Cdc42 effector protein-4 (CEP4) was recently identified by our laboratory to be a substrate of multiple PKC isoforms in non-transformed MCF-10A human breast cells. The significance of phosphorylated CEP4 to PKC-stimulated motility of MCF-10A cells was evaluated. Single site mutants at Ser residues embedded in potential PKC consensus sites (Ser^{18}, Ser^{77}, Ser^{80}, and Ser^{86}) were individually replaced with Asp residues to simulate phosphorylation. Following expression in weakly motile MCF-10A cells, the S18D and S80D mutants each promoted increased motility, and the double mutant (S18D/S80D) produced a stronger effect. MS/MS analysis verified that Ser^{18} and Ser^{80} were directly phosphorylated by PKCa in vitro. Phosphorylation of CEP4 severely diminished its affinity for Cdc42 while promoting Rac activation and formation of filopodia (microspikes). In contrast, the phosphorylation-resistant double mutant S18A/S80A-CEP4 blocked CEP4 phosphorylation and inhibited motility of MCF-10A cells that had been stimulated with PKC activator diacylglycerol lactone. In view of the dissociation of phospho-CEP4 from Cdc42, intracellular binding partners were explored by expressing each CEP4 double mutant from a tandem affinity purification vector followed by affinity chromatography, SDS-PAGE, and identification of protein bands evident only with S18D/S80D-CEP4. One binding partner was identified as tumor endothelial marker-4 (TEM4; ARHGEF17), a guanine nucleotide exchange factor that is associated with cancer-related phenotypes (1). In non-transformed MCF-10A human breast cells, PKC signaling pathways produce rearrangement of the actin cytoskeleton, increased microtubule dynamics, and activation of Rac, one of the small Rho GTPases (2–4). These pleiotropic responses impact cell morphology, proliferation, and migration, which are collectively associated with tumor growth and metastasis. The frequently observed interplay of PKC with the small Rho GTPases and their effector proteins and regulatory enzymes, e.g. guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (5), underscore the importance of the GTPases in breast cancer (6, 7). GTPase-activating proteins and GEFs enable the small GTPases to cycle between GTP-bound (active) and GDP-bound (inactive) states. Investigation of PKC substrates and their interactions with the small GTPases and their regulatory enzymes is a promising strategy for identifying potential targets for anticancer drugs (8, 9).

Protein kinase C (PKC) and its substrates participate in signaling pathways in human breast cells that are associated with cancer-related phenotypes (1). In non-transformed MCF-10A human breast cells, PKC signaling pathways produce rearrangement of the actin cytoskeleton, increased microtubule dynamics, and activation of Rac, one of the small Rho GTPases (2–4). These pleiotropic responses impact cell morphology, proliferation, and migration, which are collectively associated with tumor growth and metastasis. The frequently observed interplay of PKC with the small Rho GTPases and their effector proteins and regulatory enzymes, e.g. guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (5), underscore the importance of the GTPases in breast cancer (6, 7). GTPase-activating proteins and GEFs enable the small GTPases to cycle between GTP-bound (active) and GDP-bound (inactive) states. Investigation of PKC substrates and their interactions with the small GTPases and their regulatory enzymes is a promising strategy for identifying potential targets for anticancer drugs (8, 9).

PKC is expressed as multiple isoforms that can select specific substrates as well as shared substrates. The isoforms are subcategorized by the nature of their regulation: the conventional isoforms (α, β1/β2, and γ) are activated by diacylglycerol (DAG) and Ca^{2+}, the novel isoforms (δ, ε, η, and θ) are activated only by DAG, and the atypical isoforms (λ/ι and ζ) are activated by other lipids (e.g. ceramide) (10). PKC α and δ isoforms were shown previously to promote cancer-related phenotypes (11, 12). It is noted that a given cell line or tissue will express a characteristic PKC isoform profile that includes some, but usually not all, of these isoforms. For example, MCF-10A cells express low levels of α, δ, η, and ζ.

The traceable kinase method has been very useful in identifying PKC substrates as it provides a means to track the phosphorylated proteins produced by a specific PKC isoform that...
had been genetically engineered to bind an unnatural ATP analog (13, 14). MCF-10A cells provide a suitable system for this approach because the endogenous PKC isoforms are expressed at low levels, thereby producing a low background against which the products of the traceable mutant can be detected. Our laboratory investigated substrates of three traceable PKC isoforms (α, δ, and ζ) in MCF-10A cells (5). Findings identified similar protein phosphorylation profiles for α and δ, whereas that for ζ was radically different. Following MS/MS of selected bands, it was found that the α and δ isoforms were associated with a number of Rho GTPase effectors and regulatory proteins, including a protein identified as Cdc42 effector protein-4 (CEP4). Upon further evaluation in vitro with highly pure, recombinant CEP4 and PKC isoforms as well as in cells treated with isoform-specific shRNA reagents, CEP4 proved to be a substrate for all of them. These findings prompted the speculation that individual PKC isoforms may respond to specific stimuli but upon activation may share common protein substrates that relate to a specific phenotype.

CEP4, also known as binder of Rho GTPases-4, is one of five related isoforms, CEPs 1–5 (15), and is expressed ubiquitously in all human adult tissues (16). The existence of this family was discovered as the result of a two-hybrid screen with the GTPase TC10 as bait (15). Consequently, all five CEPs were found to bind with high affinity to the GTP-bound form of Cdc42 but did not bind to the other small GTPases (Rac and RhoA) (15, 17). Site-specific mutagenesis established that Cdc42 binds to CEP at its 16-amino acid Cdc42/Rac interaction binding (CRIB) domain (15, 18). In the limited published research available on CEPs, the phenotypes associated with these proteins depend on formation of the CEP/Cdc42 complex and its role in actin-based membrane protrusions (e.g. pseudopodia), actin filament assembly, and cell–cell contacts. There is one example of CEP3 acting independently of Cdc42 in which it associated with a septin GTPase (19). This protein forms multimeric complexes and has been implicated in cytokinesis, cell polarity, and oncogenesis. Binding of CEP3 to septins disrupted their oligomeric organization. However, this effect by CEP3 was blocked when GTP-Cdc42 was overexpressed, thus providing an example of negative regulation of CEP3 by Cdc42 (19).

