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Identification and Characterization of Protein Kinase C Substrates in Human Breast Cells

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Identification and Characterization of Protein Kinase C Substrates in Human Breast Cells

By Xin Zhao

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

2014
This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
Abstract

Identification and Characterization of Protein Kinase C Substrates in Human Breast Cells

By

Xin Zhao

Advisor: Professor Susan A. Rotenberg

Aberrations in PKC signaling can lead to the development of multiple human diseases and the most prominent association of PKC with disease has been in tumor growth and metastasis. PKC and its related pathways have been recognized as promising targets for blocking the malignancy of breast cancer cells. To better understand PKC-mediated pathway in breast cancer cells, it is important to identify the cellular substrates of PKC. The main focus of this work is to identify physiologically relevant cellular substrates of PKC in human breast cells and to characterize their roles in cancer-related phenotypes. The work to be described consists of two projects: (1) identification of a new PKC substrate that contributes to cancer-related phenotypes in human breast cells; (2) characterization of the functional significance of its phosphorylation by PKC in normal and malignant human breast epithelial cells.

In the first project, the traceable kinase method was applied to identify potential PKC substrates in human breast cells. Potential substrates included those proteins that regulate or serve as effectors for the small GTPases. In light of previous studies showing that small GTPases play an important role in PKC-induced motility, the present study focused on proteins deemed to be strong candidate substrates. From ROCK1, CEP4, PAK2 and CLASP1...
four candidates, it was demonstrated that CEP4 is in fact an intracellular PKC substrate, as shown by in vitro kinase assay and intracellular phosphorylation in human breast cells under DAG-lactone stimulation. CEP4 was found to serve as an intracellular substrate for PKC-α, −δ, and −ζ by testing the impact on intracellular CEP4 phosphorylation by PKC isoform-specific shRNA reagents or kinase-defective mutants.

In the second project, the functional significance of phospho-CEP4 was investigated in MCF-10A cells and MDA-MB-231 cells. Phospho-CEP4 stimulated cell motility, slowed the cell proliferation rate, and induced filopodia formation. In MCF-10A cells, the phosphorylation of CEP4 caused its disassociation from Cdc42. Rac1 was shown to participate in phospho-CEP4-induced cell motility. As shown by others, CEP4 does not interact with Rac1 directly. Phospho-CEP4 might interact with Rac1 directly or associate with another partner to engage the Rac1 pathway to induce cell migration. This study is the first to identify CEP4 as a PKC substrate in human breast cells. The demonstrated functional significance of phospho-CEP4 to malignant phenotypes of human breast cells could provide a predictive marker for human breast cancer.
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First and foremost I would like to express the deepest appreciation to my mentor Dr. Susan Rotenberg. The faith and enthusiasm she has for science is so infectious and her optimistic attitude motivates me to keep moving forward. I appreciate all her time and insightful ideas to make my Ph.D. experience a productive, stimulating and wonderful journey. Without her guidance and persistent help, this dissertation would not have been possible.

Second, I am deeply grateful to all my dissertation committee members, Dr. Cathy Savage-Dunn, Dr. Karl Fath, Dr. Lesley Davenport and Dr. Jimmie E. Fata. The profound knowledge they showed me in our meetings deeply motivated me to explore further in biochemical world. I would like to thank them for their time, encouragement and invaluable advice in all my committee meetings over the years.

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## Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>BORG</td>
<td>Binder of Rho GTPases</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BIM</td>
<td><em>bis</em>-Indoleylmaleimide</td>
</tr>
<tr>
<td>CEP4</td>
<td>Cdc42 effector protein</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>N, N, N’, N’-ethylenediamine-tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-<em>bis</em> (2-aminoethylether)-N,N,N’,N’-tetra-acetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>MARCKS</td>
<td>Myristoylated alanine-rich C-kinase substrate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil-containing protein kinase</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>VC</td>
<td>Vector control</td>
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Chapter 1: Introduction
1.1: PKC Structure, Classification, and Regulation

Ca$^{2+}$-activated, phospholipid-dependent protein kinase (PKC) is defined by a structurally-related family of serine/threonine protein kinases that are involved in several biological processes including proliferation, differentiation, apoptosis, adhesion and migration. All members contain a highly conserved kinase domain and regulatory domain. The kinase domain resides in the C-terminal half of the protein and consists of motifs that are required for ATP/substrate binding and catalysis. The regulatory domain is contained within the amino terminal half of the protein and is defined by an auto-inhibitory pseudo-substrate domain and one or two discrete membrane targeting domains; a C1 domain that binds diacylglycerol (DAG) or phorbol ester, and a C2 domain that binds Ca$^{2+}$ (Steinberg, 2008). This family is sub-classified into three groups of isoforms based on their cofactor requirements for activation and corresponding structural differences in their regulatory domain (Figure 1.1) (Newton, 2001). The three subclasses are: (1) conventional PKCs (cPKCs) that contains both a C1 domain that functions as a DAG binding site and a C2 domain that binds Ca$^{2+}$; (2) novel PKCs (nPKCs) are structurally similar to the cPKCs, except that they lack a Ca$^{2+}$ binding site but can be stimulated by DAG; (3) atypical PKCs (aPKCs) also lack a Ca$^{2+}$-binding C2 domain and have an atypical C1 domain that makes them insensitive to DAG but responsive to other lipids such as arachidonic acid and ceramide. The group of conventional PKC isoforms is the most highly studied and best understood subclass.

The PKC family transduces cell signals by promoting lipid hydrolysis (Nishizuka, 1995). Many known membrane receptors transmit intracellular signals through activation of PKC (Kenny et al., 2007; Lee and Bell, 1991). One known pathway for PKC activation is described in Figure 1.2 (Koivunen et al., 2006). An external signal activates a G-Protein-Coupled Receptor (GPCR), which activates the GTPase enzyme phospholipase C (PLC).
PLC cleaves phosphoinositol-4,5-bisphosphate (PIP2) into DAG and inositol-1,4,5-trisphosphate (IP3). IP3 interacts with a calcium channel in the endoplasmic reticulum (ER) thereby triggering the release of Ca\(^{2+}\) into the cytoplasm. The increase in Ca\(^{2+}\) levels activates cytoplasmic PKC which translocates to the membrane and is anchored to the plasma membrane via DAG and phosphatidylserine (PS). Activated PKC isoforms phosphorylate protein substrates in the membrane and cytosol to propagate signals throughout the cell that culminate in various biological and cancer-related phenotypes.

![Figure 1.1: Scheme of primary structures of protein kinase C family members showing domain composition and activators.](image)

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trisphosphate (IP3). IP3 interacts with a calcium channel in the endoplasmic reticulum (ER) thereby triggering the release of Ca\textsuperscript{2+} into the cytoplasm. The increase in Ca\textsuperscript{2+} levels activates cytoplasmic PKC which translocates to the membrane and is anchored to the plasma membrane via DAG and phosphatidylserine (PS). Activated PKC isoforms phosphorylate protein substrates in the membrane and cytosol to propagate signals throughout the cell that culminate in various biological and cancer-related phenotypes.

![Activation of conventional PKC by an extracellular signal via G-coupled receptors.](image)

**Figure 1.2: Activation of conventional PKC by an extracellular signal via G-coupled receptors.** PKC activation is initiated by an extracellular signaling molecule (e.g. EGF) that binds to a G-protein-linked receptor (GPCR)/receptor tyrosine kinase (RTK) in the cell membrane. Next, the binding of the signaling molecule triggers the activation of a heterotrimeric G-protein on the intracellular face of the cell membrane. The G-protein activates phospholipase C (PLC) which cleaves phosphoinositol-4,5 bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). Next, IP3 interacts with a calcium channel in the endoplasmic reticulum (ER) and causes release of Ca\textsuperscript{2+} into the cytoplasm. The increase in Ca\textsuperscript{2+} levels activates PKC. Lastly, activated PKC phosphorylates its specific substrates on serine/threonine residues (Koivunen et al., 2006).

### 1.2: Cancer Biology

Cancer consists of diseases in which abnormal cells divide without control, and remains the second most common cause of death in the United States. In the last two decades, there have
been enormous studies in understanding the cellular and molecular basis of malignancy, its initiation, promotion and progression.

In 2001, Hanahan and Weinberg described six key changes that occur in cancer which all lead to enhanced tumor growth and survival: growth factor independence, evading growth suppressors, avoiding apoptosis, maintaining replicative potential, angiogenesis and invasion/metastasis (Hanahan and Weinberg, 2011). These phenotypes are developed in different tumor types via distinct mechanisms and at various times during tumorigenesis. The expansion of migration and invasive properties are key events in the oncogenic progression of cells since they drive metastasis which is the most frequent cause of death for patients with cancer. Metastasis is a multistep process in which cancer cells disseminate from the primary tumor to distant organs (Figure 1.3) (Fidler, 2003). Greater knowledge of the mechanisms that drive motility and invasion of these tumor cells is crucial to further understand metastasis and to develop strategies to prevent it.

1.3: PKC and Cancer

PKC regulates multiple aspects of tumorigenesis, including cell proliferation, angiogenesis, metastasis, and apoptosis, making it a major regulator in malignant transformation.

Early studies showing that PKC isoforms are activated by tumor-promoting phorbol esters suggested an important role for PKC in carcinogenesis (Castagna et al., 1982; Niedel et al., 1983). Activation of PKC leads to cell-type specific effects such as proliferation, differentiation, apoptosis, migration and morphology. In different cancer types, PKC isoforms have been shown to display different expression profiles and there is evidence of isoforms having specific as well as overlapping roles during cancer progression. From the three subclasses, PKC-α, −δ and −ζ are among the most highly studied isoforms.
Figure 1.3: The main steps in the formation of metastasis. (a) Cancer transformation and tumor growth. (b) Proliferation and angiogenesis: the physiological process through which new blood vessels form from pre-existing vessels. (c) Detachment and invasion: the cancer cells detach themselves from the primary tumors and enter the bloodstream. (d) Circulation: the aberrant cells travel via the blood stream. (e) Extravasation: the cells leave the blood stream and enter into the surrounding tissues (Fidler, 2003).

Immunohistochemical studies have demonstrated over-expression of PKC-α in prostate, endometrial, high-grade urinary bladder and hepatocellular cancers (Koren et al. 2000; Langzam et al. 2001; Koren et al. 2004; Varga et al. 2004). However, immunohistochemistry of advanced stage human breast tumors revealed that in the majority of specimens PKC-α was down-regulated (Kerfoot et al. 2004). PKC-α mediates anti-proliferative signaling and plays a tumor-suppressing role in a number of cell types including intestinal, pancreatic, and mammary cells (Detjen et al., 2000; Sun and Rotenberg, 1999). However, in other models,
PKC-α promotes proliferative and tumorigenic responses (Jiang et al. 2004; Sharma et al. 2007; Wu et al. 2008). Peltonen’s group showed that the PKC inhibitor Go6976 promotes cellular junction formation and consequently inhibits invasion in cultured high grade urinary bladder carcinoma cells (Koivunen et al., 2004). Previous studies in the Rotenberg lab reported that over-expression of PKC-α in non-transformed MCF-10A human breast cells caused a pronounced increase in motility with loss of detectable E-cadherin, decreased proliferation (due to slowed passage through G1 of the cell cycle) and radical alterations in morphology (extensive and well-defined organization of actin stress fibers) (Sun and Rotenberg, 1999).

Similar to the expression of PKC-α, PKC-δ can be either up-regulated as in hepatocellular cancer (Tsai et al., 2000) or down-regulated as in urinary bladder cancer (Langzam et al., 2001). The most important function of PKC-δ is thought to be promotion of apoptosis. PKC-δ activity is known to result in many pro-apoptotic signals and shows pro-apoptotic effects in many cell lines, such as in UV-light-induced apoptosis of cultured breast cancer cells (Zeidan et al., 2008). A recent study showed that PKC-δ has a pro-survival role by suppressing the ERK1/2 pathway since depletion of PKC-δ in MDA-MB-231 human breast cancer cells was sufficient to drive these cells into apoptosis (Lonne et al., 2009).

Similarly, both tumor-promoting and tumor-suppressing roles have been attributed to PKC-ζ. Diaz-Meco’s group showed that PKC-ζ deficient mice displayed an increase in Ras-induced lung carcinogenesis, suggesting a role for this kinase as a tumor suppressor in vivo (Galvez et al., 2009). Another recent study showed that PKC-ζ inhibited colon cancer cell growth and enhanced differentiation and apoptosis, while inhibiting the transformed phenotype of these cells (Mustafi et al., 2006). For breast cancer development, PKC-ζ activates the proliferative, invasive, and metastatic potential of breast cancer cells. Studies
demonstrated that PKC-ζ was required for human breast cancer cell chemotaxis (Liu et al., 2009), and PKC-ζ overexpression in MDA-MB-468 stimulated cell motility (Sun et al., 2005). The stable overexpression of PKC-ζ in immortalized mammary epithelial cells (NMuMG) activated the mitogenic ERK pathway, leading to profound effects on the ability of NMuMG cells to proliferate, adhere, migrate, and secrete proteases (Utreger et al., 2005).

PKC isoforms apparently have overlapping, different, and even opposing biological functions in the context of proliferation, transformation, and metastasis. There is a great degree of cell type specificity which is likely due to the differential expression level of each PKC isoform as well as varying ability to access intracellular compartments where protein substrates are located.

The upstream or downstream components of PKC signaling pathways that determine its functional effects in a specific context are not completely defined. In this project, we sought to identify PKC substrates and to characterize how these substrates affect cancer-related phenotypes in human breast cell lines.

1.4: PKC Substrates

The functions of PKC isoforms in cancer-related attributes result from their actions on protein substrates in signaling pathways that support phenotypes such as motility, adhesion, and proliferation. Identification of an intracellular substrate for each kinase could provide a phospho-protein marker for detecting activity through a specific PKC-related signaling pathway and also provide a novel target for drug discovery.

Evaluation of synthetic peptides as artificial substrates of PKC led to characterization of the consensus phosphorylation motif K/R1-3(X)0-2S/T(X)0-2R/K1-3 (where X indicates any amino acid) (Kemp and Pearson 1990; Pearson and Kemp 1991). The observation that a Ser/Thr
residue flanked by basic amino acids presents a good motif for PKC phosphorylation was confirmed by the presence of this consensus sequence in many in vivo substrates. Moreover, the combined use of motif identification and database search provides an effective strategy to predict the likely substrates of a protein kinase.

Until now, very few known intracellular PKC substrates have undergone extensive characterization for their functional significance; however, there are some PKC substrates that were found related to cell motility and morphology. Some of these protein substrates are listed below.

**MARCKS:** Myristoylated alanine-rich C-kinase substrate (MARCKS) is a filamenous (F) actin crosslinking protein that has been implicated in membrane and cytoskeleton interactions and plays important roles in cell shape, motility and transmembrane transport (Hartwig et al., 1992; Herget et al., 1992, 1995; Blackshear, 1993; Taniguchi and Manenti, 1993). In mouse melanoma cells, MARCKS with pseudo-phosphorylated mutations (Ser → Asp) at Ser-152, Ser-156 and Ser-163 (the sites phosphorylated by PKC) resulted in its dissociation from the plasma membrane and cytoskeleton and translocated into the cytosol. This event facilitated the rearrangement of the actin cytoskeleton at the plasma membrane and promoted cell migration (Chen and Rotenberg, 2010).

**VASP:** Vasodilator-stimulated phosphoprotein (VASP) is a member of the Ena-VASP protein family which is involved in actin polymerization and interacts with other proteins involved in cell motility. VASP is known to undergo phosphorylation at Ser-157 by PKC (Wentworth et al., 2006). The phosphorylation of VASP plays an important role in remodeling of the actin cytoskeleton in lamellipodia (membrane ruffling), filopodia (or focal adhesions), and stress fiber formation.
MAPs: Microtubule-associated proteins (MAPs) are proteins that interact with microtubules of the cytoskeleton. Of particular interest is that MAP4 underwent phosphorylation at Ser-815 by a bovine brain preparation of conventional PKC isoforms (Mori et al., 1991). Since Ser-815 is directly involved in the binding between a microtubule and MAP4, its phosphorylation by PKC inhibits the ability of MAP4 to promote microtubule assembly.

IQGAP: IQGAPs were initially identified as an effector for Cdc42 and Rac1 (Fukata et al., 2002; Kuroda et al., 1999). IQGAPs inhibit the GTPase activity of Cdc42 and Rac1 by stabilizing their GTP-bound form, and are consistent with their involvement in actin-dependent functions such as cell shape and motility (Bashour et al., 1997). IQGAP, as an effector for Cdc42 and Rac1, was shown to be involved in the formation of cell–cell adhesion sites through binding to E-cadherin and β-catenin (Kuroda et al. 1999; Noritake et al. 2004). In addition, IQGAP proteins also participate in cancer cell invasion and metastasis. Their expression was up-regulated in various invasive human cancers and the elevated levels of IQGAP-1 were associated with a poor prognosis (Dong et al. 2006). Tandem mass spectrometry analysis (MS/MS) and immunoprecipitation experiments revealed that Ser-1443 of IQGAP1 was phosphorylated in vivo, potentially by PKC-ε (Grohmanova et al., 2004). However, IQGAP has not been further studied as a substrate of other PKC isoforms.

1.5: The Traceable Kinase Method

The Traceable Kinase Method initially developed by the Shokat laboratory offers an approach to identify direct substrates of a protein kinase (Liu et al., 1998a). In this approach, a protein kinase of interest is genetically engineered to accept a non-natural phosphate donor substrate (A*TP) that cannot be bound by the corresponding wild-type protein kinase or other protein kinases (Fig.4). Modification of the ATP binding active site entails site-directed mutagenesis at a residue (typically methionine or isoleucine) that comes closest to the N6-
amino group of the adenosine moiety of bound ATP. By replacing this residue with glycine or alanine, more space is created so that a more “bulky” ATP analogue can be accommodated. Consequently, the mutant kinase can accept a structurally modified ATP analogue that is derivatize at the N⁶-amino group. Following transfer of the γ-phosphate, direct substrates can be identified in a gel by autoradiography or by Western blot with an antibody that recognizes the phosphorylated PKC consensus site. The strength of this approach rests on the inability of the wildtype enzyme or any other protein kinase to bind the ATP analogue, therefore reducing the level of background phosphorylation. It is important to test the mutant kinase with several ATP analogues to determine which one best supports the activity of the mutant while being minimally used by the wildtype enzyme.

This strategy was successfully applied to identify the direct substrates of several protein kinases: v-Src (Liu et al., 1998a, 1998b), c-Jun N-terminal protein kinases (JNK) (Habelhah et al., 2001) and CDK2 (Polson et al., 2001). In the first application of this method, the Shokat group determined the first direct substrates of v-Src in fibroblasts (Shah and Shokat, 2002). In that study, they determined from an X-ray crystal structure of v-Src co-crystallized with ATP the appropriate residue (Ile-338) for mutagenesis due to its proximity to the N⁶-amino group in ATP. Later, Abeyweera and Rotenberg applied the Traceable Kinase Method using an ATP analogue ([γ-³²P]-phenyl-N⁶-ATP) for characterization of α6-tubulin as a new PKC substrate in MCF-10A cells with a traceable PKC-α mutant (Figure 1.5) (Abeyweera and Rotenberg, 2007). By aligning the ATP binding site of the PKC-α sequence with that of PKC-δ and PKC-ζ to identify the appropriate sites for mutagenesis, Dr. Rotenberg’s group also successfully developed and compared the phospho-protein profiles for PKC-δ, and PKC-ζ mutants, details of which are discussed in Chapter II (Chen et al., 2012).
Figure 1.4: The Traceable Kinase Method. A protein kinase of interest is engineered to accept a non-natural phosphate donor substrate (A*TP) that is poorly accepted by the wild-type protein kinase. The active site is modified so that it can accommodate a structurally modified γ-32P-labeled nucleotide A*TP. By comparing the autoradiograph with the Coomassie blue stained gel, radioactive bands are excised from the gel for identification by MS/MS (Shah and Shokat, 2003).

