Design and Characterization of a Traceable Protein Kinase Cα[†,‡]

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ABSTRACT: Protein kinase Cα (PKCα) is a critical component of pathways that govern cancer-related phenotypes such as invasion and proliferation. Proteins that serve as immediate substrates for PKCα offer potential targets for anticancer drug design. To identify specific substrates, a mutant of PKCα (M417A) was constructed at the ATP binding site such that it could bind a sterically large ATP analogue derivatized through the N^6 amino group of adenosine ([γ-^{32}P]-N^6-phenyl-ATP). Because this analogue could be utilized by the mutant kinase but not by wild-type PKCα (or presumably other protein kinase) to phosphorylate peptide or protein substrates, ^{32}P-labeled products were the direct result of the mutant PKCα. Kinetic analysis with [γ-^{32}P]-N^6-phenyl-ATP revealed that the mutant retained undiminished affinity for the peptide substrate ($K_m = 12.4\ \mu$M) and a V_{max} value (10.3 pmol/min) that was only 3-fold lower than that exhibited by the wild-type enzyme with natural ATP. However, with [γ-^{32}P]ATP, the mutant had a somewhat lower affinity ($K_m = 82.8\ \mu$M) than the wild-type enzyme ($K_m = 9.3\ \mu$M) in vitro but was competent in causing aggressive motility in nonmotile MCF-10A human breast cells (with endogenous ATP), as previously described for wild-type PKCα. The FLAG-tagged PKCα mutant was expressed in MCF-10A cells and used to co-immunoprecipitate high-affinity substrates from lysates. Immunopellets were reacted with [γ-^{32}P]-N^6-phenyl-ATP, and radiolabeled products were analyzed by SDS−PAGE and autoradiography. Mass spectrometry of selected bands identified several known substrates of PKC, thereby validating the methods used in these studies. These findings provide a foundation for future applications of this traceable PKCα mutant.

To begin to dissect the mechanism(s) through which protein kinase Cα (PKCα)[1] activity promotes its pleiotropic cellular effects, it is imperative that immediate substrates of this protein kinase be identified. Several proteins that are known to serve as PKC substrates were shown to be directly associated with microfilaments and capable of altering cytoskeletal structure. These proteins include the myristoylated alanine-rich C-kinase substrate, adducin, GAP43, fascin, and ERM (ezrin-radixin-moesin) proteins (*1*). More recently, β4-integrin was shown to disrupt cell−cell contacts (*2*). Upon phosphorylation by PKC, these substrates are thought to trigger dynamic changes in the cortical cytoskeleton that result in breakdown of cell−cell interactions and cell migration (*3*).

Studies from several laboratories have reported on cancer-related phenotypes, such as motile behavior and proliferative activity, that are produced in human breast cells as a result of PKCα overexpression (*4*−*7*). A nontransformed, nonmotile human breast cell line (*8*), MCF-10A cells offer an excellent system in which to study the phenotypic outcome of PKCα overexpression. These cells express a very low background of endogenous PKCα and acquire aggressive motility as a result of engineered expression of PKCα (*6*). This motile behavior occurs without the addition of tetradecanoyl 12-phorbol 13-acetate (TPA), suggesting that the enzyme undergoes intracellular activation. This endogenous activation offers the additional advantage of engendering phenotypic outcomes that are directly attributed to the activity of transfected PKCα rather than to undefined PKC isoforms. Furthermore, PKCα overexpression in these cells causes no change in the expression of other PKC isoforms (*6*), unlike MCF-7 cells (*4*). Thus, the nontransformed human breast cell environment offered by MCF-10A cells may be useful for identifying direct substrates of PKCα that would provide important targets for designing antimetastasis drug combinations.

Initially developed by the Shokat laboratory using the tyrosine protein kinase *v*-src (*9*−*12*), the traceable kinase method offers a much-needed approach to identify the immediate substrate(s) of an individual protein kinase. This method entails site-directed mutagenesis of the protein kinase cDNA at a site in the ATP binding domain that lies closest to the adenosine moiety of bound ATP. The mutation at this

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[1] Abbreviations: PKCα, protein kinase Cα; PKA, cAMP-dependent protein kinase; NDPK, nucleoside diphosphate kinase; WT, wild type; VC, vector control; aa, amino acid; PS, phosphatidylserine.