In the present work, CEP4 was further investigated as a PKC substrate as well as for the functional consequences of its phosphorylation. By use of site-specific mutagenesis at PKC consensus sites as well as tandem mass spectrometry (MS/MS) of CEP4 phosphorylated by PKCa in vitro, key sites of phosphorylation in CEP4 were defined. Site-directed mutants of CEP4 were used to explore the significance of phosphorylation to its interaction with Cdc42, its binding interactions with other proteins, its effects on the actin cytoskeleton, and its role in cell motility. Overall, these studies establish phosphorylated CEP4 as a component of the PKC-mediated motility signaling pathway and as a modulator of actin-based membrane protrusions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human breast epithelial MCF-10A cells were obtained from The Barbara Ann Karmanos Cancer Center. BL21 and DH5α bacterial cells, DMEM/F-12 medium, horse serum, and DNA primers were purchased from Invitrogen. Human CEP4-pCMV6 and bicistronic tumor endothelial marker-4 (TEM4) shRNA/GFP and control shRNA/GFP plasmids were obtained from Origene Inc. (Rockville, MD). EZview Red anti-FLAG M2 affinity gel beads, protease inhibitor mixture, and phosphatase inhibitor mixtures were obtained from Sigma-Aldrich. The Rac inhibitor NSC23766 and p21-activated kinase-binding domain-agarose beads were purchased from EMD-Millipore (Billerica, MA). PolyExpress DNA transfection reagent was purchased from Excellgen, Inc. (Gaithersburg, MD). The QuikChange mutagenesis kit and the InterPlay Mammalian Tandem Affinity Purification (TAP) System were obtained from Agilent Technologies (Santa Clara, CA). Cell lysis buffer, phospho-PKC substrate antibody, and the myc tag antibody were acquired from Cell Signaling Technology, Inc. (Beverly, MA). Partition-defective protein 6G (ParD6G) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TEM4 antibody and pure, recombinant CEP4 were purchased from Novus Biologicals (Littleton, CO). Pure, recombinant PKCa was obtained from Fitzgerald Industries International (North Acton, MA). The DAG-lactone reagent (JH-131E-153) was a gift from Dr. V. Marquez (National Cancer Institute, Frederick, MD).

**Plasmids**—Human CEP4 cDNA was restricted at SgfI and MluI sites in the pcMV6 plasmid. Expression from this plasmid conferred DDK and myc epitope tags at the C terminus of the recombinant protein. By use of PCR, the cDNA insert was amplified with suitable primers for subcloning into the TAP vector (Agilent Technologies) at BamHI and EcoRV restriction sites. The TEM4 shRNA or scrambled control shRNA each was co-expressed with GFP from a bicistronic vector for evaluation of transfection efficiency.

**Construction of Pseudophosphorylated or Phosphorylation-resistant Mutants**—Substitution of the Ser (S) codons in CEP4 cDNA with either Asp (D) or Ala (A) codons was carried out by the QuikChange method using PCR with two mutagenesis primers (Invitrogen) that were designed to introduce the desired mutation. In the following (forward) primers used for single or double substitutions, the changed codon is underlined: S18D, 5′-CAC-TCC-AAG-CGC-CGT-GAC-GA-GAC-CTC-AGC-3′; S77D, 5′-TCA-CTC-TCC-GAG-AAG-3′; S80D, 5′-AAA-CGC-AGT-CTC-AGC-GAC-AGA-GAG-GTC-GGC-GCC-3′; S86D, 5′-AGG-AAG-TTC-CTG-GCC-AGG-CCG-TCG-3′; S18A, 5′-CAC-TCC-AAG-CGC-CGT-GAC-GA-GAC-AGG-3′; and S80A, 5′-AAA-CGC-AGT-CTC-AGG-GAG-GAC-AGA-GAG-GTC-GGC-GCC-3′. All mutant sequences were confirmed by DNA sequencing of the entire open reading frame (Macrogen Inc., Rockville, MD).

**Cell Culture and Transfection**—MCF-10A cells were grown in 10-cm or 60-mm BD Falcon culture dishes at 37 °C and 5% CO2 in DMEM/F-12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, 0.02% Fungizone, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, and 100 ng/ml cholera toxin. Cells were replated 1 day prior to transfection so that the cell density was 80%. The medium was replaced with fresh complete medium 1 h before transfection. Plasmid DNA was mixed with the PolyExpress transfection reagent in a 1:3 ratio (DNA/reagent) and incubated...
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for 15 min at room temperature. The mixture was added to the cells and incubated at 37°C and 5% CO₂. After 18–24 h, the medium was replaced with fresh complete medium. Cells were harvested 48 h post-transfection. At this time point, the transfection efficiency was typically 60–80%.

For cells transfected with a plasmid encoding an shRNA reagent (TEM4 or scrambled control), the plasmid was transiently transfected into MCF-10A cells with PolyExpress as described above. At 48 h, a plasmid encoding one of the CEP4 mutants or the vector control was transfected, and the cells were incubated at 37°C for an additional 48 h in complete medium. As judged by the GFP signal evident throughout the cells, the transfection efficiency was typically 60–80%.