1.6: The Small Rho GTPases –Rac, Cdc42, and Rho

The small Rho GTPases comprise a family of small signaling G proteins (20-30kDa), which are members of the Ras superfamily. The most studied members are Rac1, Cdc42, and RhoA. Rho GTPases have been described as “molecular switches” that play a role in actin dynamics, cell proliferation, apoptosis, motility, gene expression and many other common cellular functions. They cycle between an active GTP-bound state and an inactive GDP-bound state (Figure 1.6) (Etienne-Manneville and Hall, 2002). In the GTP-bound form, they are able to
interact with effector or target molecules to initiate downstream responses. Hydrolysis of the GTP to GDP by GTPase activating protein (GAPs) drives these interactions while exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors (GEFs) which completes the cycle. Guanine nucleotide dissociation inhibitors (GDI) maintain small GTPases in the inactive state.

Figure 1.5: Design of ATP binding site for mutants of PKC-α. (A). Three-dimensional structure of PKA with bound ATP in which the adenosine moiety is delineated by the translucent surface. (B). Alignment of primary sequences in the vicinity of the ATP binding site for v-Src, PKA, and PKC-α. (C). In vitro phosphorylation of protein substrates co-immunoprecipitating with PKC-α mutant M417A (Abeyweera and Rotenberg, 2007).

Rac1, Cdc42 and RhoA play a vital role in cancer progression. All aspects of tumor progression related cell migration, cellular polarity, cell adhesion, cytoskeletal reorganization, and their related signal transduction pathways are controlled through the interplay between the Rho GTPases and their effectors. In Swiss 3T3 cells, constitutively activated mutants of Rho were found to induce the assembly of focal adhesion and actin stress fibers (Ridley and Hall, 1992). Rac1-regulated signal transduction pathway can
stimulate actin polymerization to form actin-rich surface protrusions, notably lamellipodia and membrane ruffles (Ridley et al., 1992). Microinjection of constitutively active Cdc42 into Swiss 3T3 cells triggered the formation of actin-rich, finger-like membrane extensions (filopodia) (Nobes and Hall, 1995). Rho GTPases also play an important role in the establishment of functional cell-cell contacts (adherents junctions and tight junctions) and in regulating the cell polarization. These facts reveal the important role of Rho GTPases in cell migration (Figure 1.7).

**Figure 1.6: The Rho GTPase cycle.** The Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. The cycle is highly regulated by three classes of protein: guanine nucleotide exchange factors (GEFs) catalyze nucleotide exchange and mediate activation; GTPase-activating proteins (GAPs) stimulate GTP hydrolysis, leading to inactivation; and guanine nucleotide exchange inhibitors (GDIs) maintain the GTPase in an inactive state. In the GTP-bound form, they are able to interact with effector or target molecules to initiate downstream responses (Etienne-Manneville and Hall, 2002).

The different Rho GTPase functions are regulated by their specific effectors that bind to the activated Rho proteins through their GTPase binding motif such as the CRIB (Cdc42/Rac1 interactive binding) domain. To date, there are approximately 40 potential effectors of Rac1, Rho and Cdc42 that were identified by using affinity chromatography and the yeast two-hybrid system. For example, ROCK (Rho-associated coiled-coil-containing protein kinase) that is an effector of Rho is required for the assembly of stress fibers and focal adhesions (Ishizaki et al., 1997). WASP and PAK, as effectors of Cdc42, are involved in the formation
of filopodia downstream of Cdc42 (Rohatgi et al., 1999; Zhao et al., 1998), whereas IQGAP1 and IQGAP2 are effectors for Rac1 and Cdc42 and function in actin polymerization (Erickson et al., 1997).

Figure 1.7: The effect of Rho GTPases in different steps of cell migration. Rho GTPases play important roles in lamellipodia or filopodia extension, formation of new adhesions, cell body contraction, tail detachment and cell polarity (Ridley, 2001).

PKC-α also is considered to be a downstream effector of Rho GTPases because PKC-α undergoes a protein-protein interaction with RhoA, Cdc42 and Rac1, and causes the phosphorylation of downstream PKC-α substrates (Flynn et al., 2000; Kamada et al., 1996; Cook et al., 2006). There is some evidence showing that the downstream substrates of PKC like MAP kinase and IQGAP are also effectors of Rho GTPases. In 1999, Dr. Rotenberg’s group found that the overexpression of PKC-α induced the formation of lamellipodia and membrane ruffling and caused a dramatic increase in cell motility (Sun and Rotenberg, 1999). These PKC-α–generated phenotypes were related to the function of Rac1 since the dominant negative mutant of Rac1 impaired PKC-induce motility by 40% whereas the dominant negative mutants of RhoA and Cdc42 were ineffective. A detailed mechanism
describing how Rho GTPases participate in PKC-induced malignant phenotypes has not yet been addressed.

1.7: Cdc42 Effector Protein-4 (CEP4)

CEP4 (Cdc42 Effector Protein-4) or Borg4 (Binder of Rho GTPase-4) is a member of the Cdc42-binding effector family. Rotenberg’s group demonstrated CEP4 is a PKC substrate in MCF-10A cells (Chen et al., 2012). Initially named MSE55 (marrow stromal/endothelial cell protein with a molecular mass of 55kDa), CEP1 was the first member of the family to be characterized. A common motif found in the majority of Cdc42 and Rac effector proteins is the conserved 16-aa CRIB motif, which serves as a binding site for Cdc42 and Rac. By searching the GeneBank™ database for similarity with the CRIB domain, MSE55 was identified to be a target protein for Cdc42 and Rac GTPase (Drechsel, 1995). In 1999, MSE55 was reported to be a Cdc42 non-kinase effector protein that mediates actin cytoskeleton reorganization at the plasma membrane in NIH 3T3 cells (Burbelo et al., 1999). By performing a large two-hybrid screen of a whole mouse embryo library with TC10 GTPase as bait, Joberty et al. discovered a new family of Cdc42/TC10 binding proteins that they called BORG (Binders Of Rho GTPases) proteins. One member of this family was identical to the previously reported protein MSE55 and was named Borg5 (Burbelo et al., 1999). Later, Dr. Burbelo’s group identified and sequenced three additional human Expressed Sequence Tags (EST) cDNA clones that contained a CRIB domain and two additional regions of amino acid homology to MSE55 by using the amino acid sequence of MSE55 to query the EST database. These cDNA clones, designated CEP2, CEP3, and CEP4, encode proteins of 210, 254, and 356 amino acids, respectively. Later, they also identified and sequenced a mouse cDNA clone designated mCEP5, encoding a protein of 150 amino acids that contains features in common with MSE55. They proposed that MSE55 was renamed as
CEP1 to better describe its structural and functional relationship to other members of the CEP family (Figure 1.8).

All CEP members contain the 16-amino acid CRIB consensus sequence which is required for binding Cdc42. Following site-directed mutagenesis to create the triple alanine mutant CEP1-D36A, P41A, H47A, it was found that these mutations eliminated the interaction of CEP1 with Cdc42, which single site mutations were insufficient to block binding. The CRIB triple mutant also showed fewer cellular extensions and more stress fibers when compared with the wildtype CEP1 in NIH 3T3 cell (Burbelo et al., 1999).

Genetically engineered overexpression of an individual CEP in fibroblast cells induced pseudopodia formation. The CEP protein acted as a Cdc42 downstream effector, and suggested a role in inducing actin filament assembly and cell shape changes. Moreover, in keratinocytes, CEPs expression caused the loss of F-actin and reduced levels of E-cadherin at adherent junctions, thereby suggesting that CEP interferes in the assembly of normal adherent junctions (Hirsch et al., 2001). CEPs are also known to associate with septins and alter septin organization to form long, thick septin filaments. GTP-Cdc42 negatively regulates septin organization by binding to CEPs, thereby inhibiting their association with septins (Joberty et al., 2001). Since 2001, there has not been any research on the CEP proteins. The molecular functions and mechanisms of individual CEPs in mammalian cells therefore remain unexplored.
Figure 1.8: The CEP family of proteins. (A). Protein sequence alignments of the CEP proteins. (B). Sequence alignment of CRIB, CI, and CII regions with other signalling molecules. (C). Polygenetic tree (Hirsch et al., 2001).
In the work to be presented, the following subjects will be addressed: (1) In Chapter 3, in a study that applied the Traceable Kinase Method (Chen et al., 2012), proteins that co-immunoprecipitate with traceable PKC-α, -β, and -ζ are regarded as potential substrates of these PKC isoforms in MCF-10A cells. From several potential PKC substrates, CEP4 was identified as an intracellular PKC substrate; (2) In Chapter 4, phosphorylation site mutants of CEP4 were constructed to determine the intracellular functional significance of its phosphorylation at PKC consensus sites and to study the mechanistic significance of phospho-CEP4 in cancer-related phenotypes in non-transformed MCF-10A cells; (3) In Chapter 5, the dominant negative effect of the phosphorylation-resistant CEP4 mutant was explored in malignant human breast cells; (4) In Chapter 6, the significance of this work is discussed.
Chapter 2: Materials and Methods
2.1 Materials

Human breast epithelial cells (MCF-10A cells) were obtained from The Barbara Ann Karmanos Cancer Center and MDA-MB-231 cells (metastatic human breast cancer cells) were from the ATCC (Manassas, VA). Competent DH5α cells, BL21 competent cells, cell culture media, antibiotics, serum, insulin, DABCO, DNA primers and secondary antibodies (FITC-conjugated goat anti-rabbit and Alexa Fluor 594-conjugated goat anti-mouse) were purchased from Life Technologies (Carlsbad, CA). The pCMV6-CEP4 construct was purchased from Origene (Rockville, MD), pGL4.5 (Luc/CMV/Hygro) vector was from Promega (Madison, WI), and the pGEX-2T-Cdc42-Q61L plasmid was obtained from Addgene (Cambridge, MA). Quick-Change Mutagenesis kit was purchased from Agilent Technologies, Inc. (Santa Clara, CA). OmicsLink™ shRNA expression clones against PKC-α, -β and -δ were purchased from GeneCopoeia™ (Rockville, MD), and a HuSH-29 shRNA expression vector targeting TEM4 (ARHGEF17) was obtained from Origene (Rockville, MD). PolyExpress in vitro DNA transfection reagent was purchased from Excellgen, Inc. (Gaithersburg, MD). Rabbit anti-CEP4 antibody was purchased from Bethyl Laboratory (Montgomery, Texas) and anti-TEM4 was obtained from Novus Biologicals (Littleton, CO). HRP-conjugated secondary antibodies and PARD6G primary antibody were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Beta-actin, phospho-PKC substrate antibody, Myc-tag mouse Ab and Myc-tag rabbit mAb were acquired from Cell Signaling Technology, Inc. (Beverly, MA), and rabbit IQGAP monoclonal antibody was from Abcam (Cambridge, MA). EZview red Anti-FLAG M2 affinity gel beads, protease inhibitor cocktail, and phosphatase inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). B-PER bacterial protein extraction reagent, bacterial protease inhibitor cocktail, glutathione-agarose and West Pico chemiluminescence reagents were purchased from Pierce Biotechnology (Rockford, IL). Rac1 inhibitor, Cdc42/Rac1 GTPase inhibitor, and bis-indoleylmaleimide
were obtained from Calbiochem-Millipore Corp. (Billerica, MA). The DAG-lactone reagent (JH-131E-153) was a kind gift from Dr. V. Marquez (NCI-Frederick, NIH).

2.2 Plasmid Construction

2.2.1 Construction of Plasmids Encoding Pseudo-Phosphorylated or Phosphorylation-Resistant Mutants of CEP4

Substitution of serine residues by either an aspartate codon (pseudo-phosphorylated residue) or alanine codon (phosphorylation-resistant residue) was carried out by the Quick-change method. The single site mutants were made in the wild type construct pCMV6-CEP4 using a commercially available site-directed mutagenesis kit (Agilent). For each mutant, two mutagenesis primers (Invitrogen) were designed and synthesized according to the desired mutation. The primers that were used to produce the pseudo-phosphorylated mutants at Ser-18, Ser-77, Ser-80 and Ser-86 are given in the table below:

<table>
<thead>
<tr>
<th>CEP4 pseudo-phosphorylated Mutants</th>
<th>CEP4 Mutants Primer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP4 S18D</td>
<td>5’ CAC-TCC-AAG-CGC-CGT-GAC-CGA-GCG-GAC-CTC-ACG 3’</td>
</tr>
<tr>
<td></td>
<td>5’ CGT-GAG-GTC-CGC-TCG-CTG-AGG-GAG-CCG-CTT-GGA-GTG 3’</td>
</tr>
<tr>
<td>CEP4 S77D</td>
<td>5’ TCA-TCT-TCC-AAA-CGC-GAC-CTC-CTG-TCC-AGG-AAG 3’</td>
</tr>
<tr>
<td></td>
<td>5’CTT-CCT-GGA-CAG-GAG-GTC-GCG-CTT-GTA-AAG 3’</td>
</tr>
<tr>
<td>CEP4 S80D</td>
<td>5’ AAA-CGC-AGT-CTC-CTG-GAC-AGG-AAG-TTC-CGG-GGC 3’</td>
</tr>
<tr>
<td></td>
<td>5’ GCC-CGG-GAA-CTT-CTT-GTC-CAG-GAG-CT-CTG-TTT 3’</td>
</tr>
<tr>
<td>CEP4 S86D</td>
<td>5’ AGG-AAG-TTC-CGG-GGC-GAC-AAG-CGG-TCA-CAG-TCG 3’</td>
</tr>
<tr>
<td></td>
<td>5’ CAG-CTG-TGA-CCG-CTT-GTC-GCC-CGG-GAA-CTT-CTT 3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CEP4 phosphorylation-resistant Mutants</th>
<th>CEP4 Mutants Primer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP4 S18A</td>
<td>5’ CAC-TCC-AAG-CGC-CGT-GCC-CGA-GCG-GAC-CTC-ACG 3’</td>
</tr>
<tr>
<td></td>
<td>5’CGT-GAG-GTC-CGC-TCG-GGC-AGC-GCG-CTT-GGA-GTG 3’</td>
</tr>
<tr>
<td>CEP4 S80A</td>
<td>5’ AAA-CGC-AGT-CTC-CTG-GCC-AGG-AAG-TTC-CGG-GGC 3’</td>
</tr>
<tr>
<td></td>
<td>5’ GCC-CGG-GAA-CTT-CTT-GGC-CAG-GAG-ACT-GCG-TTT 3’</td>
</tr>
</tbody>
</table>

Table 2.1: The single site mutation primers of CEP4 pseudo-phosphorylated and phosphorylation resistant mutants.

2.2.2 Subcloning of Myc-tagged CEP4-WT/-18D80D/-18A80A into pCTAP\_Vector for Tandem Affinity Purification (TAP)
The cDNAs for Myc-tagged WT-CEP4 and Myc-tagged mutants (~1.060 kb) were expressed from a pCMV6 vector (4.929 kb) or a pCTAP<sub>A</sub> vector (4.5 kb), followed by purification and digestion with BamHI and EcoRV. The digested products were purified on a 1% agarose gel and eluted with Wizard SV gel clean up reagents. Each eluted cDNA insert (~1.060 kb) was subcloned into BamHI/EcoRV sites of pCTAP<sub>A</sub> vector (~4.5 kb). All clones were verified by DNA sequencing (Macrogen, Inc.). At least two clones for each constructs were selected and expressed in MCF-10A cells. Following Western blot, CEP4 expression was demonstrated by evidence of a 60kDa band using rabbit monoclonal anti-Myc diluted to 1:1000 (Cell Signaling Technology, Inc.).

2.3 Transformation of Bacterial Cells and Plasmid Preparation

Competent DH5α cells were gently thawed on ice and 50 μl cells were transferred to a pre-chilled Eppendorf tube. Plasmid DNA was diluted to approximately 50 ng/μl. DNA (50ng) was added to 50 μl cells and the mixture was incubated on ice. After 30 minutes, the Eppendorf tube was subjected to 42°C heating for 45 seconds, and then placed on ice for 2 minutes to reduce the damage to DH5α cells. Pre-warmed (to 37°C) S.O.C medium (900 μl) was added to the Eppendorf tube and incubated in a water bath shaker for 2 hours at 37°C. The resulting culture (250 μl) was spread on an agar plate containing the appropriate antibiotic. The plate was incubated in a 37°C incubator for over 16 hours. Single colonies were picked and cultured for subsequent plasmid preparation. Plasmids were isolated from DH5α by use of the Genopure Plasmid Midi-prep kit (Roche, Indianapolis, IN).

2.4 Cell Culture and Transfection

2.4.1 Cell Culture
MCF-10A cells were cultured in 10-cm plates (BD Falcon) at 37°C and 5% CO₂ in DMEM:F12 media supplemented with 5% horse serum, insulin (10 μg/ml), epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), and hydrocortisone (0.5 μg/ml), and maintained with antibiotics [2% penicillin/streptomycin, and fungizone (0.5 μg/ml)]. MDA-MB-231 cells were grown in high glucose with L-glutamine DMEM (ATCC) supplemented with 10% fetal bovine serum, and antibiotics [1% penicillin/streptomycin, and fungizone (0.5 μg/ml)]. Cells were split once per week and washed with PBS. Cells were recovered from the plates by trypsin-EDTA (0.25%) treatment and sub-cultured in a 1:3 ratio every 3–4 days. For experiments in which cell lysates were prepared, cells were removed from the plates by scraping.

2.4.2 Transient Transfection

Cells were re-plated one day prior to transfection so that the cell density was 70-80% on the day of transfection. For 60-mm dishes, plasmid DNA (5 μg) was combined with PolyExpress reagent (15 μl) and incubated for 15 minutes at room temperature. The reagent/DNA (3:1) mixture was added to the cells for 18-24 hours followed by replacement with fresh complete medium. For cells transfected with PolyExpress, the transfection efficiency was typically 60%-80% at 48 hours post-transfection.

2.4.3 Stable Transfection of MDA-MB-231 Cell Line

MDA-MB-231 cells were cultured in DMEM containing L-glutamine (ATCC), 10% FBS, 0.2% fungizone, and 1% penicillin/streptomycin (Life Technologies, Inc.). Stable transfection of MDA-MB-231 cells in complete medium was carried out using Lipofectamine (Invitrogen) as transfection reagent. MDA-MB-231 cells were transfected with wildtype, S18D-80D or S18A-80A mutants of a Myc-DDK tagged CEP4 cDNA construct (8 μg) and
Lipofectamine 2000 (Invitrogen, 12 μl for each well in a 6-well plate). At 48 hours post-transfection, the cell medium was replaced with selection medium containing 1000 μg/ml G418 (50 mg/ml) for 4-5 days. Individual colonies from the transfected cells were isolated after 3-4 weeks of a gradual reduction in the concentration of the selection drug to 600 μg/ml. All stably transfected cell lines were maintained in growth medium containing G418 500 μg/ml. Cells were replaced with fresh media containing drug every 2 days. Protein expression of Myc-tagged constructs in stable cells was verified by Western blot analysis using a rabbit polyclonal anti-Myc antibody (1:1000, Cell Signaling Technology).

