Structural Properties and Mechanisms That Govern Association of C Kinase Adapter 1 with Protein Kinase C3 and the Cell Periphery*

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Association of an atypical protein kinase C (aPKC) with an adapter protein can affect the location, activity, substrate specificity, and physiological role of the phosphotransferase. Knowledge of mechanisms that govern formation and intracellular targeting of aPKC adapter protein complexes is limited. Caenorhabditis elegans protein kinase C adapter proteins (CKA1 and CKA1S) bind and target aPKCs and provide prototypes for mechanistic analysis. CKA1 binds an aPKC (PKC3) via a phosphotyrosine binding (PTB) domain. A distinct, Arg/Lys-rich N-terminal region targets CKA1 to the cell periphery. We discovered that a short segment (212GGIDNGAFHEHEI234) of the V2 (linker) region of PKC3 creates a binding surface that interacts with the PTB domain of CKA1/CKA1S. The docking domain of PKC3 differs from classical PTB ligands by the absence of Tyr and Pro. Substitution of Ile214, Asn216, or Phe219 with Ala abrogates binding of PKC3 with CKA1; these residues cooperatively configure a docking site that complements an apolar surface of the CKA1 PTB domain. Phosphorylation site domains (PSD1, residues 11–25; PSD2, residues 61–77) in CKA1 route the adapter (and tethered PKC3) to the cell periphery. Phosphorylation of Ser17 and Ser46 in PSDs 1 and 2 elicits translocation of CKA1 from the cell surface to cytoplasm. Activities of DAG-stimulated PKCs and opposing protein Ser/Thr phosphatases can dynamically regulate the distribution of adapter protein between the cell periphery and cytoplasm.

Atypical protein kinase C (aPKC) isozymes, which include mammalian PKCs ι and λ and Caenorhabditis elegans PKC3 (1–5), are involved in transmitting mitogenic, inflammatory, and anti-apoptotic signals; regulating gene expression; controlling vesicular trafficking and ion channel activities; establishing cell polarity; and mediating asymmetric cell divisions (4–12). To exert control over such diverse aspects of cell physiology, aPKCs regulate effector proteins in cytoplasm, cytoskeleton, nucleus, and at the surfaces of plasma and internal membranes (4–14). aPKCs lack structural features that mediate direct association with cytoskeleton or organelles. Thus, elucidation of alternative mechanisms that enable aPKCs to encounter and control effector proteins in discrete microenvironments is an important goal. Recent investigations (reviewed in our companion paper (45)) suggest that aPKC functions are diversified and specialized via interactions with adapter proteins (6, 14–20). Candidate adapter proteins possess two fundamental features: a tethering domain that ligates an aPKC, and a distinct targeting region that routes the adapter-aPKC complex to intracellular sites enriched in substrate-effector proteins and/or regulatory molecules that modulate phosphotransferase activity. The aPKC “recruitment” model further suggests that systematic characterization of aPKC adapter proteins will ultimately yield novel insights into regulatory properties, substrate specificities, and precise physiological roles of atypical PKCs.

The nematode C. elegans is an attractive model system for studies on aPKC adapter proteins. C. elegans physiology is regulated by signaling molecules, mechanisms and pathways that are operative in mammals (21, 22). Only one aPKC (PKC3) is encoded by the C. elegans genome (3). PKC3 is expressed and anchored at all developmental stages (3). This Ca2+- and diacylglycerol (DAG)-independent kinase is essential for the progression of embryogenesis, asymmetry in early cell divisions, and overall viability of the organism (3, 11). In 1-cell embryos, ~25% of PKC3 associates with Par-3, a multi-PDZ domain protein that is crucial for generating intracellular polarity (11, 12). Mechanisms governing formation of PKC3/Par-3 complexes, the identity of targets for bound PKC3, and the precise biochemical/physiological function of the complex remain to be defined. Association of >90% of PKC3 with cytoskeleton/membranes in embryos indicates that additional adapter proteins are expressed during early phases of C. elegans development (3). In post-embryonic C. elegans, PKC3 accumulates in a highly asymmetric fashion in intestinal, pharyngeal, and other cells (3). Polarized enrichment of PKC3 in nondividing cells that will not undergo apoptosis suggests that anchored PKC3 plays distinct roles in terminally differentiated cells. By using knowledge of the established properties of C. elegans PKC3 and reagents derived from PKC3 cDNA and protein (3), it should be possible to discover and characterize a cohort of adapter proteins that collectively diversify aPKC functions from birth to death.

