# A phorbol ester-binding protein is required downstream of Rab5 in endosome fusion

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**Abstract Previous observations indicate that a zinc and phorbol ester binding factor is necessary for endosome fusion. To further characterize the role of this factor in the process, we used an in vitro endosome fusion assay supplemented with recombinant Rab5 proteins. Both zinc depletion and addition of calphostin C, an inhibitor of protein kinase C, inhibited endosome fusion in the presence of active Rab5. Addition of the phorbol ester PMA (phorbol 12-myristate 13-acetate) reversed the inhibition of endosome fusion caused by a Rab5 negative mutant. Moreover, PMA stimulated fusion in the presence of Rab5 immunodepleted cytosol. These results suggest that the phorbol ester binding protein is acting downstream of Rab5 in endosome fusion.**



*Key words:* Endosome fusion; Endocytosis; Protein kinase C; Phorbol ester; Rab5

## 1. Introduction

Rab5 is a limiting regulator of early endosome fusion, a critical step along the endocytotic pathway that immediately follows the internalization step at the plasma membrane [1,2]. In BHK-21 cells, overexpression of Rab5 wild type (Rab5:WT) leads to increased early endosome fusion and elevated endocytosis whereas overexpression of a dominant negative Rab5 mutant (Rab5:S34N) results in decreased early endosome fusion and reduced endocytosis [1,3,4].

Interestingly, the GTPase deficient mutant (Rab5:Q79L) increases endosome fusion [4,5], suggesting that GTP hydrolysis is not required for the process. In this context, it has been proposed that Rab5 in its GTP-bound form recruits rabaptin-5 to the membrane. Rabaptin-5 binding is thought to retard GTP hydrolysis by blocking the activation of the GTPase activity of Rab5 by Rab5-GAPs. Thus, the docking/fusion machinery could remain bound to Rab5 allowing the fusion to occur [6]. The exact mechanism by which Rab5 produces all its remarkable effects on endosome fusion is still poorly understood. A connection between Rab5, Ras and phosphatidyl inositol 3-kinase (PI 3-kinase) has been suggested [7–9].

By using a cell-free assay, we have shown that *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN), a good chelator for many metal ions except $Ca^{2+}$ and $Mg^{2+}$, strongly inhibited endosome fusion. This inhibition could be reversed by addition of $Zn^{2+}$ but not by other bivalent cations, indicating that the TPEN effect was specific for $Zn^{2+}$ and that a zinc binding factor is necessary for endosome fusion [10]. We have also shown that the zinc binding factor is regulated by phorbol esters [11]. Some of the best characterized zinc and phorbol ester binding proteins belong to the PKC family [12]. The Cys-rich region of PKC, which coordinates zinc, binds the natural activator diacylglycerol as well as phorbol esters [13]. The same region also irreversibly binds the inhibitor calphostin C (CPC) [14]. When assessed in the in vitro assay, phorbol 12-myristate 13-acetate (PMA) stimulates endosome fusion whereas CPC inhibits the process in a zinc dependent fashion. Moreover, we have shown that a protein containing a PKC-like cysteine-rich domain is associated to endosomes in a zinc dependent manner and that the Cys2 region of PKCγ inhibits endosome fusion probably competing with the phorbol ester binding factor for a target molecule [11]. These results indicate that endosome fusion requires a protein with a cysteine-rich region similar to the Cys2 region of PKCγ. However, the function of the phorbol ester binding factor in endosome fusion is still unknown.

The aim of this work is to study the functional relationship between the phorbol ester binding factor and Rab5, probably the best characterized factor specifically involved in early endosome dynamics. According to the results, the factor is required for the stimulatory effect in endosome fusion of the constitutively active mutant Rab5:Q79L. Moreover, activation of the factor with phorbol ester can overcome the inhibitory effect in endosome fusion of the dominant negative mutant Rab5:S34N. Taken together, the results indicate that the phorbol ester binding factor is required downstream of Rab5 in endosome fusion.

## 2. Materials and methods

### 2.1. Materials

J774 E-clone (mannose receptor positive), a macrophage cell line, was grown to confluence in minimum essential medium containing Earle's salts and supplemented with 10% fetal calf serum. Cytosol from J774 was the high speed supernatant of a cell homogenate obtained as described [15]. Cytosols were stored at −80°C and aliquots (200 μl) were gel filtered through 1 ml Sephadex G-25 before use in the fusion assay. Rab5 immunodepleted cytosol was prepared by incubating cytosol overnight at 4°C with protein A-Sepharose-CL4B coupled to anti-Rab5 IgG monoclonal. Recombinant Rab5 wild type and mutants were prepared, purified, and prenylated as described [2]. TPEN was obtained from Molecular Probes, Portland, OR, USA. All other reagents were purchased from Sigma, St. Louis, MO, USA.

