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Overexpression of atypical protein kinase C in HeLa cells facilitates macropinocytosis *via* Src activation

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ABSTRACT

Atypical protein kinase C (aPKC) is the first recognized kinase oncogene. However, the specific contribution of aPKC to cancer progression is unclear. The pseudosubstrate domain of aPKC is different from the other PKC family members, and therefore a synthetic peptide corresponding to the aPKC pseudosubstrate (aPKC-PS) sequence, which specifically blocks aPKC kinase activity, is a valuable tool to assess the role of aPKC in various cellular processes. Here, we learned that HeLa cells incubated with membrane permeable aPKC-PS peptide displayed dilated heterogeneous vesicles labeled with peptide that were subsequently identified as macropinosomes. A quantitative membrane binding assay revealed that aPKC-PS peptide stimulated aPKC recruitment to membranes and activated Src. Similarly, aPKC overexpression in transfected HeLa cells activated Src and induced macropinosome formation. Src–aPKC interaction was essential; substitution of the proline residues in aPKC that associate with the Src-SH3 binding domain rendered the mutant kinase unable to induce macropinocytosis in transfected cells. We propose that aPKC overexpression is a contributing factor to cell transformation by interacting with and consequently promoting Src activation and constitutive macropinocytosis, which increases uptake of extracellular factors, required for altered cell growth and accelerated cell migration.

1. Introduction

A variety of signaling molecules are known to regulate and to coordinate intracellular membrane transport [1–3]. In that regard, we are actively characterizing the role of the human oncogene atypical protein kinase C (aPKC) in membrane trafficking [4]. We previously found that Src-dependent tyrosine phosphorylation of aPKC was required for aPKC association with the small GTPase Rab2 on vesicular tubular clusters and for intracellular transport in the early secretory pathway [5,6]. Src is a ubiquitous member of the nonreceptor tyrosine kinase family and is involved in several signal transduction pathways that regulate cell growth, motility, and differentiation [7,8]. This kinase contains several functional domains including the Src Homology 2 domain (SH2 domain) that binds to tyrosine phosphorylated residues and the Src Homology 3 domain (SH3 domain) that binds to proline-rich sequences. Although under steady state conditions Src is predominantly inactive, ligands may activate Src by competition and displacement of the SH2/SH3 domain interaction [9,10]. For example, in PC12 cells Src binds with aPKC through the SH3 domain, which results in aPKC phosphorylation on multiple tyrosine residues located in both the regulatory and the catalytic domain [11].

The aPKC subfamily differs in structure and function from the other PKC isoforms; that is, the lack of a calcium binding domain and only one cysteine-rich motif in the regulatory domain render aPKC insensitive to activation by calcium, diacylglycerol, and phorbol esters [12,13]. In addition, unlike the other isoforms, aPKC contains a Phox and Bem 1 domain found in adapter/scaffold proteins that functions as a protein–protein interaction module to oligomerize and organize signaling complexes within microdomains of the plasma membrane/intracellular compartments and on cytoskeletal elements [14,15]. There are no known pharmacological inhibitors that interfere with the activity of a specific PKC; therefore, synthetic peptides corresponding to the PKC pseudosubstrate domain are used routinely to determine substrate specificity and to characterize the physiological response of the phosphorylated substrate [16–20]. Indeed, we reported that the aPKC pseudosubstrate (aPKC-PS) peptide was a potent inhibitor of aPKC-dependent glyceraldehyde-3-phosphate dehydrogenase phosphorylation and prevented vesicular stomatitis virus G-protein transport from the endoplasmic reticulum to the Golgi complex in semi-intact cells [20,21]. These studies were the first to show that aPKC was required for transport in the early secretory pathway. It has also been reported that aPKC plays a role in endocytosis. For example, aPKC is a regulator of vascular endothelial growth factor receptor endocytosis and signaling in cultured endothelial cells [22]. Sanchez and coworkers found that a

Abbreviations: aPKC, atypical protein kinase C; aPKC-PS, atypical protein kinase C pseudosubstrate; act-Src, activated Src; aPKC-SC, scrambled peptide to aPKC-PS; TXrDex, Texas red conjugated dextran.

