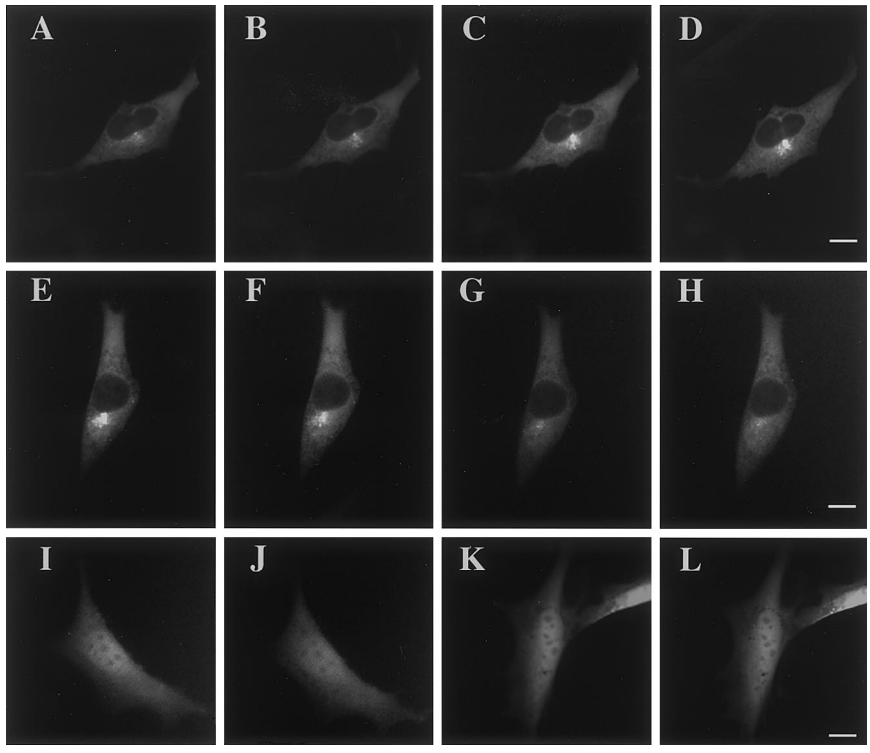

FIG. 2. Intracellular distribution of fluorescent-tagged wild-type PKD. Swiss 3T3 cells were transfected with constructs encoding GFP-PKD (A–H) or GFP (I–L) proteins and incubated at 37°C for 18 h. Cultures were then shifted from 37 to 20°C and images obtained at 0 min (A, I), 2 min (B), 5 min (C), and 7 min (D, J) after the temperature shift. Cultures maintained for 20 min at 20°C were shifted to 37°C and images obtained at 0 min (E, K), 2 min (F), 5 min (G), and 7 min (H, L) after the temperature shift. Bar, 10 μm.

plasma membrane of fibroblast and epithelial cells by a mechanism that requires its CRD (24). PKD also localizes to Golgi, cytosol and mitochondria in different cell types (12, 19–23). Although recent studies indicated that PKD is involved in Golgi organization and vesicular transport (20–22), the domain(s) responsible for its Golgi localization remained undefined. In order to identify the domain of PKD responsible for its association with the Golgi compartment, we used real-time imaging of fluorescent-tagged PKD.

The fusion of GFP tag to PKD did not produce any detectable effect on its catalytic activity, phorbol ester binding or activation loop phosphorylation as we previously reported (8, 12, 24). Swiss 3T3 fibroblasts, a model system extensively used to elucidate signaling by endogenously expressed GPCR (28), were transiently transfected with wild type and mutants PKD fused to GFP (Fig. 1) and examined 18 h later. We found that GFP-PKD was distributed throughout the cytosol with some cells showing a more pronounced signal at the perinuclear area with no or very little fluorescence detected in the nuclei of these cells (Fig. 2A). When Swiss 3T3 cells were shifted from 37 to 20°C, GFP-PKD rapidly accumulated in a single area adjacent to the nucleus (Figs. 2A–2D). As we previously demonstrated, this pattern of accumulation corresponded to localization in the Golgi compartment as revealed by colocalization of endogenous PKD and the Golgi-associated 58-kDa protein (24). The accumulation of GFP-PKD in the Golgi compartment occurred within 5 min of the temperature shift in 90% of the cells. When Swiss 3T3 expressing GFP-PKD were preincubated at 20°C for 20 min and then shifted to 37°C, the fluorescent signal previously detected at the Golgi compartment redistributed to the cytosol within 5 min (Figs. 2E–2H). In contrast, GFP was distributed throughout the cytosol and nuclei, and the temperature shifts from 37 to 20°C or 20 to 37°C had no effects on its distribution (Figs. 2I–2L).

