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

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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.

Keywords

Protein kinase C; coatomer; ADP-ribosylation factor-1 (ARF1); Receptor for activated C kinase-2 (RACK2); Golgi apparatus; phorbol ester

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

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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 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), 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 minutes and replaced with fresh 37°C DMEM, 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 minutes before permeabilization using 0.1% Triton X-100 for four minutes 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 one hour 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 Figure 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 coatomer to PKCε translocation by treating cells with or without BFA prior to activation with the phorbol ester, TPA. As expected, when the cells were treated with TPA in the absence of BFA, the GFP-PKCε redistributed and within ten minutes was found almost exclusively at the plasma membrane (Fig. 2A). In the presence of BFA, we observed that PKCε was dispersed throughout the cell consistent with the previous experiment. Nevertheless, the BFA-dispersed GFP-PKCε rapidly translocated to the cell surface within 10 minutes of TPA addition (Fig. 2A). This indicates that that ARF1-regulated coatomer function is not essential for PKCε translocation to the cell surface. Together, the effects of BFA support a role for RACK2/β′-COP in the steady-state localization of PKCε at the Golgi apparatus.

PKCε translocation in response to cell signaling has predominantly been studied in the “anterograde” direction—from the Golgi apparatus to the cell surface. It is also possible that RACK2/β′-COP plays a role during “retrograde” translocation from the cell surface back to the Golgi apparatus. We tested this by treating cells with TPA for 10 minutes and then examining PKCε distribution after removing the TPA and washing cells with fresh media (Fig. 2B). Following the TPA washout, PKCε is found to have mostly dissociated from the plasma membrane within 1 hour and is mostly localized to the Golgi apparatus within 2 hours. The slow kinetics of PKCε redistribution to the Golgi apparatus is consistent with previous reports analyzing the consequences of washing out phorbol esters and may reflect slow turnover of TPA [34]. Treating cells with a relatively high concentration of BFA (50 μM) before and during the TPA washout had no overt effect on the dissociation of PKCε from the cell surface (Fig. 2C). As expected, in the presence of BFA, the PKCε did not relocalize to the juxtanuclear region following the washout but stayed dispersed in the cytosol. The effects of BFA during “retrograde” translocation indicate that the ARF-regulated coatomer complex does not play an essential role in the dissociation reaction at the plasma membrane. ARF1 function was necessary for correct Golgi morphology and the juxtanuclear steady-state distribution of PKCε.

3.3 PKC activation does not cause overt redistribution of coatomer

Our data support the model that coatomer contributes to PKCε localization in NIH3T3 fibroblasts. As an additional test of RACK2/β′-COP's role during signal-induced PKCε translocation, we compared the distribution of RACK2/β′-COP-GFP and mCherry-PKCε in cotransfected NIH3T3 cells before and after TPA treatment (Fig. 3). At steady state, both proteins colocalized at the juxtanuclear Golgi apparatus. While the mCherry-PKCε rapidly dissociated from the Golgi apparatus and translocated to the plasma membrane after TPA addition, there was no overt change in RACK2/β′-COP-GFP distribution. This experiment does not exclude the possibility that a small fraction of the total cellular coatomer redistributes and contributes to PKCε translocation. However, it indicates that TPA-induced translocation of PKCε does not involve a dramatic redistribution of the coatomer complex or of the RACK2/β′-COP subunit alone.

3.4 PKCε can bind isolated membranes through both coatomer-dependent and – independent mechanisms

The analysis of PKCε translocation in fibroblasts suggests that PKCε can form distinct binding interactions with intracellular membranes. The steady-state localization at the Golgi apparatus appears to require a binding interaction with the ARF1-regulated pool of coatomer. By contrast translocation and localization at the plasma membrane appears to be independent of the bulk of cell coatomer. One possibility is that there is an ARF1-coatomer-dependent binding interaction with Golgi membranes at steady state, and an ARF1-coatomer-independent reaction at the plasma membrane upon activation with phorbol esters. We further characterized the requirements for coatomer during PKCε binding interactions with membranes using cell-free assays (Fig. 4). Briefly a membrane preparation was incubated with cytosol, and then reisolated away from the reaction by isopycnic centrifugation [29]. Membrane-bound proteins were then quantified by Western blotting.