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dominate-negative aPKC mutant interfered with trafficking of the epidermal growth factor receptor [23]. Additionally, E-cadherin endocytosis is inhibited by aPKC phosphorylation of Numb [24].

In this study, we found that unlike inhibition of the early secretory pathway, the aPKC-PS peptide induced the endocytic process of macropinocytosis, an actin driven, clathrin-independent pathway that mediates the non-selective internalization of solute macromolecules [25–30]. Importantly, induction of macropinocytosis was mimicked by ectopic aPKC overexpression. Although in most cell types macropinocytosis is a transient response to growth factor stimulation, constitutive macropinocytosis occurs in v-Src- or H-ras transformed cells [28–33]. Interestingly, we found that aPKC-peptide treated and aPKC-transfected HeLa cells had elevated activated Src (act-Src) and that Src–aPKC interaction was necessary for macropinocytosis to occur independent of growth factor addition. The fact that aPKC is overexpressed in a number of human cancers [4,34–36] and the apparent relationship of macropinocytosis with cell growth, motility, and enhanced nutrient uptake [30,33,37] suggests a potential mechanism by which aPKC contributes to tumorigenesis.

2. Materials and methods

2.1. Materials

aPKC pseudosubstrate peptide (Myr-SIYRRGARRWRKL(biotin) YCAN), scrambled aPKC (Myr-WRICGNKARL(biotin)RRYYSAR), and PKCα/β (Myr-RFARKGAL(biotin)RQKNVHEVKN) were synthesized by GenScript Corp. (Piscataway, NJ). aPKCι cDNA was generously provided by Dr. Trevor Biden (Garvan Institute of Medical Research, Sydney, Australia). The mouse embryo fibroblast cell line SYF was purchased from American Type Culture Collection (Manassas, VA). Primary and secondary antibodies were purchased from the indicated suppliers; anti-PKC from BD Biosciences (San Jose, CA); anti-Src and anti-phospho-Src (Tyr416) from Cell Signaling Technology, Inc. (Danvers, MA); Alexa Fluor 488 chicken anti-mouse antibody, Alexa Fluor 594 chicken anti-rabbit, and Hoechst 33342 from Life Technologies (Grand Island, NY). FITC-streptavidin was purchased from ThermoScientific (Pittsburgh, PA). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)pyrimidine (PP2) was purchased from EMD Millipore (Billerica, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Quantitative membrane binding assay

HeLa membranes (~30 μg of total protein) prepared as previously described [6,38] were added to a reaction mixture that contained 27.5 mM Hepes (pH 7.4), 2.75 mM MgOAc, 65 mM KOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU rabbit muscle creatine phosphokinase, rat liver cytosol (~25 μg total protein) and 2.0 μM GTPγS [39]. The pseudosubstrate domain peptides were added at the concentrations indicated under Results and the reaction mix incubated at 37 °C for 15 °C. The binding reaction was layered onto a 20% sucrose cushion and centrifuged at 35,000 ×*g* for 20 min at 25 °C. The proteins in the recovered pellet were separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with the appropriate primary and secondary antibodies, developed with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ), and then quantified by densitometry using the ImageQuant program (GE Healthcare) [6,40].