Cell Lysis and Western Blot—Cells were disrupted in lysis buffer (Cell Signaling Technology, Inc.) and sonicated three times each for 10 s followed by centrifugation at 10,000 × g for 10 min to remove insoluble debris. The lysate was assayed for protein concentration using a protein dye reagent (Bio-Rad).

Samples of known protein concentration were denatured with 5× SDS sample buffer (50% (w/v) glycerol, 1% SDS, 0.05% bromphenol blue, 0.4% x Tris, pH 6.8, and 2 mM dithiothreitol) followed by heating at 95°C for 5 min. Proteins were separated by 8% SDS-PAGE and transferred to a PVDF membrane (Millipore Corp.). The blot was developed with the appropriate antibodies and detected with chemiluminescence reagents (Pierce).

Immunoprecipitation—For MCF-10A cells expressing wild-type (WT) or mutant CEP4, cells were lysed in hypotonic, detergent-free lysis buffer (20 mM Tris, pH 7.4, 2 mM MgCl₂, 2 mM EGTA, 1 mM DTT, 10 μM bisindolylmaleimide, protease inhibitors (1:1000), and phosphatase inhibitors (1:100)) followed by immunoprecipitation with anti-FLAG EZview beads. Anti-FLAG beads (60 μl pre-equilibrated in cold TBS) were applied to the lystate and rotated for 2 h at 4°C. The beads were centrifuged at 8200 × g for 5 min and washed three times in hypotonic, detergent-free lysis buffer. Pellets were electrophoresed on 8% SDS-polyacrylamide gels and transferred to a PVDF membrane, and immunoreactive bands were detected with the appropriate antibody.

Binding Assay with CEP4 Mutants and Constitutively Active Q61L-Cdc42—Glutathione S-transferase (GST)-tagged Q61L-Cdc42 was expressed from a bacterial expression vector (pGEX-2T) in BL21 cells. This plasmid was provided by the laboratory of Prof. Gary Bokoch (Scripps Research Institute; [pGEX-2T] in BL21 cells). This plasmid was provided by the laboratory of Prof. Gary Bokoch (Scripps Research Institute; [pGEX-2T] in BL21 cells). This plasmid was provided by the laboratory of Prof. Gary Bokoch (Scripps Research Institute; [pGEX-2T] in BL21 cells).

MCF-10A cells expressing myc-tagged wild-type or mutant CEP4 were lysed in hypotonic, detergent-free buffer (20 mM Tris, pH 7.4, 2 mM MgCl₂, 2 mM EGTA, 1 mM DTT, 10 μM bisindolylmaleimide, protease inhibitors (1:1000), and phosphatase inhibitors (1:100)). The cell lysates were obtained by centrifugation at 10,000 × g for 10 min and precleared with 60 μl of glutathione-Sepharose beads (Fisher Scientific) by rotating at 4°C for 1 h. The precleared MCF-10A cell lysate was incubated with immobilized Q61L-Cdc42 (described above) by rotation at 4°C for 40 min. Following two washes with hypotonic, detergent-free lysis buffer, the bound protein was eluted by boiling with 1× SDS-PAGE sample buffer, resolved by 8% SDS-PAGE, and transferred to a PVDF membrane. Immunobiochemical analysis with anti-myc was used to determine the amount of myc-tagged CEP4 that was bound to Cdc42.

Isolation of Binding Partners of Pseudophosphorylated CEP4 by the TAP Method—Proteins that were physically associated with CEP4 were isolated by use of a CEP4 double mutant expressed from a TAP vector that conferred two affinity tags at the C terminus of CEP4. The affinity tags enabled binding to a streptavidin-binding resin and a calmodulin-binding resin, respectively (Agilent Technologies). MCF-10A cells grown in three 10-cm plates to 80–90% confluence were transfected with a TAP vector expressing a CEP4 double mutant (S18A/S80A or S18D/S80D). Forty-eight hours post-transfection, cells were resuspended in 1 ml of hypotonic, detergent-free lysis buffer (20 mM Tris, pH 7.4, 2 mM MgCl₂, 2 mM EGTA, 1 mM dithiothreitol, 10 μM bisindolylmaleimide, 0.1% protease inhibitor mixture, and 1% phosphatase inhibitor mixture) and disrupted by three successive freeze-thaw cycles (10 min at −150°C and 10 min in cold water). After centrifugation of the cells at 10,000 × g for 10 min, the crude cell lysate was incubated for 2 h at 4°C with 100 μl of streptavidin-binding resin (pre-equilibrated with streptavidin binding buffer). The resin and any bound proteins were washed twice with 750 μl of streptavidin binding buffer and eluted with 200 μl of streptavidin elution buffer for 1 h at 4°C. The eluate was incubated with 50 μl of calmodulin-binding resin (pre-equilibrated with calmodulin binding buffer) at 4°C for 3 h and washed twice with 750 μl of calmodulin binding buffer. The bound proteins were eluted by boiling with 1× loading buffer at 95°C for 5 min, resolved by SDS-PAGE, and stained with GelCode Blue. Bands of interest were excised and analyzed by MS/MS.

Motility Assay—Cell motility was measured by the cell sedimentation method, which required a 10-well glass slide and a 10-hole metallic manifold (Creative Scientific Methods, Inc.). The cells were applied to the slide through the manifold so that the cells form a tight concentric circle in each well. After incubation overnight at 37°C and 5% CO₂, the manifold was removed, and the cells were immediately viewed under a Nikon Diaphot microscope (t = 0). The extent of movement was determined in triplicate by measuring the difference in total area at t = 0 and t = 6 h using a camera attached to the microscope (Moticam 2000) and Motic Image software. The results of triplicate measurements were averaged.

