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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.

Keywords

atypical PKC; Src; macropinocytosis; pseudosubstrate peptide

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

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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 renders 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 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 dominant-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)RRYY SAR, and PKC α/β (Myr-RFARKGAL(biotin)RQKNVHEVKN) were synthesized by GenScript Corp. (Piscataway, NJ). aPKC ζ 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 antimouse 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 μ g 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 CaCl₂, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU rabbit muscle creatine phosphokinase, rat liver cytosol (~ 25 μ g total protein) and 2.0 μ M GTP γ S [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 $\times$ 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 (10⁵/60 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 μ l drop of DMEM/1.0% BSA/20 mM Hepes without or with the indicated peptide (20 μ M) 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 μ M 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 μ M 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 3X with PBS, permeabilized with 0.5% saponin/PBS/5% normal goat serum for 10 min, washed 3X 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 Hoeschst 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- SH3 was made by a two-step PCR procedure [20]. In the first reaction, overlapping 5' and 3' fragments were generated using pcDNA-PKC ι as the template. The 5' portion of the molecule was generated using the 5' wild-type primer (5'-GGCGAATTCATGTCCCACACGGTCGCAGGC) in combination with the 3' mutagenic oligonucleotide (5'-TCCTGCACAAGCCATCCCAGCACGTTCTGCTACACAAGCG AACACATG). The 5' mutagenic oligonucleotide (5'-CATGTGTTTCGCTTGTGTAGCAGAACGT GCTGGGATGGCTTGTGCAGGA) 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 μ g of empty vector (control) or with 5 μ g of the above constructs using Lipofectamine (Life Technologies), and then selected for 10 days in 50 μ g/ml geneticin (Life Technologies) at 37°C in a 5% CO₂ 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 ($\sim 5 \times 10^6$) cultured for 1.5 h in serum-free DMEM were incubated with 20 μ M aPKC-PS peptide or with 20 μ M 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 Na₂OV₄ 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) were incubated with 20 μ M 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 5X 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.

3. Results

We examined the intracellular location of myristoylated and biotinylated aPKC-PS peptide in HeLa cells after incubation for 5–20 min at 37°C by labeling fixed cells with FITC-conjugated avidin. Fluorescent signal for fluorescein was observed on plasma membrane ruffles (Figure 1A) and on the membranes of peripherally located cytoplasmic vacuoles of various sizes ranging from 1–3 μ m in diameter (Fig. 1B) that appeared to be derived from the plasma membrane based on their proximity (Fig. 1A, arrow). Similar peptide-containing structures were not observed in control cells (Fig. 1C) or in cells treated with a myristoylated and biotinylated scrambled peptide to aPKC-PS (aPKC-SC) (Fig. 1D) or in cells treated with a modified peptide encoding the pseudosubstrate domain of PKC α/β (data not shown) suggesting that induction of membrane ruffles and vacuoles is specific to the aPKC-PS peptide.

Because macropinocytosis has been implicated in the internalization of some cell penetrating peptides that are rich in arginine or lysine [42–44] and the large size of the peptide-labeled vacuoles, we asked if the aPKC-PS peptide induced structures are macropinosomes by analyzing the uptake of a fluid phase tracer. For these studies, HeLa cells plated on coverslips were incubated with aPKC-PS peptide or with aPKC-SC peptide in the presence of 70 kDa Texas red-dextran (TXrDex) for 15 min at 37°C. A substantial number of large and heterogeneous endocytic structures that contained fluorescent dextran were observed only in aPKC-PS incubated HeLa cells (compare Fig. 1E and 1F. Boxed area; FITC-avidin labeled structures containing TXrDex). Pre-incubation with cytochalasin D, which blocks actin polymerization and consequently macropinocytosis, prevented aPKC-PS stimulated TXrDex uptake (Fig. 1G). Similarly, pre-treatment with amiloride, a selective inhibitor of macropinocytosis [45, 46] resulted in no detectable TXrDex uptake in peptidomimetic treated cells (Fig. 1H). In contrast, aPKC-PS peptide did not influence receptor-mediated endocytosis of transferrin (data not shown). These combined results are consistent with the idea that the endocytic structures induced by the aPKC peptide are macropinosomes.