To rule out possible Golgi binding artifacts of GFP-PKD due to the presence of the fluorescent tag at the N-terminus of PKD, we analyzed the distribution of a chimeric protein between the red fluorescent protein (RFP) from *Discosoma* sp. fused to the C-terminus of PKD. We found that a temperature shift from 37 to 20°C induced the accumulation of PKD-RFP in the Golgi compartment within 5 min and that a shift back to 37°C caused its redistribution in the cytosol within 5 min (data not shown). Taken together with our previous findings (24) and other published studies (12, 19–23), the data shown in Fig. 1 suggest that the preferential localization of PKD to the Golgi compartment not only is dependent on the cell type but also very

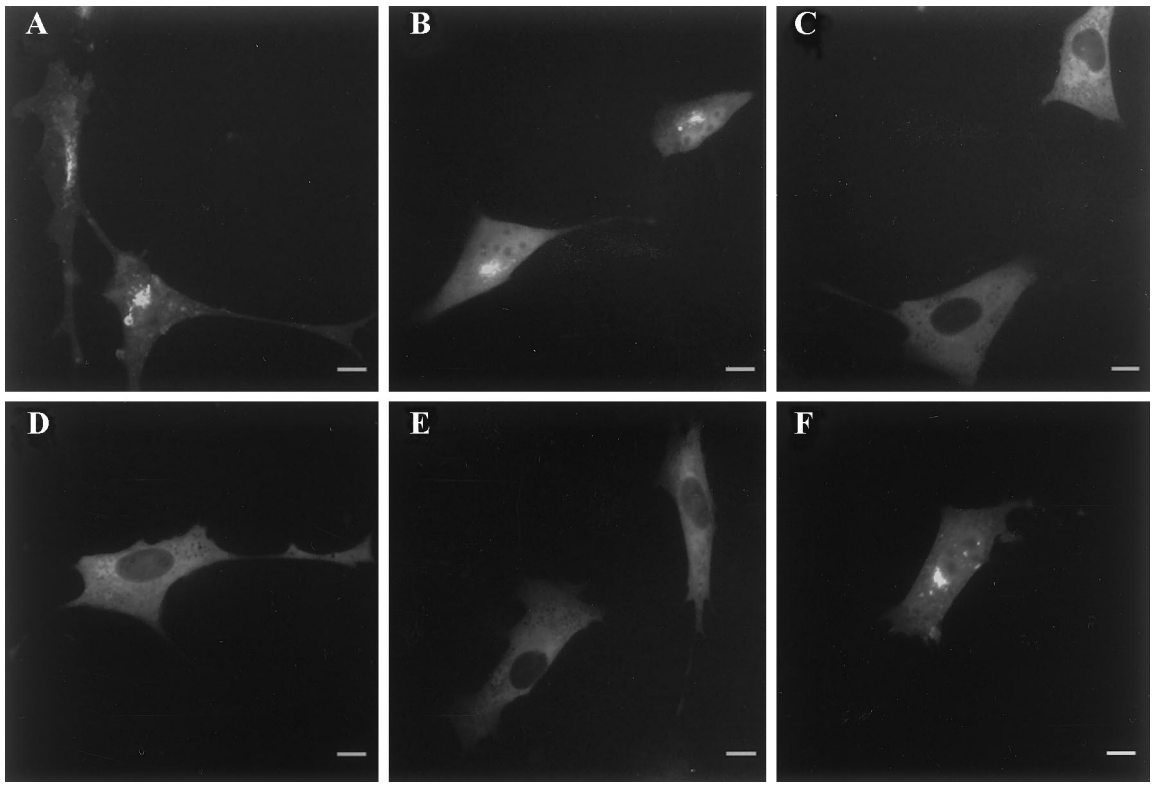

FIG. 3. Intracellular distribution of fluorescent-tagged mutant PKDs. Swiss 3T3 cells were transfected with constructs encoding GFP-PKDΔCat (A), GFP-PKDΔPH (B), GFP-PKDΔCRD (C), GFP-PKDΔCys1 (D), GFP-PKDΔCys2 (E), and GFP-PKDCRD (F) and further incubated at 37°C for 18 h before real time imaging. Cultures were shifted from 37 to 20°C and images obtained after 20 min incubation. Bar, 10 μm.

sensitive to experimental conditions, particularly temperature.