2.4.4 shRNA Transfection

Short hairpin RNA (shRNA) expression constructs specific for human PKC-α, PKC-δ, and PKC-ζ and for the scrambled control shRNA reagent were from GeneCopoeia (Rockville, MD). The shRNA-encoding plasmid (5 μg for a 60 mm plate) was transfected with PolyExpress DNA transfection reagent in a ratio of 1:3 (μg shRNA/μl transfection reagent) in serum-free medium. The reagent mixture was added to cells in 2.8 ml complete medium and incubated with cells for 96h at 37°C (5% CO₂). The bi-cistronic shRNA-encoding plasmid co-expressed the shRNA product and the green fluorescent protein (GFP), thereby allowing a determination of transfection efficiency. Protein knockdown was verified by Western blot analysis.

2.5 In vitro Protein Kinase Activity Assay

Pure recombinant CEP4 (200 ng), PAK2 (300 ng), or CLASP (300 ng) were diluted in ice cold 1X kinase buffer that contained 25 mM TRIS (pH 7.4), 10 mM MgCl₂, 0.5 mM EGTA, 1 mM DTT, and 0.1 mg/mL phosphatidyserine with either 5 μM DAG-lactone (for PKC-α and -δ) or 1 μM ceramide (for PKC-ζ). Each of the reactions was tested in vitro with pure,
recombinant PKC-α (170 ng), PKC-δ (100 ng), or PKC-ζ (166 ng). In order to ensure that PAK2 and CLASP1 were unphosphorylated prior to the kinase reaction, PAK2 and CLASP1 recombinant proteins were incubated with PP2A (25 μM) for 2h at 30°C, followed by addition of phosphatase inhibitors and incubation on ice for 30 minutes. The kinase reaction was initiated by addition of ATP (100 μM final concentration) and the mixture was incubated at 30°C for 30 min. The kinase reaction was quenched with 5X SDS sample buffer and analysis of the phosphorylation reaction was conducted by Western blot with anti-PKC substrate antibody (1:1000).

2.6 Cell Lysis and Western Blot

Cells were disrupted by sonication for 10 seconds for three times in the presence of lysis buffer [50 mM TRIS (pH 7.4), 1 mM NaCl, 2 mM EGTA, 2 mM EGTA, 1% Triton X-100, 0.1% protease inhibitors and 1% phosphatase inhibitors], and centrifuged at 10,000 x g for 10 minutes to remove insoluble material. The protein concentration was determined with Bio-Rad protein dye reagent. Samples of known protein concentration were denatured with 5X SDS sample buffer [50% glycerol (v/v), 1% SDS, 0.05% bromophenol blue, 0.4 M Tris pH 6.8, and 2 mM dithiothreitol (DDT)], followed by heating at 95°C for 5 minutes. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore Corp.), and probed with the appropriate primary antibody. Immunoreactive bands were detected with peroxidase-conjugated secondary antibody and detected by chemiluminescence (Pierce Biotechnology, Rockford, IL).

2.7 Immunoprecipitation

2.7.1 Immunoprecipitation with CEP4 Antibody

MCF-10A cells were treated with DAG-lactone (10 μM) or DMSO (0.05 % v/v) for 60
minutes at 37°C. The cells were harvested and dissociated by trypsinization followed by three washes with 5 ml complete medium plus one wash with 5 ml serum-free medium. Cell lysates were prepared by sonication 3x10 seconds in 500 μl lysis buffer [50 mM TRIS (pH 7.4), 150 mM NaCl, 5 mM EGTA and 5 mM EDTA, 1% Triton X-100, 10 μM bis-indoleylmaleimide, 0.1% protease inhibitors and 1% phosphatase inhibitors], followed by centrifugation at 10,000 x g for 10 minutes. The supernatant was pre-cleared using 30 μl protein A/G-agarose beads. The pre-cleared lysate was incubated with 2.5 μl CEP4 antibody overnight at 4°C, followed by incubation of 50 μl protein A/G agarose beads for 1 hour. The immuno-complex was obtained by centrifugation at 10,000 x g for 10 minutes and washed three times in 500 μl lysis buffer. The bound proteins were eluted with 1X SDS-PAGE sample buffer by heating at 95°C for 5 minutes. Samples were resolved by SDS-PAGE (typically 8%), transferred to a PVDF membrane, and immuno-reactive bands were detected by the appropriate primary antibody.

2.7.2 Immunoprecipitation with EZview Red ANTI-FLAG M2 Affinity Beads

MCF-10A cells expressing CEP4 wildtype or its related mutants were harvested and dissociated by trypsinization followed by three washes with 5 ml completed medium and one wash with 5 ml serum-free medium. Cell lysates were prepared by sonication 3x10 seconds in 500 μl hypotonic, detergent-free lysis buffer [20 mM TRIS (pH 7.4), 2mM MgCl₂, 2 mM EGTA and 1 mM DTT, 10 μM bis-indoleylmaleimide, 0.1% protease inhibitors, and 1% phosphatase inhibitors], followed by centrifugation at 10000 x g for 10 minutes. The supernatant was pre-cleared using 30 μl mouse IgG-agarose beads. Sixty μl of EZview red anti-FLAG M2 affinity beads (pre-equilibrated in cold TBS) were applied to the pre-cleared lysate and incubated for 2 hours at 4°C. The immuno-complex was obtained by centrifugation at 8200 x g for 5 minutes and washed three times in hypotonic, detergent-free lysis buffer.
Bound proteins were eluted in 1X SDS-PAGE sample buffer and heating at 95°C for 5 minutes. Samples were resolved by 8% SDS-PAGE gel, transferred to a PVDF membrane, and immuno-reactive bands were probed with the appropriate primary antibody.

### 2.8 Assay of Binding of CEP4 mutants to Cdc42

MCF-10A cells expressing Myc-tagged wildtype CEP4, S18D-80D-CEP4, or S18A-S80A-CEP4 were lysed in 500 µl hypotonic, detergent-free lysis buffer [20 mM TRIS (pH 7.4), 2mM MgCl₂, 2 mM EGTA and 1 mM DTT, 10 µM bis-indoylmaleimide, 0.1% protease inhibitors, and 1% phosphatase inhibitors], followed by centrifugation at 10,000 x g for 10 minutes. The supernatant was pre-cleared using 30 µl mouse IgG-agarose beads. Sixty µl of EZview red anti-FLAG M2 affinity beads (pre-equilibrated in cold TBS) were applied to the pre-cleared lysate and incubated for 2 hours at 4°C. The immuno-complex was obtained by centrifugation at 8200 x g for 5 minutes and washed three times in hypotonic, detergent-free lysis buffer. The bound proteins were eluted by 1X SDS-PAGE sample buffer by heating at 95°C for 5 min. Samples were resolved by 8% SDS-PAGE gel, transferred to a PVDF membrane, and immuno-reactive bands were probed with the appropriate primary antibody.

BL21 bacterial cells containing plasmids encoding glutathione S-transferase (GST)-tagged Cdc42-Q61L were cultured to a density of A₆₀₀ = 0.6~1.0. Expression of the fusion protein was induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 0.1 mM and the cells were cultured for an additional 3–5 hours (or overnight) at 37°C. To extract the protein from bacterial cells, the cells were collected by centrifugation at 5000 x g for 10 minutes, and the pellets were suspended with 3 ml of B-PER lysis reagent containing 0.2% (6 µl) lysozyme, 0.2% (6 µl) DNase I and 1% (30 µl) halt protease inhibitor. To separate soluble proteins from insoluble material, the resulting lysate was centrifuged at 15000 x g for 5 minutes. The supernatant was incubated with 500 µl glutathione-Sepharose affinity resin in a
Bio-Rad mini-column pre-equilibrated in wash buffer (50 mM Tris, 150 mM NaCl, pH 8.0), and the column was washed twice with 10 resin bed volumes of wash buffer. The beads with immobilized GST-Cdc42-Q61L were suspended in wash buffer and transferred to an Eppendorf tube, centrifuged at 8000 x g for 5 minutes, and the buffer was removed. The pre-cleared MCF-10A cell lysate (described above) was incubated with immobilized GST-Cdc42-Q61L by rotation at 4°C for 40 minutes. After two washes with hypotonic, detergent-free lysis buffer, the bound protein was eluted by boiling with 1X SDS-PAGE sample buffer, resolved by 8% SDS-PAGE, and transferred to a PVDF membrane. Anti-myc rabbit mAb was used to determine the amount of myc-tagged CEP4 that had bound to Cdc42.

Figure 2.1: Photomicrographs of human breast epithelial cells during the cell sedimentation method. Bright field micrographs were recorded with a phase contrast microscope (A, B), and with a fluorescence microscope (C, D) to visualize GFP-expressing cells. Images shown in A and C were captured immediately after the manifold had been removed (t=0), and images shown in B and D are images of the cells shown in A and C captured 6 hours later. The area of each circle was measured with Motic Image software.
2.9 Motility Assay

The extent of cell movement was evaluated by the cell sedimentation method (CSM Inc.). This assay was carried out with a 10-well glass slide and a 10-hole manifold. The cells (~8x10³) were applied in 1 µl to the slide through one of the holes of the manifold so that the cells sedimented as a tight concentric circle in the corresponding well. After incubation overnight at 37°C (5% CO₂), the manifold was removed and the cells were detected under a Nikon Diaphot microscope (Moticam 2000) at t = 0 and t = 6 hours. To study the effect on motility of a drug (DAG-lactone, Cdc42 inhibitor, or Rac inhibitor), the medium was replaced at t=0 with fresh medium containing the specific reagent. The extent of movement was determined by measuring the change in total area over 6 hours using Motic Image software. Each experiment was performed in triplicate and averaged. Three independent experiments were performed.

2.10 Proliferation Assay

Cell proliferation was measured by using Alamar Blue (Life Technologies) in a fluorescence-based assay that can be used to assess survival and proliferation of mammalian cells. Cells were added to a 96-well plate at a density of 1x10³ cells/µl complete medium and incubated at 37°C in a 5% CO₂ atmosphere overnight to allow attachment. On day 2, Alamar blue was added to each well to a final concentration of 4% v/v (7.2µl /180µl). Wells containing 180 µl growth medium without any cells served as the blank. After incubation for 3 hours, the fluorescence of each well was measured using a plate reader (SpectraMax M5 Microplate Reader) set at λ_ex = 530 nm and λ_em = 590 nm and a gain of 40. The plate was returned to the incubator until the next time point.

2.11 Immunocytochemistry
2.11.1 F-actin Staining

For F-actin experiments, MCF-10A cells were cultured overnight on poly-lysine-coated coverslips and transfected with the Myc-tagged CEP4 vector control or one of the CEP4 double mutant constructs (S18D-S80D, S18A-S80A). Forty-eight hours post-transfection, each coverslip was washed twice in 2 ml PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA and 2 mM MgCl$_2$, pH 6.9). The cells were fixed for 15 minutes with 3.7% paraformaldehyde in 0.1 M PBS (pH 7.4). Non-specific sites were blocked for 15 minutes at 37°C with immuno-blocker (0.1% sodium azide and 2% BSA in TBST). After blocking, the cells were incubated 1.5 hours at 37°C with Myc-tagged rabbit mAb diluted to 1:300 (Cell Signaling Technology), followed by FITC-conjugated goat anti-rabbit secondary antibody diluted to 1:300 (Santa Cruz Biotechnology). F-actin was stained with rhodamine-phalloidin diluted to 1:300 (Cytoskeleton) for 1 hour at 37°C and nuclei were stained with Hoechst 33342 stain diluted to 1:1000 (Invitrogen) for 5 minutes at 37°C before mounting. Imaging was performed with a Plan-Neofluor 63X oil immersion objective lens on a Zeiss Axio Imager-M2 upright fluorescence microscope.

2.11.2 IQGAP and E-cadherin Staining

MCF-10A cells transfected with the myc-tagged CEP4 vector control or one of the CEP4 mutant constructs were analyzed for localization and expression of IQGAP or E-cadherin. Forty-eight hours post-transfection, each coverslip was washed twice in 2 ml PBS and the cells were fixed for 10 minutes with 4% PFA in 0.1 M PBS (pH 7.4). Depending on the purpose of the experiment, cells were stained with mouse/rabbit monoclonal anti-Myc diluted to 1:300 (Cell Signaling Technologies), rabbit monoclonal anti-IQGAP diluted to 1:200 (Abcam), or mouse monoclonal anti-E-cadherin diluted to 1:150 overnight (Santa Cruz Biotechnology), followed by either FITC-conjugated goat anti-rabbit (green, 1:300) or Alexa
Fluor 594-conjugated goat anti-mouse secondary antibodies (red, 1:300). Cells were mounted on clean glass slides with DABCO and sealed. Images were acquired by a 63x1.4NA or a 100X oil immersion objective lens using the Axio Imager-M2 upright fluorescence microscope (Zeiss).

2.12 Tandem Affinity Purification (TAP) Method

CEP4-associated proteins were isolated by using tandem affinity purification (TAP) by following the Inter-Play Mammalian TAP System protocol (Agilent Technologies). The TAP method entailed purification of a protein complex under non-detergent conditions by two consecutive affinity columns (streptavidin and calmodulin affinity resins). The resulting elute was detected on a Gelcode Blue-stained SDS-PAGE gel, and selected protein bands were analyzed by mass spectrometry. MCF-10A cells were transfected with the TAP empty vector, TAP-CEP4-S18A/S80A, or TAP-CEP4-S18D/S80D. After 48 hours, cells were suspended in 1 ml lysis buffer containing 1% of protease inhibitor and 1% of phosphatase inhibitor, and lysed by three successive rounds of freezing the cells at -150°C for 10 minutes, followed by thawing in cold water for 10 minutes. After removal of cell debris by centrifugation at 16,000 x g for 10 minutes, the crude cell lysates were incubated with 100 μl streptavidin resin (pre-equilibrated with streptavidin binding buffer) for 2 hours at 4°C. The resin containing bound protein was washed twice with 750 μl streptavidin binding buffer and the proteins were eluted with 200 μl streptavidin elution buffer by incubating with rotation for 1 hour at 4°C. The elutes were applied to 50 μl calmodulin resin (pre-equilibrated with calmodulin binding buffer) at 4°C for 3 hours and washed twice with 750 μl calmodulin binding buffer. The bound proteins were eluted by boiling with 1X SDS-PAGE loading buffer at 95°C for 5 minutes, and resolved by 8% SDS-PAGE. The identities of eluted proteins were revealed by mass spectrometry analysis of selected bands that appeared with the S18D/S80D-CEP4.
mutant but not with the S18A/S80A-CEP4 mutant. The gel was stained with Gelcode Blue (Pierce), and bands of interest were excised for identification by mass spectrometry.

2.13 Pull-Down Assay of Activated Small GTPases

Activation of small GTPases was measured in lysates of MCF-10A cells using commercially available buffers (Cytoskeleton, Inc.). Briefly, cells (transfected with either CEP4 18D/80D or CEP4 18A/80A) were disrupted in a lysis buffer (50 mM TRIS, pH 7.5, 10 mM MgCl2, 0.5 M NaCl, 2% Igepal) supplemented with phosphatase and protease inhibitors followed by centrifugation at 13,000 x g at 4 °C for 10 min. Agarose beads conjugated to either PAK-binding domain (PBD) (Millipore Corp.) or Rho-binding domain (RBD) (Cytoskeleton, Inc.) were used in a pull-down assay to isolate activated G-proteins. Cell lysates with a total protein content of 600 μg of protein were incubated with PAK-PBD beads (10 μg) or RBD (25 μg) beads at 4°C for 1h. The beads were washed once with wash buffer (25 mM TRIS, pH 7.5, 30 mM MgCl2 and 40 mM NaCl) followed by centrifugation at 4°C. The entire sample was loaded and bound proteins were separated by 12% SDS-PAGE and immunoblotted with anti-Rac or anti-Cdc42 (Cell Signaling Technology), or anti-RhoA (Cytoskeleton, Inc.).

2.14 Mass Spectrometry

Identification of proteins was performed at the Keck Foundation Mass Spectrometry Resource Laboratory at the Yale University Medical Center. Gel-resolved bands were excised and proteins were digested in situ with trypsin. The resulting peptides were analyzed by LC–MS/MS on a 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 required that two or more MS/MS spectra matched those
of the same protein entry in the database, and that matched peptides corresponded to tryptic peptides in the protein.

2.15 Statistical Analysis

Each experiment was performed a minimum of three times. Changes in area for cell migration measurements between groups were assessed with Student’s $t$ test. Data are expressed as mean ± standard deviation.
Chapter 3: Identification of Novel Substrates of Protein Kinase C Isoforms in Human Breast Cells
3.1: Introduction

PKC was first identified more than three decades ago as a calcium- and phospholipid-dependent serine/threonine-specific protein kinase. Since its discovery, PKC has been studied and recognized as a major player in a wide variety of cellular processes, and has become increasingly attractive for oncology research (Tsai et al., 2000; Teicher, 2006; Martiny-Baron and Fabbro, 2007; Urtreger et al., 2012).

The discovery of PKC as a major intracellular receptor for tumor promoting phorbol esters suggested the importance of PKCs in tumor initiation and progression (Castagna et al., 1982; Niedel et al., 1983). Over-expression or down-regulation of PKC isoforms have been described in different types of cancer or at different stages of tumor development, which makes it difficult to assign a particular role for individual PKC isoforms in cancer. This complexity suggests that PKC isoforms have specific as well as overlapping roles during cancer progression. In previous studies conducted by the Rotenberg lab, over-expression of wildtype PKC-α in non-transformed, non-motile human breast MCF-10A cells led to suppressed cell proliferation, dramatically increased cell motility, and radical alternations in cell morphology (large, disaggregated, and flat, with formation lamellipodia, membrane ruffling and extensive actin fibers) (Sun and Rotenberg, 1999). In human breast cells, PKC-δ is known to promote migration (like PKC-α) or survival (unlike PKC-α), as well as other aspects of the malignant phenotype (Kiley et al., 1999; Lønne et al., 2009; Mccracken et al., 2003). In contrast, some recent studies has shown that PKC-ζ is considered to be a novel tumor suppressor (Galvez et al., 2009; Nazarenko et al., 2010). In NMuMg human breast cells, PKC-ζ apparently operated through different signaling pathways because stable overexpression of PKC-ζ showed higher clonogenic ability and protease secretion and a significantly lower migration rate (Urtreger et al., 2005).
The role of PKC isoforms in cancer progression results from phosphorylation of substrates lying on signaling pathways that govern cancer-related phenotypes. However, each isoform may interact with its unique substrates or may share substrates with other PKC isoforms. Identification of an intracellular substrate for which a functional significance can be established would provide a phospho-protein marker for detecting activity through a PKC-related signaling pathway and a novel target for drug discovery. At present, very few intracellular protein substrates have been identified that have functional significance. The identification and characterization of novel PKC isoform-specific intracellular substrates has great importance for understanding the mechanisms about how each PKC isoform affects cell behavior and its specific role in cancer progression.

In this study, PKC-α, -δ, and -ζ were selected for analysis since they are representative of conventional, novel, and atypical subclasses of PKC, respectively. Knowledge of their substrates and whether they are shared or specifically selected by a single isoform, would clarify whether isoform-specific mechanisms are operating, and thereby inform strategies for the design of anti-cancer drugs. We applied the traceable kinase method to identify novel substrates of PKC-α, -δ, and -ζ in human breast cells. Because the over-expression of PKC-α induced motility and formation of lamellipodia and membrane ruffling in non-transformed MCF-10A cells (Sun and Rotenberg, 1999), these phenotypes suggested the participation of specific small GTPases (Ridley and Hall, 1992; Nobes and Hall, 1995; Schmitz et al., 2000). Based on these previous findings, PKC isoforms may interact with GTPase-related effector and regulatory proteins.