2.1 aPKC Pseudosubstrate Domain Promotes aPKC Recruitment to Membranes and Src Activation

We previously reported that aPKC-PS peptide (unmodified form, no biotin or myristoyl group) promoted aPKC recruitment to membranes when introduced into our quantitative membrane-binding assay [21]. For this assay, salt-washed HeLa cell membranes were incubated with rat liver cytosol and GTP γ S without or with increasing aPKC-PS peptide concentration, and then the pellet containing membranes and associated proteins was subjected to SDS-PAGE and Western blot analysis [39, 40]. Likewise, the modified peptide stimulated aPKC binding to HeLa membranes in a dose dependent manner (Fig. 2A). In contrast, comparable doses of scrambled peptide added to the binding assay failed to recruit aPKC above the control level. These results prompted us to evaluate the intracellular distribution of aPKC in the presence and absence of aPKC-PS peptide. In control cells, the kinase had a predominantly punctate appearance and immunolocalized throughout the cytosol and in proximity to the nucleus (Fig. 2B) whereas aPKC-PS peptide incubated cells

contained peripherally located vacuoles that co-labeled with aPKC and with the peptide (boxed area; Manders coefficient 0.967).

Based on these observations, we predicted that aPKC-interacting proteins would also exhibit a dose-dependent recruitment to peptide-treated membranes. Because aPKC binds directly to Src [11, 47] the blot from the binding assay was reprobbed for activated Src (act-Src), as determined by an antibody that specifically recognizes phosphorylated Tyr-416 (pTyr-416) [6, 48]. As we observed for aPKC, the peptidomimetic caused a dose dependent increase in membrane bound act-Src while supplementing the assay with the scrambled peptide had no effect (Fig. 3A). Similarly, incubation of intact HeLa cells for 15 min with increasing amounts of membrane permeable aPKC-PS induced a concomitant increase in endogenous activated Src (Fig. 3B).

Several studies have reported that v-Src activation triggers constitutive membrane ruffling and macropinocytosis [31, 32, 49]. Therefore, we examined the intracellular location of act-Src in control and aPKC-PS peptide incubated cells by indirect immunofluorescence. As we observed for aPKC, act-Src was found on large vacuoles (~1–3 μ m) that co-distributed with aPKC (boxed area; Manders coefficient 0.892) in aPKC-PS peptide treated cells (Fig. 3C). To determine if c-Src kinase activity was required for the induction of the aPKC-PS/Src labeled vacuoles, HeLa cells were pre-incubated with the Src-specific tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)pyrimidine (PP2) for 1 h at 37°C, and then peptide added and incubated for an additional 20 min. As shown in Fig. 3C, peptide and PP2 treated cells did not display vacuoles/macropinosomes containing aPKC/act-Src. To further establish that act-Src was necessary for generating the aPKC-PS induced phenotype, the triple-knockout mouse embryo fibroblast cell line SYF that lacks Src and the related tyrosine kinases Yes and Fyn was incubated with peptide [50]. We did not observe any PKC-PS peptide labeled vacuoles in SYF cells, however both aPKC and peptide co-distributed on membrane ruffles (Fig. 3C, arrow). These combined observations demonstrate that aPKC-PS peptide both requires and stimulates Src kinase activity for macropinosome formation.

To learn if the peptide was binding directly to Src and causing its activation, we took advantage of the attached biotinylated moiety to enrich for peptide-binding proteins. HeLa cells were exposed to peptide for 30 min at 37°C, and then the total cell lysate was incubated with streptavidin beads to pull-down aPKC peptide bound proteins. Src like aPKC was detected only in the pull-down of aPKC-PS peptide incubated cells (Fig. 3D). However, purified act-Src was not recovered on the beads indicating that the interaction with the peptide is indirect (Fig. 3E).