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site replaces an amino acid residue that has a long aliphatic side chain (e.g., isoleucine or methionine) with either glycine (R = H) or alanine (R = CH_3), thereby removing steric hindrance in the vicinity of the N^6-amino group of the adenosine moiety. The resulting mutant protein should retain catalytic activity and substrate specificity for both ATP and protein substrates but, unlike all other protein kinases in the cell, can productively bind and utilize an analogue of ATP that is derivatized at the N^6-amino group (e.g., N^6-phenyl-ATP). As a result, phosphorylation of protein substrates in lysates or subcellular fractions that are treated with this ATP analogue can be attributed exclusively to the activity of the mutant protein kinase. Thus, the traceable kinase method provides a more efficient means of identifying direct protein substrates of a protein kinase of interest than older, more labor-intensive approaches.

The amino acid (aa) residue that corresponds to that site mutated in *υ*-src (I338) by the Shokat group had been identified initially in the X-ray crystal structure of c-src as one of three residues (V337, I338, and E339) that are physically within a 5 Å sphere of the N^6-amino group of the adenosine moiety of bound ATP. In a mutant in which I338 was replaced with an Ala residue, successful interaction with an unnatural ATP analogue was demonstrated by in vitro catalytic assay (*9*), and it proved to be one of the highest-affinity mutants identified by phage display (*10*). Importantly, the mutation in the ATP binding domain of *υ*-src had no observable effect on substrate specificity, as shown with a peptide library (*12*). The Shokat laboratory employed this mutant to identify *υ*-src phosphorylation sites on Dok-1 and showed that these sites are important for binding RasGAP and Csk, both negative effectors of src signaling (*11*). Since the introduction of the traceable kinase method, a few laboratories have used it to pursue protein substrates of highly significant protein kinases such as JNK (*13*), ERK2 (*14*), and Raf-1 (*15*).

Most studies that employed the traceable kinase method were guided by an available three-dimensional (3D) structure of the kinase that had been cocrystallized with bound ATP/Mg^{2+}, thereby enabling the identification of nearby side chains. For PKCα and its related isoforms, a 3D structure recently became available for PKC*θ* (*16*), but the ATP binding site was unoccupied. Nevertheless, most kinase catalytic domains have a high degree of sequence homology at the ATP binding domain and therefore have a similar topography in this region. This fact is likely a reflection of their similar chemistry with ATP despite their recognition of different protein substrates. In this first report, we demonstrate the design, expression, and characterization of a traceable PKCα mutant. This work lays the foundation for subsequent studies that will employ this mutant to search for PKCα substrates in different cultured cell lines.

EXPERIMENTAL PROCEDURES

Materials. Cell culture serum, growth factors, media, and DNA sequencing primers were purchased from Invitrogen, Inc. (Carlsbad, CA). Antisera for PKCα, protein A/G−agarose beads, and chemiluminescent reagents were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FLAG-tagged mouse monoclonal antibody, QuikChange mutagenesis reagents, and the pCMV4 vector were purchased from Stratagene (La Jolla, CA). Duracryl was obtained from Genomic Solutions (Ann Arbor, MI). Immobilon-P transfer membranes were obtained from Millipore Corp. (Bedford, MA). Agarose-conjugated FLAG M-2 antibody (E-Z view beads), phosphatase inhibitor cocktail, protease inhibitors, and NDPK were obtained from Sigma-Aldrich (St. Louis, MO). ^{25}Ser peptide (RFARKGSLRQKNV) was custom synthesized by GenScript Corp. (Piscataway, NJ). Fugene 6 was acquired from Roche Applied Science (Indianapolis, IN). Gelcode Blue was purchased from Pierce Co. (Rockford, IL). N^6-Phenyl-ADP and N^6-phenyl-ATP were purchased from Axxora (San Diego, CA), and [γ-^{32}P]GTP was acquired from Perkin-Elmer Life and Analytical Sciences (Boston, MA).

Mutagenesis. The cDNA of bovine PKCα (a gift from P. J. Parker, Cancer Research UK London Research Institute, London, U.K.) was subcloned into a pCMV4 vector at EcoRI and XhoI sites. Expression from this vector confers a FLAG tag (DYKDDDDK) at the C-terminus of the product PKCα. Replacement of the active site Met (M417) with a Gly or Ala residue was performed by the QuikChange method. The following primers were used: 5′-GAC CGG CTG TAC TTC GTC GGC GAG TAC GTC AAC GGC-3′ for M417G and 5′-GAC CGG CTG TAC TTC GTC GCG GAG TAC GTC AAC GGC-3′ for M417A. Each mutation was verified by sequencing the entire open reading frame (North Shore University Hospital Core Facility, Manhasset, NY).