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*Abbreviations:* BSA, bovine serum albumin; CPC, calphostin C; DAG, diacylglycerol; PA, phorbol 13-monoacetate; PMA, phorbol 12-myristate 13-acetate; GST-Rab5 proteins, glutathione *S*-transferase fusion proteins; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine

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*2.2. In vitro endosome fusion assay*

Early endosomes were prepared by loading J774 E-clone macrophages with aggregated monoclonal anti-dinitrophenol (DNP) mouse IgG (via the Fc receptor) or with DNP-β-glucuronidase (via the macrophage mannose receptor) as previously described [15]. To obtain endosome enriched fractions, postnuclear fractions were diluted 15-fold in homogenization buffer and centrifuged sequentially at $35\,000\times g$ for 1 min and at $50\,000\times g$ for 5 min. The second pellet was used for fusion reactions. Fusion between two populations of endosomes containing either the antibody or the enzyme was performed as previously described [15,16]. The fusion buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH pH 7.0, 1 mM dithiothreitol, 1.5 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, 8 mM creatine phosphate, 31 U/ml creatine phosphokinase, and 0.25 mg/ml DNP-BSA) was supplemented with gel filtered cytosol, Rab5 immunodepleted cytosol, CPC, $ZnCl_2$, PMA, phorbol 13-monoacetate (PA), *N*-ethylmaleimide (NEM), or GST-Rab proteins, as required. The samples were incubated at 37°C for 45 min and the reaction was stopped by cooling on ice.

## 3. Results

### *3.1. TPEN and CPC inhibit the Rab5 stimulated endosome fusion*

To address whether the stimulation of endosome fusion promoted by Rab5 requires the zinc and phorbol ester binding factor previously described [10,11], we examined the effect of the zinc chelator TPEN and CPC, a membrane permeant inhibitor of several PKC isoforms, in the in vitro endosome fusion assay. CPC inhibits PKC by irreversibly oxidizing the phorbol ester binding site, which coordinates zinc, in a light dependent manner [17].

The in vitro assay does not require addition of exogenous Rab5, which is present on the membranes and cytosol included in the fusion reaction. However, if endosomes are preincubated with 60 mM EDTA for 10 min at 25°C and washed by sedimentation prior to the assay, fusion becomes fully dependent on exogenously added activated Rab5 (Rab5+GTPγS or Rab5:Q79L) (Fig. 1AFig. 2A). Activated Rab5 can support fusion at very low concentrations of cytosol. We have used this approach to show that Rab5 dependent fusion is inhibited by TPEN and CPC. Fig. 1A shows that fusion among EDTA washed endosomes cannot occur even in the presence of the non-hydrolyzable GTP analog GTPγS, indicating that the amount of endogenous cytosolic Rab5 included in this set of experiments is not enough to support fusion. When recombinant Rab5:WT and GTPγS are added together, fusion proceeds. However, if TPEN or CPC is also added, fusion is blocked. TPEN is a good chelator for many metal ions, except $Ca^{2+}$ and $Mg^{2+}$. Fig. 1B shows that addition of $Zn^{2+}$ reverses the effect of TPEN, but only in the presence of recombinant Rab5:WT and GTPγS. Addition of $Zn^{2+}$ in the absence of Rab5:WT and GTPγS produced only a minor recovery of endosome fusion, ruling out the possibility that the ion could trigger a Rab5 independent fusion process.

GTPγS could activate several GTPases besides Rab5. Therefore, to assess the requirement of the factor under conditions where only Rab5 was active, we studied the effect of CPC on endosome fusion in the presence of Rab5:Q79L, a GTPase defective mutant of Rab5. It has been shown that Rab5:Q79L stimulates in vitro endosome fusion to the same extent as Rab5:WT in the presence of GTPγS [5]. Fig. 2A shows that Rab5:Q79L stimulates fusion among EDTA washed endosomes and that CPC addition inhibits fusion but only when zinc and CPC are added together. The zinc requirement for CPC inhibition of endosome fusion has been previously described [11].