2.2 aPKC Overexpression in HeLa Cells Stimulates Macropinocytosis

The induction of macropinocytosis generally occurs within 30 sec of growth factor addition, and then vanishes [28, 30, 51]. In contrast, specialized cell types such as macrophages and dendritic cells or Src transformed cells undergo constitutive formation of macropinosomes that occurs absent of growth factor [28, 30, 31, 51]. Because the aPKC-PS peptide; 1) increased aPKC membrane binding, 2) caused Src activation, and 3) induced macropinosome formation independent of growth factor, we hypothesized that an increase in

the intracellular level of aPKC would facilitate constitutive macropinocytosis. To that end, HeLa cells were transfected with His6-tagged aPKC cDNA, selected for growth in geneticin for 10 days, and then the resistant cells analyzed by indirect immunofluorescence light microscopy. Large vacuoles that co-labelled with anti-His6-aPKC and anti-act-Src (Fig. 4A) were observed in the kinase overproducing cells that were similar to the endocytic structures found in aPKC-PS peptide treated cells. Notably, act-Src appeared as intense fluorescent patches on the cytoplasmic face of the vacuoles (Fig. 4A). The uptake of fluorescent dextran by aPKC overexpressing cells relative to mock-transfected (empty vector) control cells (Fig. 4B) suggested that the TXrDex labeled structures are newly formed [52]. We found that preincubation with amiloride significantly reduced the number of TXrDex labeled elements in the aPKC transfected cells (Fig. 4B).

Based on an earlier report that the aPKC proline-rich motif located within residues 98–114 binds to the Src-SH3 domain [11] a substitution mutant was constructed (PKC- SH3) replacing the four prolines in that stretch of sequence with an alanine. HeLa cells transfected with His6-aPKC- SH3 did not display large endocytic vacuoles comparable to those found in aPKC-WT overexpressing cells (Fig. 4C) and failed to internalize TXrDex (Fig. 4C). Interestingly, immunoblot analysis of total cell lysate from control and transfected cells showed that aPKC overexpression resulted in elevated act-Src levels (~ 2 fold increase) compared to the basal level of act-Src in mock transfected control cells (Fig. 4A). In contrast, the His6-aPKC-SH3 transfected cells did not possess an increased level of act-Src (Fig. 4C). These collective results support the notion that the large endocytic elements are macropinosomes that require aPKC-Src interaction and activation for formation.

3. Discussion

Peptidomimetics corresponding to protein-protein interacting domains and regulatory domains serve as valuable tools to elucidate protein function and subsequently biological processes [53–55]. For example, the lack of PKC specific inhibitors has resulted in the use of synthetic peptides corresponding to the PKC pseudosubstrate domain, which inhibit kinase activity. This autoinhibitory sequence blocks the substrate binding cavity and disengagement allows substrate binding and subsequent phosphorylation [56, 57]. Similarly, the pseudosubstrate domain peptide does not interfere with ATP binding but maintains the kinase in the membrane associated active state [58, 59]. The pseudosubstrate domain of the aPKC family is different from the other PKC family members and resides downstream and adjacent to the proline-rich motif located within residues 98–114 that binds to the Src-SH3 domain [11, 56, 57]. We have successfully used this peptide to block kinase activity in a cell free assay and to demonstrate a role for aPKC in the early secretory pathway using membrane permeabilized cells [21]. Unlike those protocols in which the plasma membrane is not a barrier to peptide uptake, here we employed N-terminal acetylated aPKC-PS to promote plasma membrane incorporation and subsequent translocation across the lipid bilayer. We never observed any significant diffuse cytoplasmic fluorescence over a period of 1 h after peptide treatment at 37°C or in cells incubated with peptide at 4°C (data not shown), indicating that aPKC-PS peptide was internalized by an endocytic process. The peptide and aPKC associated with the plasma membrane where ruffles formed and after

internalization was found on the surface of large heterogeneous endocytic vesicles that we showed by using pharmacological probes and solute uptake to be macropinosomes.

Although the exact mechanism used by the aPKC-PS peptide to induce macropinocytosis is not yet elucidated, the results of this study strongly suggest that the effect is attributable to persistent Src activation that requires interaction with aPKC. It has been previously reported that act-Src binds to aPKC [47]. This finding would explain the significant increase of membrane-associated act-Src obtained after aPKC-PS peptide treatment of membranes. Indeed, peptide promoted aPKC recruitment to membranes both *in vitro* and *in situ*. Similarly, ectopic aPKC overexpression in HeLa cells resulted in a significant increase in the basal level of act-Src (~ 2 fold increase) compared to the nontransfected control cells.