The Rotenberg lab applied the Traceable Kinase Method using an ATP analogue ([γ-32P]-phenyl-N6-ATP) to identify substrates of a traceable PKC-α mutant (Abeyweera and Rotenberg, 2007). Expression of this mutant in MCF-10A cells led to the identification by
MS/MS of several potential substrates in MCF-10A human breast cells. Most recently, the Rotenberg lab developed a traceable mutant for each of three PKC isoforms (-α, -δ and -ζ) and analyzed their phospho-protein profiles in parallel. The high affinity substrates were isolated by co-immunoprecipitation of the traceable PKC isoform mutants or wild type using anti-FLAG (for PKC-α/-β) or anti-PKC-ε (for PKC-ζ containing a short ε-tag). Those substrates that co-immunoprecipitated with the traceable kinase mutants and underwent phosphorylation following addition of the ATP analogue N6-phenyl-ATP (but minimally by the wildtype enzyme) were detected by Western blot using the PKC substrates antibody. This antibody specifically detects the phosphorylated PKC consensus site and the phospho-protein profile for each traceable PKC, shown in Figure 3.1. Selected bands were excised from a companion SDS-PAGE gel containing the same samples run in parallel, and the proteins contained in those bands were identified by mass spectrometry.

The MS/MS results are listed in Table 3.1A which details many of the identified proteins, such as the small G-proteins and their accessory proteins that were judged to be related to cancer-related phenotypes. Some proteins were already known to be PKC substrates such as IQGAP (Grohmanova et al., 2004), MAP4 (Mori et al., 1991) and VASP (Wentworth et al., 2006) (Table 3.1B). Other proteins were considered as novel candidate substrates like ROCK1/2, CEP4, PAK2 and CLASP. In this Chapter, the objective was to determine which of these novel candidates function as PKC substrates in MCF-10A cells.
Figure 3.1: Phosphoprotein profiles of WT and traceable PKC isoforms. MCF-10A transfectants expressing epitope-tagged WT or a traceable mutant of each PKC isoform were lysed in detergent-free medium, and each isoform was immunoprecipitated, as described in Materials and Methods. This step facilitated the isolation of high-affinity substrates of each PKC isoform. Following the addition of N6-phenyl-ATP to each immunoprecipitated enzyme under activating conditions (phosphatidylinerine for PKC-α and -δ and ceramide for PKC-ζ), phosphorylated products were resolved via SDS-PAGE and stained with Gelcode Blue. Western blot analysis of an aliquot of each sample (representing 25% of the total sample) was conducted with a duplicate gel, and the resulting blot was probed with PKC substrate antibody (1:1000). Each WT–traceable mutant pair was aligned with the corresponding Gelcode blue-stained gel that serves as the loading control. Bands excised for MS/MS analysis are indicated with a caret (<). Each phospho-protein profile is representative of three independent experiments (Chen et al., 2012).
Table 3.1: Proteins that co-immunoprecipitate with traceable PKC isoforms. A. Proteins were identified by MS/MS, as described in the Materials and Methods. The Mascot protein score is indicated next to each detected protein. Those proteins that contain one or more potential PKC phosphorylation sites at either partial or full consensus sequences are shown in bold. B. Proteins identified by MS/MS that had been previously identified as either a substrate or binding protein of PKC are shown along with the site of PKC phosphorylation (given in parentheses) (Chen et al., 2012).

3.2: Results

Based on the list of proteins identified by MS/MS, the possible PKC candidate substrates were narrowed down to four proteins: CEP4 (43 kDa), PAK2 (63 kDa), CLASP1 (170 kDa) and ROCK-1 (200 kDa) which co-immunoprecipitated with either PKC-α or PKC-δ. These four proteins were selected for the following reasons: first, the primary structures of these proteins contain at least one partial or complete consensus site for phosphorylation by PKC;
second, they are all related to small Rho GTPases and are known to play a functional role in cell morphology and cell motility.

ROCK is a major downstream effector of small GTPase RhoA. ROCK is involved in cell morphology and cell migration by regulating actin organization (Riento and Ridley, 2003). The Cdc42 Effector Protein-4 (CEP4)/Borg4 is a member of the Cdc42-binding protein family. CEP4 induces pseudopodia formation by acting as a Cdc42 effector, which suggests a role in inducing actin filament assembly and cell shape changes (Joberty et al., 1999; Burbelo et al., 1999; Hirsch et al., 2001). P21-activated protein kinase (PAK) is activated by Rac1 and Cdc42 and is therefore an effector of these GTPases. PAK is known to be involved in numerous biological functions, including cytoskeletal organization, cell motility, cell growth and apoptosis (Bokoch, 1998; Coniglio et al., 2008; Siu et al., 2010). CLASP1 (CLIP-associating protein) is a microtubule (MT) plus-end binding protein that associates with CLIP-170 and CLIP-115 (cytoplasmic linker proteins) (Galjart, 2005). CLASPs can induce local stabilization of MTs and regulate MTs dynamics in polarized cells in response to signaling cues (Lansbergen et al., 2006; Watanabe et al., 2009).

An investigation of ROCK1 demonstrated that ROCK1 is not a PKC substrate. This conclusion was based on the absence of phosphorylation of ROCK1 by a pure, recombinant PKC isoform (-α, –δ, or -ζ) in vitro, and by the absence of phosphorylation in MCF-10A cells following DAG-lactone treatment (Figure 3.2) (Chen, X. Ph.D. Thesis, 2010).
Figure 3.2: In vitro and intracellular kinase assay with ROCK. (Chen, X. Ph.D. Thesis, 2010)

Figure 3.3: In vitro kinase assay of CEP4, PAK2 or CLASP-1. Pure recombinant CEP4 (700 ng), CLASP (1 μg) and PAK2 (300 μg for the whole cell lysate) were analyzed for phosphorylation by individual PKC isoforms in vitro. Each protein was tested in vitro with pure, recombinant PKC-α (170 ng), PKC-δ (100 ng), or PKC-ζ (166 ng). In order to ensure that CLASP1 was dephosphorylated prior to performing the kinase reaction, it was treated with PP2A for 2h at 30°C, following which phosphatase inhibitors were added and the protein was kept on ice for 30 min. The reaction was conducted in 1X kinase buffer, which contained 0.1 mg/mL phosphatidylserine with either 5 μM DAG-lactone (for PKC-α and -δ) or 1 μM ceramide (for PKC-ζ). The reaction was initiated by the addition of 100 μM ATP and incubated at 30°C. After 30 min, each reaction was quenched with sample buffer and the analysis of phosphorylation was conducted by Western blotting with anti-PKC substrate antibody (1:1000).
Figure 3.4: Testing of phosphorylation of potential PKC substrates in MCF-10A cells. Cells were treated with DAG-lactone (10 μM) or DMSO (0.05%, v/v) for 1 hour at 37°C. The cells were harvested and dissociated by trypsinization followed by three washes with 5 ml complete medium and one wash with 5 ml serum-free medium. Cell lysates were prepared by sonication 3x10 seconds in lysis buffer [50mM TRIS (pH 7.4), 150 mM NaCl, 5 mM EGTA and 5 mM EDTA, 1% Triton X-100, 10 μM bis-indoleylmaleimide, 0.1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail], followed by centrifugation at 10000 x g for 10 minutes. The supernatant was pre-cleared using 30 μl protein A/G-agarose beads for 1 h. The pre-cleared lysate was aliquotted into three Eppendorf tubes (200 ng/tube), followed by incubation with 5 μl anti-CEP4 antibody (Bethyl Labs), 5 μl anti-PAK2 antibody (Cell Signaling Technology), or 5 μl anti-CLASP1 antibody (Epitomics) overnight at 4°C. The immunocomplexes were collected by incubation of 50 μl protein A/G-agarose beads for 1 hour at 4°C and centrifugation at 10000 x g for 10 minutes. The pellets were analyzed by Western blot with the PKC substrates antibody (1:1500) that recognizes the phosphorylated PKC substrate consensus site in phospho-CEP4, or CEP4 antibody (1:2000) to establish equivalent protein loading.
3.2.1 Testing of Phosphorylation of Potential PKC Substrates in Vitro

To determine if CEP4, PAK2 or CLASP1 are PKC substrates in vitro, the pure recombinant protein of each was obtained and evenly divided into different kinase reactions and subjected to phosphorylation with PKC-α, -δ or -ζ in the presence of ATP and the appropriate activator. Phosphorylated products were detected by Western blot with the PKC substrate antibody. CEP4 and CLASP1 recombinant proteins were purchased from Novus Biologicals (Littleton, CO). PAK2 protein was obtained by immunoprecipitation from MCF-10A cells using PAK2 antibody. In Figure 3.3, CEP4 and PAK2 each underwent phosphorylation by PKC-α, and -δ in vitro; in addition CEP4 was a substrate for -ζ in vitro. However, CLASP1 was shown to be phosphorylated even without addition of a protein kinase. In order to ensure that the recombinant protein CLASP1 was unphosphorylated, we treated CLASP1 with protein phosphatase 2A (PP2A) prior to the kinase reaction. However, the result was not improved (Figure. 3.3).

3.2.2 Testing of Phosphorylation of Potential PKC Substrates in MCF-10A Cells

To confirm the validity of the in vitro results, intracellular phosphorylation state of each potential substrate was analyzed following its immunoprecipitation from MCF-10A cells treated with or without DAG-lactone. The MCF-10A cell line is a non-tumorigenic, non-transformed human mammary epithelial cell line. These cells express low levels of PKC-α and offer a low background of substrate phosphorylation. DAG-lactone, an activator of conventional and novel PKC isoforms, activates conventional and novel PKC isoforms in MCF-10A cells, and thereby promotes the phosphorylation of PKC substrates.

MCF-10A cells were first treated with DAG-lactone (10 μM) for 30 min or DMSO as the vehicle control. Following the preparation of lysates, the endogenous protein was
immunoprecipitated with an antibody corresponding to CEP4, PAK2, or CLASP-1, and the pellet was analyzed by western blot using the phospho-(Ser) PKC substrate antibody and a protein-specific antibody that detects the total level of expression of the protein being tested. Of these proteins, only CEP4 showed strong phosphorylation in response to DAG-lactone in MCF-10A cells (Figure 3.4A). In contrast, PAK2 and CLASP1 showed undetectable phosphorylation in MCF-10A cells after stimulation by DAG-lactone (Figure 3.4 B, C). Based on the result from the in vitro and intracellular kinase assays, it was concluded that CEP4 is a PKC substrate in MCF-10A cells.

3.2.3 Stoichiometry of Phosphorylation of Intracellular CEP4 Relative to the Total CEP4

After confirming that CEP4 undergoes phosphorylation in MCF-10A cells in response to DAG-lactone, we sought to quantitate the ratio of phospho-CEP4/total CEP4 in unstimulated and stimulated cells in order to establish the number of PKC phosphorylation sites. The ratio of intracellular phospho-CEP4 relative to total CEP4 was determined by reference to known amounts of the pure, recombinant CEP4 protein standard that had been phosphorylated by pure, recombinant PKC-α in vitro. A time course of in vitro phosphorylation demonstrated that maximal phosphorylation of pure, recombinant CEP4 was achieved after 2 hours at 30°C (Figure 3.5A). The phosphorylated product was subsequently used as a standard for quantitating the phosphorylation of intracellular CEP4. The experiment consisted of a Western blot containing known picomole (pmole) amounts of standard phospho-CEP4 run in the gel with samples of immunoprecipitated CEP4 from MCF-10A cells that had been treated for 1 hour with DAG-lactone (10 μM). For comparison with the total amount of CEP4, a duplicate blot was performed with identical samples and known amounts of pure, recombinant unphosphorylated CEP4. Each blot was probed with either the PKC substrates
antibody or CEP4 antibody to detect phospho-CEP4 or total CEP4 protein, respectively. Quantitation of phospho-CEP4 or total CEP4 triplicate samples was performed with ImageJ by comparing the samples with known amounts of standard CEP4 or phospho-CEP4 protein present in the same blot (Figure 3.5B). The ratio of phospho-CEP4 to total CEP4 in DAG-lactone-treated cells was calculated to be $3.2 \pm 0.5$ pmol, the average of two independent experiments (each consisting of triplicate measurements). This finding implies that in response to DAG-lactone, PKC isoforms phosphorylate an average of three to four sites in CEP4.

3.2.4 Interference of Intracellular CEP4 Phosphorylation by shRNA-Encoding Plasmids of PKC Isoforms

To evaluate whether CEP4 serves as an intracellular substrate for one or more PKC isoforms, MCF-10A cells were transfected with individual shRNA-encoding plasmids for isoform -α, -δ, or -ζ, and their interference in CEP4 phosphorylation was evaluated under conditions of DAG-lactone stimulation. Each shRNA-encoding plasmid featured co-expression of GFP so that the transfection efficiency could be readily determined. Following a 48-hour incubation period, the transfection efficiency was found to be >70%. However, a period of 96 hours was required to produce maximal knockdown of each PKC isoform. The single plasmids used for PKC-α and -δ had been validated by the manufacturer for their ability to knockdown their intended target and were effective at knocking down expression of an isoform by 60−100% in MCF-10A cells (Figure 3.6A). Of four constructs obtained for PKC-ζ, two (ζ-2 and ζ-3) were effective in achieving knockdown of PKC-ζ (by 80−90%) in MCF-10A cells (Figure 3.6A). In Figure 3.6C, it was confirmed that the PKC-ζ shRNA (ζ-2 and ζ-3) successfully knocked down the expression of PKC-ζ and had no effect on PKC-α and- δ.
Figure 3.5: Stoichiometry of intracellular phospho-CEP4 to total CEP4 in unstimulated and stimulated cells. Phosphorylation of pure, recombinant CEP4 (1 mg) was conducted with pure, recombinant PKC-α (0.74 mg) (Sigma-Aldrich), as shown in panel A, and was complete after 2 hours. Each time point consisted of 200 ng (4 pmol) of CEP4 protein. (B) Intracellular phosphorylation of CEP4 was tested in MCF-10A cells cultured in 60 mm plates and treated for 1 hour with 10 μM DAG-lactone or DMSO (0.1%, v/v) as the vehicle control. Whole cell lysates were prepared in 0.5 ml of 50 mM TRIS-HCl, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1% Triton X-100, 10 μM bis-indoleylmaleimide, phosphatase inhibitors, and protease inhibitors. Lysates were pre-cleared with a 1:1 combination of rabbit IgG-agarose and protein A/G-agarose, and CEP4 protein was immunoprecipitated with 3 μg CEP4 antibody and protein A/G-agarose. Western blotting of the immunopellets was used to assess CEP4 phosphorylation with the PKC substrate antibody (1:1500). To demonstrate equivalent protein loading and to determine the total amount of CEP4 protein in each sample, a duplicate blot was probed with anti-CEP4 (1:2000). The results are representative of two independent experiments. The ratio of intracellular phospho-CEP4 relative to total CEP4 in each sample was determined with ImageJ and by reference to the known standards for phospho-CEP4 or CEP4. Standard phospho-CEP4 that was produced after 2 hours by pure, recombinant PKC-α in panel B and represents the maximally phosphorylated protein.

In DAG-lactone-treated cells, silencing of each individual isoform led to a substantial inhibition of CEP4 phosphorylation relative to the scrambled control (SC) shRNA plasmid. When compared with that of SC-treated cells, inhibition of CEP4 phosphorylation averaged 60% with either the PKC-α or PKC-δ shRNA reagent, and >85% with PKC-ζ shRNA (ζ-2), where each value represents the average of three independent experiments (Figure 3.6B).
These findings suggest that each of the three PKC isoforms phosphorylates CEP4 in the intracellular environment.

**Figure 3.6:** Knockdown of individual PKC isoforms blocks CEP4 phosphorylation. MCF-10A cells were cultured in six-well plates and transiently transfected with a 3:1 mixture of PolyExpress reagent (6 μl) and a plasmid (2μg) encoding shRNA that targeted a single PKC isoform (α, δ, or ζ). Following incubation of the cells at 37 °C for 96 hours, lysates were prepared and normalized for total protein. An aliquot of each lysate (30 μg) was applied for demonstration of knockdown of each isoform using Western blotting by use of an isoform-specific antibody, as shown in A. The remaining portion of each lysate (100 μg) was immunoprecipitated with anti-CEP4 (3 μg), and the resulting immunopellets were analyzed in duplicate Western blots for either phosphorylated CEP4 (PKC substrate antibody, 1:1500 dilution) or for total CEP4 protein (anti-CEP4, 1:2000 dilution), as shown in B. In order to demonstrate the specificity of shRNA reagent for PKC-ζ, the expression of PKC-α, -δ and -ζ was analyzed with Western blot staining with PKC PKC-α (1:1000), -δ (1:1000) and -ζ (1:1000) using equal amount of lysates (30 μg), as shown in C. Western blots are representative of three independent experiments.

### 3.2.5 Interference of Intracellular CEP4 Phosphorylation by Kinase-Defective Mutants of PKC Isoforms
In a complementary approach, MCF-10A cells were transfected with individual kinase-dead (KD) hemagglutinin (HA)-tagged mutants for isoform -α (K368R), -δ (K376R), or -ζ (K281M), and their interference in CEP4 phosphorylation was evaluated under conditions of DAG-lactone stimulation. In each lysate, the expression of a kinase-dead mutant was verified by Western blot with an HA-tag antibody (Figure 3.7). Lysates were immunoprecipitated with anti-CEP4, and the resulting immunopellets were analyzed for phosphorylated CEP4 and for total CEP4 protein by Western blotting. The results, shown in Figure 3.7, reveal a substantial dominant negative effect on CEP4 phosphorylation by the KD mutants of PKC -α and -δ averaging 40−70% for three independent experiments. However, no significant interference (<10%) was observed with KD-PKC-ζ that may have had limited access to CEP4. The agreement of the shRNA and kinase-dead mutant experiments for PKC-α and -δ is further evidence that CEP4 is a substrate for both isoforms.

Figure 3.7: Kinase-dead mutants for PKC-α and -δ interfere with phosphorylation of CEP4. MCF-10A cells were cultured on 60-mm plates and transfected with a 3:1 mixture of PolyExpress reagent (15 μl) and plasmid (5 μg) encoding the HA-tagged, kinase-dead (KD) mutant for each PKC isoform (KDα, KDδ, or KDζ) or the vector control (VC). Expression of HA-tagged KD mutants was verified in each whole cell lysate (50 μg/lane) by probing with the anti-HA tag (1:1000). CEP4 was immunoprecipitated from each cell lysate with anti-CEP4, and the pellets were evaluated for phosphorylated CEP4 (P-CEP4) by Western blotting with the PKC substrate antibody (1:1500). An equivalent CEP4 content for all samples was established by probing immunopellets in a duplicate blot with anti-CEP4 (1:2000). The results are representative of three independent experiments.
3.3: Discussion

In a recent application of the traceable kinase method, we defined substrate profiles of the PKC isoforms in MCF-10A cells. Some proteins that co-immunoprecipitated with traceable PKC-α, -δ and -ζ had been already identified as PKC substrates (e.g. VASP, IQGAP-1, MAP4, and α-tubulin) which validates the effectiveness of the traceable kinase method with these cells (Table 3.1). Likewise, some proteins that co-immunoprecipitated with traceable kinase mutants were potential substrates in MCF-10A cells because they contained one or more PKC consensus sites (Table 3.1). Several proteins that are GTPase-related and have a known effect on cancer-related phenotypes, namely ROCK1, CEP4, PAK2 and CLASP1, were tested to determine whether they serve as PKC substrates both in vitro and in MCF-10A cells.