A yeast two-hybrid interaction screen yielded a unique C. elegans cDNA that encodes two novel PKC3-binding proteins (45). These proteins, which were named protein kinase C adapter 1 (CKA1) and CKA1S, are expressed throughout the life span of C. elegans and accumulate near the inner surface of...
plasma membranes in vivo and in transfected cells (45). In highly polarized epithelial cells, CKA1 and CKA1S are differentially targeted to the lateral plasma membrane surface (near tight junctions). CKA1 (593 amino acids) contains a positively charged N-terminal region (residues 1–89) that precedes a phosphotyrosine binding (PTB) domain (residues 90–231) and a unique central/C-terminal segment (residues 232–593). The minimal fragment of CKA1 polypeptide that tethers PKC3 corresponds to the intact PTB domain (45). Deletion mutagenesis-transfection experiments indicate that the N-terminal region of CKA1 governs routing of the PKC3 adapters to the cell periphery (45). Two distinct, exceptionally basic clusters of amino acids (residues 11–25 and residues 61–77) seem to play important roles in targeting and anchoring CKA1. The composition and arrangement of amino acids within the clusters generate sequence motifs that resemble classical phosphorylation site domains (PSDs) of the ubiquitous MARCKS protein and MARCKS-related proteins (29, 30). Utilization of an alternative initiation codon in CKA1 mRNA results in the synthesis of CKA1S (45). The amino acid sequence of this adapter isoform is identical with residues 45–593 in CKA1. Therefore, CKA1S lacks the first PSD but retains all other structural and functional features of CKA1. Our current model proposes that a PTB domain and PSDs in CKA1/CKA1S collaborate in a novel mechanism by which the CKA1 N-terminal region (including PSDs) routes the adapter protein to the cell periphery. To address these topics, we attempted to answer a series of fundamental questions. (a) What structural features enable PKC3 to engage the CKA1/CKA1S PTB domain? (b) Does PKC3 contain a phosphotyrosine that is essential for formation of a stable complex with CKA1 or CKA1S? (c) Are the adapter proteins candidate substrates (or substrate-effectors) for PKC3 and/or DAG-activated PKCs? (d) Are Ser and Thr residues within the N-terminal regions of CKA1 and CKA1S phosphorylated? (e) If so, what is the functional significance of N-terminal phosphorylation? We now report the results and conclusions from experiments designed to answer the questions posed above.

**EXPERIMENTAL PROCEDURES**

**Deletion and Site-directed Mutagenesis—**Deletion mutagenesis was performed on PKC3 cDNA via the polymerase chain reaction, as previously described (31, 32, 45). For N- or C-terminal deletions of the regulatory region of PKC3 (nucleotides 1–699), the 5’ and 3’ ends of cDNAs encoding desired segments of PKC3 were extended with NotI and BamHI restriction sites, respectively, and cloned into the yeast expression vector pAS1. In separate reactions, SpeI and NotI restriction sites were appended at the 5’ and 3’ ends of cDNAs encoding desired fragments of PKC3. Amplified cDNAs were cloned into the mammalian expression vector pEBG (33). This enabled expression of GST partial PKC3 fusion proteins in hamster AV-12 cells (see “Results and Discussion” for details). Amino acid substitutions were introduced into full-length CKA1 and partial PKC3 polypeptides by PCR-based site-directed mutagenesis (QuickChange™ kit, Stratagene). Mutagenesis reactions were carried out according to the manufacturer’s instructions. All mutants were verified by DNA sequencing.

**Cell Culture and Transfections—**Hamster AV-12 cells were grown as described previously (35, 45). Cells were transfected with GST partial PKC3 transgenes (inserted in recombinant pEBG vectors) via calcium phosphate precipitation as reported previously (34, 35). The same methodology was used to introduce wild type and mutant CKA1 and CKA1S transgenes (inserted in recombinant pRcCMV vectors (45)) into AV-12 cells. Stable transfectants were obtained by selection with 1 mg/ml G418 for 14 days (34).

**Purification of GST PKC3 Fusion Proteins—**AV-12 cells were transfected with expression plasmids encoding various GST partial PKC3 fusion proteins. Cells were harvested 24 h after transfection and lysed with 20 mM Tris-HCl, pH 7.5, containing 0.25 mM diethiothreitol, 0.15 mM NaCl, 1 mM EDTA, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 10 µg/ml trypsin inhibitor, 10 mM benzamidine HCl, 100 µg/ml pefabloc, and 0.5% (w/v) Triton X-100. GST-PKC3 transfectants (from one 10-cm plate) were isolated and purified by incubation with 35 µl of G-Sepharose 4B beads at 4°C for 1 h. Subsequently, the beads were pelleted (3,000 × g) and washed twice with 1 ml of lysis buffer. One-third of the beads was mixed with protein (250 µg) extracted from AV-12 cells that were transfected with a CKA1/CKA1S transgene. Various concentrations (0.05 µM to 2 mM) of peptides (SGGGIDNGAFHEHEI or AHNIFGISVNIIDN) were incubated at 4°C in competition binding experiments. Samples were incubated at 4°C for 1 h. Beads were pelleted at 3,000 × g, and the supernatant solution was removed. The beads were washed 5 times by suspension in 1 ml of 10 mM sodium phosphate, pH 7.4, containing 0.15 mM NaCl and 0.1% (w/v) Tween 20, and centrifugation at 3,000 × g. Bound proteins were released from the beads by boiling in 25 µl of SDS gel loading buffer, fractionated by denaturing electrophoresis, and transferred to an Immobilon P membrane as described previously (3, 36). Blots were probed with anti-CKA IgGs, and antigen-IgG complexes were visualized by an enhanced chemiluminescence procedure as reported previously (3, 34).