Synthesis and Isolation of [γ-^{32}P]-N^6-Phenyl-ATP. Radioactive [γ-^{32}P]-N^6-phenyladenosine triphosphate ([γ-^{32}P]-N^6-phenyl-ATP) was prepared by using bovine nucleoside diphosphate kinase (NDPK) to transfer $^{32}P_i$ from [γ-^{32}P]GTP to N^6-phenyladenosine diphosphate (N^6-phenyl-ADP), by adapting a published method (*17*). The reaction medium consisted of buffer [20 mM Tris (pH 7.4), 5 mM EDTA, and 1 mM DTT], 0.02 unit of NDPK, 250 μCi of [γ-^{32}P]-GTP, and 0.833 mM N^6-phenyl-ADP. The [γ-^{32}P]-N^6-phenyl-ATP product that resulted over a period of 1 h at 30 °C was isolated by HPLC using a Vydac quaternary amine anion exchange column (4.6 mm inside diameter × 250 mm length). The mobile phase consisted of the following: A = NaH_2PO_4/$NaHPO_4$ (1:1 molar ratio) at 50 mM in water, adjusted to pH 2.8 with acetic acid, and B = NaH_2PO_4/$NaHPO_4$ (1:1 molar ratio) at 250 mM, adjusted to pH 2.9 with acetic acid. The gradient started at 0% B for 2 min, then was ramped to 60% B, followed by a linear gradient from 60 to 100% B for 20 min, and held at 100% B for 50 min. The flow rate was 2.0 mL/min, and peak detection was carried out at 288 nm. The moderately broad peak corresponding to [γ-^{32}P]-N^6-phenyl-ATP eluted between 51 and 57 min. This radioactive product was shown to coelute with the authentic, nonradioactive N^6-phenyl-ATP standard. Peak material was immediately neutralized to pH 7 with 10 N NaOH, and an aliquot was analyzed for radioactive content by β-scintillation counting. On the day of each experiment, nonradioactive N^6-phenyl-ATP was added as a carrier to produce a 100 μM working solution with specific radioactivity in the range of 600−850 cpm/pmol.

Cell Culture and Transfection. Midpassage MCF-10A human breast epithelial cells were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI). MCF-10A cells were cultured as previously described (*8*). Transient transfection was carried out with 4 μg of cDNA complexed with Fugene 6 for 6 h in serum-free medium, followed by

addition of the normal serum component and incubation at 30 °C in 5% CO_2. After 48 h, the cells were harvested.

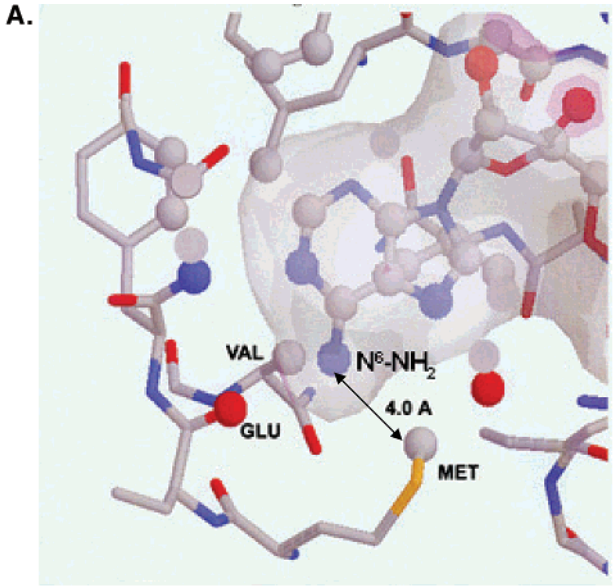
Assay of PKCα Catalytic Activity in Vitro. MCF-10A cells were harvested by trypsinization followed by three washes with complete medium. These steps facilitated cell dissociation and disruption and proved to be superior to simple cell scraping. Lysates were prepared from transfected MCF-10A cells in the presence of hypotonic detergent-free ip buffer [20 mM Tris (pH 7.4), 2 mM EGTA, 2 mM $MgCl_2$, and 1 mM dithiothreitol] containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 10 ng/mL leupeptin, and 10 ng/mL soybean trypsin inhibitor) and phosphatase inhibitors. Immunoprecipitation of mutant or wild-type PKCα proteins was carried out with an anti-FLAG−agarose conjugate for 2 h at 4 °C. For the negative control, immunoprecipitation was carried out with lysates prepared from cells that had been transfected with the empty vector. Immunocomplexes were collected by centrifugation at 8800*g* and 4 °C for 30 s, and the pellets were resuspended, rotated in 0.5 mL of ip buffer at 4 °C for 5 min, and centrifuged at 8800*g* for 30 s. This wash step was carried out twice, and the third wash was performed with 0.5 mL of 2× kinase assay buffer [100 mM Tris-HCl (pH 7.4), 1.0 mM $CaCl_2$, 20 mM MgAc, and 1 mM DTT] containing protease inhibitors and phosphatase inhibitors. For each immunopellet, the beads were aliquotted equally into triplicate tubes. The reactions were initiated by addition of [γ-^{32}P]-N^6-phenyl-ATP or [γ-^{32}P]ATP to the indicated concentration, and the final concentrations of the assay components in a 60 μL reaction volume were 33 mM Tris (pH 7.4), 0.33 mM $CaCl_2$, 6.66 mM MgAc, 0.33 mM DTT, 0.083 mg/mL PS, and 53.3 μM ^{25}Ser peptide. This synthetic peptide substrate (RFARKGSLRQKNV) is the modified pseudosubstrate sequence for PKCα (*18*). The assay was carried out for 10 min at 37 °C. In pilot studies, this time period was observed to lie on the linear portion of the progress curve. Each reaction was quenched by applying a 50 μL aliquot to a 2 cm × 2 cm square of phosphocellulose paper that was immediately dropped into a 1 L beaker of water. After all of the reactions had been quenched, the papers were washed five times with water, and each paper was analyzed by scintillation counting (*6*). Radioactivity values were averaged and corrected for background activity provided by vector control assays.