Immunocytochemistry—Cells were cultured overnight on polylysine-coated coverslips and then transfected with the myc-tagged CEP4-S18D/S80A or -S18D/S80D constructs. Each coverslip was washed twice in 2 ml of PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂, pH 7.4). The mixture was added to the cells and incubated at 37°C and 5% CO₂. After 18–24 h, the medium was replaced with fresh complete medium. Cells were harvested 48 h post-transfection.
Characterization of CEP4 Proteins Mutated at PKC Consensus Sites—In an initial approach to define the site(s) of CEP4 phosphorylation, we evaluated Ser/Thr residues lying within PKC consensus sites. Whereas a canonical consensus site consists of a Ser or Thr flanked by one or two cationic residues (20), a partial site would be that in which only one side of the phosphorylated site is adjacent to a cationic residue(s). Upon inspection of the CEP4 primary sequence, Ser18, Ser77, Ser80, and Ser86 were found to be embedded in either a complete PKC consensus site (Ser18) or partial site (Ser77, Ser80, and Ser86) and were clustered in the N-terminal region (Fig. 1A). Site-directed mutagenesis of each Ser codon to an Asp codon created a negative charge, thereby simulating the presence of a phosphate group at this position in the protein. Each pseudophosphorylated mutant was expressed bearing both FLAG and myc epitope tags. Following transient transfection, whole cell lysates were prepared in which an individual serine (S) was replaced with an Asp (D) residue followed by its expression in MCF-10A human breast cells. To demonstrate phosphorylation of pure, recombinant GST-tagged CEP4 (synthesized by a wheat germ system), the protein (1.1 μg) was phosphorylated at 30 °C for 2 h by pure human PKCα (1 μg) in vitro (5). The phosphorylated product and untreated control CEP4 were resolved by 8% SDS-PAGE (0.75 mm thickness). After staining with GelCode Blue, the gel slices corresponding to phospho-CEP4 or untreated CEP4 were digested with endo-Lys-C and endo-Glu-C. To identify proteins that co-purified with TAP-CEP4, gel-resolved bands of interest were excised and digested in situ with trypsin. For these experiments, the resulting peptides were analyzed by LC-MS/MS on an LTQ Orbitrap mass spectrometer. All MS/MS spectra were searched against the NCBI database with a probability or significance threshold of p < 0.05 using the automated Mascot algorithm. Identification of a protein required that two or more MS/MS spectra matched peptides of the same protein entry in the database. MS analysis was performed at the MS and Proteomics Resource at the Yale School of Medicine.

Statistical Analysis—Data are expressed as the mean ± S.D. Each experiment was performed a minimum of three times. The difference between groups was assessed with the Student’s t test.

RESULTS

Characterization of CEP4 Proteins Mutated at PKC Consensus Sites—In an initial approach to define the site(s) of CEP4 phosphorylation, we evaluated Ser/Thr residues lying within PKC consensus sites. Whereas a canonical consensus site consists of a Ser or Thr flanked by one or two cationic residues (20), a partial site would be that in which only one side of the phosphorylated site is adjacent to a cationic residue(s). Upon inspection of the CEP4 primary sequence, Ser18, Ser77, Ser80, and Ser86 were found to be embedded in either a complete PKC consensus site (Ser18) or partial site (Ser77, Ser80, and Ser86) and were clustered in the N-terminal region (Fig. 1A). Site-directed mutagenesis of each Ser codon to an Asp codon created a negative charge, thereby simulating the presence of a phosphate group at this position in the protein. Each pseudophosphorylated mutant was expressed bearing both FLAG and myc epitope tags. Following transient transfection, whole cell lysates were analyzed for levels of mutant expression by Western blot analysis of whole cell lysates (50 μg/lane) was performed with anti-myc (1:1000). Anti-β-actin (1:5000) was used to establish equivalent sample loading. C, each mutant CEP4 encoding plasmid was transfected into MCF-10A cells, and after 48 h, cell motility over 6 h was measured as described under “Experimental Procedures.” The values are reported as the average of triplicate measurements ± S.D. and are representative of three independent experiments. Statistical analysis was performed with the Student’s t test that compared each selected mutant with the VC condition (**, p < 0.00001). Error bars represent S.D.
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PAGE could be detected as two bands that had a slightly higher molecular mass than the untreated protein (75 kDa) (Fig. 2A). A Western blot of the reaction product (Fig. 2B) demonstrated that CEP4 displayed a single strong phosphorylation signal when detected with an antibody (Cell Signaling Technology, Inc.) that specifically recognized the phosphorylated PKC consensus site (PKC substrate antibody). In contrast, untreated CEP4 gave no detectable phosphorylation signal. For each condition, the stained band at ~75 kDa was analyzed by MS/MS. The results of this analysis (Table 1) revealed phosphopeptides in PKCα-treated CEP4 that contained phosphorylated Ser18 and Ser80 as well as additional sites of phosphorylation (see “Discussion”). MS/MS spectra for phospho-Ser18 and phospho-Ser80 are shown in Fig. 2C.

In light of the mutagenesis and MS/MS results, preparation of the S18D/S80D (D/D) double mutant and the corresponding phosphorylation-resistant S18A/S80A (A/A) double mutant was carried out. Their expression following transient transfection of MCF-10A cells is shown in Fig. 3A. Western blot analysis demonstrated that each double mutant was equivalently expressed. In Fig. 3B, cells transfected with the double mutants were assayed for motility and showed that the D/D mutant produced 2.5-fold higher motility relative to the A/A mutant, which produced basal motility comparable with that of the vector control.