In order to identify the domain(s) of PKD responsible for its Golgi association, different GFP-tagged PKD mutants were expressed transiently in Swiss 3T3 cells and the distribution of these molecules was monitored in live cells incubated at 20°C. We employed this temperature since it facilitates the detection of Golgi associated PKD, as demonstrated in Fig. 1. Analysis of the distribution of the fusion proteins with mutations in the C-terminus (GFP-PKDS916A) or catalytic domain of PKD (GFP-PKDS744A/S748A) showed no defect in their Golgi association (data not shown). Moreover, deletion of the complete catalytic domain of PKD did not interfere with the Golgi association of GFP-PKDΔCat but rather increased its Golgi accumulation (Fig. 3A), even at 37°C (data not shown). Overall these data demonstrated that the catalytic activity of PKD is not involved in its Golgi association.

The analysis of agonist-induced membrane translocation of classic PKCβII demonstrated that this process is mediated by the cooperative interaction of C1 and C2 regions (29). PKD does not possess a C2 region but contains a PH domain, located between the CRD and the catalytic domain of PKD. This domain could be involved in the Golgi association of PKD since PH domains are modular protein motifs that mediate protein–protein as well as protein–lipid interactions (30, 31). Analysis of the distribution of a fusion protein consisting of GFP and a PH domain deleted PKD (GFP-PKDΔPH) showed Golgi association, despite a partial nuclear localization of this fusion protein (Fig. 3B), indicating that the PH domain did not mediate this association.

The N-terminal regulatory region of PKD contains, in addition to a PH domain, a CRD comprising of a tandem repeat of cysteine-rich zinc-finger like motifs which confers high affinity binding to phorbol esters and plays a negative role in the regulation of the catalytic activity of PKD (1, 2, 6, 7). These cysteine rich-motifs of PKD, referred to as cys1 and cys2, are not equivalent. While the cys2 motif is responsible for the majority of the phorbol ester binding, both *in vivo* and *in vitro,* the function of the cys1 motif is unknown (7). In addition, the cys2 domain is responsible for its plasma membrane translocation after GPCR agonist stimulation (24). PKCδ and PKCϵ, two novel PKCs that are Ca^{2+}-independent and DAG-responsive, have been detected in the plasma membrane as well as in the Golgi compartment (32–38). In the case of PKCϵ, the tandem repeat of cysteine-rich zinc-finger like motifs that form the C1 domain mediates its interaction with

Golgi (34, 35). Because this C1 domain is similar to the CRD present in PKD (39) we investigated whether the deletion of the CRD would interfere in the interaction between PKD and Golgi. The GFP-PKDΔCRD mutant contains a deletion of the entire CRD domain of PKD, which prevents phorbol ester/DAG binding and interaction with the plasma membrane (7, 24). As shown in Fig. 3C, GFP-PKDΔCRD was distributed evenly throughout the cytosol of Swiss 3T3, with no apparent association with the Golgi compartment.

Deletion of the cys1 or cys2 domains of PKD also prevented the association of GFP-PKDΔCys1 and GFP-PKDΔCys2 with the Golgi compartment (Figs. 3D and 3E, respectively), suggesting that both the cys1 and cys2 domains mediate this interaction. The mutant GFP-PKDP287G, that contains a proline to glycine substitution at position 287 within the cys2 domain, was also defective in Golgi association (data not shown). This mutation which prevents from phorbol ester/DAG binding to PKD, blocks the translocation of PKD to the plasma membrane (7, 24). These results identified the CRD of PKD as the domain responsible for its Golgi association. Further support for this conclusion was obtained by analyzing the distribution of a fusion protein consisting of GFP and the CRD of PKD (GFP-PKDCRD). Although this fusion protein was associated with the plasma membrane and evenly distributed throughout the cytosol and nuclei, GFP-PKDCRD was preferentially concentrated in the Golgi compartment (Fig. 3F), even at 37°C (data not shown). A summary of the Golgi association properties of the different mutants analyzed is shown in Fig. 1.

The function and regulation of different signaling molecules depends on their specific subcellular localization, which is mediated by specific domains present in those proteins (26). In this study we demonstrated that the association of PKD with the Golgi compartment requires the CRD of PKD but not its catalytic domain. The targeting of PKD to the plasma membrane is also mediated by its CRD, further defining this domain as a localization signal able to target PKD to multiple subcellular compartments. It is tempting to speculate that the mechanism(s) regulating the interaction of PKD with the Golgi compartment may be similar to that regulating the association of PKD to the plasma membrane.