Radiolabeling of Co-Immunoprecipitated Proteins. Lysates of cells that had been transfected with cDNA encoding FLAG-tagged WT PKCα or M417A PKCα or the empty pCMV4 vector were prepared as described above. Immunoprecipitation was carried out with the anti-FLAG−agarose conjugate for 2 h at 6 °C, and the pelleted material was washed twice with ip buffer containing 50 mM NaCl and a third time with kinase buffer. To initiate the radiolabeling reaction, [γ-^{32}P]phenyl-ATP was added to a final concentration of 42 μM at time zero. The reaction proceeded for 30 min in a 30 °C water bath and was quenched by addition of sample buffer and heating at 95 °C for 5 min. After phosphorylated products were resolved by SDS−PAGE (8% Duracryl), the gel was stained with Gelcode Blue, dried onto blotting paper, and autoradiographed for 2 weeks at −20 °C. Prior to mass spectrometry, individual bands were excised, rehydrated, and stored at −20 °C.

Western Blot. Cells were lysed in 50 mM Tris (pH 7.4), 5 mM EDTA, 5 mM EGTA, 15 mM 2-mercaptoethanol, 1% Triton X-100, protease inhibitors, and phosphatase inhibitors. Lysates were resolved by 8% SDS−PAGE and subsequently transferred electrophoretically to a membrane (Immobilon-P). The membrane was blocked overnight in 5% reconstituted milk. Immunochemical assay was carried out for 2 h with a mouse monoclonal antibody (Stratagene) that recognizes the FLAG epitope in the recombinant PKCα proteins. For detection, the secondary IgG conjugated to horseradish peroxidase was incubated with the blot for 1 h and developed via chemiluminescence.

Motility Assay. Cell motility was analyzed by monitoring the extent of cell movement by the cell sedimentation method. The cells were applied to a 10-well slide through a 10-hole manifold (CSM, Inc., Phoenix, AZ) that restricts sedimentation of cells to a small, circumscribed area. Upon removal of the manifold, the cells radiated outwardly over an 8 h period. The cells were viewed under an inverted Nikon Diaphot microscope that was attached to a digital camera (Moticam 2000) and a computer equipped with Motic Images Plus 2.0. The extent of movement was determined by measuring the change in total area (in square micrometers) occupied by the cells.

Protein Identification by Mass Spectrometry. Gel-resolved proteins were digested with trypsin and batch-purified on a reversed-phase microtip, and resulting peptide pools were individually analyzed by matrix-assisted laser desorption ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS) (UltraFlex TOF/TOF, Bruker, Bremen, Germany) for peptide mass fingerprinting, as described previously (*30*, *31*). Selected peptide ions (*m*/*z*) were taken to search the human segment of a "nonredundant" protein database (NR; 3 717 264 entries on August 22, 2006; National Center for Biotechnology Information, Bethesda, MD), utilizing the PeptideSearch algorithm (M. Mann, personal communication; an updated version of this program is available as "PepSea" from MDS-Denmark). A molecular mass range up to twice the apparent molecular weight (as estimated from the electrophoretic relative mobility) was covered, with a mass accuracy restriction of <40 ppm and a maximum of one missed cleavage site allowed per peptide. To confirm PMF results with scores of ≤40, mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis of the same prepared samples, using the UltraFlex instrument in "LIFT" mode. Fragment ion spectra were recorded to search NR using the MASCOT MS/MS Ion Search program (*32*), version 2.0.04 for Windows (Matrix Science Ltd., London, U.K.). Any tentative confirmation (MASCOT score of ≥30) of a PMF result thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data. This work was performed at the Microchemistry and Proteomics Core Facility at Memorial Sloan-Kettering Cancer Center (New York, NY). One PKCα substrate (EF1α1) was identified by a similar protocol at the Keck Foundation Mass Spectrometry Resource Laboratory at Yale Cancer Center (New Haven, CT). This facility performed in-gel tryptic digestion, followed by LC−MS/MS analysis on a Waters Q-Tof mass spectrometer. All MS/MS spectra were searched using the automated MASCOT algorithm. Identification required that two or more spectra matched the same protein entry in the database.