It is possible that the peptidomimetic binds directly to anionic lipids in cell membranes via the basic residues, as do many cell penetrating peptides that are rich in arginine and lysine [42, 60–62]. In that case, aPKC-PS peptide could serve as a platform to associate with and maintain the kinase in the unfolded active conformation on cellular membranes, and thereby exposing the SH3 binding domain in aPKC for interaction with Src. This idea is consistent with our finding that aPKC-PS peptide binds directly to aPKC but indirectly to Src through aPKC association. Because we did not detect macropinosome formation or Src binding with and/or to the scrambled peptide that has comparable charged residues, the exact amino acid sequence must be important to stimulate the endocytic process.

aPKC has been reported to play a role in endocytosis: Src-aPKC associates with the nerve growth factor receptor TrkA in PC12 cells [11, 63]. It has also been shown that overexpression of a dominant-negative aPKC mutant inhibited trafficking of the epidermal growth factor receptor from late endosomes to lysosomes (Sanchez *et al.*, 1998). Additionally, aPKC phosphorylation of Numb prevents E-cadherin internalization [24]. Recently, aPKC was reported to regulate vascular endothelial growth factor receptor endocytosis [22]. aPKC kinase activity has also been demonstrated to play a pivotal role in an unique phagocytic pathway utilized by *Helicobacter pylori* that was inhibited by myristoylated aPKC-PS peptide [64]. Phagocytosis shares many features with macropinocytosis, however distinct molecular mechanisms are utilized by each process and by specific cell-types, which could explain why the peptide had an opposite effect from what we observed in HeLa cells [51].

Macropinocytosis is an important physiological process [30, 51]. Although in most cell types macropinocytosis is a transient response to growth factors or phorbol esters, constitutive macropinocytosis occurs in Src-transformed cells suggesting a role of this endocytic process in cell transformation [31, 32]. In many transformed cells, macropinocytosis occurs in a constitutive manner to increase uptake of extracellular fluids/nutrients required for heightened metabolic activity of the cancer cell [33] and to promote cell mobility [65]. While Src is better known for inducing malignant transformation of numerous cell types, aPKC overexpression facilitates tumorigenesis in a variety of cancers [66–73]. Moreover, aPKC has been shown to regulate the actin cytoskeleton in association with the other partitioning-defective (Par) complex proteins [74–77]. Unlike the clathrin-dependent endocytic pathway, actin dynamics is required for macropinocytosis. Once a

macropinosome has formed, the structure undergoes a maturation process that varies dependent on the cell-type but utilizes the trafficking machinery employed in other endocytic pathways [30]. In HeLa cells, the bulk of the macropinosome membrane is recycled back to the cell surface, which requires Par-3, Par-6, CDC42, and aPKC [27, 78, 79]. Therefore, aPKC may have a dual function in macropinocytosis in which association with Src results in Src activation and stimulation of the endocytic event whereas membrane retrieval of nondegraded macropinosome membrane might utilize aPKC kinase activity. We propose that this process is accentuated and occurs at a high frequency in cancer cells, which overexpress both kinases, contributing to tumor malignancy.

4. Conclusions

Our findings have identified a new intracellular role for aPKC in the endocytic process of macropinocytosis. Increased plasma membrane associated aPKC promoted by either the addition of the aPKC pseudosubstrate peptide or by aPKC ectopic overexpression resulted in increased aPKC-Src interaction and subsequent Src activation, which induced macropinosome formation. Both kinases are found on the newly formed macropinosomes and therefore may have a dual function in the endocytic pathway by first promoting macropinosome formation (closure) and subsequently contributing to macropinosome stability.

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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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Fig. 1.

PKC-PS peptide stimulates formation of macropinosomes. HeLa cells were incubated with 20 μ M aPKC-PS for 5 min (A) or 20 min (B), and then FITC-avidin added to detect the peptide. Membrane ruffles labeled with aPKC-PS (A, arrow). The induction of large vesicles (macropinosomes) is specific to aPKC-PS. (C) HeLa cells (control) incubated with FITC-avidin. (D) HeLa cells incubated for 20 min with aPKC-SC peptide. (E) HeLa cells (control) incubated for 20 min with 1 mg/ml TXrDex (F) HeLa cells co-incubated with aPKC-PS peptide and TXrDex for 20 min. Boxed areas show large vesicles containing TXrDex in the

lumen and aPKC-PS on the vesicle membrane. (G) HeLa cells pretreated with cytochalasin D for 30 min, and then incubated for 20 min with aPKC-PS peptide and TXrDex. (H) HeLa cells pretreated with amiloride for 30 min were incubated for 20 min with aPKC-PS peptide and TXrDex. Scale bar, 5 μ m. A representative image is shown from five independent experiments.