Similar results were obtained with unwashed endosomes and saturating concentrations of cytosol. Under these conditions, fusion occurs without the addition of recombinant Rab5. However, addition of Rab5:Q79L produced a significant enhancement in endosome fusion. CPC inhibited both the basal and the Rab5 stimulated endosome fusion (Fig. 2B) but only in the presence of zinc. These data suggest that a zinc dependent factor sensitive to CPC is required for endosome fusion. Moreover, since Rab5 in the presence of GTPγS and the constitutively activated Rab5:Q79L putatively

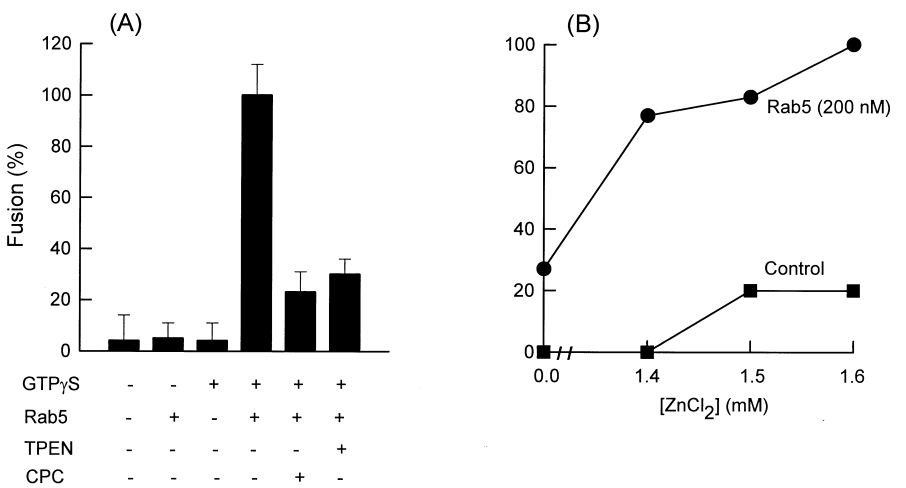


Fig. 1. Inhibition of Rab5 stimulated endosome fusion by TPEN and CPC. Postnuclear supernatants were incubated with 60 mM EDTA for 10 min at 25°C. The fractions were then diluted in homogenization buffer and endosome enriched fractions were obtained as described in Section 2. A: Endosome fusion was assessed at low cytosolic concentrations (0.1 mg/ml) and tested in the following conditions: in the presence or absence of 40 μM GTPγS, 200 nM Rab5:WT, 3 mM TPEN and 0.5 μM CPC plus 0.3 mM $ZnCl_2$ as described [11]. Fusion was expressed as a percentage of the value observed in the presence of 40 μM GTPγS plus 200 nM Rab5. B: Endosome fusion was assessed at low cytosolic concentrations (0.1 mg/ml) and tested in fusion buffer containing 3 mM of TPEN and increasing concentrations of $ZnCl_2$ in the presence (●) or absence (■) of 70 μM GTPγS plus 200 nM Rab5:WT. Fusion was expressed as a percentage of the maximum value observed. The results shown are representative of at least three experiments.

