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ARF1-regulated coatomer directs the steady-state localization of protein kinase C epsilon at the Golgi apparatus

Tabitha A. Peterson, Mark Stamnes *

Department of Molecular Physiology & Biophysics, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA 52242, USA

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ABSTRACT

Protein kinase C epsilon (PKCε) contributes to multiple signaling pathways affecting human disease. The function of PKCε requires it to undergo changes in subcellular distribution in response to signaling events. While the mechanisms underlying this translocation are incompletely understood, it involves the receptor for activated C kinase protein (RACK2/β′-COP). This receptor also functions as a vesicle coat protein in the secretory pathway where it is regulated by the small GTP-binding protein ADP-ribosylation factor, ARF1. We inhibited ARF1 activation to test the requirement for RACK2/β′-COP in PKCε localization in NIH3T3 fibroblasts. We found that steady-state localization of PKCε at the Golgi complex requires ARF1-regulated RACK2/β′-COP function. By contrast, we did not observe any defects in phorbol ester-induced translocation when ARF1 was inhibited. We also found that PKCε bound to isolated membranes through two distinct mechanisms. One mechanism was dependent upon RACK2/β′-COP while a second was RACK2/β′-COP-independent and stimulated by phorbol esters. Finally, we show that RACK2/β′-COP affects the subcellular distribution of a constitutively active form of PKCε, in a manner similar to what we observed for wild-type PKCε. Together, our data support a role for RACK2/β′-COP in the steady-state localization of PKCε at the Golgi apparatus, which may be independent of its role during PKCε translocation to the cell surface.

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1. Introduction

Protein kinase C epsilon (PKCε) contributes to signal transduction pathways that regulate a diverse range of cellular processes including proliferation, differentiation, metabolism, and intracellular trafficking [1–3]. PKCε acts as an oncogene in vitro and may serve as a tumor biomarker [4,5]. Signaling pathways that can protect heart cells from ischemic damage also rely on PKCε-mediated phosphorylation [6–8]. The contribution of PKCε to multiple signaling pathways and disease states arises in part through its ability to localize to different sites within the cell. Depending on the cell type and signaling state, PKCε can adopt distinct subcellular localization, for example at the nucleus, mitochondria, plasma membrane, Golgi apparatus or actin microfilaments [9–12]. Remarkably, PKCε distribution can change rapidly during signal transduction. In heart cells, activation of adenosine receptors causes PKCε to redistribute to sarcomeres [8,13,14]. PKCε localizes to the Golgi apparatus in fibroblasts, but rapidly dissociates from the Golgi membrane and translocates to the cell surface or nucleus when PKC-signaling is activated by addition of phorbol esters [10,15–17].

The subcellular distribution and translocation of PKCε relies on targeting motifs within its N-terminal regulatory domain. The epsilon isoform belongs to the novel subclass of PKC that contains N-terminal regulatory C2-like and C1 domains separated from the C-terminal kinase domain by a hinge region [2]. The C1 domain binds diacylglycerol and other lipids to affect activation and localization. The C1 domain is also the binding site for carcinogenic phorbol esters that are potent activators of the conventional and novel sub-types of PKC [3]. The C1 domain may also influence PKCε localization through protein interactions, for example with the Golgi-apparatus cargo receptor protein, p23 [18,19]. C2 domains are a second major targeting motif shared by many PKC isoforms. PKCε has a C2-like domain upstream of the C1 domain. Unlike conventional PKCs, the C2-domain of PKCε does not bind calcium. The C2 domain confers binding to localization receptors called RACKs (receptor of activated C kinase) [1–3,20]. Each PKC isoform binds with unique affinity to RACKs; PKCε binds preferentially to RACK2 or εRACK [20]. The binding interaction between RACK2 and PKCε contributes to cardioprotective signal transduction [6–8,21,22]. Peptides that inhibit the binding interaction between RACK2 and PKCε also influence tumor cell growth [4,23].