As shown by in vitro kinase assay, pure recombinant PKC-α, -δ or -ζ each phosphorylated pure, recombinant CEP4 (Figure 3.3). In MCF-10A cells, CEP4 also underwent phosphorylation in response to DAG-lactone (Figure 3.4A). It was concluded that only CEP4 was a PKC substrate in MCF-10A cells. With regard to the other three candidates, ROCK1 was not a PKC substrate since it did not undergo phosphorylation in vitro and in MCF-10A cells (X. Chen, Ph.D. Thesis, 2010) (Figure 3.2). Recombinant PKC isoforms -α and -δ proteins phosphorylated PAK2 but not CLASP1 recombinant protein (Figure 3.3). However, in MCF-10A cells, neither PAK2 nor CLASP1 showed any increased phosphorylation following stimulation by DAG-lactone (Figure 3.4B, 3.4C). Thus, ROCK1, PAK2, and CLASP1 are not PKC substrates in MCF-10A cells. Their co-immunoprecipitation with traceable PKC mutants suggests that they are nonetheless physically associated with PKC in the intracellular environment.
It is notable that there are five structurally related human CEP isoforms, of which CEP4 is the only member to possess a canonical PKC consensus site at Ser-18 (two pairs of cationic residues flanking a serine or threonine residue). CEP4 was phosphorylated in a stoichiometric ratio of almost 4:1 (moles of phospho-CEP4 per mole of CEP4) in DAG-lactone stimulated MCF-10A cells (Figure 3.5). Therefore, it is likely that there are additional sites being modified by DAG-stimulated PKC isoforms. Inspection of the human CEP4 primary sequence shows that in addition to a full consensus site at Ser-18, there are three partial consensus sites (two cationic residues on one side of a serine residue) located at Ser-77, Ser-80, and Ser-86. The significance of these proposed sites of phosphorylation to CEP4 function provides an avenue for further investigation that will be explored in Chapter 4.

In MCF-10A cells, phosphorylation of CEP4 in response to DAG-lactone was shown to be impaired by individual isoform-specific shRNA reagents or kinase-dead mutants of either PKC-α or PKC-δ (Figure 3.6, 3.7). DAG-lactone, a cell-permeable PKC activator, selectively activates cPKC (PKC-α) and nPKC (PKC-δ), but does not stimulate aPKC (PKC-ζ) (Garcia-Bermejo et al., 2002). The loss of DAG-stimulated CEP4 phosphorylation by PKC-ζ knockdown was surprising because it implied that this isoform, which is insensitive to DAG, phosphorylates CEP4 only in DAG-treated control cells. The possibility that, in addition to PKC-ζ, the two PKC-ζ shRNA reagents also silenced DAG-sensitive PKC isoforms was considered. However, no effect on either PKC-α or PKC-δ expression accompanied PKC-ζ knockdown (Figure 3.6C). A plausible explanation is that DAG is known to cause recruitment of PKC-ζ to the plasma membrane via DAG-stimulated PKC isoforms that consequently activate the MAP kinase cascade, as recently reported for bronchial epithelial cells stimulated with phorbol esters. It is further noted that blockade of DAG-stimulated CEP4 phosphorylation by silencing of native PKC-ζ (Figure 3.6C) is inconsistent with the
lack of interference by the kinase-dead PKC-ζ mutant (Figure 3.7). A reasonable explanation is that, unlike the native enzyme, the kinase-dead mutant had limited access to CEP4. For example, kinase-dead PKC-ζ may not have been able to bind CEP4 because its substrate binding site was already occupied by a high-affinity substrate whose release depended upon being phosphorylated. Nevertheless, the outcome produced by silencing native PKC-ζ by each of two different shRNA reagents clearly demonstrated the role of this isoform in CEP4 phosphorylation. The efforts taken here to establish which of the three PKC isoforms phosphorylate CEP4 in the intracellular environment has led us to conclude that CEP4 serves as a substrate for all three PKC isoforms.

A number of studies over the past few years have revealed that the Rho GTPases and their effector proteins play important roles in human breast cancer, especially in invasion and metastasis. An earlier study conducted by the Rotenberg laboratory (Sun and Rotenberg, 1999) demonstrated that Rho GTPases were functionally involved in phenotypes produced by PKC-α overexpression in MCF-10A cells. In that initial study, a dominant negative mutant of Rac1 inhibited PKC-α-induced motility by 40%. In another example, the knockdown of PKC-ε in MDA-MB-231 cells, an aggressive breast cancer cell line with elevated PKC-ε levels, was shown to significantly decrease tumor metastasis. Moreover, PKC-ε deficient clones were found to have lower Rho GTPase protein levels and activation (Pan et al., 2005).

In this chapter, CEP4, a Cdc42 effector protein, was shown to be a PKC substrate in MCF-10A cells. One avenue for further investigation will be to determine if PKC-mediated phosphorylation of CEP4 alters its binding affinity for activated Cdc42, thereby modulating CEP4 and Cdc42 signaling pathways and producing one or more phenotypes previously attributed to PKC in human breast cells. This objective will be pursued in the next chapter (Chapter 4).
Chapter 4: Characterization of The Functional Significance of CEP4 in MCF-10A Cells
4.1: Introduction

RhoA protein was first identified by Richard Axel’s group in 1985 due to its homology to Ras in *Aplysia* (Madaule and Axel, 1985). Since that time, at least 20 additional Rho-related family members have been identified in mammals. Rho, Rac and Cdc42 are the most extensively studied members due to their significance in regulating the organization of actin filaments during morphogenesis and migration of mammalian cells (Hall, 1994; Nobes and Hall, 1995, 1999; Schmitz et al., 2000). Rho GTPases act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state in order to direct signaling pathways that produce cellular responses such as motility.

![Rho Family GTPases in Breast cancer](image)

*Figure 4.1: Rho Family GTPases in Breast cancer.* Rho Family GTPases are involved in different stages of cancer progression: dedifferentiation and upregulation of uncontrolled proliferation, angiogenesis, invasion and metastasis (Zhang and Nie, 2011).
Rho GTPase signaling pathways are thought to contribute to human breast cancer initiation and progression by regulating growth, angiogenesis, invasion and metastasis of breast cancer cells (Figure 4.1). Altered expression of a Rho GTPase, changes in its activation status, or changes in the expression level of its effector proteins, all were found to occur in human breast cancer. RhoA was found overexpressed in breast tumor tissues but not in the normal tissues (Fritz et al., 2002). Microarray analysis showed that over-expression of RhoC genes in MCF-10A breast cells was associated with invasion and metastasis (Wu et al., 2004). Immunohistochemical staining of Rac1 showed weak expression in benign breast disease but high expression levels in primary breast tumors (Schnelzer et al., 2000). Furthermore, the Rac/p21 activated kinase (PAK) signaling pathway is essential for receptor tyrosine kinase ErbB2-mediated transformation of human breast epithelial cancer cells (Muthuswamy and Chernoff, 2011). In addition, PAK2, an effector protein of both Rac1 and Cdc42, was implicated in breast carcinoma cells invasion and migration by regulating formation of focal adhesion and lamellipodia (Coniglio et al., 2008). Cdc42 is also known to be involved in human breast carcinogenesis. For example, the expression of a dominant negative mutant of Cdc42 reduced the number of focal contacts, inhibited colony formation in soft agar and affected cell growth in vivo (Fritz et al., 1999). The activation of the downstream effectors-PAK2 and N-WASP by Cdc42 is also important for amoeboid movements (Gadea et al., 2008). In addition, Cdc42-mediated activation of MRCK was required for collective migration of carcinoma cells (Gaggioli et al., 2007). However, several recent studies suggested Cdc42 may function as a tumor suppressor. The Zuo group showed that Cdc42 negatively regulated intrinsic migration/invasion of highly aggressive breast cancer cells (MDA-MB-231 and C3L5 cell line) (Zuo et al., 2012). Although Rho GTPases have been implicated in cellular transformation in breast cancer, their role in cancer development is complicated and may be cell type-specific.
In 1999, the Rotenberg group showed that in MCF-10A cells engineered to over-express PKC-α, the dominant negative forms of Rac1, Rho or Cdc42 can each eliminate the formation of stress fibers. However, only the dominant negative form of Rac1 suppressed the motility phenotype induced by over-expressed PKC-α. This finding indicated that Rac1 participates in PKC-directed signaling pathways. In 2004, Gutierrez’s group demonstrated that the phosphorylation of IQGAP (an effector of Cdc42 and Rac1) by PKC-ε enhanced its binding with Cdc42-ND (nucleotide-depleted) and made the phosphorylated IQGAP1 behave as a Cdc42 inhibitor (Grohmanova et al., 2004). In 2014, the Rotenberg group reported that the phosphorylation of α-tubulin by PKC induces the activation of Rac1 and also stimulates Rac1-dependent cell motility (De et al., 2014). Thus, PKC-mediated phosphorylation can modulate the motility signaling pathway by involving the small GTPases.

A recent study from the Rotenberg lab demonstrated that CEP4 is also a PKC substrate in MCF-10 cells (Chen et al., 2012). CEP4 was first reported to be a novel Cdc42 effector protein together with other members of the CEP family in 2001 (Hirsch et al., 2001). CEPs were showed to bind strongly to immobilized GST-Cdc42 but not to Rac1. CEPs interacted with Cdc42 through their CRIB (Cdc42/Rac interactive binding) domain and functioned as downstream effector proteins in regulating actin reorganization and alteration of cell morphology.

In the present work, MCF-10A cells was chosen as a model since this cell line expresses low levels of PKC isoforms so that the function of CEP4 and its phosphorylation site mutants can be studied against a low background. In this Chapter, the significance of CEP4 phosphorylation to PKC-related phenotypes is explored.

4.2: Results
4.2.1 Preparation and Characterization of Single Site Mutants of CEP4 in MCF-10A Cells

Among the five members of the CEP family, CEP4 is the only member that contains PKC consensus sites. In a determination of the number of sites phosphorylated by PKC, the ratio of phospho-CEP4 to total CEP4 in DAG-lactone treated cells was between 3:1 and 4:1 (Chen et al., 2012). That initial calculation suggested the possibility that there is more than one site that undergoes phosphorylation by DAG-stimulated PKC isoforms.

Potential PKC sites were identified by the presence of a consensus sequence, which consists of a Ser or Thr flanked by one or two basic residues. Four sites were identified at Ser-18, Ser-77, Ser-80, and Ser-86 (Figure 4.2A). In order to investigate the functional significance of these candidate phosphorylation sites in CEP4, a pseudo-phosphorylated (Ser → Asp) mutant of each site was constructed by site-directed mutagenesis. Upon identification of the phenotypically significant site(s), a phosphorylation-resistant mutant (Ser → Ala) was also developed in order to determine whether it has a negative impact on a PKC-associated phenotype (motility, proliferation or morphology).

A pCMV6 plasmid encoding the wildtype CEP4 was obtained from a commercial source and used for these studies. The plasmid conferred FLAG and Myc tags to the CEP4 protein upon expression. Plasmids encoding CEP4-S18D, CEP4-S77D, CEP4-S80D or CEP4-S86D were prepared and verified by a commercial sequencing facility. Following transfection of each construct into MCF-10A cells, the expression level of each mutant (~48kDa) was demonstrated by Western blot analysis with a Myc antibody (Figure 4.2B). To determine the functional significance of each mutated site, a migration assay was performed with each pseudo-phosphorylated mutant individually expressed in MCF-10A cells. MCF-10A cells were transfected with CEP4-S18D, CEP4-S77D, CEP4-S80D, CEP4-S86D, or the vector
control. By use of a 10-well glass slide pre-coated with 1% BSA, the cells were seeded into the well through a cell sedimentation manifold (8000 cells/well) to establish a tight circular monolayer. Following overnight incubation and subsequent removal of the manifold, each circle was imaged at t = 0h and t = 6h with Motic Image software. The numerical difference in the areas at t=0h and t=6h were plotted graphically, as shown in Figure 4.2C. The CEP4-S80D transfectants exhibited the strongest motile behavior that had a 2-fold higher motility as compared with the CEP4-WT. Cells expressing the CEP4-18D mutant displayed the second strongest motility that was 1.3-fold higher as compared to the WT-CEP4, whereas the CEP4-88D mutant exhibited a level of motility that was equivalent to the WT and the CEP4-77D mutant showed no enhancement whatsoever. These results demonstrated that CEP4-S18D and CEP4-S80D had the strongest effects on motile behavior and implied that Ser-18 and Ser-80 are the sites of PKC phosphorylation having functional significance.

4.2.2 Preparation and Testing of Phosphorylation-resistant Mutants of CEP4

To study the functional significance of phosphorylation at Ser-18 and Ser-80, phosphorylation-resistant mutants were created as single site mutants (S18A, S80A) and as a double mutant (S18A-80A). The expression level of the three mutant proteins in MCF-10A transfectants was confirmed by Western blot (Figure 4.3A). Next MCF-10A transfectants were assessed for a change in their motility by the cell sedimentation method. At t = 0 h, the medium were replaced with fresh medium containing 10 μM DAG-lactone or 0.05% DMSO (v/v) as a control. DAG-lactone stimulates endogenous PKC activity that in turn enhances motility of MCF-10A cells. Motility values were calculated by the difference in the areas occupied by the cells at t=0h and t=6h, and the results were plotted in Figure 4.3B. The S18A-80A double mutant was the most effective in inhibiting DAG-lactone stimulated motility by 50%.
Figure 4.2: Preparation and characterization of CEP4 pseudo-phosphorylated single site mutants. (A) Amino terminal sequence of human CEP4 where the PKC consensus sites (full or partial) are underlined and the serine residues that could serve as potential phosphorylation sites (red) are identified by their position. (B) Myc-tagged CEP4 mutants were prepared in which an individual serine (S) was replaced with aspartate (D) residue, followed by its expression in MCF-10A human breast cells. To demonstrate expression of the mutant proteins relative to a vector control (VC), 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 judge equivalent sample loading. (C) For assay of cell motility, each mutant CEP4-encoding plasmid was transfected into MCF-10A cells. After 48 h, the transfected cells were plated on 10-well glass slide and incubated overnight at 37°C. Cell motility was measured in triplicate by the cell sedimentation assay (described in the ‘Methods’). Images were acquired by a 4X objective at t = 0 h and t = 6 h. The difference in the area between the two time points was averaged. 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 the VC condition with each selected mutant (**P < 0.00001).

The next step was to assess the impact of Ser-18 and Ser-80 on the extent of CEP4 phosphorylation in MCF-10A cells under PKC-activated conditions. MCF-10A cells were transfected with VC, CEP4-WT, CEP4-S18A, CEP4-S80A or CEP4-S18A/80A. Following transfection, the cells were treated with DAG-lactone (10 μM) or DMSO for 1h at 37°C. The
cells were lysed in hypotonic detergent-free IP buffer in order to avoid the interference of detergent during the immunoprecipitation and the samples were normalized for total protein content. CEP4 protein was immunoprecipitated from each sample with EZ view anti-FLAG beads, and the immunopellets were analyzed by Western blot.

Figure 4.3: Expression of phosphorylation-resistant CEP4 mutants block DAG-lactone-induced cell motility. A. Plasmids encoding CEP4-WT, CEP4-18A, CEP4-80A and CEP4-18A/80A were transfected into MCF-10A cells. A representative Western blot of whole cell lysates (50 µg per lane) showed a similar level of CEP4 expression with Myc antibody (1:1000). A β-actin antibody (1:5000) was used to establish equivalent sample loading. B. CEP4 WT and mutants were tested for their impact on motility of MCF-10A cells treated with 10 µM DAG-lactone. The results are representative of three independent experiments. 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).
Figure 4.4: Expression of phosphorylation-resistant CEP4 mutants inhibit DAG-lactone-stimulated phosphorylation of CEP4. A. Wildtype (WT)-CEP4 or single site and double site mutants corresponding to S18A, S80A, or S18A/S80A-CEP4 were transfected into MCF-10A cells. After 48h incubation at 37°C, the cells were treated with DAG-lactone (10 μM) or DMSO (0.05% v/v) for 1h at 37°C, followed by preparation of a whole cell lysate (150 μg/sample) with detergent-free lysis buffer [20 mM TRIS (pH 7.4), 2mM MgCl2, 2 mM EGTA and 1 mM DTT, 10 μM bis-indoleylmaleimide, 0.1% protease inhibitors, and 1% phosphatase inhibitors]. The lysate was pre-cleared using 30 μl mouse IgG-agarose beads, followed by immunoprecipitation with 60 μl anti-FLAG agarose beads (described in ‘Methods’). The pellets were analyzed by Western blot with PKC substrate antibody (1:1500) that recognizes the phosphorylated PKC substrate consensus sites in phospho-CEP4, or CEP4 antibody (1:2000) as loading control. B. Quantitation of the signal intensity was carried out with Image J software.

Phospho-CEP4 was detected with the PKC substrate antibody (detects the phosphorylated PKC consensus site) and the expression of total CEP4 was detected with human CEP4 antibody. In Figure 4.4A, the results showed that CEP4-WT was strongly phosphorylated when the cells were stimulated by DAG-lactone. Compared to DAG-lactone stimulated phosphorylation of CEP4-WT, the single site phosphorylation-resistant mutants – (S18A or S80A) each suppressed the DAG-lactone-induced phosphorylation of CEP4 by 75-80%. However, expression of the CEP4S18A/80A mutant eliminated the DAG-lactone-induced phosphorylation signal to below that of the basal condition (VC). These results were
quantified by Image J shown in Figure 4.4B. Taken together, Ser-18 and Ser-80 appear to be the major phosphorylation sites having functional significance. Therefore, CEP4 18D/80D, (the pseudo-phosphorylated double mutant), and CEP4 18A/80A (the phosphorylation-resistant double mutant), were used in subsequent experiments to investigate their phenotypic effects in MCF-10A cells.

4.2.3 Evaluation of CEP4 Mutants in Additional Phenotypes

Previous studies reported that over-expression of PKC-α in non-transformed MCF-10A human breast cells causes a pronounced increase in motility with loss of detectable of E-cadherin, decreased proliferation (due to slow passage through G1 of the cell cycle) and radical alterations in morphology (extensive and well-defined organization of actin stress fibers) (Sun and Rotenberg 1999). In view of the importance of PKC activity to cancer-related phenotypes, pseudo-phosphorylated CEP4 was explored as the agent that produces these phenotypes.

4.2.3.1 Proliferation Assay of CEP4 Mutants in MCF-10A Cells

Overexpression of PKC-α in MCF-10A cells was previously shown to inhibit cell proliferation by slowing the passage of cells through the G1 stage of the cell cycle (Sun and Rotenberg, 1999). To determine if phospho-CEP4 similarly inhibits cell proliferation, an assay with Alamar Blue was performed on MCF-10A cells that expressed a double mutant, CEP4-18A/80A or CEP4-18D/80D. In addition to staining cells, the Alamar Blue reagent acts as a fluorimetric indicator of metabolic activity. MCF-10A cells were transfected with a CEP4 double mutant or the vector control. After 48h at 37°C, the transfectants were re-plated into 96-well plates, and the Alamar Blue reagent was added to each well to a final concentration of 4% (v/v).
Figure 4.5: The impact of phosphorylation-resistant/pseudo-phosphorylated CEP4 mutants on proliferation in MCF-10A cells. Plasmids encoding CEP4-18A/80A and CEP4-18D/80D were transiently transfected into MCF-10A cells. Forty-eight hours post-transfection, the cells were re-plated into 96-well plates. The proliferation assay of the transfectants was assessed using the Alamar Blue reagent, as described in the “Methods” section. The fluorescence intensity was measured each day for 4 days.