B.

v-src	(333)	P-I-Y-I-V-**I**-E-Y-M-S-K	(343)
PKA	(115)	N-L-Y-M-V-**M**-E-Y-V-P-G	(125)
PKCα	(412)	R-L-Y-F-V-**M**-E-Y-V-N-G	(423)
M417G		R-L-Y-F-V-**G**-E-Y-V-N-G	
M417A		R-L-Y-F-V-**A**-E-Y-V-N-G	

FIGURE 1: Design of ATP binding site mutants of PKCα. (A) Three-dimensional structure of PKA with bound ATP in which the adenosine moiety is delineated by the translucent surface. The proximity of the M120 to the adenosine moiety is illustrated. Molecular modeling was performed with Protein Explorer (PDB entry 1ATP). (B) Alignment of primary sequences in the vicinity of the ATP binding site for *v*-src, PKA, and PKCα. M417 in PKCα corresponds to M120 of PKA and I338 of *v*-src. Site-specific mutants at M417 that were prepared for this study are shown.

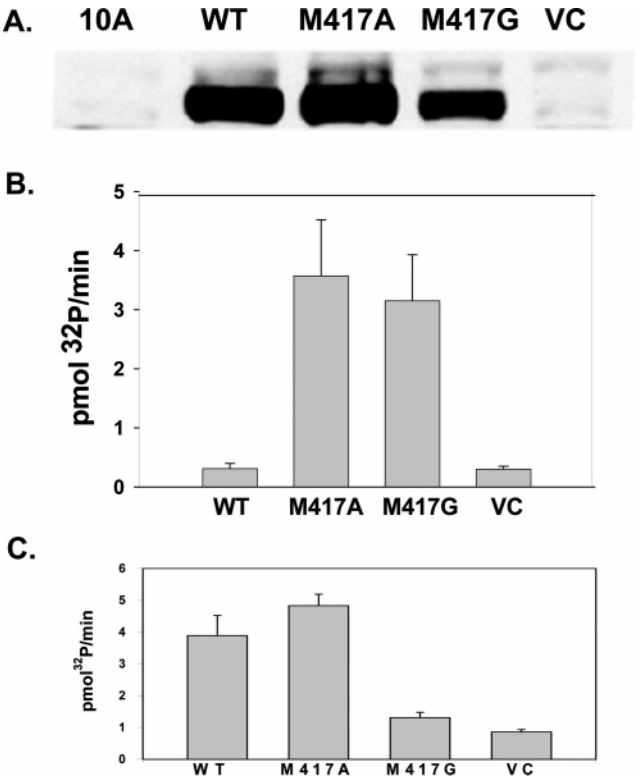


FIGURE 2: Expression and catalytic activity of PKCα mutants in MCF-10A cells. (A) Lysates of parental MCF-10A cells and transfectant cells were analyzed by Western blot (30 μg protein/lane) and detected with a FLAG tag antibody. (B) Cells that were transfected with plasmids encoding WT PKCα, the M417A or M417G mutant, or the vector control (VC) were immunoprecipitated with an anti-FLAG−agarose conjugate. Immunopellets were analyzed in triplicate for phosphotransferase activity with ^{25}Ser peptide and 42 μM [γ-^{32}P]phenyl-ATP or (C) with 42 μM [γ-^{32}P]ATP, as described in Experimental Procedures. Each figure is representative of three independent experiments.