**Phosphorylation of GST-PSD Fusion Proteins—**Phosphorylated PSD1 and PSD2 fragments (residues 1–63 and PSD2 (residues 59–108) from CKA1 were synthesized via PCR and cloned into the bacterial expression plasmid pGEX-KG (37). This enabled synthesis in Escherichia coli of two fusion proteins, GST-PSD1 (designated PSD1’) and GST-PSD2 (PSD2’), that contain potential PKC phosphorylation sites. The soluble fusion proteins were purified to near-homogeneity by affinity chromatography as described previously (38). Purified GST-PSD1’ (2 µg) was mixed with [γ-32P]ATP (100–200 cpm/pmol), 5 mM MgCl2, 0.5 mM EGTA, 1 mM diethiothreitol, 20 µg/ml phosphatidylserine). After a 4–6-min incubation at 30°C, reactions were terminated by adding 0.2 volume of 5% gel loading buffer and proteins were size-fractionated by denaturing electrophoresis (10% gel) (36). Phosphorylated PSD1’ and PSD2’ proteins were visualized by autoradiography. Radiolabeled proteins were excised from the gel, and 32P radioactivity was measured in a scintillation counter. Kd and Vmax values for PSD1’ and PSD2’ were calculated from measurements of the rate of incorporation of 32P radioactivity into the fusion proteins (see legend for Fig. 6). Mutant PSD1’ and PSD2’ proteins were generated by sitewise mutagenized, expressed in E. coli, purified, and phosphorylated as described below.

**Other Experimental Procedures—**Descriptions of production and affinity purification of antibodies, yeast two-hybrid protein interaction assays, denaturing electrophoresis, Western immunoblot analysis, DNA sequencing, immunoprecipitations, and immunofluorescence analysis of the intracellular distribution of wild type and mutant CKA1 and CKA1S proteins are provided in the accompanying paper (45).

**RESULTS AND DISCUSSION**

**The CKA1-Binding Site in PKC3 Contains Six Core Amino Acids and Lacks Tyr—**N- and C-terminal boundaries of the segment of PKC3 that engages the PTB domain of CKA1 were mapped by employing deletion mutagenesis in concert with yeast two-hybrid protein interaction assays. Both full-length PKC3 and a large N-terminal fragment of the kinase (residues 1–233, designated PKC3-N233) avidly bound to the PTB domain (Fig. 1, A and B). In contrast, a segment of PKC3 that encompasses amino acids 234–597 was not a PTB ligand (data not shown). Thus, the catalytic domain (residues 250–515, Ref. 3) and C-terminal region (residues 516–597) of PKC3 (Fig. 1C) do not mediate binding with CKA1/CKA1S. Elimination of sequences corresponding to the pseudosubstrate site (residues 109–121), the Cys-rich regulatory region (residues 128–177), and/or the unique N-terminal portion (residues 1–89) of PKC3 failed to disrupt coupling with the CKA1 PTB domain (Fig. 1A, Δ6, Δ7, Δ9, Δ10, Δ12, and Δ13 and Fig. 1B, 111–223, 173–233, and 212–233). Only mutations that deleted all or part of a segment of PKC3 bounded by Gly212 and Ile224 abolished teth...
Mammalian cells were used to directly and independently test conclusions drawn from assays performed in the yeast two-hybrid system. The expression plasmid pEBG contains a multienzyme cloning site that is preceded by the strong, constitutive elongation factor 1α promoter and a GST gene. This enables expression of GST fusion proteins in transfected cells. Complementary DNAs encoding amino acids 111–218, 111–227, or 111–233 from PKC3 were cloned into the pEBG vector. Each recombinant pEBG construct and a CKA1S transgene were then introduced into hamster AV-12 cells. Transfected cells accumulated similar amounts of each GST partial PKC3 fusion protein (Fig. 2A, lanes 2–4) and CKA1S (not shown). PKC3 fusion proteins were isolated and purified from cell extracts by binding with GSH-Sepharose 4B beads. The CKA1S transgene was then introduced into the yeast two-hybrid system, and the resulting yeast cell lines were tested for their abilities to interact with the CKA1 PTB domain via yeast two-hybrid complementation assays (++ indicates that a strong signal was produced by transformed yeast within 30 min of initiation of the β-galactosidase assay). The N- and C-terminal boundaries of the fragments are marked with numbers that correspond to positions of amino acids within the full-length sequence of PKC3 (3) (GenBank™ accession number AF02566). Results are presented in a diagrammatic representation of the domain organization of PKC3. V1–V3 are variable regions that are not conserved in other PKC isoforms. D shows the amino acid sequence of the segment of PKC3 that couples with CKA1/CKA1S (PTB domain).
PKC3 to bind with CKA1 and CKA1S. Mutation of other residues (as typified by His222 to Ala) had no effect on association of PKC3 with its cognate PTB domain. The data suggest that residues 111–227 from PKC3 (GST-PKC3-(111–227)) contains an Asn216 to Ala substitution affects CKA1-mediated targeting of intact aPKC to the cell surface (Fig. 4C, panels 2 and 3). Thus Asn216, a critical core component of the CBSP module (Figs. 3 and 4A), is essential for competitive docking of fusion protein ligand with membrane-associated CKA1 in situ. Results in Fig. 4C strongly support the idea that the CBSP region of PKC3 (initially identified by in vitro binding assays) is a ligand for the CKA1 PTB domain in the context of intact cells.