The plates were incubated at 37°C, 5% CO₂ and daily measurements were made for 4 days. Since the fluorescence intensity is sensitive to temperature, the plates were allowed to equilibrate to room temperature for 30 min prior to fluorescence measurement using a 96-well plate reader at λ<sub>ex</sub> = 530 nm and λ<sub>em</sub> = 590 nm. The plates were returned to the incubator until the next time point. Cell proliferation rates displayed no significant difference in MCF-10A cells expressing a mutant CEP4 or VC (Figure 4.5). The lack of effect may be due to CEP4 mutants having been transiently transfected into MCF-10A cells whereby the expression was highest at 48h. Thus, when cells were subsequently plated into a 96-well plate post-transfection and monitored for 4 days to measure the proliferation, the transfected cells may have lost the plasmid as the assay progressed. Therefore, this proliferation assay may not
be an accurate representation of the impact on proliferation by the mutants. The best way to characterize a proliferation effect is to work with stably transfected cell lines, as will be discussed in Chapter 5.

4.2.3.II Immunocytochemistry of MCF-10A Cells Expressing Double Mutants of CEP4

The actin cytoskeleton has a fundamental role in various cellular processes such as migration, morphogenesis, cytokinesis, endocytosis and phagocytosis. Small Rho GTPases have been studied as components that regulate and organize actin structure (Hall, 1994; Nobes and Hall, 1995; Ridley and Hall, 1992). In NIH-3T3 fibroblasts cells, CEPs were shown to induce pseudopodia formation through the Cdc42 pathway and was visualized by F-actin staining (Hirsch et al., 2001). In primary keratinocytes, CEP2 and CEP5-expressing cells showed less F-actin staining at the adherent belt and displayed thin stress fibers that extended throughout the cell body, similar to the effect caused by the constitutively active form Cdc42-Q61L (Hirsch et al., 2001). On the basis of these results, CEPs were proposed to act as downstream effectors of Cdc42 that reorganize the actin cytoskeleton and produce cell morphology changes. However, in Madin-Darby canine kidney cells (MDCK), Cdc42 affected the cortical actin structure and distribution of the actin cytoskeleton at cell tight junctions but there were undetectable CEP-induced changes in actin organization, suggesting that CEP and Cdc42 acted independently (Rojas et al., 2001). Therefore, we examined whether the distribution of F-actin and the morphology of cells were altered by the expression of the phosphorylation site mutants of CEP4 in MCF-10A cells.

Vector control (VC) cells or those expressing CEP4 WT, CEP4-18D/80D or CEP4-18A/80A were cultured for 24h under serum-free conditions on coverslips in 6-well plates. The cells were stained with Myc antibody to recognize the cells transfected with a CEP4 plasmid (shown in green). The distribution of F-actin and the cell morphology were determined by
incubating fixed and permeabilized cells with rhodamine-phalloidin (shown in red) and examining stained cells with a Zeiss fluorescence microscope (Figure 4.6A). After transfection, CEP4 expression was identified in the cytoplasm and at the cell membrane, regardless of whether CEP4 was expressed as the wildtype or mutant protein. In CEP4-18D/80D expressing cells, actin filaments were well-defined throughout the entire cell body (Figure 4.6A). In approximately 85% of cells expressing CEP4-18D/80D, we observed filopodia which are long, thin actin filaments that project from the cell surface. Approximately 50% of CEP4-WT-expressing cells also showed these structures. In contrast, only 10% of cells expressing CEP4-18A/80A displayed filopodia, similar to the vector control cells (Figure 4.6B). In summary, these observations indicated that the actin structure in MCF-10A cells is responsive to the expression of pseudo-phosphorylated CEP4.
A. VC | WT | 18A/80A | 18D/80D

- **CEP4**
- **ACTIN**
- **NUCLEUS**
- **BRIGHT FIELD**
- **MERGED**

B. Fraction of cells with filopodia

![Bar chart showing the fraction of cells with filopodia for different conditions.](chart.png)

- **VC**
- **CEP4-WT**
- **CEP4-18A-80A**
- **CEP4 18D-80D**

**Note:** The bar chart indicates a significant difference between **CEP4 18D-80D** and the other conditions, as denoted by the asterisks. **VC** and **CEP4-WT** show lower fractions compared to the other conditions.
Figure 4.6: Pseudo-phosphorylated CEP4 mutants induce filopodia formation in MCF-10A cells. A. MCF-10A cells were transfected with Myc-tagged wildtype (WT)-CEP4, CEP4-18D/80D or S18A/S80A. Twenty-four hours post-transfection, the medium was replaced with serum-free media and the cells were incubated overnight. On the second day, the serum-starved cells were fixed by 4% paraformaldehyde. CEP4 expression was detected by rabbit monoclonal antibodies against Myc epitope tag (1:300) followed by staining with FITC-conjugated goat anti-rabbit IgG (1:300). F-actin was visualized by staining with rhodamine-phalloidin (100 nM). Images were acquired at 63x objective. Bar, 10μm. B. Cells expressing filopodia are represented as a fraction of the total number of transfected cells, where a total of 50 cells were analyzed per condition over three independent experiments. 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 for cells expressing A/A and D/D mutants (**P < 0.0001).

IQ-domain GTPase-activating proteins (IQGAPs) comprise a family of multi-domain proteins that act as a scaffold for numerous interacting partners while transducing extracellular signals that influence mitogenic, morphological and migratory cell behavior. IQGAP1 is the most studied member of the family due to its role in actin reorganization that affects cell-cell adhesion, cell polarization and cell migration (Johnson et al., 2009). There is mounting evidence showing that IQGAP1 contributes to cancer progression by regulating the signaling pathway in cell proliferation and stimulation of cell motility and invasion (Johnson et al., 2009; White et al., 2009). IQGAP1 was initially identified as a Cdc42/Rac1 effector protein and was also demonstrated to participate in Cdc42/Rac1- induced cell migration and invasion (Fukata, 1996; Mataraza et al., 2003). IQGAP forms a ternary complex with activated Cdc42 or Rac1 and the adenomatous polyposis coli (APC) protein leading to regulate actin structure (Watanabe et al., 2004). Since we observed filopodia formation and enhanced cell motility with the expression of phospho-CEP4, we were interested in determining whether phosphorylation of CEP4 modulates actin structure and cell migration by affecting localization of IQGAP1. To address this question, we transfected MCF-10A cells with CEP4-18A/80A or CEP4-18D/80D (shown in red) and stained with IQGAP1 antibody (shown in green). As shown in Figure 4.7A and 4.7B, CEP4-18D/80D expression was correlated with the association of IQGAP1 with cell-cell contacts for non-migrating cells and
at the leading edge and filopodia of moving cells. In contrast, CEP4-18A/80A expression was correlated with primarily a cytoplasmic location of IQGAP1, with a much lower level of IQGAP evident at cell-cell junctions and at the cell surface (Figure 4.7A and 4.7B). It is noted that expression of neither CEP4 mutant affected the total level of IQGAP1 expression (Figure 4.7C). However, the results suggested that the phosphorylation of CEP4 might interfere in the sub-cellular distribution of IQGAP. In this regard, phosphorylation of CEP4 may activate the Cdc42/Rac1 pathway.

E-cadherin is a calcium-dependent cell-cell adhesion molecule with important roles in cell adhesion and migration. PKC-α overexpression in MCF-10A cells resulted in the loss of detectable E-cadherin (Sun and Rotenberg, 1999), and expression of Cdc42-Q61L and CEPs also resulted in reduced levels of E-cadherin at adherens junctions (Hirsch et al., 2001). Therefore, we performed immunocytochemistry to study the distribution of E-cadherin at adherens junctions in cells expressing a CEP4 double mutant. As shown in Figure 4.8, no detectable difference was found in the distribution or expression of E-cadherin (shown in red) between the two mutants (shown in green) in MCF-10A cells.
Figure 4.7: IQGAP immunofluorescence at the cell leading edge and cell-cell junctions of CEP4 mutants expressing MCF-10A cells. Myc-tagged CEP4-S18D/80D or CEP4-S18A/S80A were transfected into MCF-10A cells and fixed with paraformaldehyde 48h after transfection. CEP4 expression (shown in red) was detected by a mouse monoclonal antibody against the Myc epitope tag (1:300) followed by staining with Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (1:300). IQGAP was visualized by staining with rabbit monoclonal anti-IQGAP (shown in green) at a 1:200 dilution followed by staining with FITC-conjugated goat anti-rabbit IgG (1:300). A. Images show the location of IQGAP near cell-cell junctions only in cells expressing CEP4-18D/80D. B. Images show IQGAP signals near the cell leading edge and associated filopodia only in cells expressing CEP4-18D/80D. Images were acquired with a 63x objective. Scale bar is 10 μm. C. MCF-10A cells were transfected with Myc-tagged CEP4-WT, CEP4-S18D/80D or CEP4-S18A/S80A. The WT transfectants were treated with or without DAG-lactone (10 μM). Whole cell lysates were prepared and analyzed by Western blot (75 μg/lane). The expression level of IQGAP was detected by anti-IQGAP (1:1000).
Figure 4.8: E-cadherin immunofluorescence at cell-cell contacts of CEP4 mutants in MCF-10A cells. Myc-tagged CEP4-WT, CEP4-S18D/80D or CEP4-S18A/S80A were transfected into MCF-10A cells and fixed 48h post-transfection. CEP4-18D/80D and CEP4-18A/80A were detected with rabbit anti-Myc antibody (1:300) followed by staining with FITC-conjugated goat-anti rabbit antibody (1:300). E-cadherin was detected with mouse monoclonal anti-E-cadherin diluted to 1:150 followed by staining with Fluor 594-conjugated goat anti-mouse secondary antibodies (red, 1:300). Images were acquired with a 63x objective. Scale bar is 10 μm. C. MCF-10A cells were transfected with Myc-tagged CEP4-WT, CEP4-S18D/80D or CEP4-S18A/S80A. The wildtype transfectants were treated with or without DAG-lactone (10 μM). Whole cell lysates were prepared and analyzed by Western blot (75 μg/lane). The expression level of E-cadherin was detected by anti-E-cadherin (1:1000).
4.2.4 Phosphorylation of CEP4 Alters Its Binding Affinity for Cdc42

A previous report demonstrated CEPs bind to Cdc42 in a GTP-dependent fashion, but not to Rac1 or Rho (Hirsch et al., 2001). Other studies have suggested that PKC-mediated phosphorylation influences the binding activity of accessory proteins (like GDI or effector proteins) that regulated the activation state of Rho GTPase (Grohmanova et al., 2004; Mehta et al., 2001). The next step was to test the hypothesis that phosphorylation of CEP4 alters its affinity for Cdc42. We used glutathione-agarose beads to bind constitutively active glutathione-S-transferase (GST)-tagged-Cdc42Q61L that had been prepared from bacterial cells. MCF-10A cells expressing the Myc-tagged CEP4-WT (treated with or without DAG-lactone) or expressing CEP4 18A/80A or CEP4-18D/80D were prepared into whole cell lysates. Each lysate was incubated with the immobilized GST-Cdc42Q61L; the beads were washed, and the material remaining on the beads was analyzed by Western blot with anti-Myc. It was observed that DAG-lactone treatment-induced phosphorylation of CEP4-WT or the pseudo-phosphorylated mutant each exhibited a very weak interaction with immobilized GST-Cdc42Q61L. Whereas WT-CEP4 isolated from untreated cells or the phosphorylation-resistant CEP4 mutant each displayed strong binding affinity for Cdc42-Q61L. The results suggested that phosphorylation of CEP4 greatly diminishes its affinity for Cdc42, presumably causing the release of Cdc42 to other downstream effectors. Since the pseudo-phosphorylated mutant of CEP4 also produced enhanced cell motility and induced filopodia, it is possible that phosphorylated CEP4 interacts with other pathways to stimulate cell motility after its release from Cdc42.
Figure 4.9: Binding of CEP4 double mutants to constitutively active GST-Cdc42-Q61L. MCF-10A cells were transfected with Myc-tagged WT plasmid or a mutant-encoding CEP4 plasmid. Where indicated, the WT-CEP4-expressing cells were treated with 10 μM DAG-lactone or DMSO (0.05%, v/v). Following bacterial expression and immobilization of GST-Cdc42-Q61L on glutathione beads, a MCF-10A cell lysate containing WT or mutant CEP4 was incubated with the beads for 40 min. After centrifugation for 5 minutes at 10,000 x g and washing with detergent-free lysis buffer three times, the pellets were analyzed by Western blot with anti-Myc (1:1000) to determine the amount of bound CEP4 protein in each condition. The blot was also analyzed with anti-Cdc42 (1:5000) to establish an equivalent loaded amount of pelleted Cdc42-Q61L.

4.2.5 Phosphorylated CEP4 Activates Rac1

PKC-mediated phosphorylation of CEP4 produces elevated cell motility which had been previously attributed to PKC in MCF-10A human breast cells. An earlier study from our laboratory demonstrated that Rac1 was functionally involved in the motility phenotype produced by PKC-α expression in these cells (Sun and Rotenberg, 1999). Since we had identified that phosphorylation of CEP4 causes its disassociation with Cdc42, it was of interest to determine whether Rac1 was involved in phospho-CEP4-induced cell motility. For this purpose, cell migration of cells expressing CEP4-18D/80D was measured under conditions of Rac1 inhibition using the small molecule inhibitor NSC23766. This cell-permeable molecule prevents Rac1 activation to the GTP-bound form by inhibiting binding of Rac1-specific guanine nucleotide exchange factors (GEFs), thereby keeping Rac1 in the GDP-bound (inactive) state. In Figure 4.10B, it was observed that the Rac1 inhibitor significantly inhibited migration induced by CEP4-18D/80D transfectants by 95%. Cdc42 has been implicated in both stimulatory and inhibitory effect on cell motility. The phospho-CEP4 induced motility might also result in a Cdc42-stimulated pathway. Therefore, we also tested
the impact of a Cdc42 inhibitor ML141 on cell migration of MCF-10A cells. ML141 is cell permeable allosteric compound that acts as a potent, selective, reversible and non-competitive inhibitor of Cdc42 GTPases. However, ML141 did not suppress the cell motility but rather stimulated this phenotype regardless of which plasmid had been transfected into MCF-10A cells (Figure 4.10A). These results suggested that Rac1 (but not Cdc42) participates in phosho-CEP4-induced cell migration in MCF-10A cells.

Figure 4.10: The effect of a Rac1 inhibitor (NSC23766) and Cdc42 inhibitor (ML141) on motility of MCF-10A cells transfected with CEP4 mutants. A. Triplicate samples of transfectants expressing mutant CEP4 proteins or the vector control (VC) were treated with or without 5 µM ML141 (in DMSO) for 8 h during the entire period (8 h) that motility was being measured. The results are representative of three independent experiments. 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 Cdc42 inhibitor (*P < 0.0001). B. Triplicate samples of transfectants expressing mutant CEP4 proteins or the vector control (VC) were treated with or without 50 µM NSC23766 (in water) for 6 h during the entire period (6 h) that motility was being measured. The results are representative of three independent experiments. 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 Rac1 inhibitor (*P < 0.0001, **P < 0.000001).
We next examined whether the Rac1 inhibitor also affected the phospho-CEP4-induced alterations of morphology in MCF-10A cells. To address the role of Rac1 in the formation of filopodia, we treated CEP4-18D/80D transfectants with 50 μM Rac1 inhibitor for 2 hours, followed by staining of the cells with rhodamine-phalloidin. Fluorescence microscopy (Zeiss) indicated that compared with untreated cells expressing CEP418D/80D, of which 85% displayed filopodia, only 10% of cells exhibited filopodia after treatment with the Rac1 inhibitor. It is noted that only 50% of cells exhibited filopodia after treatment with the Cdc42 inhibitor. These results indicated that phospho-CEP4 induces cell surface projections by a mechanism that involves Rac1, and that Cdc42 contributes to a lesser extent to the formation of these structures.

**Figure 4.11:** The effect of a Rac1 inhibitor (NSC23766) or Cdc42 inhibitor (ML141) on actin structure of MCF-10A cells transfected with CEP4 mutants. MCF-10A cells expressing Myc-tagged pseudo-phosphorylated CEP4 mutant were treated with or without 50 μM NSC23766 or 5 μM ML141 for 2 h, fixed with paraformaldehyde, and stained with rhodamine-phalloidin (100 nM) to detect F-actin. Transfected cells were identified by GFP fluorescence with Myc-tagged rabbit mAb (1:300) as described in the ‘Methods’ section. Images were acquired with a 63x objective. Scale bar is 10 μm.
In order to investigate whether phospho-CEP4 activates small GTPases (especially Rac1), a RhoA/Rac1/Cdc42 activation assay was performed. MCF-10A cells expressing CEP4-18D/80D or CEP4-18A/-80A were lysed and each lysate was incubated with either PAK-PBD agarose (EMD-Millipore) to pull down any activated Rac1 and Cdc42, or Rhotekin-agarose to pull down activated RhoA. Proteins present in pulled-down samples were resolved by SDS-PAGE (12%) and probed with an antibody specific for Rac1, Cdc42, or RhoA. As shown in Figure 4.12, cells expressing the CEP4-S18D-80D mutant exhibited stronger signals for GTP-Rac1 than cells expressing the CEP4 18A-80A mutant or vector control. In contrast, GTP-Cdc42 and GTP-RhoA signals were not affected by expression of either CEP4 mutant. Similar to previous findings with α6-tubulin mutants (De et al., 2014), there were negligible activated Cdc42 signals in all conditions; whereas RhoA was strongly activated in all conditions (data not shown). Thus, the expression of phospho-CEP4 stimulated the activation of Rac1.

Figure 4.12: Western blot analysis of the level of activated Rac1, in response to CEP4 mutant expression. CEP4-18A/80A (A/A) or CEP4-18D/80D (D/D) cDNA plasmids were transfected into MCF-10A cells. Cell lysates were prepared as described in the ‘Methods’ and each cell lysate (600 μg) was incubated with PAK-PBD beads (20 μg) for 2 h at 4°C with rotation. After washing the pellets, the material was resolved by 12% SDS-PAGE and western transfer, and the blot was probed with anti-Rac (Cell Signaling Technology; 1:500).

4.2.6 Identification of CEP4-Associated Proteins by the Tandem Affinity Purification (TAP) Method

Since phospho-CEP4 could stimulate Rac1, phospho-CEP4 might directly interact with Rac1 or interact with some other protein that induces the activation of Rac1. In an effort to identify
and characterize novel phospho-CEP4-interacting proteins, we carried out an analysis using TAP (tandem affinity purification) methodology. The TAP method entails the purification of a protein complex of interest under conditions that preserve protein-protein interactions. For this procedure two affinity purification tags are employed - a streptavidin binding peptide (SBP) and a calmodulin binding peptide (CBP), each of which is immobilized on a resin (Figure 4.13). Any binding proteins eluted from the second column (CBP) are resolved by SDS-PAGE and the gel is stained with Gelcode Blue. In our experiment, bands that were evident with the CEP4-S18D/S80D mutant but absent with the CEP4-S18A/S80A mutant were excised and analyzed by mass spectrometry (MS/MS).