RESULTS

Design, Expression, and Characterization of ATP Binding Site Mutants of PKCα. To determine the site for mutagenesis in the PKCα catalytic domain, we used the crystal structure for the catalytic subunit of cAMP-dependent protein kinase (PKA) since this catalytic domain had been cocrystallized with ATP/Mg^{2+} (*19*) and is 43% homologous in sequence with the catalytic domain of PKCα (*20*, *21*). Homology modeling of the PKCβ catalytic domain with the PKA structure as a scaffold resulted in a 3D structure that could be superimposed with PKA (*22*). In view of the high degree of sequence and tertiary structure homology between PKCα and PKA, the 3D structure of the ATP binding domain of the PKA crystal structure (Figure 1A) was used to identify aa residues in PKCα that lie in the vicinity of the N^6-amino group of the adenosine moiety (translucent boundary). Of those residues that come closest to the N^6-amino group is a Met side chain (M120) that lies within 4 Å; all other nearby atoms either are more distant or comprise the peptide backbone. In this critical region of the PKA catalytic subunit, aa 115−125 align closely with aa 412−423 in PKCα whereby M120 in PKA corresponds to M417 in PKCα. (With the exception of PKCζ, which has an Ile residue, all other PKC isoforms have a Met residue at this position.) When primary sequences for PKA and PKCα are compared with the *v*-src sequence used in the model study of Shokat (*9*), both Met residues align with I338, the site of mutagenesis in that study (Figure 1B).

Site-directed mutants of PKCα in which a Gly (G) or Ala (A) codon was substituted for the M417 position were prepared. Using wild-type PKCα that had been inserted into a pCMV4 vector (confers a FLAG tag at the C-terminus), mutant cDNA of PKCα was prepared by a PCR-based method (see Experimental Procedures). The resulting mutant cDNA constructs (M417G and M417A) were transiently transfected into MCF-10A cells and compared with parallel transfections of the wild-type (WT) PKCα or the empty vector (VC). A Western blot developed with the FLAG antibody (Figure 2A) demonstrated a high level of stable expression of both mutants that was similar to the level of expression of WT PKCα. A cell lysate for each PKCα protein was subjected to immunoprecipitation with anti-FLAG agarose beads, and the resulting immunopellets were aliquotted to triplicate tubes. The samples were assayed for catalytic activity in the presence of [γ-^{32}P]phenyl-ATP (Figure 2B). The results show that only the mutant PKCα enzymes were able to catalyze phospho transfer with [γ-^{32}P]-phenyl-ATP. Under these in vitro conditions, WT PKCα exhibited very low activity with this radiolabeled ATP analogue that was comparable to the negligible activity associated with the vector control. This important finding established that substitution of M417 with either a Gly or

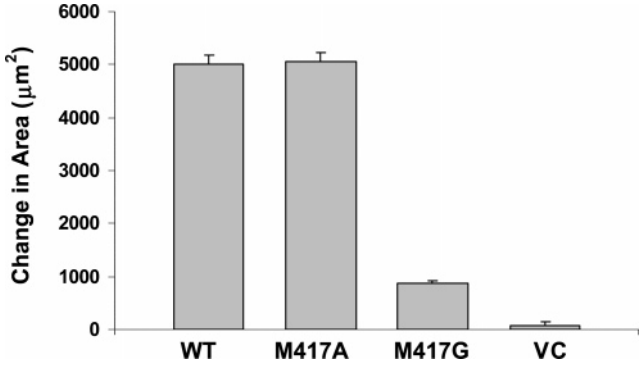


FIGURE 3: Determination of the competence of M417A and M417G PKCα mutants to engender motility in MCF-10A cells. The motility of MCF-10A transfectants was determined with each mutant PKCα, WT PKCα, or the vector control (VC), as described in Experimental Procedures. All values are the average of triplicate measurements and are representative of three independent experiments.

Ala residue endows PKCα with the ability to utilize [γ-^{32}P]-phenyl-ATP.

Identical assays were conducted to determine whether enlargement of the adenosine binding pocket altered the ability of the PKCα mutants to bind low micromolar concentrations of natural [γ-^{32}P]ATP. As shown in Figure 2C, the M417A mutant retained high catalytic activity that was comparable to or exceeded that of the WT enzyme. It is notable, however, that the M417G mutant exhibited much weaker activity with [γ-^{32}P]ATP.

To assess the intracellular competence of the PKCα mutants to induce cell motility, MCF-10A cells were transfected with each mutant construct or WT PKCα, and cell motility was analyzed by the cell sedimentation method (see Experimental Procedures). As shown in Figure 3, a high level of motility was induced by M417A that was equivalent to that produced with WT PKCα. However, expression of the M417G mutant only weakly induced cell movement. In these cell-based experiments, the PKCα mutants were obligated to use endogenous ATP as a phosphoryl donor. Thus, their different abilities to induce motile behavior were apparently due to their unequal ability to utilize endogenous ATP (Figure 2C). This functional difference identified M417A as the better molecular tool because it successfully reproduced the level of motility engendered by WT PKCα. Furthermore, in view of this intracellular competence, the specificity of M417A PKCα for protein substrates was likely to be unchanged by the mutation at the ATP binding site.