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REFERENCES

1. Valverde, A. M., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1994) Molecular cloning and characterization of protein kinase D: A target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc. Natl. Acad. Sci. USA* **91,** 8572–8576.
2. Johannes, F. J., Prestle, J., Eis, S., Oberhagemann, P., and Pfizenmaier, K. (1994) PKCu is a novel, atypical member of the protein kinase C family. *J. Biol. Chem.* **269,** 6140–6148.
3. Rozengurt, E., Sinnett-Smith, J., and Zugaza, J. L. (1997) Protein kinase D: A novel target for diacylglycerol and phorbol esters. *Biochem. Soc. Trans.* **25,** 565–571.
4. Waldron, R. T., Iglesias, T., and Rozengurt, E. (1999) The pleckstrin homology domain of protein kinase D interacts preferentially with the eta isoform of protein kinase C. *J. Biol. Chem.* **274,** 9224–9230.
5. Iglesias, T., and Rozengurt, E. (1998) Protein kinase D activation by mutations within its pleckstrin homology domain. *J. Biol. Chem.* **273,** 410–416.
6. Iglesias, T., and Rozengurt, E. (1999) Protein kinase D activation by deletion of its cysteine-rich motifs. *FEBS Lett.* **454,** 53–56.
7. Iglesias, T., Matthews, S., and Rozengurt, E. (1998) Dissimilar phorbol ester binding properties of the individual cysteine-rich motifs of protein kinase D. *FEBS Lett.* **437,** 19–23.
8. Waldron, R. T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D., and Rozengurt, E. (2001) Activation loop Ser^{744} and Ser^{748} in protein kinase D are transphosphorylated in vivo. *J. Biol. Chem.*, in press.
9. Zugaza, J. L., Waldron, R. T., Sinnett-Smith, J., and Rozengurt, E. (1997) Bombesin, vasopressin, endothelin, bradykinin, and platelet-derived growth factor rapidly activate protein kinase D through a protein kinase C-dependent signal transduction pathway. *J. Biol. Chem.* **272,** 23952–23960.
10. Abedi, H., Rozengurt, E., and Zachary, I. (1998) Rapid activation of the novel serine/threonine protein kinase, protein kinase D by phorbol esters, angiotensin II and PDGF-BB in vascular smooth muscle cells. *FEBS Lett.* **427,** 209–212.
11. Paolucci, L., and Rozengurt, E. (1999) Protein kinase D in small cell lung cancer cells: Rapid activation through protein kinase C. *Cancer Res.* **59,** 572–577.
12. Matthews, S., Iglesias, T., Cantrell, D., and Rozengurt, E. (1999) Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP-PKD fusion protein: Dissociation from PKD activation. *FEBS Lett.* **457,** 515–521.
13. Van Lint, J. V., Sinnett-Smith, J., and Rozengurt, E. (1995) Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. *J. Biol. Chem.* **270,** 1455–1461.
14. Van Lint, J., Ni, Y., Valius, M., Merlevede, W., and Vandenheede, J. R. (1998) Platelet-derived growth factor stimulates protein kinase D through the activation of phospholipase Cgamma and protein kinase C. *J. Biol. Chem.* **273,** 7038–7043.
15. Zugaza, J. L., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1996) Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. *EMBO J.* **15,** 6220–6230.
16. Matthews, S. A., Pettit, G. R., and Rozengurt, E. (1997) Bryostatin 1 induces biphasic activation of protein kinase D in intact cells. *J. Biol. Chem.* **272,** 20245–20250.
17. Sidorenko, S. P., Law, C. L., Klaus, S. J., Chandran, K. A., Takata, M., Kurosaki, T., and Clark, E. A. (1996) Protein kinase C mu (PKC mu) associates with the B cell antigen receptor complex and regulates lymphocyte signaling. *Immunity* **5,** 353–363.
18. Iglesias, T., Waldron, R. T., and Rozengurt, E. (1998) Identification of in vivo phosphorylation sites required for protein kinase D activation. *J. Biol. Chem.* **273,** 27662–27667.
19. Storz, P., Hausser, A., Link, G., Dedio, J., Ghebrehiwet, B., Pfizenmaier, K., and Johannes, F. J. (2000) Protein kinase C

[micro] is regulated by the multifunctional chaperon protein p32. *J. Biol. Chem.* **275,** 24601–24607.