Recent studies prompted a revision of concepts regarding the binding of ligands by PTB domains (25–28, 39). The initial postulate that PTB domains couple with NPxY motifs (X corresponds to any amino acid and pY indicates phospho-Tyr) is now recognized as a specific example of a more general consensus. The majority of PTB ligands do not contain phospho-Tyr and several PTB domain-partner proteins lack both Pro and Tyr in their binding sites.

Assembly and Targeting of CKA1-PKC3 Complexes

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A, CKA1S. are essential for avid binding with the PTB domain of CKA1/CKA1S. A, amino acids in the CKA1 binding region of PKC3 were altered by site-directed mutagenesis as indicated under “Experimental Procedures.” Wild type and mutant GST fusions that contain residues 111–227 from PKC3 were coexpressed with CKA1 and CKA1S in transfected AV-12 cells. Binding of adapter proteins to mutant and wild type GST partial PKC3 proteins was assessed by a combination of affinity purification of the complexes on GSH-Sepharose 4B beads and subsequent Western immunoblot analysis (see “Experimental Procedures” and the legends for Figs. 2 and 3). Proteins eluted from the beads were size-fractionated by denaturing electrophoresis, transferred to an Immobilon membrane, and probed with anti-CKA1/CKA1S antibodies. Lane 1 received proteins coisolated with the wild type CKA1 binding region of PKC3. Other samples include proteins coisolated with CKA1 binding regions of PKC3 that were mutated as follows: Ile214 to Ala (lane 2); Asn216 to Ala (lane 3); Phe217 to Ala (lane 4) and His224 to Ala (lane 5). IgG-CKA1 and IgG-CKA1S complexes were visualized by enhanced chemiluminescence methodology. Signals recorded on x-ray film are shown. A, a Western blot probed with anti-GST IgGs reveals that similar amounts of wild type and mutant GST-PKC3 fusion proteins were expressed and isolated for each binding assay. Samples applied to lanes 1–5 are duplicates of samples used for lanes 1–5 in A. Only the relevant portion of the blot is shown. C, AV-12 cells were transfected with transgenes encoding both CKA1 and PKC3. In addition, cells were cotransfected with a recombinant pEBG vector that contained a transgene encoding either GST partial PKC3 (amino acids 111–227) wild type protein (panel 1); GST partial PKC3 (amino acids 111–227) mutant protein (Asn216 to Ala, panel 2); or GST alone (panel 3). Western blots (probed with anti-GST IgGs) revealed that transfected cells accumulate similar amounts of GST fusion proteins or GST (data not shown). Cells were fixed, permeabilized, and examined by confocal immunofluorescence microscopy as previously reported (35). Fluorescence signals indicating the intracellular location of full-length PKC3 were obtained by using affinity-purified anti-PKC3 IgGs (3) and secondary antibodies tagged with fluorescein isothiocyanate. Anti-PKC3 IgGs bind with epitopes located between residues 477 and 597 at the C terminus of the atypical kinase (3). These epitopes are not present in the competing GST partial PKC3 fusion proteins. No signals were observed after blocking with excess purified antigen or with preimmune serum.