Fig. 2. aPKC-PS peptide stimulates aPKC membrane binding (A) Salt-washed membranes were incubated with increasing concentrations of aPKC-PS peptide or aPKC-SC peptide for 15 min at 37°C. Immunoblot of the membrane pellet with associated proteins was probed with anti-aPKC. (B) HeLa cells were incubated without or with 20 μ M aPKC-PS peptide for 15 min at 37°C. The fixed cells were immunolabeled with anti-aPKC (control) and with FITC-avidin (peptide). Scale bar, 5 μ M. A representative blot and images are shown for three independent experiments.

Fig. 3. aPKC-PS peptide indirectly causes Src activation. (A) Salt-washed HeLa cell membranes were incubated with increasing concentrations of aPKC-PS peptide or aPKC-SR peptide for 15 min at 37°C. The membrane pellet with associated proteins was subjected to SDS-PAGE and the western blot probed with anti-pSrc (Tyr416). (B) HeLa cells (5×10^5 75 mm dish) were incubated in serum-free media for 1.5 h at 37°C. The media was changed to DMEM/0.5% BSA containing 20 μ M aPKC-PS peptide or aPKC-SR peptide, and then the cells incubated for 15 min at 37°C. The media was replaced with DMEM containing protease

inhibitors and Na⁺ vandate, and then cells scraped off the dish with a rubber police, collected by centrifugation, and the pellet resuspended in sample buffer/Na⁺ vandate/ protease inhibitor cocktail. Equal total protein (40 mg) for each peptide dose was separated by SDS-PAGE and the western blot probed, as above. (C) HeLa cells were incubated without or with 20 μ M aPKC-PS peptide for 15 min at 37°C, fixed, and then stained with anti-aPKC (cont) and with anti-aSrc (Tyr410) (+peptide). Boxed area show large vesicles co-labeled with aPKC and aSrc (Manders coefficient of 0.892). HeLa cells pretreated with PP2 were incubated with aPKC-PS peptide for 15 min, fixed and stained with anti-aPKC. SYF cells incubated with aPKC-PS for 15 min at 37°C were fixed and stained with anti-aPKC and FITC-avidin. (D) HeLa cells were incubated with 20 μ M aPKC-PS peptide or with 20 μ M aPKC-SC peptide for 30 min at 37°C, and the post-nuclear cytosol subjected to a pull-down assay using streptavidin-conjugated beads. (E) Alternatively, purified His6-aPKC and or purified act-Src was incubated with 20 μ M aPKC-PS peptide or aPKC-SC, as above. Bead-bound proteins were resolved by SDS-PAGE followed by transfer to nitrocellulose. The blot was probed with indicated primary antibodies. Representative blots and images are shown for three independent experiments.

Fig. 4.

Macropinosome formation is stimulated in aPKC overexpressing HeLa cells. (A) HeLa cells 10 days post-transfected with empty vector (mock) or with His6-aPKC were co-stained with anti-aPKC and anti-pSrc (Tyr416). Total cell lysate (100 mg/ml) from pooled HeLa cells transfected as above, was separated by SDS-PAGE followed by transfer to nitrocellulose. The blot was probed with indicated primary antibodies, developed with ECL, and then quantitated by densitometry. The ratio of the densitometric values obtained for aPKC and pSrc (Tyr416) to the values obtained for tubulin (loading control) was plotted. (B) HeLa cells (mock transfected) or His6-aPKC expressing cells were incubated for 20 min with 70 kDa TXrDex. His6-aPKC expressing cells were pretreated with amiloride for 30 min, and then incubated with TXrDex for 20 min. (C) HeLa cells transfected with His6-aPKC- SH3 were co-stained with anti-His6 and DAPI. His6-aPKC- SH3 expressing HeLa cells were incubated with 70 kDa TXrDex for 20 min. Inset; cells that failed to take-up TXrDex expressed the aPKC mutant. Total cell lysates (100 mg/ml) from pooled HeLa cells transfected with empty vector or with His6-aPKC- SH3 were analyzed, as above.