We found that both GTPγS, a non-hydrolyzable GTP analog, and TPA stimulated PKCε binding to membranes in vitro (Fig. 4A and B). We confirmed that GTPγS activates ARF1-dependent coatomer binding in vitro, however, we did not observe an increase in coatomer binding in the presence of TPA (Fig. 4C). The fact that coatomer and PKCε binding are correlated upon addition of GTPγS, but not TPA, indicates that GTPγS- but not TPA-dependent PKCε binding to membranes is mediated by coatomer. We tested this possibility by examining the effects of inhibiting ARF1 and coatomer binding with BFA. We found that the GTPγS-activated binding of PKCε was inhibited by BFA whereas the TPA-dependent binding was unaffected by BFA (Fig. 4A and B).

Others and we showed previously that the calcium chelator BAPTA causes coatomer to dissociate from membranes both in cell-free binding assays and within intact cells [35,36]. Hence, we determined whether BAPTA affected PKC binding. As with BFA, we found that BAPTA inhibited the GTPγS-activated PKCε binding to membranes but not the TPA-activated binding (Fig. 4D). The results with BFA and BAPTA support a role for coatomer in GTPγS-stimulated, but not TPA stimulated PKCε binding to membranes. Nevertheless, BFA and calcium chelators could affect PKCε binding by disrupting factors other than ARF1 and coatomer. To address this, we have examined the ability of PKCε to bind membranes from cytosol that had been depleted of the high-molecular-weight coatomer complex. Intact cytosol supported both TPA and GTPγS-dependent binding whereas the coatomer-depleted cytosol supported TPA-dependent, but not GTPγS-dependent binding (Fig. 5). Upon quantifying and averaging three experiments, we found that GTPγS stimulated PKCε binding 7.6 fold in intact cytosol but only 2.6 fold with depleted cytosol. By contrast, TPA stimulated binding to a similar extent in intact and depleted cytosol, 10.8 and 9.7 fold respectively.

Our results using the binding assay support the model that PKCε can interact with membranes through two distinct mechanisms. One mechanism is dependent on the previously characterized targeting receptor RACK2/β′-COP, functioning as part of the ARF1-regulated coatomer complex. A second membrane binding mechanism is independent of RACK2/β′-COP and can be stimulated by TPA.

3.5 Coatomer can influence localization of kinase-active PKCε

Some models for PKC signaling suggest that the kinase is stored at one site in the cell as an inactive pool which then undergoes translocation to a new site following activation [2,37]. RACKs, defined as receptors for “active” C kinase, are anticipated to participate in the targeting or translocation of the active PKC pool. Indeed, RACK2/β′-COP is implicated in the translocation and targeting of active PKCε in fibroblasts and cardiomyocytes [7,8,17,20,22,37]. In this regard, we were interested in whether RACK2/β′-COP as part of the coatomer complex only binds an inactive pool of PKCε stored at the Golgi apparatus, or whether it also interacts with active PKCε. A constitutively active mutant form of PKCε(A159E) was shown previously to localize to the Golgi apparatus [15]. We treated GFP-PKCε(A159E)-expressing fibroblasts with BFA to determine whether the Golgi localization of active PKCε was dependent on ARF1-regulated coatomer. Similar to the wild-type PKCε (Fig. 1), GFP-PKCε(A159E) was found to dissociate from the Golgi apparatus within 15 minutes after BFA addition (Fig. 6A). Interestingly, GFP-PKCε(A159E) still undergoes translocation to the cell surface in response to TPA both in the presence and absence of BFA (Fig. 6B). This result indicates that TPA-induced translocation involves mechanisms that are independent of the binding interaction with coatomer and activation of the kinase domain.

Our data, using isolated membranes and using intact cells, support a model with multiple membrane receptors required for PKCε targeting and translocation. The RACK2/β′-COP contributes most significantly to Golgi localization in fibroblasts, whereas phorbol-ester-induced binding to the plasma membrane may rely more extensively on a RACK2/β′-COP-independent receptor. Our results indicate that PKCε could in principle exist in a kinase-active form not only at the cell surface but also complexed with coatomer at the Golgi apparatus.