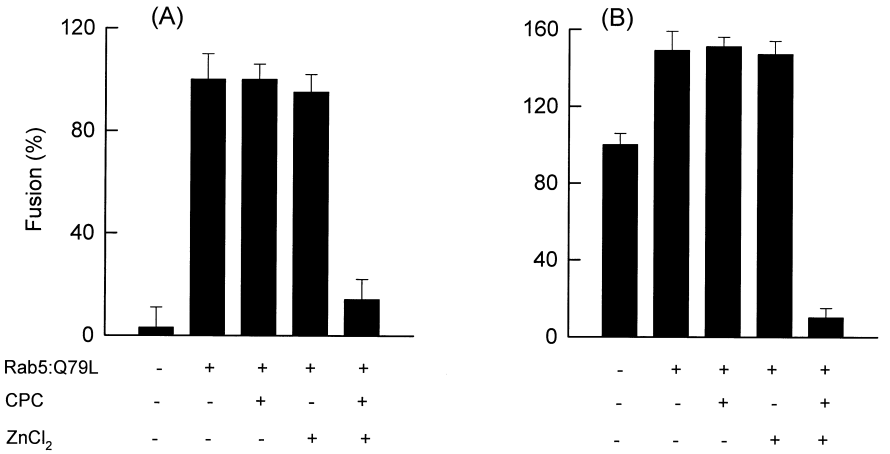


Fig. 2. Inhibition of Rab5:Q79L stimulated endosome fusion by CPC. A: Postnuclear supernatants were incubated with 60 mM EDTA for 10 min at 25°C. The fractions were then diluted in homogenization buffer and endosome enriched fractions were obtained as described in Section 2. Endosome fusion was assessed at low cytosolic concentrations (0.1 mg/ml) and tested in the in the presence or absence of 300 nM Rab5:Q79L, 0.5 μM CPC, and 0.3 mM $ZnCl_2$. Values are expressed as a percentage of maximum value observed. The data are representative of at least four independent experiments. B: Fusion among endosomes that were not treated with EDTA was assessed in the presence of 0.7 mg of cytosolic protein/ml and in the presence or absence of 300 nM Rab5:Q79L, 0.5 μM CPC, and 0.3 mM $ZnCl_2$. Values are expressed as a percentage of control fusion without any addition. The data are representative of at least four independent experiments.

bypass the activation steps, the inhibition of endosome fusion obtained indicates that the zinc binding protein is required after the activation of Rab5 or in addition to Rab5 activation (Figs. 1 and 2).

### *3.2. Phorbol ester reverses the inhibitory effect of Rab5:S34N on in vitro endosome fusion*

The results obtained with TPEN and CPC suggest that the zinc binding factor is required for the stimulatory effect of Rab5 on endosome fusion. Two models could account for these observations: (i) a sequential model where the factor is part of the pathway activated by Rab5, or (ii) a parallel model where the zinc binding factor is required in addition to Rab5 activation for endosome fusion to occur. To distinguish between these two possibilities, we took advantage of a dominant negative mutant of Rab5 (Rab5:S34N). If the factor were in a parallel pathway, its activation, via the addition of phorbol esters, would not overcome the inhibition of in vitro endosome fusion by Rab5:S34N.

Early endosome and cytosol preparations were incubated with 300 nM Rab5:S34N and/or 100 nM PMA. As shown in Fig. 3A, PMA produced a stimulatory effect on endosome fusion under control conditions [11]. Incubation with Rab5:S34N strongly inhibited fusion. Interestingly, the inhibition by Rab5:S34N was completely reversed by PMA (Fig. 3A). This observation is consistent with a role of the phorbol ester binding protein downstream of Rab5.

To confirm the observations obtained using Rab5:S34N, the effect of PMA was assessed in the presence of a Rab5 immunodepleted cytosol. Rab5 immunodepletion substantially inhibited endosome fusion (Fig. 3B). Similar to the effect observed with Rab5:S34N, the inhibition obtained using Rab5 immunodepleted cytosol was reversed by PMA. A less active analog PA was inactive, indicating that the stimulatory effect of PMA is due neither to the lipidic nature of the compound nor to the solvent (Fig. 3B).

The observations that phorbol ester can promote fusion with Rab5 immunodepleted cytosol or in the presence of Rab5:S34N are consistent with a sequential model, i.e. the PMA binding protein is acting downstream of Rab5.

Table 1
Requirements of endosome fusion in the presence of PMA

| Experimental conditions | Percentage of endosome fusion | S.D. |
|---|---|---|
| No addition | 100 | 12 |
| PMA | 138 | 8 |
| PMA−cytosol | 5 | 5 |
| PMA−KCl | 18 | 4 |
| PMA−ATP | 9 | 6 |
| PMA+staurosporine | 126 | 13 |
| PMA+chelerythrine | 124 | 9 |
| PMA+Ro 31-8220 | 127 | 10 |
| PMA+NEM | 16 | 5 |

Endosome fusion (1 mg of cytosolic protein/ml) was tested in the following conditions: PMA, 100 nM PMA; −cytosol, minus cytosolic proteins; −KCl, minus salt; −ATP, minus ATP regenerating system (i.e., ATP, creatine phosphate, and creatine phosphokinase); +staurosporine, plus 10 μM staurosporine; +chelerythrine, plus 10 μM chelerythrine; +Ro 31-8220, plus 10 μM Ro 31-8220; +NEM, vesicles and cytosol were incubated for 15 min at 4°C with 1 mM NEM before the fusion reaction, and excess NEM was quenched with 2 mM dithiothreitol. Values are expressed as a percentage of control fusion without any addition. The data represent the means ± S.D. of at least three independent experiments.