To determine whether there were additional sites of phosphorylation produced by PKC, the phosphorylation-resis-

![FIGURE 2. In vitro phosphorylation of GST-tagged human CEP4 by PKCα. A, pure, recombinant CEP4 (1.2 μg) was subjected to a phosphorylation reaction with PKCα (1 μg) for 2 h at 30 °C as described under “Experimental Procedures.” A negative control consisted of CEP4 included for the same time period in the absence of PKCα and ATP. The samples were resolved by 8% SDS-PAGE, and the gel was stained with GelCode Blue. The stained band occurring at 75 kDa was excised and submitted for analysis by MS/MS. B, samples (0.3 μg) from A were analyzed for phosphorylation by Western blotting by probing with anti-PKC substrates (1:1500) followed by probing with anti-CEP4 (1:2000) to establish equivalent sample loading. C, MS/MS spectra document that PKCα treatment produced phosphorylation of Ser18 and Ser80.]

![FIGURE 3. Expression of double site CEP4 mutants and their effects on motility. A, expression of CEP4 mutants bearing either D/D or A/A was analyzed by Western blot of whole cell lysates (50 μg/lane) with anti-myc (1:1000) or anti-β-actin (1:5000) to establish equivalent sample loading. B, each double mutant of CEP4 was transfected into MCF-10A cells, and an assay of cell motility over 6 h was carried out. The values are reported as the average of triplicate measurements ± S.D. and are representative of three independent experiments. Statistical analysis was performed with the Student’s t test that compared A/A and D/D mutants (**, p < 0.00001). Error bars represent S.D.]

| TABLE 1 | MS/MS analysis of phosphopeptides derived from phosphorylated CEP4 |
| Score | Expectation | Peptide sequence | Position | Start | End |
| 56.48 | 0.00093 | R̅GE̅P̅D̅H̅D̅E̅Q̅D̅E̅Q̅P̅S̅S̅S̅S̅S̅S̅K̅ | Ser-74 | 57 | 75 |
| 24.24 | 0.36 | R̅G̅L̅S̅L̅S̅K̅ | Ser-80 | 76 | 82 |
| 22.99 | 2 | R̅L̅V̅S̅S̅V̅ | Ser-14 | 6 | 15 |
| 17.93 | 8.2 | R̅E̅G̅P̅D̅E̅Q̅D̅E̅Q̅P̅S̅S̅S̅S̅S̅K̅ | Ser-72 | 57 | 75 |
| 15.14 | 14 | R̅R̅G̅A̅D̅L̅T̅E̅A̅ | Ser-18 | 16 | 25 |

Human CEP4 was phosphorylated by PKCα in vitro and analyzed by MS/MS as described under “Experimental Procedures.” From a total of 31 peptides (representing 64.3% coverage), the phosphorylated peptides that contained a PKC consensus site are reported, none of which were found in peptides from untreated CEP4 (42.7% coverage). Underlined residues represent the sites of phosphorylation that included Ser-80 and Ser-18.
thermore, the signal was decreased to basal levels by each of WT-CEP4 from cells treated with DAG-lactone as compared with unstimulated cells represented the basal condition. The pellets containing CEP4 protein were analyzed by Western blotting with an antibody that recognizes the phosphorylated PKC substrate consensus site (phospho-CEP4) or CEP4 antibody to determine equivalent loading. Numerical values represent the intensity of phospho-CEP4 signals relative to the VC signal (=1.0) as measured with ImageJ. These values are representative of three independent experiments. A, WT-CEP4 or phosphorylation-resistant mutants corresponding to S18A, S80A, or S18A/S80A in CEP4 were expressed as FLAG-tagged proteins in MCF-10A cells. Where indicated, the cells were treated with DAG-lactone (10 μM) or DMSO (0.05%, v/v) followed by preparation of a whole cell lysate (150 μg/sample) and immunoprecipitation with FLAG-agarose antibody (“Experimental Procedures”). The pellets containing CEP4 protein were analyzed by Western blotting with an antibody that recognizes the phosphorylated PKC substrate consensus site (phospho-CEP4) or CEP4 antibody to determine equivalent loading. Numerical values represent the intensity of phospho-CEP4 signals relative to the VC signal (=1.0) as measured with ImageJ. These values are representative of three independent experiments. A, WT-CEP4 or a related mutant was tested in a 6-h motility assay of MCF-10A cells treated with 10 μM DAG-lactone. The values are reported as the average of triplicate measurements ± S.D. and are representative of three independent experiments. Statistical analysis was performed with the Student’s t test that compared each mutant with the VC condition (*, p < 0.01; **, p < 0.0001). Error bars represent S.D.

The impact of the single site and double phosphorylation-resistant mutants was also reflected in the degree to which each mutant decreased the motility of cells stimulated by DAG-lactone (Fig. 4B). Blockade of each site individually resulted in substantially decreased motility that was further diminished by the double mutant, thus demonstrating the functional significance of each of the two sites.

**Phosphorylation of CEP4 Promotes Its Release from Constitutively Active Q61L-Cdc42**—Because CEP4 is known to bind activated (GTP-bound) Cdc42 with high affinity, a central question was whether phosphorylation of CEP4 influenced its ability to engage Cdc42 (15, 17). We addressed this question by using the constitutively active mutant of Cdc42 (Q61L-Cdc42), a mutant form that is known to bind CEP4 (15). Following bacterial expression, GST-Q61L-Cdc42 was semipurified by affinity chromatography on glutathione-Sepharose beads. A whole cell lysate was prepared from cells expressing the D/D mutant, A/A mutant, or VC. The lysate was added to the beads (collected as a pellet). Similarly, to stimulate CEP4 phosphorylation via PKC activation, cells expressing WT-CEP4 were treated with 10 μM DAG-lactone or DMSO (0.05%, v/v) followed by preparation of cell lysates. Any myc-tagged CEP4 protein that had adsorbed to the immobilized Q61L-Cdc42 was subsequently detected by SDS-PAGE/Western blotting with anti-myc.