α-helical turn conformation (27, 28), in which side chains and main chains are oriented to complement a binding pocket (surface) presented by the Numb-like, β-sandwich PTB domain of CKA1/CKA1S (25–28, 45). Properties of the CKA1 PTB domain are described in the accompanying paper (45). The PTB-binding segment of PKC3 is embedded within the V2 “linker” region of the kinase (Fig. 1C). The linker connects N-terminal pseudosubstrate and Cys-rich regulatory regions with the C-terminal phosphotransferase domain, thereby enabling physiological control of enzyme activity (1–3). The discovery that the linker region contains a site involved in tethering and targeting PKC3 to a specific intracellular location reveals a second, previously unappreciated function for this segment of the atypical kinase.

All other candidate binding/scaffold proteins for aPKCs (discussed by Zhang et al. (45)) couple with either the catalytic domain or pseudosubstrate/Cys-rich regions of aPKCs (15–17, 40). In contrast, our results assign a unique functional role to a region of aPKCs that was previously presumed to be a structural element. A comparable PTB-binding site is not evident in the V2 region of Ca2+- and/or DAG-activated, C. elegans PKCs 1 and 2 (41, 42). This suggests that the PTB-binding site in PKC3 contributes to both the complexity and specificity of aPKC-mediated signal transduction.

PKC3 Phosphorylates Ser17 and Ser65 in the PSDs of CKA1—CKA1 contains two N-terminal PSD-like regions (amino acids 11–25 and 61–77 in Fig. 6A) that are potential targets for PKC3-mediated phosphorylation. When CKA1S, which contains only the second PSD site, was isolated from transfected AV-12 cells, it served as a good substrate for PKC3 in vitro (Fig. 6B, lanes 1 and 2). However, coexpression of PKC3 and CKA1S transgenes yielded a CKA1S polypeptide that was a poor substrate for purified PKC3 (Fig. 6B, lanes 1 and 2). Thus, CKA1S was evidently phosphorylated by overexpressed PKC3 in the milieu of intact cells. Inspection of the PSD-1 (amino acids 11–25, Fig. 6A) and PSD-2 (amino acids 61–77, Fig. 6A) regions disclosed that Ser17 and Ser65 are in near-optimal sequence contexts for the phosphotransferase activity of the nPKC. Wild type and mutant GST fusion proteins that include residues 1–63 (GST-PSD1) and residues 59–108 (GST-PSD2) were synthesized in E. coli and purified to near-homogeneity by affinity chromatography. Vmax and Km values for PKC3-catalyzed phosphorylation of wild type GST-PSD1 and GST-PSD2 (Fig. 6, D and E) were similar to values obtained (Km = 1.3 μM, Vmax = 2.5 pmol/min) for a PKC3 pseudosubstrate-derived peptide (YRKRSGRRKKK), which corresponds to residues 112–123 in PKC3 except for the substitution of Ala116 with Ser, see Ref. 3). GST alone is not phosphorylated by PKC3. The kinetic constants indicate that the PSD1 and PSD2 domains are excellent substrates for PKC3. The efficiency of phosphorylation of these targets may be substantially amplified by the tethering of the kinase to the PTB domains of intact CKA1 and CKA1S adapter proteins. Substitution of Ala for Ser17 or Ser65 abolished the abilities of PSD1’ and PSD2’, respectively, to serve as PKC3 substrates (Fig. 6C). Mutation of other Ser residues in PSD1’ and PSD2’ (e.g. Ser26 to Ala, Fig. 6C) failed to diminish incorporation of 32P radioactivity. Thus, each of the

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**Fig. 4.** Identification of individual amino acids in PKC3 that are essential for avid binding with the PTB domain of CKA1/CKA1S. A, amino acids in the CKA1 binding region of PKC3 were altered by site-directed mutagenesis as indicated under “Experimental Procedures.” Wild type and mutant GST fusions that contain residues 111–227 from PKC3 were coexpressed with CKA1 and CKA1S in transfected AV-12 cells. Binding of adapter proteins to mutant and wild type GST partial PKC3 proteins was assessed by a combination of affinity purification of the complexes on GSH-Sepharose 4B beads and subsequent Western immunoblot analysis (see “Experimental Procedures” and the legends for Figs. 2 and 3). Proteins eluted from the beads were size-fractionated by denaturing electrophoresis, transferred to an Immobilon membrane, and probed with anti-CKA1/CKA1S antibodies. Lane 1 received proteins coisolated with the wild type CKA1 binding region of PKC3. Other samples include proteins coisolated with CKA1 binding regions of PKC3 that were mutated as follows: Ile214 to Ala (lane 2); Asn216 to Ala (lane 3); Phe217 to Ala (lane 4) and His224 to Ala (lane 5). IgG-CKA1 and IgG-CKA1S complexes were visualized by enhanced chemiluminescence methodology. Signals recorded on x-ray film are shown. B, a Western blot probed with anti-GST IgGs reveals that similar amounts of wild type and mutant GST-PKC3 fusion proteins were expressed and isolated for each binding assay. Samples applied to lanes 1–5 are duplicates of samples used for lanes 1–5 in A. Only the relevant portion of the blot is shown. C, AV-12 cells were transfected with transgenes encoding both CKA1 and PKC3. In addition, cells were cotransfected with a recombinant pEBG vector that contained a transgene encoding either GST partial PKC3 (amino acids 111–227) wild type protein (panel 1); GST partial PKC3 (amino acids 111–227) mutant protein (Asn216 to Ala, panel 2); or GST alone (panel 3). Western blots (probed with anti-GST IgGs) revealed that transfected cells accumulate similar amounts of GST fusion proteins or GST (data not shown). Cells were fixed, permeabilized, and examined by confocal immunofluorescence microscopy as previously reported (35). Fluorescence signals indicating the intracellular location of full-length PKC3 were obtained by using affinity-purified anti-PKC3 IgGs (3) and secondary antibodies tagged with fluorescein isothiocyanate. Anti-PKC3 IgGs bind with epitopes located between residues 477 and 597 at the C terminus of the atypical kinase (3). These epitopes are not present in the competing GST partial PKC3 fusion proteins. No signals were observed after blocking with excess purified antigen or with preimmune serum.