4. Discussion

PKCε has many cell-type- and context-specific signaling roles that include contributions to alcohol-addictive behavior, insulin secretion, cardioprotective signal transduction, and cell growth [3,6,8,15,38,39]. In order to play diverse roles, PKCε must respond to multiple upstream activating signals, phosphorylate multiple substrates, and localize to multiple sites within the cell [2]. This is accomplished in part by the ability of PKC family members to form context-specific binding interactions with proteins and second messengers. Many of these binding events are directed through specific motifs in the N-terminal regulatory domains (C1 and C2) of PKCε.

Unlike conventional PKCs where the C2 domain is critical as a calcium sensor, the most important role for the C2-like domain of the novel PKCs such as PKCε may be to bind to the RACKs [2]. The binding interaction between PKCε C2 domain and RACK2/β′-COP has been implicated in many PKCε-dependent signaling processes [4,6-8,21-23]. Indeed, the isotype-specific inhibitors and stimulators of PKCε function ostensibly by modulating the PKCε/RACK2-binding interaction [31]. The identification of a RACK for PKCε that had also been extensively characterized as a transport vesicle coat protein at the Golgi apparatus was surprising. The relationship between β′-COP's role in trafficking and in PKCε signaling

is still not clear. Given that coatomer is enriched at the Golgi apparatus, one would expect that coatomer contributes to PKCε localization in the juxtanuclear/Golgi apparatus region as is observed at steady state in fibroblasts. However, multiple studies implicate the β′-COP binding interaction in translocation to sites where coatomer would not necessarily be enriched such as the plasma membrane and muscle sarcomeres [8,14,17,31]. The goal of this study was to determine how the binding interaction between PKCε and RACK2/β′-COP contributes to localization and translocation during signal transduction.

For the case of PKCε distribution in fibroblasts, our results support a role for coatomer in the steady-state localization PKCε at the Golgi apparatus, rather than a direct role in the translocation to the cell surface. Specifically, we found that acute disruption of ARF1-regulated coatomer in intact fibroblasts caused PKCε to dissociate from the membranes while having little effect on phorbol-ester-induced translocation to the cell surface. This interpretation is corroborated by our finding that while PKCε can be recruited to isolated membranes in a coatomer-dependent manner with GTPγS, phorbol-ester-dependent binding appeared to be coatomer independent.

We cannot rule out from our intact-cell data that coatomer contributes to both Golgi localization and translocation to the cell surface. For instance, a minor pool of RACK2/β′-COP, not obvious by fluorescence microscopy and independent of ARF regulation, could function directly as a RACK for translocation to the cell surface. It could also be that Golgi localized RACK2/β′-COP influences post-translational modifications on PKCε such as phosphorylation or nitration that subsequently affect intracellular distribution. Nevertheless, the intact cell data, when taken together with our demonstration of RACK2/β′-COP-indpendent membrane binding induced by TPA, indicate that the most parsimonious interpretation of our results is that RACK2/β′-COP serves as a RACK for targeting PKCε to the Golgi apparatus while there are separate targeting mechanisms that are responsible for enrichment at the plasma membrane.

Many binding interactions with PKCε have been previously described that could mediate the RACK2/β′-COP-independent binding. For example, binding interactions between the C1 domain and membrane diacylglycerol also play a central role in PKCε distribution and activation [2,3]. PKCε is unique among PKC isoforms in that it has a short actin-binding motif within the C1 domain [2,40]. Other integral membrane proteins such as the putative cargo receptor p23 also bind to the C1 domain [18,19]. PKCε can be targeted to caveolae at the cell surface [41]. Finally, a large repertoire of signaling molecules, for example PDLIM5, TRAM, and the 14-3-3 protein bind and affect the activity of PKCε [2].