As can be seen in Fig. 5, intracellular phosphorylation of CEP4 via DAG-lactone treatment showed decreased binding to Q61L-Cdc42. Similarly, the D/D mutant bound weakly to Q61L-Cdc42 in contrast with the strongly bound A/A mutant. These results implied that phosphorylation or pseudophosphorylation of CEP4 at Ser18 and Ser80 releases it from activated (GTP-bound) Cdc42.

**Binding Partners of Pseudophosphorylated CEP4**—To determine potential binding partners of phosphorylated CEP4, TAP was used. Each double mutant was subcloned into the TAP vector to generate CEP4 bearing two affinity peptide tags (at the C terminus) that permit two consecutive affinity chromatography steps on streptavidin-binding and calmodulin-binding resins, respectively. Lysis and chromatography steps were performed under detergent-free conditions that preserved protein-protein interactions between CEP4 and its unknown binding partners (22). Following the second chromatographic step, proteins immobilized on the beads were resolved by SDS-PAGE, and the gel was stained with GelCode Blue. Fig. 6A demonstrates the expression of each TAP mutant. As shown in Fig. 6B, the D/D mutant revealed three major bands (44, 115–125, and 250 kDa) that were unique to proteins binding to the D/D mutant. Each band was excised and submitted for MS/MS analysis (data not shown). One identified protein (125-kDa band) was TEM4 (also known as ARHGEF17), which interacts with the small GTPases. Another identified protein (250-kDa band) was PARD6G, which is involved in cell polarity and serves as an

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**FIGURE 4.** Phosphorylation-resistant CEP4 mutants block DAG-lactone-stimulated phosphorylation of CEP4 and cell motility. A, WT-CEP4 or phosphorylation-resistant mutants corresponding to S18A, S80A, or S18A/S80A in CEP4 were expressed as FLAG-tagged proteins in MCF-10A cells. Where indicated, the cells were treated with DAG-lactone (10 μM) or DMSO (0.05%, v/v) followed by preparation of a whole cell lysate (150 μg/sample) and immunoprecipitation with FLAG-agarose antibody (“Experimental Procedures”). The pellets containing CEP4 protein were analyzed by Western blotting with an antibody that recognizes the phosphorylated PKC substrate consensus site (phospho-CEP4) or CEP4 antibody to determine equivalent loading. Numerical values represent the intensity of phospho-CEP4 signals relative to the VC signal (=1.0) as measured with ImageJ. These values are representative of three independent experiments. A, WT-CEP4 or a related mutant was tested in a 6-h motility assay of MCF-10A cells treated with 10 μM DAG-lactone. The values are reported as the average of triplicate measurements ± S.D. and are representative of three independent experiments. Statistical analysis was performed with the Student’s t test that compared each mutant with the VC condition (*, p < 0.01; **, p < 0.0001). Error bars represent S.D.

**FIGURE 5.** Binding of CEP4 double mutants to constitutively active GST-Q61L-Cdc42. MCF-10A cells were transfected with a plasmid encoding either the myc-tagged WT or mutant CEP4. Where indicated, the cells expressing WT-CEP4 were treated with 10 μM DAG-lactone or DMSO (0.05%, v/v). Following bacterial expression and immobilization of GST-Q61L-Cdc42 on glutathione beads, an MCF-10A cell lysate containing WT or mutant CEP4 was incubated with the beads for 40 min with rotation at 4 °C. After washing the pellets with detergent-free lysis buffer, the bound proteins were eluted by boiling with 1× sample buffer and resolved by 8% SDS-PAGE. To determine the amount of CEP4 that had bound to GST-Q61L-Cdc42, the resulting Western blot was analyzed with anti-myc (1:1000). The blot was also analyzed with anti-Cdc42 (1:5000) to establish the equivalent amount of pelleted Q61L-Cdc42 in each sample. The results are representative of three independent experiments.
adapter that (via PAR3) binds GTP-bound Cdc42, Rac1, and atypical PKCζ/λ (23–25). In view of their co-purification with D/D-CEP4 but not the A/A-CEP4 mutant, it is possible that these proteins co-localized with phospho-CEP4 in the cell.

Both TEM4 and PARD6G were validated as direct binding partners of CEP4 by performing co-immunoprecipitation of FLAG-tagged CEP4 mutants under the same non-detergent conditions as performed with the TAP partners. Each pellet was subjected to SDS-PAGE and Western blotting and then probed with the corresponding antibody. When immunoprecipitated with the FLAG-tagged D/D-CEP4, strong bands were detected for PARD6G (60 kDa) and TEM4 (180 kDa) (Fig. 7A). In contrast, when immunoprecipitation was carried out with a lysate expressing the phosphorylation-resistant A/A-CEP4 mutant or VC, these bands were weak or undetectable. The results were consistent with a direct binding interaction between phospho-CEP4 and PARD6G or TEM4. It was further noted in these experiments that D/D-CEP4 co-immunoprecipitated a substantially lower amount of Cdc42 than did the A/A mutant, consistent with the lower affinity of phospho-CEP4 for Cdc42 as found in vitro with recombinant Q61L-Cdc42 (Fig. 5).

Because of previous reports that TEM4 activates RhoA GTPase, binds actin, and promotes migration (26–28), a possible role for this GEF in phospho-CEP4-mediated motility was addressed by use of a TEM4-specific GFP-shRNA-encoding plasmid. Knockdown of TEM4 was achieved with this reagent as shown in Fig. 7B. While TEM4 knockdown was in effect, transfection of MCF-10A cells with each CEP4 double mutant or VC was performed by assay of cell motility. As shown in Fig. 7C, silencing of TEM4 resulted in inhibition by almost 40% of the motile behavior induced by the D/D-CEP4 mutant. Furthermore, the motility of these cells was decreased to the level of motility of cells co-expressing the A/A-CEP4 mutant and TEM4 shRNA. Interestingly, TEM4 silencing had a small stimulatory effect on motility of cells expressing A/A-CEP4, suggesting that TEM4 suppresses some element in the unstimulated pathway. Overall, these results implicate the TEM4-phospho-CEP4 complex as an active component in the PKC-induced motility signaling pathway.