**Fig. 5.** Comparison of peptide sequences that engage the PTB domains of various signaling/regulatory proteins. Peptide sequences of PTB domain ligands are aligned as described by Meyer et al. (39). Residues thought to be essential for stable binding of partner proteins with the indicated PTB domains are enclosed in rectangles. The PTB domain ligands for Shc and IRS-1 PTB domains contain phospho-Tyr, whereas other ligands are not phosphorylated. In the consensus sequence X indicates any amino acid, ψ corresponds to an aromatic or large aliphatic hydrophobic amino acid. Mutation of any of the residues marked with asterisks abrogates the ability of PKC3 to bind with CKA1.

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**Table 1.** Assembly and Targeting of CKA1-PKC3 Complexes

| Source of PTB domain | Binding Partner | Sequence of PTB ligand |
|----------------------|-----------------|-----------------------|
| SHC                 | EGF R           | SLNNDYQQ             |
| IRS-1               | Ins R           | ASSNPWLS             |
| JIP-1, 2            | Rho GEF         | PFINAEGH             |
| XI1                 | APP             | GYRENTPYK             |
| mNumb               | LMK             | GLINDPAKT             |
| dNumb               | NAK             | GFSNASFED             |

**Consensus**

| CKA1/CKA1S | PKC3 |
|-------------|------|
| GYXNXY/F    | GIDNGAFHE * * * * |

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N-terminal PSDs of CKA1 contain a unique target site for PKC3-catalyzed phosphorylation.

The Intracellular Distribution of CKA1/CKA1S Is Altered by Introducing or Eliminating Negative Charge at Residues 17 and 65—Basic and large hydrophobic amino acids in prototypic PSDs cooperatively promote association of proteins with plasma membrane through a combination of electrostatic and apolar interactions (43, 44). A high density of positive charge provided by clustered Arg and Lys residues (e.g., see residues 11–25, 61–77, Fig. 6A) enables binding with anionic head groups of phospholipids. The hydrocarbon interior of the membrane bilayer simultaneously accommodates and stabilizes insertion of side chains of large hydrophobic amino acids that are critical components of PSDs (43, 44).

Mutated CKA1 transgenes were generated and then expressed in AV-12 cells to determine consequences of introducing or eliminating negative charge in PSD1 and PSD2 (Fig. 6C). A CKA1 protein that contains Ser17 to Ala and Ser65 to Ala substitutions is efficiently routed to the cell periphery, leaving only a modest amount of the adapter in other cell compartments (Fig. 7A). In contrast, conversion of both Ser17 and Ser65 to Glu residues (which mimic phospho-Ser by carrying negative charge) promotes accumulation of CKA1/CKA1S in the cytoplasm (Fig. 7C). The strikingly distinct patterns of localization of CKA1Ala17–Ala65 and CKA1Glu17–Glu65 strongly suggest (a) nonphosphorylated PSD regions of CKA1 govern targeting
of the adapter protein (and tethered PKC3) to plasma/membrane-cortical cytoskeleton and (b) N-terminal phosphorylation of Ser\(^{17}\) and/or Ser\(^{65}\) disrupts crucial electrostatic and hydrophobic bonds, elicits disengagement of CKA1 (and presumably CKA1-PKC3 complexes) from a cell surface microenvironment, and promotes re-distribution of the adapter to the cytoplasm and/or cytoplasmic organelles. A key implication of these results is that the location of the wild type adapter protein may be dynamically regulated by N-terminal phosphorylation and dephosphorylation. Consistent with this idea, the intracellular localizations of wild type and mutant CKA1 proteins were obtained by using anti-CKA1 IgGs and secondary antibodies tagged with fluorescein isothiocyanate. No signals were observed after blocking with excess purified antigen or with preimmune serum.