As our study was restricted to a fibroblast cell line, it must be noted that RACK2/β′-COP could serve as a RACK for targeting to sites other than the Golgi apparatus in different cell types. For example, RACK2/β′-COP was shown to localize to sarcomeric myofibrils in cardiomyocytes [8,14]. PKCε was recruited to the RACK2/β′-COP on sarcomeres upon activation of adenosine receptor signaling. Our finding that coatomer contributes to the localization of a kinase-active mutant form of PKCε also supports the model that the binding interaction with RACK2/β′-COP contributes not just to a storage pool but also to the subcellular distribution of PKCε following its activation through signaling cascades. It will

be important to test independently the role of coatomer both at steady state and during signal-dependent translocation of PKCε in specialized cells such as tumor-derived cells and myocytes.

To summarize, our results indicate that PKCε localization and translocation may rely on multiple RACKs and targeting signals. Further studies will be necessary to define all of the multiple protein-protein binding interactions that specify the distribution and translocation of PKCε during signal transduction. This knowledge will advance our understanding of the contribution of PKCε to many human diseases including diabetes, cancer, and cardioprotective signal transduction.

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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

Highlights

>Localization of PKCε and RACK2/β′-COP at the Golgi apparatus required ARF1 activity. >Phorbol-ester-induced translocation of PKCε was independent of ARF1 activation. > PKCε bound to membranes using RACK2/β′-COP-dependent and – independent mechanisms.

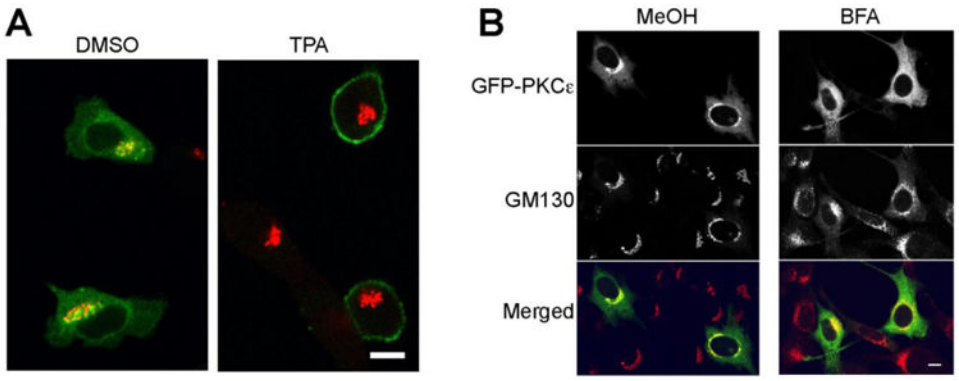

Figure 1.

The localization of PKCε at the Golgi apparatus is sensitive to the ARF1 inhibitor, BFA. NIH3T3 fibroblast cells were transfected with GFP-PKCε (green) (A) Cells were treated for 10 min with DMSO (control) or with 100nM of the phorbol ester, TPA (B) Cells were treated for 15 min with methanol (MeOH) as a control or with 10μM brefeldin A (BFA). Cells were fixed and stained for GM130 (red). Images were taken using a confocal electron micrograph. Representative images are shown for experiments that were repeated at least three times with similar results. The size bar corresponds to 10 μm.

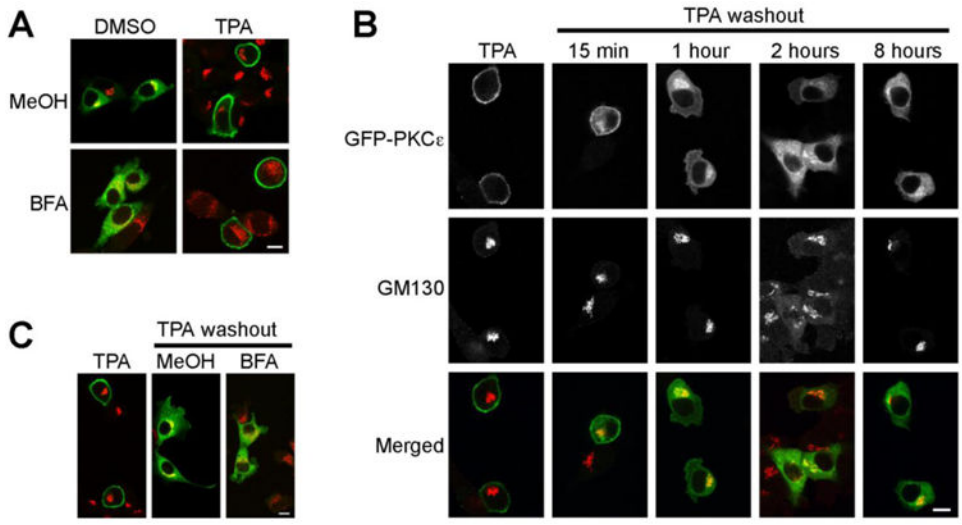

Figure 2.