![Figure 4.13: Schematic diagram of purification of protein complexes by the Tandem Affinity Purification method.](image)

To purify proteins by the TAP protocol, cell lysates were applied to the streptavidin resin, and eluted with streptavidin elution buffer. The eluate is applied to a calmodulin resin and eluted. The eluate from this second column is analyzed by SDS-PAGE (Agilent Technologies).
To prepare for this experiment, the full length of human Myc-tagged CEP418D/80D or 18A/80A (1000 bp) was cloned into the pCTAP vector (4500 bp) so that tandem affinity purification (TAP) tags consisting of a streptavidin-binding peptide and a calmodulin-binding peptide were fused in-frame to its COOH terminal of the CEP4 mutant. At least three clones for each mutant were selected and expressed in MCF-10A cells to confirm the expression of the TAP proteins. The pCTAP-CEP4-18D/80D or pCTAP-CEP4-18A/80A was transfected into MCF-10A cells and the expression was confirmed by Western blot with anti-Myc (Figure 4.14A). One clone from each of the two constructs was expressed in MCF-10A cells followed by preparation of cell lysates (from three 10-cm plates at 80-90% confluence) by the freeze/thaw method. Lysates were applied sequentially to the affinity columns, as described in the Methods, and as shown schematically in Figure 4.13. The samples were resolved by 12% SDS-PAGE gel and stained with Gelcode Blue. When the patterns of protein bands were compared between TAP-CEP4-18A/80A and TAP-CEP4-18D/80D samples, three major bands at 250 kDa, 125 kDa and 44 kDa (indicated by red boxes) were unique to the TAP-CEP4-18D/80D sample (Figure 4.14B).

To identify those proteins that specifically associated with CEP4-18D/80D, the observed 250 kDa, 125 kDa and 44 kDa bands were analyzed by MS/MS. The results are shown in Table 4.1. Potential associations between phospho-CEP4 and TEM4 or PARD6 were identified since the two proteins are related to GTPase activity. To demonstrate direct interactions with CEP4-S18D/S80D, co-immunoprecipitation (Co-IP) experiments were performed with FLAG-tagged CEP4 mutant proteins using anti-FLAG-agarose. Western blot with specific antibodies against the target protein was used to show the presence of the suspected binding partner in the pellet. Interactions between phospho-CEP4 and PARD6, and phospho-CEP4 and TEM4 were detected since PARD6 and TEM4 signals were much stronger in CEP4-18D/80D complexes (Figure 4.15). In this experiment, we also observed that CEP4-18A/80A
interacted strongly with endogenous Cdc42 whereas the CEP4-S18D/S80D bound weakly to Cdc42 (Figure 4.15). This finding was in agreement with the in vitro GST-Cdc42-Q61L binding assay that showed that phospho-CEP4 only weakly associated with Cdc42. These co-immunoprecipitation results suggested that phospho-CEP4 co-localizes and interacts with TEM4 or PARD6 in intact cells.

Figure 4.14. Tandem affinity purification (TAP) of pseudo-phosphorylated CEP4 and its binding proteins. Mutants of CEP4 were sub-cloned into a TAP vector that upon expression conferred two affinity tags (peptide sequences corresponding to specific sites in streptavidin and calmodulin) for affinity chromatography. The plasmids were transfected into MCF-10A cells and the resulting cell lysates (3 mg/sample) were applied sequentially to two affinity binding columns. The second eluate was resolved by 8% SDS-PAGE and detected by staining with Gelcode Blue. (A) Western blot demonstrating expression of the TAP-CEP4 mutants as compared with the vector control (VC) using anti-Myc (1:1000). (B) Gelcode Blue-stained 8% SDS-PAGE gel showing that the pseudo-phosphorylated CEP4 (D/D) co-purified with several protein bands not found with the phosphorylation-resistant CEP4 (A/A) mutant or VC samples. Bands excised for subsequent MS/MS analysis are indicated with a carat (<). The results are representative of two independent experiments.
Table 4.1: MS/MS analysis of proteins that co-immunoprecipitated with TAP-CEP4-18D/80D. Gel-resolved bands were excised and proteins were digested in situ with trypsin. The resulting peptides were analyzed by LC–MS/MS on a 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. (Trypsinolysis and MS/MS analysis were performed at the Keck Protein Core Facility at Yale University Medical Center.)

<table>
<thead>
<tr>
<th>Gel band</th>
<th>Proteins Detected</th>
<th>Score</th>
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<tbody>
<tr>
<td>75 kDa</td>
<td>WNK4</td>
<td>27</td>
<td>Ser/Thr protein kinase</td>
</tr>
<tr>
<td>125 kDa</td>
<td>TEM4 (ARHGEF17)</td>
<td>24</td>
<td>GEF for Rho GTPases</td>
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<tr>
<td></td>
<td>CLIP-1</td>
<td>27</td>
<td>microtubule plus-end binding protein</td>
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<tr>
<td></td>
<td>RAS guanyl releasing protein-1 (GRP1)</td>
<td>46</td>
<td>DAG-regulated GEF</td>
</tr>
<tr>
<td></td>
<td>GTP-binding protein Di-RAS2 (DIRA2)</td>
<td>31</td>
<td>low GTPase activity</td>
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<tr>
<td></td>
<td>TBC9B</td>
<td>33</td>
<td>GAP for Rab GTPase</td>
</tr>
<tr>
<td>250 kDa</td>
<td>PARD6G (PAR6-gamma)</td>
<td>31</td>
<td>adapter that links atypical PKCα/τ to Rac1/Cdc42</td>
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</tbody>
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Figure 4.15: Western blot verification of protein-protein interactions with CEP4 mutants. A. MCF-10A cells were transiently transfected with each CEP4 (A/A or D/D) or the vector control (VC). Following preparation of cell lysates (500 µg/sample), co-immunoprecipitation of each CEP4 mutant was performed with anti-FLAG under non-detergent conditions in order to evaluate binding proteins. Immunoprecipitated pellets were analyzed by Western blot with either anti-TEM4 (1:500), anti-PARD6G (1:500) or anti-Cdc42 (1:500) for studying the protein-protein interaction, and anti-CEP4 (1:2000) as a loading control.
4.2.7 Characterization of The Function of Phospho-CEP4-associated Protein TEM4 and Evaluation of Its Potential Role in Rac1 Activation

In order to further elucidate the unknown mechanism underlying Rac1 activation in phospho-CEP4 induced migration, we performed a TAP experiment to identify the phospho-CEP4-associated binding proteins, among which were included TEM4 (tumor endothelial marker 4) and PARD6 (partitioning defective homolog 6 gamma). TEM4 (tumor endothelial marker 4), also known as ARHGEF17, was identified as a tumor marker protein whose expression was elevated during cell-induced angiogenesis in endothelial cells (Croix et al., 2000). TEM4 was previously described as a cytoskeleton binding protein and also associated with the E-cadherin-catenin complex. In confluent cells, TEM4 localizes to cell–cell contacts or actin stress fibers (Croix et al., 2000). In 2013, Ngok’s group found that TEM4 is a novel Rho-specific GEF (guanine nucleotide exchange factor) in Madin-darby canine kidney (MDCK) cells. The down-regulation of TEM4 in HUVECs and MDCK cells resulted in defective cell-cell junctions and impaired cell morphology (Ngok et al., 2013). Most importantly, TEM4 can regulate endothelial cell migration. Mitin’s group reported that TEM4 provides essential control of the actin cytoskeleton network, ensures proper membrane protrusion of the leading edge, and regulates cell migration (Mitin et al., 2013). PARD6G is a 376 amino acid adaptor protein involved in cell polarization and asymmetrical cell division processes. When atypical PKC and PARD6G are expressed with constitutively active Rac1, the proteins co-localize to membrane ruffles. PARD6 also functions as a key adaptor that links Par3 to atypical PKCs and Cdc42 GTPase (Noda et al., 2001). These interactions are important for the formation or maintenance of tight junctions in MDCK epithelial cells (Joberty et al., 2000).

Based on these previous studies that showed that TEM4 activates RhoA GTPase, binds actin, and promotes the migration phenotype, TEM4 was selected for further study as a direct
binding partner of phospho-CEP4. Thus, TEM4 was evaluated for a role in phospho-CEP4-mediated cell motility. The TEM4-specific GFP-shRNA-encoding plasmid was introduced into MCF-10A cells and the knockdown of TEM4 was achieved with this reagent, as shown in Figure 4.16A. While TEM4 knockdown was in effect, MCF-10A cells were transfected with CEP4-18D/80D, CEP4-18A/80A, or VC, and cell migration was assessed. As shown in Figure 4.16B, 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 expressing the A/A-CEP4 mutant. This result implicates the TEM4/phospho-CEP4 complex as an active component in the PKC-induced motility signaling pathway.

The next step was to determine whether phosphorylated CEP4 acts through TEM4 to activate Rac1. For this purpose, cells were transfected with a CEP4 mutant (or VC) and either TEM4-shRNA or the scrambled control shRNA. Following preparation of cell lysates, pull-down of activated Rac1 was performed with PAK binding domain (PBD)-agarose beads that specifically bind the activated form of Rac1. Western blot analysis was conducted with a Rac antibody (recognizes Rac isoforms 1, 2, and 3). As shown in Figure 4.17, when cells expressed the scrambled control shRNA, the D/D-CEP4 mutant produced a strongly activated Rac signal as compared with the A/A-CEP4 mutant, or the VC. However, when TEM4 was silenced, D/D-CEP4 expression did not result in an elevated Rac signal. This finding implicated phosphorylated CEP4 or the phospho-CEP4/TEM4 complex in the mechanism by which Rac was activated.
Figure 4.16: Differential effects of TEM4 knockdown on migration of CEP4 double mutants in breast cells. (A) Demonstration that a shRNA reagent knocks down TEM4 expression in MCF-10A cells. Cells were transfected with the GFP-shRNA-TEM4 plasmid (shRNA) or the GFP-scrambled control plasmid (SC). After 48 h, cells were transfected with the pseudo-phosphorylated CEP4 double mutant (D/D), the phosphorylation-resistant CEP4 double mutant (A/A), or the vector control (VC). Forty-eight h later, cells were lysed, the samples were resolved on a 6% SDS-PAGE gel, and analyzed for TEM4 expression by Western blot (75 µg/lane) with anti-TEM4 (1:500 dilution). The experiment was performed three times with identical results. (B) MCF-10A transfectants were tested for motility in triplicate by detecting fluorescent cells expressing either the GFP-shRNA-TEM4 signal (red bars) or GFP-shRNA-SC (scrambled control) blue bars). Fluorescent images were recorded on a Leica fluorescence microscope at t = 0 and t = 6 h and the images were quantitated by Motic Image software. 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 the scrambled control and shRNA condition for each mutant (*P < 0.0001, ** P < 0.000001).

Figure 4.17: Pseudo-phosphorylated CEP4 or TEM4 activates Rac1. Following expression of either shRNA-TEM4 or the scrambled control shRNA (SC), Rac1 activation was measured in a pull-down assay with 600 µg of a lysate prepared from cells co-transfected with the shRNA reagent and CEP4-S18D/S80D (D/D), CEP4-S18A/S80A (A/A), or the empty vector (VC). 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.
4.3: Discussion

In the foregoing chapter, CEP4 was characterized as a new substrate of PKC in MCF-10A human breast cells. The functional significance of phosphorylated CEP4 to specific signaling events and cancer-related phenotypes were studied. The phosphorylation of CEP4 induced cell motility which had not been explored in any previous studies of CEP4. Inspection of the human CEP4 primary sequence shows that there are four independent PKC consensus site located at Ser-18, Ser-77, Ser-80, and Ser-86. Of these four sites, pseudo-phosphorylated mutants of CEP4 at only Ser-18 and/or Ser-80 stimulated cell migration (Figure 4.2C). In complementary experiments, expression of the CEP4-18A/80A (phosphorylation-resistant) mutant had a dominant negative effect on DAG-lactone stimulated cell motility in MCF-10A cells (Figure 4.3B). Moreover, the expression of the phosphorylation-resistant mutant CEP4-18A/80A suppressed DAG-lactone stimulated phosphorylation of endogenous CEP4 protein (Figure 4.4). These results implicated Ser-18 and Ser-80 as the primary phosphorylation sites of CEP4 that modify its intracellular functions. The phosphorylation of CEP4 at the both sites was found to induce the dissociation of CEP4 from constitutively active Cdc42 (Figure 4.9), and produced elevated cell motility and actin-based membrane protrusions (filopodia) in MCF-10A cells (Figure 4.6). In the presence of inhibitors of Cdc42 or Rac1, we found that Rac1 (but not Cdc42) participates in phosho-CEP4-induced cell migration in MCF-10A cells and filopodia formation (Figure 4.10 and 4.11). The finding that phosho-CEP4 operated independently of Cdc42 to produce these phenotypes, revealed a new dimension in the function and regulation of this effector protein.

Earlier studies characterized the CEPs as proteins that bind GTP-bound Cdc42 with high affinity (Hirsch et al., 2001). Cdc42 is bound at the CRIB domain in CEP4 which is located between Ile27 – Leu53. Therefore, the two sites of phosphorylation identified here (Ser18,
Ser80) occurred on nearby serine residues that flank this region. In the absence of an available 3-D structure of CEP4, we can only speculate that the negative charge introduced by phosphorylation of these Ser residues significantly alters the 3-D structure of the CRIB domain resulting in severely diminished binding by activated (GTP-bound) Cdc42. Notably, the other four CEP isoforms do not possess PKC phosphorylation sites (Hirsch et al., 2001), implying that they are refractory to regulation by PKC.

Since we had identified that phosphorylation of CEP4 causes its disassociation with Cdc42, it was of interest to determine whether Cdc42 (upon its release from phospho-CEP4) was a participant in one or both CEP4-induced phenotypes. Although principally known to promote cell polarity, Cdc42 was reported to suppress migration of breast cancer cells (Zuo et al., 2012). In the present study, the Cdc42 inhibitor (EMD-Millipore) resulted in a dramatic stimulation of cell motility regardless of the phosphorylation state of CEP4. These results implied that a typical role of Cdc42 in MCF-10A cells is to suppress motility. Interestingly, the Cdc42 inhibitor also eliminated 50% of D/D-CEP4-induced filopodia. Thus, Cdc42 did not play a role in phospho-CEP4 mediated cell motility. However, Rac1 activity was a crucial element in the mechanism of CEP4.

Both phenotypic effects (the elevated cell motility and filopodia formation) were impaired by treatment with a Rac1 inhibitor (Figure 4.10 and 4.11), and suggested that Rac1 is functionally involved in phospho-CEP4-induced phenotypes. Interestingly, a recent study from our lab described a model in which PKC-mediated phosphorylation of α-tubulin promotes numerous, stably elongating MTs that by an unknown mechanism leads to the formation of GTP-Rac1 and unknown downstream events that promote cell movement (De et al., 2014). Moreover, an earlier study from our lab showed that Rac1 was also functionally involved in the motility phenotype produced by PKC-α expression in MCF-10A cells (Sun
and Rotenberg, 1999). Taken together, our present findings add to this model by including another PKC substrate that activates Rac1.

Previous studies demonstrated that key components (e.g. integrins) that promote filopodia formation were also shown to have an important role in cell-cell adhesion (Mattila and Lappalainen, 2008). Moreover, filopodia were also shown to contribute to cancer progression and metastasis (Arjoner et al., 2011). In our study, the formation of filopodia participates in phospho-CEP4 induced cell motility, and their role in the CEP4 mechanism provides an area for future investigations.

It is evident that phosphorylation provides a regulatory mechanism that frees CEP4 to engage in other protein-protein interactions that contribute to motile behavior. The nature of these interactions was addressed by using the TAP method. With this approach, we identified TEM4 (ARHGEF17) as a protein that co-purifies and co-immunoprecipitates with D/D-CEP4 from whole cell lysates (Figures 4.14 and 4.15), suggesting that they co-localize in the cell. TEM4 was linked to the regulation of actin structure (Mitin et al., 2013; Ngok et al., 2013), and to migration by inhibiting actinomyosin contractility at the leading edge of a moving cell (Ngok et al., 2013). As a GEF, TEM4 is thought to activate GDP-GTP exchange in RhoA but not in Rac1 or Cdc42 (Mitin et al., 2013). It is possible that in MCF-10A cells, TEM4 or the phospho-CEP4/TEM4 complex interacts with an additional unknown component(s) (rather than Rac1 directly) that in turn produces Rac1 activation. The important finding that knockdown of TEM4 was sufficient to suppress both motility and Rac1 activation induced by the D/D-CEP4 mutant to levels produced by the A/A-CEP4 mutant (Figure 4.16 and 4.17), implicates TEM4 or the TEM4/phospho-CEP4 complex as a component of the PKC-stimulated signaling pathway. That motility and filopodia formation are downstream consequences of Rac1-mediated signaling was indicated by their almost complete elimination.
following treatment with the Rac1-specific inhibitor NSC23766. Because the inhibitor and Rac antibody used in these studies did not differentiate between Rac1 and Rac3, we cannot exclude the co-incident involvement of these isoforms, both of which had previously been implicated in metastatic phenotypes of human breast cells (Baugher et al., 2005).

Taken together, our findings support a model (Figure 6.1) 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.
Chapter 5: Characterization of CEP4 Mutants in a Malignant Human Breast Cell Line
5.1: Introduction

Several protein kinase C (PKC) isoforms were shown to influence different cellular processes that contribute to the malignancy of human breast cancer cells. The MDA-MB-231 human breast cancer cell line is one of the most frequently used cell models for human breast cancer. These cells are referred to as “triple negative” because they lack EGF-receptors, estrogen receptors, and progesterone receptors, making the cancers they generate difficult to control (Cailleau et al., 1974). These metastatic cells are intrinsically motile and express high endogenous levels of active PKC isoforms of which PKC-α is the most abundant. To further explore the functional significance of phosphorylation of CEP4 at Ser-18 and Ser-80, the phosphorylation-resistant mutant CEP4-18A/80A was prepared and a possible dominant-negative effect on motility by this mutant was studied in MDA-MB-231 breast tumor cells.

5.2: Results

5.2.1 Generating a MDA-MB-231 Stable Cell Line Expressing Pseudo-Phosphorylated or Phosphorylation-Resistant Double Mutants of CEP4

To study the impact of CEP4 mutants, MDA-MB-231 cells were stably transfected with either CEP4-18A/80A or CEP4-18D/80D. The two CEP4 mutants or vector control were transfected with lipofectamine reagent and the cells were cultured in a selective medium containing geneticin (G418-sulfate) at a concentration of 500 μg/ml. At least two clones were selected and the expression of CEP4-18A/80A or CEP4-18D/80D in these clones was confirmed by Western blot (Figure 5.1). Strong signals were detected near 50 kDa with Myc-antibody that were not present in cells stably transfected with the vector control.
**Figure 5.1: Expression of pseudo-phosphorylated and phosphorylation-resistant CEP4 mutants in MDA-MB-231 cells.** MDA-MB 231 cells were transfected with CEP4-18D/80D, CEP4-18A/80A and the empty pCMV6 vector (VC) using lipofectamine transfection reagent (8μg:10 μl). To generate stable cell lines, the transfected cells were selected with G418 500 μg /ml. Cell extracts from each stable transfectants were analyzed by Western blot and identified by probing with anti-Myc antibody (1:1000). β-actin (1:5000) served as the loading control.

### 5.2.2 Characterization of Motility of MDA-MB-231 Cells That Stably Express CEP4 Mutants

Since phospho-CEP4 induced cell migration in MCF-10A cells, we sought to determine whether the CEP4-18A/80A mutant produced a dominant negative effect on the intrinsic cell motility of stable transfectants of MDA-MB-231 cells. The cells were seeded through a cell sedimentation manifold at 7000 cells/well to establish a tight circular confluent monolayer on a 10-well glass slide pre-coated with 1% BSA. Sixteen hours after seeding, the images of circle were recorded, followed by another round of image acquisition after 6h. Measurements were performed using Motic Image software. The difference in the average area of the circles recorded at t=0h and t=6h was plotted graphically in Figure 5.2. Our results indicated that the MDA-MB-231 cells stably expressing CEP4-18A/80A exhibited a pronounced inhibition of migration (by 70%) when compared to cells expressing the pseudo-phosphorylated CEP4-18D/80D mutant, and fell below the value measured for the vector control.
Figure 5.2: Phospho-CEP4 enhances cell motility in MDA-MB 231 stable cells. MDA-MB 231 cells were stably transfected with CEP4-18D/80D, CEP4-18A/80A and pCMV6 (vector control). Motility of each transfectant was measured in triplicate by the cell sedimentation assay (described in the ‘Methods’), and the values were averaged. Student’s t-test was used to determine the statistical significance between the CEP4-18D/80D mutant and the vector control (* P<0.001) with n= 9, where n = number of concentric circles measured per condition.