**Mechanisms that govern phosphorylation of Ser\(^{17}\) and Ser\(^{65}\) remain to be determined. PKC3 phosphorylates these residues *in vitro* and (apparently) in transfected cells (that contain an elevated level of the aPKC) (Fig. 6B). However, wild type CKA1/CKA1S is enriched at the cell periphery in stably transfected cells (Fig. 7B). Moreover, both CKA1 and PKC3 are targeted to the periphery of cells that were cotransfected with a molar ratio of transgenes (CKA1:PKC3 = 3) that yields an excess of aPKC tethering sites (45). These results suggest that phosphorylation of adapter protein by tethered endogenous aPKCs or modest amounts of sequestered recombinant PKC3 are tightly regulated. This could be achieved through the dominant (regulated) activity of protein phosphatases, the presence of modulators that inhibit phosphorylation of Ser\(^{17}\) and Ser\(^{65}\) via interactions with the kinase or CKA1, or incorporation of adapter-PKC dimers into a multiprotein complex that sterically precludes interactions between the PKC3 catalytic domain and the N terminus of CKA1.**

## Association of CKA1/CKA1S with The Cell Periphery Is Enhanced by a PKC Inhibitor and Disrupted by Phorbol Ester or Protein Ser/Thr Phosphatase Inhibitors—Mechanisms that govern phosphorylation of Ser\(^{17}\) and Ser\(^{65}\) *in situ* remain to be determined. PKC3 phosphorylates these residues *in vitro* and (apparently) in transfected cells (that contain an elevated level of the aPKC) (Fig. 6B). However, wild type CKA1/CKA1S is enriched at the cell periphery in stably transfected cells (Fig. 7B). Moreover, both CKA1 and PKC3 are targeted to the periphery of cells that were cotransfected with a molar ratio of transgenes (CKA1:PKC3 = 3) that yields an excess of aPKC tethering sites (45). These results suggest that phosphorylation of adapter protein by tethered endogenous aPKCs or modest amounts of sequestered recombinant PKC3 are tightly regulated. This could be achieved through the dominant (regulated) activity of protein phosphatases, the presence of modulators that inhibit phosphorylation of Ser\(^{17}\) and Ser\(^{65}\) via interactions with the kinase or CKA1, or incorporation of adapter-PKC dimers into a multiprotein complex that sterically precludes interactions between the PKC3 catalytic domain and the N terminus of CKA1. In addition, PSD1 and PSD2 are exceptionally good substrates for Ca\(^{2+}\) and/or DAG-activated PKCs. Thus, another possibility is that Ser\(^{17}\) and/or Ser\(^{65}\) are in *vivo* targets for classical PKC or novel PKC isoforms rather than aPKCs.

**Effects of DAG-activated PKCs and protein Ser/Thr phosphatases on intracellular targeting of CKA1 and CKA1S were assessed in AV-12 cells that stably express the wild type adapters (Fig. 7B). Incubation of cells with TPA (12-tetradecanoylphorbol 13-acetate), a phorbol ester that selectively activates endogenous DAG-dependent PKCs, caused substantial depletion of adapter protein from the cell surface and promoted a concomitant increase in the cytoplasmic content of CKA1/CKA1S (Fig. 8A). Treatment with a pair of protein Ser/Thr phosphatase inhibitors (okadaic acid and cantharidin) or a combination of these inhibitors with TPA completely eliminated the enriched pool of CKA1/CKA1S at the cell periphery and elicited a homogeneous dispersal of adapter protein in cytoplasm (and/or internal membranes) (Fig. 8, B and C). Thus, compounds that are expected to promote phosphorylation of Ser\(^{17}\) and Ser\(^{65}\) elicit a pattern of CKA1/CKA1S distribution that was previously observed for the CKA1 Glu\(^{17}\)–Glu\(^{65}\) mutant (Fig. 7C). These observations support the idea that incorporation of phosphate at Ser\(^{17}\) and Ser\(^{65}\) in CKA1 (or the equivalent of Ser\(^{65}\) in CKA1S) serves as a molecular switch that enables dissociation of PKC3 adapters from the vicinity of plasma membrane.**

In contrast, GF-109203X, an inhibitor of DAG-dependent PKCs, enhanced accumulation of CKA1/CKA1S at the cell surface and diminished the level of cytoplasmic adapter protein (Fig. 5D). Inhibition of endogenous PKCs, which is expected to reduce phospho-Ser\(^{17}\) and phospho-Ser\(^{65}\) content in CKA1, yields an intracellular pattern of adapter protein localization that was previously documented for the CKA1 Ala\(^{17}\)–Ala\(^{65}\) mutant (Fig. 7A). This result is consistent with the concept that dephosphorylated PSDs play a central role in targeting/anchoring CKA1/CKA1S to plasma membrane. Concerted binding of Arg and Lys with anionic plasma membrane phospholipids and insertion of co-clustered large hydrophobic side chains into the apolar interior of the lipid bilayer are evidently optimized when negative charge is reduced or excluded at residues 17 and 65.