Interestingly, RACK2 has been identified previously as the β′-COP subunit of coatomer, a heteroheptameric transport vesicle coat complex [20]. The coatomer complex is highly enriched on membranes at the Golgi apparatus and the Golgi/ER-intermediate compartment

Abbreviations: ARF1, ADP-ribosylation factor-1; BAPTA, [1,2,-bis(o-aminophenoxy) ethane-N,N,-N′,N′, tetra-acetic acid]; BFA, brefeldin A; COP, coat protein; DMEM, Dulbecco's modified Eagle's media; ER, endoplasmic reticulum; GFP, green fluorescent protein; GTP, Guanosine-5′-triphosphate; PBS, phosphate buffered saline; PKC, protein kinase C; RACK, receptor for activated C kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate

* Corresponding author. Tel.: +1 319 335 7858; fax: +1 319 335 7330.
E-mail address: mark-stamnes@uiowa.edu (M. Stamnes).

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where it contributes to the formation of COPI-type transport vesicles. Coatomer has been best characterized as the coat protein for COPI vesicles that mediate transport within the Golgi complex and retrograde trafficking from the Golgi to the endoplasmic reticulum [24]. The connection, if any, between RACK2/β′-COP function in vesicular transport and in PKCε signal transduction is unclear.

The role for RACK2/β′-COP as a receptor that targets activated PKCε is incompletely understood. The RACK2/β′-COP-PKCε binding interaction is implicated in translocation to the cell surface in fibroblasts. For example, peptides and point mutations predicted to promote the binding interaction with RACK2/β′-COP facilitate PKCε translocation to the plasma membrane [17]. RACK2/β′-COP has also been implicated in PKC localization to the Z disk on sarcomeres in cardiomyocytes [8,11]. However, the coatomer complex including, RACK2/β′-COP, is predominantly localized to the Golgi apparatus in most cell types. This localization would seem more consistent with a role for RACK2/β′-COP in the steady-state distribution of PKCε at the juxtanuclear Golgi apparatus. We have now used PKCε distribution in cultured fibroblasts and cell-free membrane binding assays to better define the contribution of the RACK2/β′-COP subunit of coatomer to PKCε localization and translocation.

2. Materials and methods

2.1. Materials

The following antibodies were used in these studies: rabbit anti-ε-COP [25], rabbit anti-PKCε C-15 (Santa Cruz Biotechnology, Santa Cruz, California), and mouse anti-GM130 (BD Transduction Laboratories, Franklin Lakes, New Jersey). TPA was obtained from Cell Signaling Technology (Danvers, Massachusetts). Lipofectamine 2000 was obtained from Life Technologies (Carlsbad, California). Brefeldin A and GTPγS were obtained from Sigma-Aldrich (St. Louis, Missouri). Superdex 200 beads were obtained from GE Healthcare Biosciences (Piscataway, NJ).

2.2. Cell culture

NIH3T3 cells were grown on glass cover slips in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin-streptomycin, and non-essential amino acids. Subconfluent cultures were transfected with plasmids encoding GFP-PKCε, mCherry-PKCε, GFP-PKCε(A159E), and/or β′-COP-GFP as indicated in the figure legends using Lipofectamine 2000. For the translocation assay, NIH3T3 cells were treated with TPA and/or brefeldin A (BFA) at the indicated concentrations and times. Control cells were treated with solvents, either DMSO or methanol alone. To measure retrograde translocation, the media containing TPA was removed after 10 min and replaced with fresh 37 °C DMEM, and the cells were then incubated at 37 °C for the indicated times.