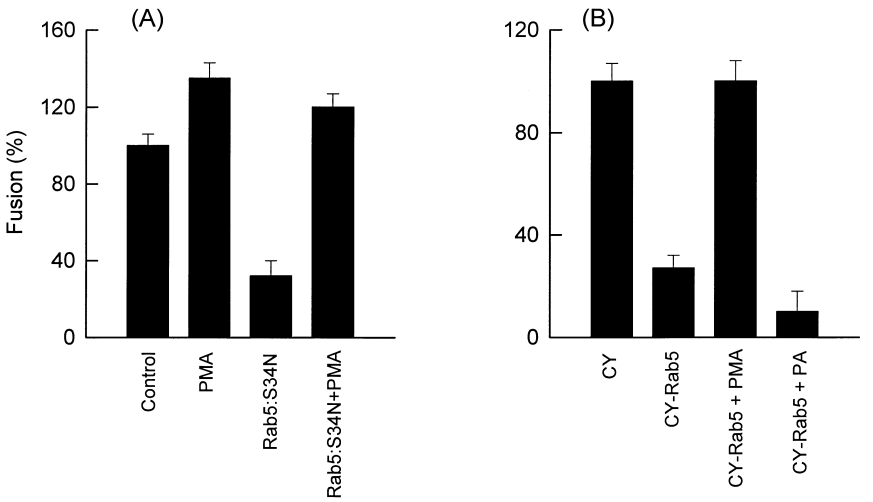


Fig. 3. Effect of PMA on Rab5:S34N and anti-Rab5 inhibited endosome fusion. A: Endosome fusion was assessed in the presence of 0.7 mg/ml of cytosolic protein, without further addition (Control), 100 nM PMA (PMA), 300 nM Rab5:S34N (Rab5:S34N), and 100 nM PMA plus 300 nM Rab5:S34N (Rab5:S34N+PMA). Values are expressed as a percentage of control fusion without any addition. The data are representative of at least four independent experiments. B: Endosome fusion was assessed in the presence of 0.7 mg/ml of control cytosol (Cytosol), 0.7 mg/ml cytosol immunodepleted of Rab5 (CY-Rab5), CY-Rab5 plus 100 nM PMA (CY-Rab5+PMA), and CY-Rab5 plus 100 nM PA (CY-Rab5+PA). Values are expressed as a percentage of the fusion observed with control cytosol without any addition. The data are representative of at least four independent experiments.

*3.3. Characterization of the PMA dependent endosome fusion*

The fact that a less active phorbol ester analog was inactive in the fusion assay suggests that the PMA effect on fusion is specific. However, to rule out the possibility of a non-specific effect on fusion, endosome fusion in the presence of PMA was characterized. Table 1 shows that fusion in the presence of PMA requires cytosol, KCl, ATP and that it is NEM sensitive. These results indicate that the effect of PMA on endosome fusion is specific, requiring all the factors normally associated with regulated vesicle fusion. Moreover, the PMA effect was inhibited neither by 10 μM staurosporine or 10 μM chelerythrine, both inhibitors of the catalytic domain of PKC, nor by the PKC ATP binding site inhibitor Ro 31-8220, suggesting that a PKC-like kinase activity is not required.

## 4. Discussion

Membrane trafficking among intracellular organelles requires the assembly and disassembly of macromolecular complexes that mediate the formation of transport vesicles from donor compartments, allows for their movement through the cytoplasm followed by high fidelity membrane docking, and fusion with acceptor compartments. Several components of this machinery have been identified. However, the process is still far from being completely characterized. Several well defined factors, such as Rab GTPases, are required for transport but have not yet been precisely configured in the model. Rabs have been suggested to ensure the directionality of SNAREs binding [18–21]. Recently, it has been proposed that Rab proteins transiently interact with the t-SNARE allowing the formation of the v/t-SNARE complex by displacement of a negative regulator [22].

In this study, we have shown that a phorbol ester binding factor is required for the effect of both wild type Rab5 and the constitutively activated Rab5 mutant Rab5:Q79L, indicating that the factor is not necessary for Rab5 activation. In addition, the fact that activation of the phorbol ester binding factor by PMA can overcome the inhibitory effect of the dominant negative mutant Rab5:S34N suggests that the factor is acting downstream of Rab5. We cannot rule out the possibility that the inhibitors used (CPC and TPEN) could inactivate one factor required for Rab5 effects and PMA could activate another factor that promotes fusion by a Rab5 independent mechanism. However, we have observed that fusion between endosomes in the presence of PMA has several characteristics similar to those described for Rab5 dependent fusion (i.e. it is NEM sensitive and ATP, KCl, and cytosol dependent). In addition, in a previous report, we have shown that PMA is inhibitory in the presence of the Cys2 region of PKCγ under conditions that support Rab5 dependent fusion [11]. This observation indicates that conditions that inhibit PMA regulated fusion also inhibit Rab5 dependent fusion suggesting that both factors are not acting in independent pathways.