ARF-regulated coatomer function is not required for the translocation to or dissociation of PKCε from the plasma membrane. NIH3T3 fibroblast cells were transfected with GFP-PKCε (green). (A) Cells were first treated with MeOH (control) or 50μM BFA for 30 minutes followed by a 10 min treatment with DMSO (control) or the phorbol ester, TPA. (B) Retrograde translocation of PKCε. Cells were treated for 10 min with TPA, followed by a TPA washout for the indicated time points. (C) Cells were treated for 10 min with TPA followed by a 2-hour TPA washout containing DMSO (control) or 50μM BFA. Cells from A, B, and C were fixed at indicated time points and stained for GM130 (red). Images were taken using a confocal electron micrograph. Representative images are shown for experiments that were repeated at least three times with similar results. The size bar corresponds to 10 μm.

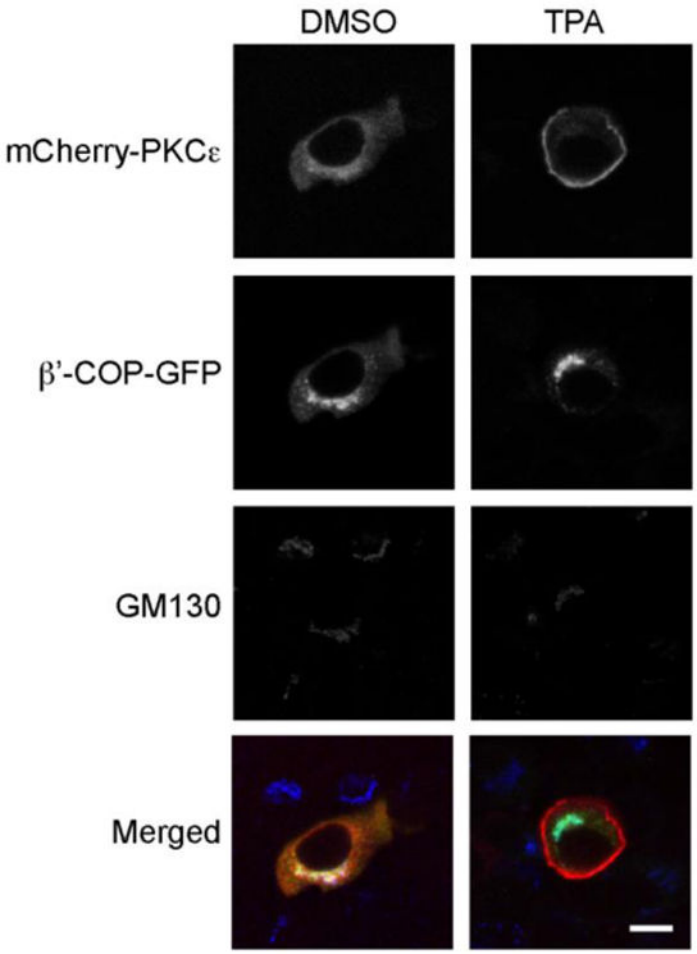

Figure 3.

PKCε activation does not cause overt redistribution of coatomer. NIH3T3 fibroblast cells were co-transfected with mCherry-PKCε (red) and β′-COP-GFP (green). Cells were treated 10 min with DMSO (control) or 100nM of the phorbol ester, TPA. Following the 10 min treatment, cells were fixed and stained for GM130 (blue). Images were taken using a confocal electron micrograph. Representative images are shown for experiments that were repeated at least three times with similar results. The size bar corresponds to 10 μm.