5.2.3 Characterization of Proliferation Assay for CEP4 Mutants in MDA-MB-231 Stable Cells

As previously shown, the CEP4 mutants in MCF-10A cells did not reveal any difference in the cell proliferation rate (Figure 4.5). Since this lack of an effect may have been the outcome of transient transfection, the proliferation assay was performed on the stably transfected MDA-MB-231 cells. Vector control, CEP4-18D/80D or CEP4-18A/80A stable transfectants were plated into a 96-well plate. Cell proliferation was measured in a time-dependent manner. It was noted that CEP4-18A/80A transfectants proliferated faster than CEP4-18D/80D. This finding suggested that phosphorylation of CEP4 suppresses cell proliferation and was reminiscent of the suppression of proliferation engendered by PKC-α over-expression in MCF-10A cells (Sun and Rotenberg, 1999).
5.2.4 Generation of MDA-MB-231/luciferase Stable Cell Lines Expressing CEP4 Mutants

In order to study the effects of CEP4 mutants in a mouse model, we prepared stable cell lines that constitutively express a CEP4 mutant while co-expressing firefly-luciferase protein in order to track the mutants in vivo.

MDA-MB-231 cells that stably express CEP4-18D-80D or CEP4-18A-80A (expressed from a vector bearing a neomycin-resistance gene) were transfected with a pGL4.50 vector containing the luc gene and the hygromycin-resistance gene. The transfection was conducted according to the manufacturer’s instructions for lipofectamine 2000. Hygromycin was used to select for stable clones 48h after transfection. We screened the resulting clones at different concentrations of hygromycin (100~500 µg/ml). Finally, the optimal concentration for
growing the stable cells was judged to be 400 \mu g/ml for hygromycin and 500 \mu g/ml for G418.

Isolation of clonal cells (10^5 cells/ml) was carried out to identify the positive clones by the presence of luciferase activity (Bright Glo™ luciferase assay, Promega). Briefly, 100 \mu l of each cell suspension was added per well of a 96-well plate in triplicate. Following an overnight incubation (37°C, 5% CO_2), 100 \mu l Bright Glo™ reagent (prepared according to the manufacturer’s protocol) was added to each well and the cells were incubated for 5 minutes at room temperature. The mixture of reagents and complete medium served as a blank, and reagents added to MDA-MB-231 parental cells (lacking luciferase and CEP4 mutants) served as a negative control. The bioluminescence intensity of each clone was measured with a 96-well plate reader equipped with a luminometer (SpectraMax M5 microplate reader). In Figure 5.4A, the luminescence data indicated successful stable expression of luciferase protein in MDA-MB-231 cells expressing either Myc-tagged CEP4-18D/80D or CEP4-18A/80A mutants. Western blot result probed with anti-Myc confirmed that the positive clones retained the Myc-tagged CEP4 mutants (Figure 5.4B).
Figure 5.4: Luminescence assay with MDA-MB-231 cells stably expressing luciferase and either CEP4-18A/80A or CEP4-18D/80D. A. Cells stably expressing either of the two CEP4 mutants were transfected with luciferase (Material and Methods), and maintained with hygromycin (400 μg/ml) along with G418 (500 μg/ml). The stable cells were plated into a 96-well plate and the luminescence intensity was recorded by a 96-well plate reader. The luminescence intensity for each construct was plotted relative to the basal luminescence of MDA-MB-231 parental cells which were assigned an arbitrary value of 1 (100%). The values are reported as the average of triplicate measurements ± s.d. B. Whole cell lysates (100 μg per sample) were prepared to confirm that the stable cell lines still exhibited CEP4 mutant expression. The blot was probed with polyclonal rabbit Myc antibody (1:1000, Cell Signaling Technology) to detect the signals of Myc-tagged CEP4. β-actin (1:5000, Cell Signaling Technology) was used as a loading control.
5.3: Discussion

In this section, we generated MDA-MB-231 stable cell lines carrying the pseudo-phosphorylated and phosphorylation-resistant CEP4 mutants. The results showed a dominant negative effect on migration by CEP4-18A/80A (phosphorylation-resistant) in MDA-MB-231 cells. For proliferation, CEP4-18A/80A stimulated cell proliferation compared to the other constructs. Since the overexpression of PKC-α was previously observed to slow down the proliferation of MCF-10A cells (Sun and Rotenberg, 1999), the present finding implied that phospho-CEP4 also suppresses the proliferation rate. The CEP4-18D-80D and CEP4-18A-80A stable cells will be used to study the behavior of these cells in a three-dimensional proliferation assay. Three-dimensional (3D) cultures provide a powerful tool that simulates the in vivo environment (Kenny et al., 2008; Liu et al., 2013). It will be used to study the differences of the two CEP4 mutants on cell proliferation and invasion and to predict their behavior in vivo. We also generated a MDA-MB-231 stable cell line carrying CEP4-18D-80D/luc or 18A-80A/luc that will be used for assessing tumor growth and metastasis in a mouse model. Stable expression of the luciferase gene will enable tracking of the development and spread of breast tumors generated by MDA-MB-231 transfectants and will reveal the impact of CEP4 phosphorylation on these parameters.
Chapter 6: Concluding Statement and Future Directions
6.1: Concluding Statement

In the late 1970s, Nishizuka and co-workers discovered a new enzyme, Ca\(^{2+}\)-activated, phospholipid-dependent protein kinase – protein kinase C (PKC), which is one of the most studied enzymes in biology (Nishizuka, 1986). As the major intracellular receptor of tumor-promoting phorbol esters, protein kinase C (PKC) has been studied for decades for its potential role in carcinogenesis and has been recognized as a potentially important target for cancer therapeutics (Koivunen et al., 2006; Teicher, 2006; Martiny-Baron and Fabbro, 2007; Urtreger et al., 2012). PKC isoforms were shown to have different expression profiles and variable functions during cancer progression of a particular cancer type. PKC isoforms were shown to be involved in controlling cell proliferation, cell apoptosis, cell polarity and cell motility in mammalian epithelial cells. Notably, the deregulation of these pathways by PKC has been observed in breast cancer (Gökmen-Polar et al., 2010; Lønne et al., 2010; Urtreger et al., 2012). Therefore, PKC is a promising target for blocking breast cancer malignancy. However, the fact that PKC activity consists of multiple structurally-related isoforms makes it more difficult to establish the role of a single PKC isoform in breast cancer progression. For the conventional isoforms, PKC-\(\alpha\) is still considered as an appropriate target for cancer therapies especially due to its role in metastasis (Langzam et al., 2001; Kerfoot et al., 2004; Koivunen et al., 2004; Varga et al., 2004). Representing the novel PKC isoforms, PKC-\(\delta\) inhibits cell apoptosis and promotes metastasis of human breast cancer (Kiley et al., 1999; Varga et al., 2004; Urtreger et al., 2005; Zeidan et al., 2008; Lønne et al., 2009). Regarding the potential role of atypical PKC isoforms in breast cancer, evidence links PKC-\(\zeta\) to increased proliferative, invasive and metastatic potential of breast cancer cells (Urtreger et al., 2005; Mustafi et al., 2006; Galvez et al., 2009; Nazarenko et al., 2010). Hence, PKC-\(\alpha\), -\(\delta\) and -\(\zeta\) are prominent targets for breast cancer therapy and predictive markers for breast
cancer aggressiveness. In view of the importance of PKC activity to cancer progression, very little is known about PKC substrates that impact cellular transformation. The identification of physically relevant substrates for each of the three PKC isoforms is very important for a complete understanding of the mechanisms for isoform-specific PKC regulation in malignancy and thereby could reveal strategies for the design of an anti-cancer drug.

In the present work, the focus was on the study of PKC substrates in phenotypes of human breast cancer. The “traceable kinase method” was successfully applied to identify PKC substrates. This method was introduced in 1997 by K. Shokat who initially used it to identify protein substrates of v-Src (Shah et al., 1997; Liu et al., 1998b). Several laboratories have since applied this method to determine the substrates of key protein kinases such as JNK, ERK2, and Raf-1 (Liu et al., 1998a; Ting et al., 2001; Shah and Shokat, 2002; Böhmer and Romeis, 2007). In the adaptation of this approach to the study of PKC substrates, our traceable kinase method involved expression followed by co-immunoprecipitation of the PKC-α, -δ or –ζ traceable kinase mutants along with any high-affinity substrates bound by them in MCF-10A human breast cells (Chen et al., 2012). This non-transformed, non-motile human breast cell line expresses low levels of PKC-α, -δ, and -ζ isoforms therefore providing an ideal substrate-rich environment in which to identify their protein substrates in response to a PKC activator (DAG-lactone).

By applying the traceable kinase method, we defined the potential substrate profiles of three PKC isoforms in MCF-10A human breast cells. Because previous studies demonstrated that the small GTPase Rac1 has an impact on PKC induced motility, effector and accessory proteins related to this class of signaling molecule were of primary interest. Among the potential substrates, we selected ROCK1, CEP4, CLASP1 and PAK2 as our candidates since they are small GTPase accessory proteins and have been linked to cell motility, invasion or
metastasis. The in vitro kinase assays ruled out the possibility of ROCK1 and CLASP1 (Figures 3.2, 3.3). Intracellular phosphorylation assays demonstrated that only CEP4 underwent phosphorylation in response to DAG-lactone (Figure 3.4). For PAK2, we found that PKC-α and -δ phosphorylated PAK2 in vitro but not in non-transformed MCF-10A cells treated with DAG-lactone. However, in view of the potential of PAK to undergo PKC-mediated phosphorylation, a role for phospho-PAK in the invasiveness of breast cancer cells should be further explored. As a result of these experiments, CEP4 was pursued as a PKC substrate in human breast cells.

CEP4 belongs to a family of poorly understood effector proteins of Cdc42. To date, the CEP proteins had been studied as a Cdc42 effector protein in NIH-3T3 fibroblasts and keratinocytes for their role in regulating the actin cytoskeleton (Joberty et al., 1999; Burbelo et al., 1999; Hirsch et al., 2001). This study is the first to identify CEP4 as an intracellular PKC substrate and to characterize the function of phospho-CEP4 in cancer-related phenotypes of human breast cells.

In the present work, we found that CEP4 serves as substrate for all three PKC isoforms (PKC-α, −δ, and -ζ) in MCF-10A cells. Measurements of the stoichiometry of phosphorylation showed that there might be three to four phosphorylation sites in CEP4 (Figure 3.6, 3.7). By inspection of the CEP4 primary sequence, we found there are four potential phosphorylation sites – Ser-18, Ser-77, Ser-80 and Ser-86 (Figure 3.5). Among the four sites we tested, Ser-18 and Ser-80 were found to be the sites whose phosphorylation impacts CEP4 function (Figure 4.2, 4.3, 4.4). Therefore, CEP4 was prepared as a pseudo-phosphorylated mutant (S18D/S80D)-CEP4 to establish the function of phospho-CEP4, and S18A/80A was constructed as a phosphorylation-resistant mutant to block the effect of PKC on associated phenotypes. As shown in Chapter 4, CEP4-S18D/80D stimulated cell motility.
and induced filopodia in MCF-10A cells (Figure 4.2, 4.6). To further explore the mechanism of the phospho-CEP4-induced phenotype, we determined whether phosphorylation of CEP4 influences its affinity for Cdc42. The result demonstrated that phospho-CEP4 is largely dissociated from Cdc42 (Figure 4.9). This finding opened up two lines of inquiry: (1) the released Cdc42 could become activated, resulting in stimulated cell motility and filopodia; or (2) phospho-CEP4 itself could interact with other proteins involved in cell motility and formation of filopodia. To examine the two possibilities, pseudo-phosphorylated CEP4 was tested on motility in the presence of a Cdc42 inhibitor-ML141. The outcome indicated that Cdc42 seems not to be involved in motility and might be related to a limited extent in filopodia formation (Figure 4.10A and 4.11). Based on our previous studies, Rac1 was functionally involved in the motility phenotype produced by PKC-α expression in MCF-10A cells. There is therefore a strong possibility that Rac1 is also involved in phospho-CEP4-mediated motility. In order to test this idea, we investigated the function of Rac1 in phospho-CEP4-related phenotypes. A Rac1 inhibitor was found to eliminate cell motility and the formation of filopodia produced by phospho-CEP4 (Figure 4.10B, 4.11). Although it was shown previously that CEP4 does not bind constitutively active Rac1, an enhanced binding affinity of pseudo-phosphorylated CEP4 for constitutively active Rac1 remains an untested possibility. Alternatively, proteins associated with phospho-CEP4 (and not unphosphorylated CEP4) might interact with Rac1 and thereby regulate Rac1. In this regard, we found that TEM4 interacts directly with phospho-CEP4 (Figure 4.14 and 4.15). Interestingly, the knockdown of TEM4 was sufficient to suppress both motility and Rac1 activation induced by the D/D-CEP4 mutant to levels produced by the A/A-CEP4 mutant (Figure 4.16 and 4.17). Thus, TEM4/phospho-CEP4 complex plays an important role in phospho-CEP4 induced activation of Rac1 and cell motility.
The studies presented in this dissertation are innovative because they are the first to identify CEP4 as a PKC substrate and the first indication of the functional significance of phospho-CEP4 in human breast cells. This study provides a model that describes how PKC activation in human breast cell lines unleashes a sequence of events that result in cell movement. The phosphorylation of a PKC substrate such as CEP4 is likely to promote its association with other proteins that culminate in Rac1 activation (Figure 6.1). The following proposed studies may further elucidate the complete model and test our findings in an animal model.

Figure 6.1: Model of PKC-mediated phosphorylation of CEP4 and its consequences. PKC isoforms phosphorylate CEP4 leading to its dissociation from Cdc42. Phospho-CEP4 binds to TEM4 that in turn stimulates GDP-GTP exchange in Rac1 and leads to downstream events supporting motility and filopodia formation.

6.2: Future Studies

6.2.1: Evaluation of the Functional Significance of IQGAP1 and PAK2 as PKC Substrates
IQGAP1 is an actin binding protein involved in regulating various cellular processes like organization of the actin cytoskeleton, cellular adhesion and cell migration (Brandt and Grosse, 2007; Jadeski et al., 2008; Johnson et al., 2009; White et al., 2009). IQGAP1 is also considered as an oncogene, since it participates in cancer cell invasion and metastasis, and its expression is up-regulated in various invasive human cancers (Dong et al. 2006). IQGAP1, acting as an effector protein for Cdc42 and Rac1 effector, inhibits the GTPase activity of Cdc42 and Rac1 by stabilizing their GTP-bound form, and is consistent with their involvement in actin-dependent functions such as cell shape and motility (Bashour et al. 1997). IQGAP1 also has been identified as a PKC substrate that undergoes phosphorylation at Ser-1443 in cells (Grohmanova et al., 2004). As a PKC substrate, IQGAP1 has not yet been studied. Since one phosphorylation site has been discovered, the pseudo-phosphorylated and phosphorylation-resistant mutants can be prepared and the functional significance (to motility and organization of actin filaments) will be investigated in MCF-10A cells. The possibility of additional phosphorylation sites can also be explored by mutation of PKC consensus sites.

PAK2 belongs to the p21-activated protein kinase (PAK) family which is known to be involved in numerous biological functions, including cytoskeletal organization, cell motility, cell growth and apoptosis. The PAK family members are serine/threonine protein kinases that serve as major downstream targets of the small G proteins Cdc42 and Rac1 (Bagrodia and Cerione, 1999). Evidences have been shown that PAK2 is required for the generation of focal adhesions and is implicated in controlling membrane and cell shape. However, in the present work, PAK2 was phosphorylated in vitro but not in MCF-10A cells. The potential of phospho-PAK2 in motile cells such as metastatic cancer cells provides an avenue for future investigation.
Figure 6.2: Known and potential PKC substrates have been characterized in the Rotenberg lab.

6.2.2: Elucidation of the PKC signaling pathway

Because of the importance of PKC in cancer metastasis, it is essential to identify PKC substrates and to interpret the role of substrates as components of PKC signaling pathway(s). In view of the multi-focal effects of PKC in cancer cells, the understanding of a PKC signaling pathway greatly facilitates the discovery of anti-cancer drugs and therapies. In an effort to elucidate the components of the PKC signaling pathway, recent studies from the Rotenberg lab identified α–tubulin as a PKC substrate (De et al. 2014). Phosphorylation of α-tubulin at a single site (Ser165) results in its increased incorporation into microtubules that promotes increased interactions by microtubules with the membrane that in turn produced activation of Rac1 and increased motility (De et al., 2014). That D/D-CEP4 co-purifies with CLIP1, a microtubule plus-end binding protein, suggests a possible interaction of phospho-CEP4 with elongating microtubules that produce Rac1 activation. Whatever the mechanism, Rac1 serves as a point of convergence by at least two PKC substrates, underscoring the importance of Rac1-associated GEFs (and GAPs) to both motility signaling and membrane protrusions in breast cancer (Parri and Chiarugi, 2010; Sosa and Kazanietz, 2012). In view of the fact that α-tubulin and CEP4 are both highly abundant PKC substrates that promote
signaling through Rac1, we speculate that their co-incident phosphorylation by PKC could lead to continuous GDP-GTP exchange on Rac1 that consequently gives rise to aggressive cell movement. Future work will try to investigate other PKC substrates such as MARCKS, PAK2, and IQGAP1 to determine whether they also contribute to this pathway. Moreover, after elucidation of the PKC signaling pathway, inhibition of one or more certain targets in this pathway by rationally designed inhibitors can be tested for its inhibition of PKC induced cell migration and evaluated for preventing cancer metastasis in an animal model.

6.2.3: Investigation of CEP4 or Other PKC Substrates in a Three Dimensional (3D) Assay and Its Contribution to Mammary Tumor Development

A 3D cell culture is an artificially-created environment that closely resembles natural tissue (Liu et al., 2013). A three-dimensional 3D matrix retains the biophysical and biomechanical signals that affect cell functions such as proliferation, migration, adhesion and gene expression (Kenny et al., 2008; Graf and Boppart, 2010). Therefore, in order to study the effect of phospho-CEP4 on cell migration, morphogenesis, and proliferation, a 3-D cell culture assay is predictive of the impact of CEP4 within the in vivo environment.

The MDA-MB-231-\textit{Luc} stable cell lines expressing CEP4-18A/80A or CEP4-18D/80D were generated and will be used to track tumorigenesis and metastasis of these cells in an orthotopic mouse model. MDA-MB-231-\textit{Luc} cells bearing either CEP4-18A/80A or CEP4-S18D/S80D will be implanted into the fat pads of a nude mouse. The growth and spread of the resulting tumors will be monitored using the luciferase reporter gene \textit{in vivo} by bioluminescence imaging technology (IVIS). Isolation of the resulting tumors will be followed by establishing Myc-CEP4 mutant expression by Western blot, RT/PCR, and immunohistochemistry (Frick et al., 2013). The animal work will help to establish the role of phospho-CEP4 in vivo.
Chapter 7: References