It has been shown that zinc binding domains are critical regions that can mediate specific protein-protein interactions [23]. The phorbol ester binding factor may directly associate with Rab5. It has already been mentioned that the Rab5 homolog Vps21p interacts with the zinc binding protein Vps8p [24]. EEA1 is a hydrophilic protein present in cytosol and early endosome membrane fractions. At its C-terminus, the protein presents a cysteine-rich motif that is crucial for its colocalization with the GTPase deficient mutant of Rab5 [25]. In addition, it has been proved that EEA1 directly interacts with both the PI(3)K product phosphatidylinositol 3-phosphate and Rab5, linking PI(3)K function to Rab5 regulation of endosome fusion [9]. Even though this new Rab5 interacting protein binds zinc, it is unlikely that it would interact with phorbol esters. The known phorbol ester binding domains are highly similar and it has been shown that point mutations on the zinc binding domain of PKC completely abolished the binding of phorbol ester [27]. A sequence alignment between the zinc and phorbol ester binding domain of rat PKCα and

both the FYVE finger [26] and the $C_2H_2$ zinc binding domains from EEA1 has not shown significant similarity (Blast 2.0.6).

Stenmark et al. [28] have shown that Rab5 in its GTP-bound form binds rabaptin-5. This protein inhibits Rab5-GAP activity of membranes and may regulate the GTP-GDP cycle of Rab5 [6]. Recently, Horiuchi et al. [29] have described a novel Rab5 GDP/GTP exchange factor and Xiao et al. [30] have shown that the tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GAP. Since none of these proteins contain a zinc binding domain and presumably do not bind phorbol esters, the phorbol ester binding factor may be acting downstream of these proteins or may interact with Rab5 in conjunction with them. A GDI displacement factor that releases endosomal Rab GTPases from Rab-GDI has also been described [31]. Nevertheless, our experiments clearly indicate that the PMA binding protein is acting after the Rab5 activation step, therefore, after the action of a GDI displacement factor.

The nature of the phorbol ester binding factor is at present unknown. The best characterized proteins that bind phorbol esters belong to the PKC family. However, some other proteins can bind phorbol esters with high affinity [32]. PKC and PKC-like molecules have been conspicuously implicated in both the exocytotic and endocytotic pathways. PMA is believed to regulate the movement of a number of cell surface receptors in a PKC sensitive fashion. Thus, PMA has been demonstrated to stimulate the transcytosis and apical recycling of the polymeric immunoglobulin receptor in MDCK cells, potentially involving either the α and/or ε isozymes [33]. PMA has also been suggested to induce rapid endocytosis and down modulation of the chemokine receptor CXCR4 that is required, together with CD4, for the entry of T cell line adapted HIV-1 [34]. Moreover, two laboratories have directly involved phorbol ester binding factors in vesicular transport by using in vitro assays. Fabri et al. [35] have shown that the export of the vesicular stomatitis virus glycoprotein from the endoplasmic reticulum requires a phorbol ester binding protein. Simons et al. [36,37] have shown that the generation of vesicles from purified Golgi fractions requires a protein kinase C-like factor. Interestingly, both groups have reported that a kinase activity is not necessary for the effect of the factor(s). Consistent with the results observed in the exocytotic pathway, a kinase activity is probably not directly involved in the effect of the phorbol ester binding factor acting downstream of Rab5, as inferred from the lack of effect of several inhibitors of the catalytic activity of PKC. This observation does not exclude the possibility that the phorbol ester binding factor could be a member of the PKC family because some functions of PKC proteins are not related to the kinase activity of these enzymes. A relevant example is the synergistic activation of phospholipase D (PLD) by PKCα and small GTPases, such as rho and ARF, in a process independent of the kinase activity of PKCα [38]. Phosphatidic acid, the product of PLD activation has been implicated in a cascade of events leading to the formation of a membrane microdomain enriched in acidic phospholipids that can promote membrane destabilization and fusion [39].

The factor necessary for the Rab5 effect on endosome fusion can be related to other phorbol ester binding proteins. Beta2-chimaerin is a high affinity phorbol ester receptor and a p21rac-GTPase activating protein [32], indicating that members of the chimaerin family can interact with small GTPases. Further work will be required to identify and characterize the PMA binding protein required for the effect of Rab5 in endosome fusion.

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