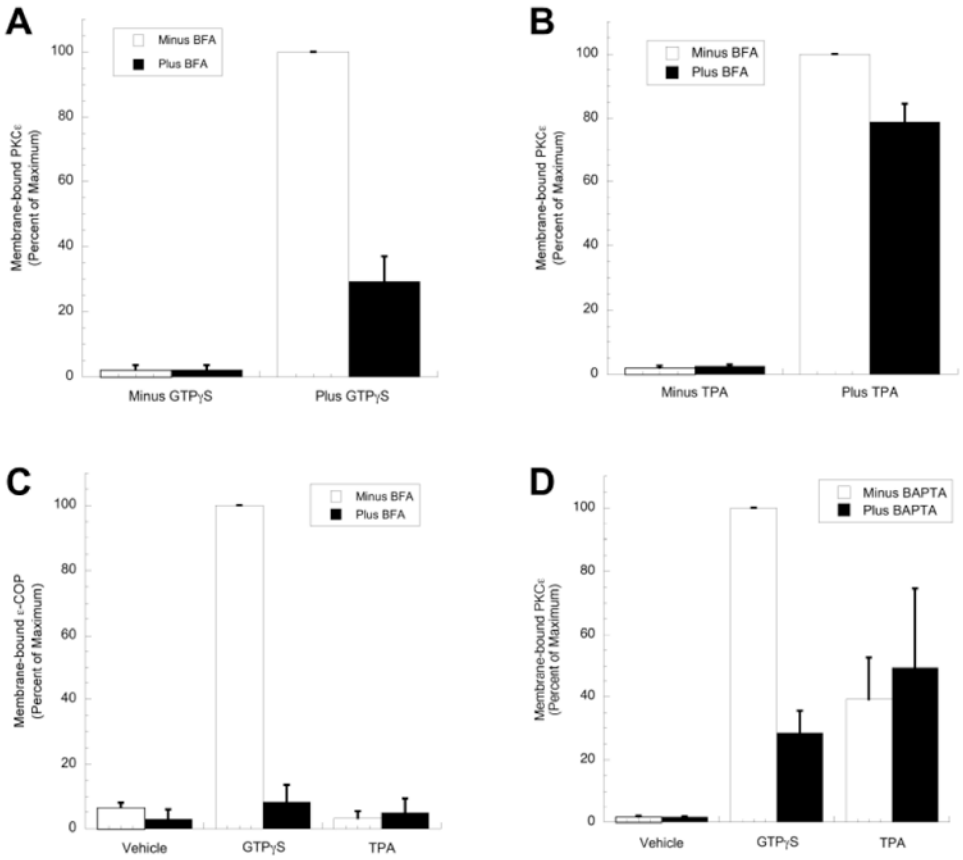

Figure 4.

PKCε binds isolated membranes through coatomer-dependent and –independent mechanisms. Cell-free membrane binding assays were conducted as described in materials and methods. The levels of membrane bound PKCε was determined following incubations with and without GTPγS (A) or TPA (B) in the absence of presence of BFA (C) Plotted is the average level of membrane bound ε-COP treated with vehicle, GTPyS, or TPA in the absence or presence of BFA. (D) The level of membrane bound PKCε was determined following incubations containing vehicle, GTPyS, or TPA in the absence or presence of BAPTA Plotted are the average levels from three independent experiments. Bars indicate the standard error.

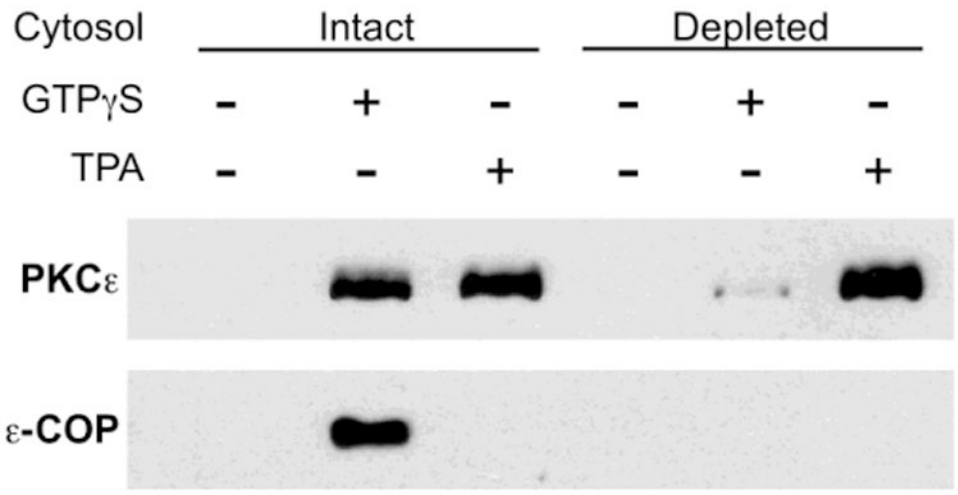

Figure 5.