**Rac Activation Is Induced by Expression of Pseudophosphorylated CEP4**—In view of our previous findings that PKC activity promotes motility by activating Rac (4), we considered the possible involvement of Rac in CEP4-mediated phenotypes. To determine whether phospho-CEP4-induced motility relies on Rac activation, MCF-10A cells expressing the D/D-CEP4 mutant were treated with a Rac-specific inhibitor (NSC23766). This drug blocks the binding of GEFs, thereby preventing GDP-GTP exchange and Rac activation (29). As can be seen in Fig. 8A, highly motile cells expressing the D/D-CEP4 mutant were inhibited by 95% in the presence of 50 µM inhibitor, thereby implicating activated Rac1 in the mechanism of cell movement. It is noted that for both Cdc42 and RhoA pulldown assays showed that neither of the CEP4 mutants resulted in a changed

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**FIGURE 6. TAP of pseudophosphorylated CEP4 and its binding partners.** Mutants of CEP4 were subcloned into a TAP vector that upon expression conferred epitope tags for isolating each mutant and its associated proteins by affinity chromatography as described under "Experimental Procedures." A plasmid encoding a CEP4 mutant was transfected into MCF-10A cells, and following affinity chromatography, bound proteins were resolved by SDS-PAGE and detected by GelCode Blue staining. A, Western blot analysis of whole cell lysates containing the TAP-CEP4 mutants or the VC probed with anti-myc (1:1000). B, a GelCode Blue-stained 8% SDS-polyacrylamide gel showing that the pseudophosphorylated CEP4 (D/D) was associated with several protein bands not found in the phosphorylation-resistant CEP4 (A/A) or VC samples. Bands excised for subsequent MS/MS analysis are indicated with <.<. The results are representative of two experiments.

**FIGURE 7. Analysis of protein-protein interactions with CEP4 mutants.** A, MCF-10A cells were transiently transfected with each CEP4 (A/A or D/D) or the VC. Following preparation of cell lysates (500 µg/sample), co-immunoprecipitation of each CEP4 mutant was performed with anti-FLAG under non-detergent conditions to pull down binding proteins. Pellets were analyzed by Western blot with anti-TEM4 (1:500), anti-PARD6G (1:500), anti-Cdc42 (1:500), or anti-CEP4 (1:2000). The experiment was performed twice with identical results. B, demonstration that an shRNA reagent knocks down TEM4 expression in MCF-10A cells. Cells were transfected with the GFP-shRNA-TEM4 plasmid or the GFP-SC plasmid. After 48 h, cells were transfected with the pseudophosphorylated CEP4 double mutant (D/D), the phosphorylation-resistant CEP4 double mutant (A/A), or the VC. Forty-eight hours later, cells were lysed, and the samples were resolved by 6% SDS-PAGE and analyzed for TEM4 expression by Western blotting (75 µg/lane) with anti-TEM4 (1:500 dilution). The experiment was performed three times with identical results. C, MCF-10A transfectants were tested for motility in triplicate by detecting fluorescent elegant bar graph showing the average of replicate measurements ± S.D. and are representative of three independent experiments. Statistical analysis was performed with the Student’s t test that compared the scrambled control and shRNA condition for each mutant (*, p < 0.0001; **, p < 0.00001). Error bars represent S.D.
Phosphorylation of CEP4 by PKC Drives Motility

FIGURE 8. Pseudophosphorylated CEP4 or TEM4 activates Rac1. A, pseudophosphorylated CEP4-induced motility is eliminated by treatment of cells with NSC23766, an inhibitor of Rac1. Transfectant MCF-10A cells expressing a double mutant of CEP4 (D/D or A/A) or VC were treated with (solid bars) or without (hatched bars) 50 μM NSC23766 (in water) during the entire period of the 6-h motility assay. The values are reported as the average of triplicate measurements ± S.D. Statistical analysis was performed with the Student’s t test that compared values obtained with and without the inhibitor (*, p < 0.001; **, p < 0.0001). B, following expression of either TEM4 shRNA or the SC shRNA, Rac1 activation was measured in a pulldown assay with 600 μg of a lysate prepared from cells co-transfected with the shRNA reagent plus D/D, A/A, or VC as described under “Experimental Procedures.” Western blot analysis was performed with anti-Rac (1:500). Total Rac was determined with 70 μg of each whole cell lysate. The results are representative of three independent experiments. Error bars represent S.D.

FIGURE 9. Pseudophosphorylated CEP4 mutants induce formation of filopodia in MCF-10A cells. A, cells were transfected with a CEP4 mutant (A/A or D/D) or the VC followed by fixation with paraformaldehyde as described under “Experimental Procedures.” myc-tagged CEP4 expression was detected with anti-myc rabbit monoclonal antibody (1:300) followed by staining with FITC-conjugated goat anti-rabbit IgG (1:300) (green signals). F-actin was visualized by staining with rhodamine-phalloidin (100 nM) (red), and nuclei were visualized with Hoechst stain (blue). Images were acquired with a 63× objective. Scale bar, 10 μm. Where indicated, MCF-10A cells expressing the D/D mutant were treated with or without 50 μM NSC23766 (in water) for 2 h followed by fixation. The results are representative of a minimum of three independent experiments. B, for each condition, the fraction of transfected cells exhibiting filopodia was averaged over three independent experiments. Fifty cells were analyzed per condition. Statistical analysis was performed with the Student’s t test that compared values obtained for cells expressing A/A- and D/D-CEP4 mutants (**, p < 0.0001). Error bars represent S.D. Inh, inhibitor.