Kinetic analysis was used to compare the performance characteristics of WT PKCα and the M417A mutant with natural ATP or the N^6-phenyl-ATP analogue. As given in Table 1, the WT enzyme exhibited a K_m for ATP of 9.3 μM, whereas the M417A mutant had a K_m of 82.8 μM for ATP. In addition, both mutant and WT enzymes displayed equivalent V_{max} values with ATP. When tested with [γ-^{32}P]-phenyl-ATP, the mutant enzyme exhibited a K_m of 12 μM, indicating that the affinity for N^6-phenyl-ATP was comparable to the affinity of WT PKCα for natural ATP. These values were on par with the substrate affinity previously reported for a rat brain preparation of endogenous PKC α/β isoforms (K_m = 24 μM) that was measured with the same peptide substrate (*23*). It was noted that the V_{max} value of the M417A mutant with [γ-^{32}P]phenyl-ATP was decreased by only 3-fold when compared with that of WT PKCα with

Table 1: Kinetic Constants of Recombinant Wild-Type and Mutant PKCα Activities[a]

	ATP		N^6-phenyl-ATP	
	V_{max} (pmol/min)	K_m (μM)	V_{max} (pmol/min)	K_m (μM)
WT	33.5 ± 23	9.3 ± 1.5	nd[b]	nd[b]
M417A	30.5 ± 10	82.8 ± 11	10.3 ± 7	12.4 ± 0.4

[a] Catalytic activities were measured with immunoprecipitated WT or the M417A mutant in triplicate 10 min assays at 30 °C consisting of kinase buffer, ^{25}Ser peptide (32 μM), and either [γ-^{32}P]ATP (5−30 μM) or [γ-^{32}P]-N^6-phenyl-ATP (4−10 μM). The results were corrected for background activity, averaged, and analyzed by a double-reciprocal plot (*29*). Each value shown is the average of two independent experiments. [b] Not determined.

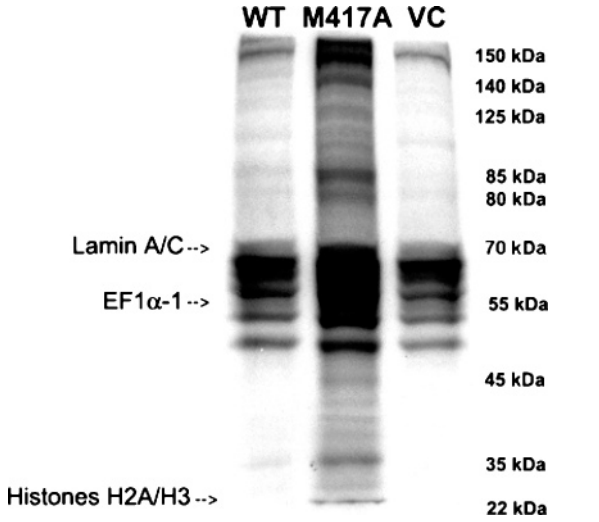


FIGURE 4: In vitro phosphorylation of protein substrates co-immunoprecipitating with M417A. In triplicate tubes containing immunopelleted M417A, reactions were initiated by addition of [γ-^{32}P]phenyl-ATP and the reaction progressed for 30 min at 30 °C and thereafter was quenched by addition of sample buffer and heating at 95 °C for 5 min. The samples were resolved by 7% SDS−PAGE, dried on blotting paper, and autoradiographed for 2 weeks at −20 °C. A representative autoradiogram is shown.

[γ-^{32}P]ATP (Table 1). By contrast, a 40-fold decrease in V_{max} was reported for the corresponding *v*-src mutant assayed with N^6-cyclopentyl-ATP, as compared with WT *v*-src analyzed with ATP (*9*). These results indicate that the mutation at M417 in PKCα does not cause substantial differences in enzyme performance that would impair the ability to bind substrate and to convert it into product at a reasonable rate.