2.3. Morphological assay/dextran uptake

HeLa cells (10^5/60 mm dish) grown in Dulbecco's Modified Eagle Medium (DMEM)/5% fetal bovine serum (FBS) were plated overnight on coverslips, and then transferred to serum-free DMEM for 1.5 h. The coverslips were inverted and placed in a 50 μl drop of DMEM/1.0% BSA/20 mM Hepes without or with the indicated peptide (20 μM) for 0–60 min at 37 °C, and then transferred to 4% formaldehyde/phosphate buffered saline (PBS) for 20 min at RT. For macropinosome visualization, cells plated on coverslips as above, were incubated without or with 20 μM PKC-PS peptide and 1 mg/ml TX-red-conjugated 70 kDa dextran (Molecular Probes, Grand Island, NY) in serum-free media for 15 min at 37 °C. In some experiments, cells were pretreated with 100 μM 5-(N-ethyl-N-isopropyl)amiloride (Sigma-Aldrich) diluted in serum-free DMEM media at 37 °C for 30 min prior to peptide and dextran addition. At the end of the incubation, cells were rinsed five times with ice cold PBS and immediately fixed in 4% formaldehyde. The fixed cells were washed 3× with PBS, permeabilized with 0.5% saponin/PBS/5% normal goat serum for 10 min, washed 3× with PBS, and then blocked for 1 h in PBS/5% normal goat serum. The cells were then incubated for 30 min at RT with the indicated primary antibody, washed with PBS, incubated with the appropriate fluorescent conjugated secondary antibodies for 30 min at RT, washed, stained for 10 min with Hoechst 33342, washed with PBS, mounted in Mowiol (EMB Millipore) containing DABCO (Sigma-Aldrich), and then viewed with a Zeiss Axio Imager epifluorescence microscope (Carl Zeiss, Gottingen, Germany) and photographed with an AxioCam MRm camera (Zeiss Microimaging, Thornwood, NY) using Axio Vision Z-stack software and colocalization module (Zeiss Microimaging) [39].

2.4. Constructs and transfection

His6-aPKC WT was prepared, as previously described [20,41]. His6-aPKC-ΔSH3 was made by a two-step PCR procedure [20]. In the first reaction, overlapping 5′ and 3′ fragments were generated using pcDNA-PKCι as the template. The 5′ portion of the molecule was generated using the 5′ wild-type primer (5′-GGCGAATTCATGTCCCACACGGTCGC AGGC) in combination with the 3′ mutagenic oligonucleotide (5′-TCCT GCACAAGCCATCCCAGCACGTTCTGCTACACAAGCGAACACATG). The 5′ mutagenic oligonucleotide (5′-CATGTGTTCGCTTGTGTAGCAGAACGTG CTGGGATGGCTTGTGCAGGA) and the 3′ wild-type anti-sense oligonucleotide (5′-CCGAATTCGGATCAGACACATTCTTCTGC) were used to generate the 3′ portion of the molecule. The two PCR products were combined to generate the full-length substitution mutant in a second reaction using the 5′ wild type and 3′ anti-sense primers, and then ligated into pcDNA4/HisMax-Topo (Life Technologies). The mutant sequence was verified by DNA sequence analysis. For expression studies, HeLa cells were plated overnight without or with coverslips (5×10^5 cells/60 mm dish), transfected with 5 μg of empty vector (control) or with 5 μg of the above constructs using Lipofectamine (Life Technologies), and then selected for 10 days in 50 μg/ml geneticin (Life Technologies) at 37 °C in a 5% CO_2 incubator. The pooled geneticin resistant cells were processed for indirect immunofluorescence or total cell lysates prepared for SDS-PAGE and Western blot analysis.

2.5. Peptide binding assay

HeLa cells (~5×10^6) cultured for 1.5 h in serum-free DMEM were incubated with 20 μM aPKC-PS peptide or with 20 μM aPKC-SC peptide in DMEM/1% BSA for 30 min at 37 °C, shifted to ice, washed in PBS, and then lysed in RIPA buffer containing 1 mM Na_2OV_4 and protease inhibitor cocktail set 1 (EMB Millipore). The post-nuclear cytosol was incubated with streptavidin-conjugated sepharose beads (Cell Signaling Technology, Inc.) for 5 h at RT with end-over-end mixing. Alternatively, purified His6-aPKC [21] and/or purified act-Src (EMD Millipore) was incubated with 20 μM aPKC-PS peptide or aPKC-SC, as above. The beads were collected by centrifugation in a tabletop microfuge at 10,000 rpm for 5 min, and then washed 5× with 1 ml RIPA. The bound proteins were eluted from the beads by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE followed by transfer to nitrocellulose.