80% of transfectants as well as substantially higher numbers of filopodia than did expression of the A/A mutant. The difference in cell surface protrusions by WT-CEP4 and the A/A mutant correlated with their effects on motility (Fig. 3C) and their level of intracellular phosphorylation (Fig. 4A). Treatment of D/D-CEP4 transfectants with a Rac inhibitor (NSC23766) eliminated all actin-based features and correlated with the loss of motility (Fig. 8A). These observations showed that phospho-CEP4 produced activated Rac, and this was the mechanism that caused increased filopodia and motility of MCF-10A cells.

DISCUSSION

In this study, CEP4 was characterized as a new substrate of PKC in non-transformed MCF-10A human breast cells. Site-directed mutants of CEP4 that had been pseudophosphorylated at both Ser18 and Ser80 of this abundant Cdc42-binding protein were found to engender cell motility, a previously unrecognized...
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FIGURE 10. Model of PKC-mediated phosphorylation of CEP4 and its consequences. DAG-sensitive PKC isoforms phosphorylate CEP4 at Ser\(^{18}\) and Ser\(^{80}\), leading to its dissociation from Cdc42. Phospho-CEP4 binds TEM4 that in turn stimulates GDP-GTP exchange in Rac1 and leads to downstream events supporting motility and formation of filopodia. A Rac1 inhibitor (I) (NSC23766) that specifically blocks GDP-GTP exchange on Rac1 interrupts this process, leading to complete inhibition of motility induced by phospho-CEP4. GAPs, GTPase-activating proteins.

function of CEP4. Ser\(^{18}\) and Ser\(^{80}\) were subsequently validated by MS/MS (Fig. 2C) as direct target sites of PKCa in vitro (Table 1). An important outcome of phosphorylation at these sites was that CEP4 lost its affinity for constitutively active Cdc42 in vitro (Fig. 5) and in cells (Fig. 7A), consequently leading to the acquisition of cell motility (Fig. 3) and the development of actin-based membrane protrusions (filopodia) (Fig. 9). The finding that phospho-CEP4 operated independently of Cdc42 to produce these phenotypes revealed a new dimension in the function and regulation of this effector protein. It is noted that the other four CEP isoforms do not possess PKC phosphorylation sites (17), suggesting that they are refractory to regulation by PKC.

Earlier studies characterized the CEPs as proteins that bind GTP-bound Cdc42 with high affinity (15, 17). Cdc42 is bound at the CRIB domain in CEP4 that is located between Ile27 and GTP-bound Cdc42 with high affinity (15, 17). Cdc42 is bound at PKC sites (17), suggesting that they are refractory to regulation by other four CEP isoforms do not possess PKC phosphorylation and regulation of this effector protein. It is noted that the other four CEP isoforms do not possess PKC phosphorylation sites (17), suggesting that they are refractory to regulation by PKC.

During the course of these experiments, Cdc42 was also considered as a participant in one or both CEP4-induced phenotypes following its release from phospho-CEP4. Although principally known to promote cell polarity, Cdc42 was recently reported to suppress migration of breast cancer cells (33). This finding contrasts with the promotility effects of activated Rac1 (34). In the present study, there was no evidence that Cdc42 is involved in the PKC-mediated motility phenotype in agreement with an earlier report (4). It was further noted that adding a Cdc42 inhibitor (EMD-Millipore) resulted in a dramatic increase in cell motility regardless of the phosphorylation state of CEP4.\(^{4}\) These results implied that activated Cdc42 suppresses motility of MCF-10A cells. Interestingly, the Cdc42 inhibitor also eliminated 50\% of D/D-CEP4-induced filopodia.\(^{4}\) Thus, although our investigations continue to support a major role for activated Rac in driving cell motility, it is likely that Rac and Cdc42 each contributes to cell surface protrusions.

A previous report from our laboratory (5) showed that PKCa, PKCa\(_{\text{δ}}\), and PKCa\(_{\text{ε}}\) (representing the subcategories of conventional, novel, and atypical PKC isoforms, respectively) can each carry out phosphorylation of CEP4 in MCF-10A cells. In the
present work, DAG-lactone was used to activate DAG-dependent PKC isofoms to produce intracellular phospho-CEP4 (Figs. 4 and 5). The question of whether the same sites in CEP4 are recognized by DAG-independent isofoms such as PKCζ was not addressed in the foregoing studies. In this regard, we note that the co-localization of D/D-CEP4 with a PAR6 protein (PAR6ΔG), an adapter that comes into contact with Cdc42 and PKCζ (via PAR3), suggests a means by which an interaction between dephosphorylated CEP4 and activated PKCζ may occur, particularly in human breast cells (24). Taken together, our findings suggest a model (Fig. 10) of an emerging PKC signaling pathway in which PKC-mediated phosphorylation of CEP4 causes its release from GTP-Cdc42 followed by formation of a complex of phospho-CEP4 with a GEF (e.g. TEM4) that results in Rac1 activation, membrane protrusion, and cell movement.

In an earlier effort to elucidate the components of the PKC signaling pathway, we identified α-tubulin as a PKC substrate (35). Phosphorylation of α-tubulin at a single site (Ser165) resulted in its increased incorporation into microtubules that promoted increased interaction by microtubules with the membrane, activation of Rac, and increased motility (3). Apparently, Rac serves as a point of convergence for at least two PKC substrates, underscoring the importance of Rac-associated GEFs (and GT Pase-activating proteins) to both motility signaling and membrane protrusions in breast cancer (6, 7). In view of the fact that α-tubulin and CEP4 are both highly abundant PKC substrates that promote signaling through Rac, we speculate that their coincident phosphorylation by PKC could lead to continuous GDP-GTP exchange on Rac that consequently gives rise to aggressive cell movement.

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