In Vitro Phosphorylation of Proteins Co-Immunoprecipitating with M417A. The ability of M417A to utilize [γ-^{32}P]-phenyl-ATP efficiently makes possible the identification of PKCα-specific substrate proteins in MCF-10A cell lysates. The M417A mutant of PKCα was immunoprecipitated from transfectant cell lysates with FLAG antibody to "pull down" any substrate proteins bound to it, thus favoring high-affinity substrates. For the negative controls, lysates prepared from cells expressing either WT PKCα or the empty vector were immunoprecipitated with anti-FLAG in parallel. Following reaction of each immunopellet with [γ-^{32}P]phenyl-ATP, ^{32}P products were analyzed by one-dimensional SDS−PAGE and autoradiography. As shown in Figure 4, several strong signals were detected in the sample containing the M417A mutant that were weak or absent in immunopellets containing WT PKCα or vector control samples. It is noted that there were

strong bands that occurred reproducibly in the range of 47−60 kDa in the vector control immunopellets that were also present in the WT PKCα and M417A samples. Although significant background signals were also detected in other studies in which this method was used (*9*−*15*), the source of this background remains unclear (see Discussion).

For each of three unique bands produced by M417A at 15, 50, and 85 kDa, a moderate to strong ^{32}P signal was present. Selected bands were excised from the gel and submitted for analysis by mass spectrometry. Proteins in selected bands were identified as histone H2A and histone H3 (∼15 kDa), eukaryotic translation elongation factor 1-α1 (EF1-α1, 50 kDa), and lamin A/C (∼69 kDa). Histones H2 and H3 are known to undergo phosphorylation in vitro by PKC (*24*, *25*), as is EF factor 1 (*26*). PKCα is also known to interact with the nuclear membrane proteins lamin A and lamin C (*27*, *28*) and specifically phosphorylates lamin C at Ser572 (*27*). Thus, these results confirm that known substrates of PKCα can be identified by the methods used here.

DISCUSSION

In this study, a traceable PKCα mutant was developed and characterized. The site of mutation in the traceable kinase was identified at M417 since it aligned with I338 in *υ*-src, Shokat's model enzyme, and with M120 in PKA, a close relative of PKCα. Aided by the availability of the three-dimensional structure of PKA that had been cocrystallized with ATP-Mg^{2+}, we could directly verify the proximity of M120 in PKA to the N^6-amino group of the adenosine moiety (Figure 1A). Mutation of M417 to an Ala residue resulted in a mutant PKCα that (i) reacts with [γ-^{32}P]phenyl-ATP while WT PKCα cannot (Figure 2B), (ii) has an affinity for phenyl-ATP that is similar to that of the WT enzyme for the natural ATP (Table 1), (iii) selectively phosphorylates protein substrates that were previously identified as substrates of native PKCα (Figure 4), and (iv) is competent in producing a phenotype (i.e., motility) that was previously ascribed to PKCα in MCF-10A cells (Figure 3) (*6*).

The high background of radioactive bands produced in immunopellets obtained from control samples (Figure 4) deserves further comment. Although the source of the background in MCF-10A cells remains undefined, it is probably not due to co-immunoprecipitation of endogenous PKCα (via self-dimerization) since this isoform is expressed at exceedingly low levels, and its presence would have been evident in peptide-based catalytic assays of immunopellets containing either WT PKCα or vector control samples (Figure 2B). However, these catalytic assays would not have detected the presence of protein substrates. One plausible explanation is that immunoprecipitation with the FLAG antibody in detergent-free isotonic buffer pulls down other protein kinases that persist as nonspecifically bound proteins, despite multiple washes of the immunopellet. This possibility is supported by the increased number and intensity of radioactive bands produced with natural [γ-^{32}P]ATP in an immunopellet isolated from vector control-treated cells (results not shown). The few high-intensity bands observed in control samples with [γ-^{32}P]phenyl-ATP (Figure 4) suggest that there are native protein kinases that can utilize phenyl-ATP. One possible approach to suppressing the activity of other protein kinases is to add nonradioactive ATP to the labeling reaction mixture along with [γ-^{32}P]phenyl-ATP, coupled with the use of the M417G mutant, which weakly utilizes ATP (Figure 2C) and therefore would preferentially bind the radioactive ATP analogue. Another option is to use a different epitope tag such as GST whose isolation can be carried out with glutathione−agarose beads instead of an antibody. If a high background of radioactive bands obscures the detection of bands that are unique to the traceable mutant, then resolution of radiolabeled products by two-dimensional SDS−PAGE can be performed. Over and above these technical considerations, it should be emphasized that the high background observed here with MCF-10A cells is not likely to occur with all cell lines. Indeed, similar analyses being conducted in this laboratory with human prostate cells (LNCaP cells) produce much lower background levels.

The foregoing analysis demonstrates that this traceable mutant of PKCα reproduces the performance characteristics of the WT enzyme and verifies that it can be used successfully to identify substrates of this enzyme. Overall, the methods and strategies described here for nontransformed human breast cells will provide a foundation for similar studies to be conducted with cancer cells and other cell model systems.

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