Coatomer-depleted cytosol supports TPA-dependent, but not GTPγS-dependent, binding of PKCε to membranes. Cell-free assays were carried out as described in materials and methods with intact or coatomer-depleted cytosol. GTPγS, TPA, or vehicle were added as indicated. The experiment was repeated three times with similar results. Shown is a representative western blot probed with anti-PKCε and anti-ε-COP antibodies.

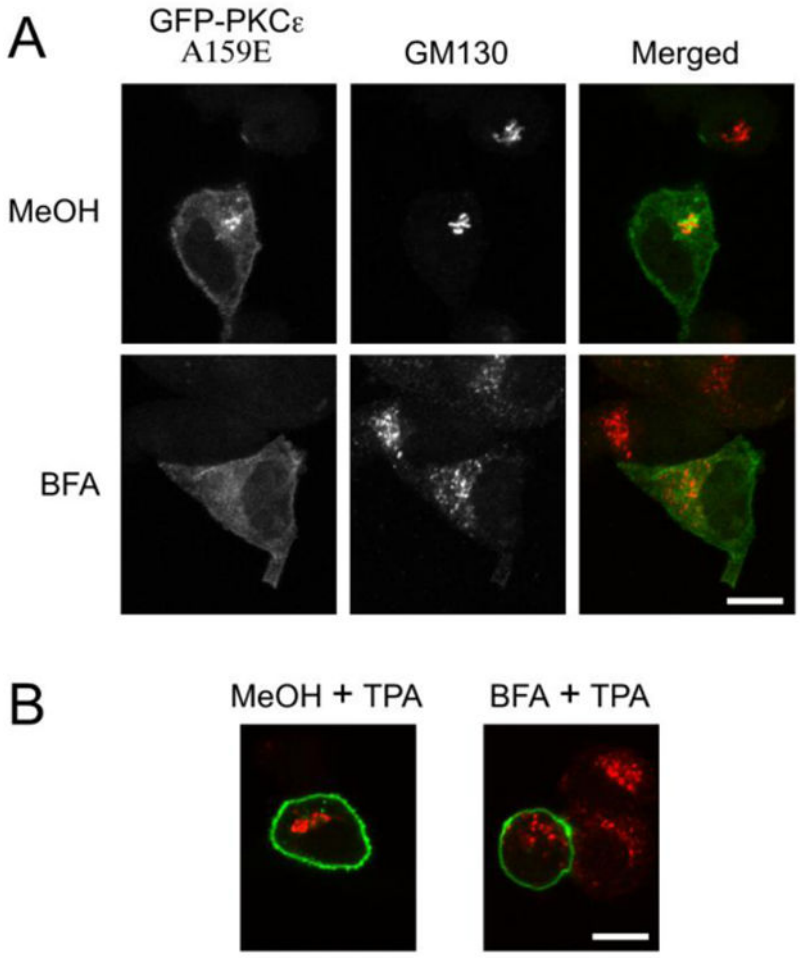

Figure 6.

ARF-regulated coatomer contributes to the localization of constitutively active PKCε. NIH3T3 fibroblast cells were transfected with GFP-PKCε(A159E) (green). (A) Cells were treated for 15 min with MeOH (control) or 10μM brefeldin A (BFA). (B) Cells were first treated with MeOH (control) or 50μM BFA for 30 minutes followed by a 10 min treatment with the phorbol ester, TPA. Cells were fixed and stained for the Golgi apparatus marker GM130 (red). Representative confocal micrographs are shown of three independent experiments. The size bar corresponds to 10 μm.