20. Liljedahl, M., Maeda, Y., Colanzi, A., Ayala, I., Van Lint, J., and Malhotra, V. (2001) Protein kinase D regulates the fission of cell surface destined transport carriers from the *trans*-Golgi network. *Cell* **104,** 409–420.
21. Jamora, C., Yamanouye, N., Van Lint, J., Laudenslager, J., Vandenheede, J. R., Faulkner, D. J., and Malhotra, V. (1999) Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D. *Cell* **98,** 59–68.
22. Prestle, J., Pfizenmaier, K., Brenner, J., and Johannes, F. J. (1996) Protein kinase C mu is located at the Golgi compartment. *J. Cell Biol.* **134,** 1401–1410.
23. Matthews, S. A., Iglesias, T., Rozengurt, E., and Cantrell, D. (2000) Spatial and temporal regulation of protein kinase D (PKD). *EMBO J.* **19,** 2935–2945.
24. Rey, O., Young, S. H., Cantrell, D., and Rozengurt, E. (2001) Rapid protein kinase D translocation in response to G protein-coupled receptor activation: Dependence on protein kinase C. *J. Biol. Chem.*, in press.
25. Jaken, S. (1996) Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.* **8,** 168–173.
26. Teruel, M. N., and Meyer, T. (2000) Translocation and reversible localization of signaling proteins: A dynamic future for signal transduction. *Cell* **103,** 181–184.
27. Newton, A. C. (1997) Regulation of protein kinase C. *Curr. Opin. Cell Biol.* **9,** 161–167.
28. Rozengurt, E. (1986) Early signals in the mitogenic response. *Science* **234,** 161–166.
29. Feng, X., Becker, K. P., Stribling, S. D., Peters, K. G., and Hannun, Y. A. (2000) Regulation of receptor-mediated protein kinase C membrane trafficking by autophosphorylation. *J. Biol. Chem.* **275,** 17024–17034.
30. Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996) PH domains: Diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* **85,** 621–624.
31. Gibson, T. J., Hyvönen, M., Musacchio, A., Saraste, M., and Birney, E. (1994) PH domain: The first anniversary. *Trends Biochem. Sci.* **19,** 349–353.
32. Wang, Q. J., Fang, T. W., Fenick, D., Garfield, S., Bienfait, B., Marquez, V. E., and Blumberg, P. M. (2000) The lipophilicity of phorbol esters as a critical factor in determining the pattern of translocation of protein kinase C delta fused to green fluorescent protein. *J. Biol. Chem.* **275,** 12136–12146.
33. Wang, Q. J., Bhattacharyya, D., Garfield, S., Nacro, K., Marquez, V. E., and Blumberg, P. M. (1999) Differential localization of protein kinase C delta by phorbol esters and related compounds using a fusion protein with green fluorescent protein. *J. Biol. Chem.* **274,** 37233–37239.
34. Lehel, C., Oláh, Z., Jakab, G., Szállási, Z., Petrovics, G., Harta, G., Blumberg, P. M., and Anderson, W. B. (1995) Protein kinase C epsilon subcellular localization domains and proteolytic degradation sites. A model for protein kinase C conformational changes. *J. Biol. Chem.* **270,** 19651–19658.
35. Lehel, C., Olah, Z., Jakab, G., and Anderson, W. B. (1995) Protein kinase C epsilon is localized to the Golgi via its zinc-finger domain and modulates Golgi function. *Proc. Natl. Acad. Sci. USA* **92,** 1406–1410.
36. Lehel, C., Olah, Z., Mischak, H., Mushinski, J. F., and Anderson, W. B. (1994) Overexpressed protein kinase C-delta and -epsilon subtypes in NIH 3T3 cells exhibit differential subcellular localization and differential regulation of sodium-dependent phosphate uptake. *J. Biol. Chem.* **269,** 4761–4766.
37. Cacace, A. M., Guadagno, S. N., Krauss, R. S., Fabbro, D., and Weinstein, I. B. (1993) The epsilon isoform of protein kinase C is an oncogene when overexpressed in rat fibroblasts. *Oncogene* **8,** 2095–2104.
38. Mischak, H., Goodnight, J. A., Kolch, W., Martiny-Baron, G., Schaechtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H., and Mushinski, J. F. (1993) Overexpression of protein kinase C-delta and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. *J. Biol. Chem.* **268,** 6090–6096.
39. Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997) Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci.* **6,** 477–480.