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\(^2\) L. Zhang, H. Feng, and C. S. Rubin, unpublished observations.
AV-12 cells that stably express a wild type CKA1 transgene were incubated with 1 μM TPA (A); 500 nM okadaic acid plus 10 μM cantharidin (B); or 5 μM GF109203X (2-(3-dimethylaminopropyl)-indol-3-yl)-3-(1H)-indol-3-yl) maleimide), a potent PKC inhibitor (D). The intracellular distribution of CKA1/CKA1S was then determined by confocal immunofluorescence microscopy as described by Li et al. (35). E, AV-12 cells that stably express CKA1 and CKA1S were incubated with 10 μM cantharidin plus 0.5 μM okadaic acid (for 20 min) or without drugs. Cells were lysed in 20 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaF, 5 μM cantharidin, and 20 mM microcystin. After centrifugation at 125,000 × g for 60 min at 4 °C, supernatant solutions (which contain proteins from cytoplasm) were collected. Membrane-associated CKA1/CKA1S proteins were solubilized from the pelletted particulate fraction of AV-12 cells by extraction with lysis buffer supplemented with 2% Triton X-100. Detergent-soluble proteins were collected in the supernatant after a second round of centrifugation (125,000 × g for 60 min at 4 °C), and detergent-soluble protein fractions were size-fractionated by denaturing electrophoresis and visualized on x-ray film (see Fig. 6B). Lanes 1 and 2 received duplicate samples of adapter proteins derived from the particulate (membranes and cytoskeleton) fraction of control cells; lanes 3 and 4 contained duplicate samples of cytoplasmic adapter proteins derived from cells treated with cantharidin and okadaic acid. Parallel Western immunoblot analyses showed that each lane of the gel was loaded with similar amounts of CKA1/CKA1S proteins (data not shown). Results presented in lanes 3 and 4 were replicated when cytoplasmic CKA1/CKA1S proteins (~10% of total CKA1/CKA1S) from control cells were analyzed. Membranes and cytoskeleton from drug-treated cells contained negligible amounts of adapter proteins.

The predicted relationship between intracellular location and status of PKC phosphorylation sites in CKA1/CKA1S was directly assessed. In control (untreated) AV-12 cells, adapter proteins accumulate at the cell periphery. CKA1 and CKA1S isolated from untreated AV-12 cells are excellent substrates for purified PKCs (Fig. 8E, lanes 1 and 2). Thus, PKC target sites are available in adapter proteins associated with the cell periphery. When cells are incubated with protein Ser/Thr phosphatase inhibitors, CKA1 and CKA1S are uniformly distributed throughout the cytoplasm (Fig. 8B). Purified PKCs catalyzed only minimal phosphorylation of immunoprecipitated adapter proteins derived from the cytoplasmic compartment (Fig. 8E, lanes 3 and 4). Thus, most of the PKC target sites in cytoplasmic CKA1/CKA1S are phosphorylated in situ.

Distribution of CKA1/CKA1S between the cell periphery and cytoplasm appears to be dynamically regulated by levels and activities of endogenous DAG-stimulated PKCs and opposing protein Ser/Thr phosphatases in a model cell system. Further development of this model will entail the following: (a) identification of individual DAG-activated PKC isoforms that control adapter protein localization and (b) rigorous testing of the idea that phosphorylation of Ser17 and/or Ser65 in CKA1 constitutes a key regulatory switch in intact cells. Nevertheless, the currently available data suggest a novel and potentially important consequence of this mode of regulation. The localization and function of an atypical PKC may be rapidly altered by hormones or growth factors that activate phospholipases Cβ or Cγ, thereby initiating DAG-mediated signal transduction. In such a pathway DAG-activated PKCs would serve as upstream regulators that channel signals carried by a lipid second messenger to a DAG-independent kinase. A pertinent speculation is that colocalization of sites of DAG synthesis and CKA1/CKA1S protein in plasma membrane could promote targeted accumulation of DAG-activated PKCs. When kinase activity exceeds local protein phosphatase activity, N-terminal phosphorylation of DAG-activated PKC would proceed, thereby triggering translocation of aPKC-adapter complexes. These possibilities are topics of ongoing studies.

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Structural Properties and Mechanisms That Govern Association of C Kinase Adapter 1 with Protein Kinase C3 and the Cell Periphery
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