2.3. Immunofluorescence

The cells were washed with PBS and fixed with 4% paraformaldehyde. They were quenched with 50 mM ammonium chloride for 10 min before permeabilization using 0.1% Triton X-100 for 4 min at room temperature. The cells were washed three times with PBS and blocked with 2% donkey serum in PBS at room temperature for 30 min. Appropriate dilutions of the primary antibodies in PBS plus 0.2% donkey serum and 0.1% Tween 20 were added to cells for 1 h at room temperature. The cells were washed three times with PBS and incubated with secondary antibodies. The cells were washed again three times, mounted on slides, and analyzed by confocal microscopy (model LSM-510; Carl Zeiss MicroImaging, Thornwood, New York).

2.4. Membrane-binding assays

Rat-liver membranes and bovine-brain cytosol were prepared as described previously [26,27]. For Fig. 6, coatomer-depleted cytosol was prepared as described previously [28]. Briefly, bovine brain cytosol was fractionated by gel filtration using Superdex 200 beads. The fractions containing the high molecular weight coatomer complex were identified by Western blot analysis. The non-coatomer-containing fractions were recombined and concentrated to generate depleted cytosol. Intact cytosol was prepared by recombining and concentrating all of the column fractions.

The membranes and cytosol were incubated with a final reaction volume of 0.2 ml as described previously [29]. GTPγS and TPA were included in the reactions at the indicated concentrations. For the experiments using BFA the membranes and cytosol were preincubated separately such that the final BFA concentration was 400 μM. Preincubation with methanol alone (2% final) was carried out as a control. Following the incubation, the membranes were reisolated from the reaction by flotation through an isopycnic sucrose gradient [29]. Fractions were recovered from the top and analyzed by Western blotting. Western blot signals were quantified by densitometry and normalized to standard curves generated from a serially diluted cytosol [30].

3. Results

3.1. Dissociation from Golgi membranes is not sufficient for translocation to the cell surface

NIH3T3 fibroblasts provide a good model to study the molecular mechanisms underlying PKCε localization and its regulation. In these cells, the epsilon isoform localizes to the Golgi apparatus at steady state, but undergoes nearly complete translocation to the plasma membrane in response to PKC-activating phorbol-esters [15,16]. As expected, we observed that the GFP-PKCε localized to the juxtanuclear Golgi apparatus at steady state when expressed transiently in NIH3T3 cells (Fig. 1A). Furthermore, we confirmed that the GFP-PKCε undergoes rapid redistribution to the cell surface upon addition of the phorbol ester, TPA (Fig. 1A).

RACK2/β′-COP has been previously described as a receptor directing PKCε translocation in response to cell signaling [17,31]. However, RACK2/β′-COP localizes to the Golgi apparatus as part of the coatomer transport-vesicle-coat complex and could also contribute to steady-state PKCε localization. Coatomer localization to the Golgi apparatus requires activation of the small GTP-binding protein ARF1 [32]. We tested whether RACK2/β′-COP, as part of the coatomer complex, contributes to PKCε steady-state localization by treating the GFP-PKCε-expressing cells with BFA (Fig. 1B). BFA inhibits nucleotide exchange on ARF1 causing both ARF1 and coatomer to rapidly dissociate from the Golgi membranes [33]. We used a relatively low BFA concentration (10 μM) and short time point (15 min.) that dissociated ARF1 and coatomer from the membrane yet caused only modest disruption of Golgi morphology. Following BFA treatment, GFP-PKCε no longer localized to the Golgi apparatus and appeared dispersed throughout the cytoplasm (Fig. 1B). GFP-PKCε did not appear enriched at the plasma membrane in the presence of BFA alone, indicating that dissociation from the Golgi apparatus is not sufficient for redistribution to the plasma membrane. The effects of BFA indicate that PKCε localization in cells can be regulated by ARF-family GTP-binding proteins. Furthermore, it suggests that the interaction between PKCε and the RACK2/β′-COP subunit of coatomer can contribute to the steady-state localization of PKCε at the Golgi apparatus.

3.2. ARF-regulated coatomer function is not required for PKCε binding and dissociation from the plasma membrane

RACK2/β′-COP is implicated as a receptor that promotes translocation of PKCε upon activation [15,16]. We tested the contribution of

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