PKCζ-mediated phosphorylation controls budding of the pre-chylomicron transport vesicle

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Summary

Dietary triacylglycerols are absorbed by enterocytes and packaged in the endoplasmic reticulum (ER) in the intestinal specific lipoprotein, the chylomicron, for export into mesenteric lymph. Chylomicrons exit the ER in an ER-to-Golgi transport vesicle, the pre-chylomicron transport vesicle (PCTV), which is the rate-limiting step in the transit of chylomicrons across the cell. Here, we focus on potential mechanisms of control of the PCTV-budding step from the intestinal ER. We incubated intestinal ER with intestinal cytosol and ATP to cause PCTV budding. The budding reaction was inhibited by 60 nM of the PKC inhibitor Gö 6983, suggesting the importance of PKCζ in the generation of PCTV. Immunodepletion of PKCζ from the cytosol and the use of washed ER greatly inhibited the generation of PCTVs, but was restored following the addition of recombinant PKCζ. Intestinal ER incubated with intestinal cytosol and [γ-^{32}P]ATP under conditions supporting the generation of PCTVs showed the phosphorylation of a 9-kDa band following autoradiography. The phosphorylation of this protein correlated with the generation of PCTVs but not the formation of protein vesicles and was inhibited by depletion of PKCζ. Phosphorylation of the 9-kDa protein was restored following the addition of recombinant PKCζ. The association of the 9-kDa protein with proteins that are important for PCTV budding was phosphorylation dependent. We conclude that PKCζ activity is required for PCTV budding from intestinal ER, and is associated with phosphorylation of a 9-kDa protein that might regulate PCTV budding.

Key words: PKCζ, Pre-chylomicron transport vesicle, Protein phosphorylation, Chylomicron

Introduction

Intestinal epithelial cells (enterocytes) absorb, process and secrete dietary fat in the form of triacylglycerol-rich chylomicrons. The rate-limiting step in this multi-event process is the exit of nascent chylomicrons from their site of synthesis, the endoplasmic reticulum (ER) (Mansbach and Dowell, 2000). The nascent chylomicrons leave the ER in a specialized vesicle, the pre-chylomicron transport vesicle (PCTV), that transports them uni-directionally to the cis-Golgi (Kumar and Mansbach, 2nd, 1999). There are important differences between PCTVs and vesicles that transport newly synthesized proteins (hereafter referred to as 'protein vesicles') from the ER to the Golgi. The large chylomicrons require that PCTVs are 250 nm or more in diameter (Siddiqi et al., 2003). By contrast, protein vesicles are smaller (60-80 nm) (Matsuoka et al., 1998). Further, PCTV do not require coatomer protein-II (COPII) proteins for the budding from ER membranes, but COPII proteins are required for the budding of protein vesicles (Siddiqi et al., 2003).

Five different cytosolic proteins, Sar1, Sec23, Sec24, Sec13 and Sec31, are involved in COPII-complex formation on the ER membrane, which leads to sorting of cargo and the eventual budding of protein vesicles (Barlowe et al., 1994). Phosphorylation of COPII proteins might mediate their function. Sar1 is an initiator of protein-vesicle formation, and its binding to the ER membrane can be regulated by a protein kinase (Aridor and Balch, 2000). Further, the phosphorylation of Sec31 might also be required for protein-vesicle budding (Salama et al., 1997).

Although our previous data have shown that ATP is required for PCTV budding, the role of protein kinases in this event has not been examined (Siddiqi et al., 2003). The significant differences between the formation of PCTVs and protein vesicles suggest that a protein kinase different from that controlling the generation of protein vesicles controls the budding of PCTVs. In other vesicle systems, specific kinases have been identified with particular transport vesicles (Tisdale, 2000; Westermann et al., 1996). In this context, an important role for protein kinase C (PKC) has been demonstrated in the formation of protein-carrying vesicles from distinct sub-cellular organelles that include Golgi vesicles and vesicular tubular clusters (VTCs) (Simon et al., 1996; Tisdale, 2000; Tisdale and Artalejo, 2006). On the basis of their structures and activation requirements, PKCs have been divided into three categories by Nishizuka et al.: (1) conventional PKC (cPKC) isoforms (α, β1, β2 and γ) that are both Ca^{2+} and diacylglycerol (DAG) dependent, (2) novel PKC (nPKC) isoforms (δ, ε, η, θ and μ) that are DAG dependent but do not need Ca^{2+} and (3) atypical PKC (aPKC) isoforms (ζ, ι and λ) that are Ca^{2+} and DAG independent (Nishizuka, 1992).

The aim of the present investigation was to identify protein kinases and phosphorylated substrates that regulate PCTV budding. In this report, we show that using a combination of specific inhibitors, recombinant proteins and immunodepletion studies, the aPKC isoform PKCζ regulates PCTV budding. In addition, we describe that the phosphorylation of an unknown 9-kDa protein corresponds to the PCTV-budding event, and the association of the 9-kDa protein with proteins required for PCTV budding depends on its phosphorylation.

Results

Inhibitors of PKC inhibit PCTV budding

ATP is required for the budding of PCTVs from intestinal ER membranes (Siddiqi et al., 2003), which suggests that the budding event is associated with the activity of a protein kinase. Our first major goal was, therefore, the identification of the protein kinase involved. Protein kinase A (PKA) has been described to be involved in the budding of vesicles from the trans-Golgi network (Muniz et al., 1997) but not in the generation of vesicles from the ER. Protein kinase B (PKB) has been associated with ER to Golgi transport of SREBP and SCAP, proteins involved in cholesterol synthesis regulation (Du et al., 2006). To determine the involvement of this kinase in PCTV budding, we used the PKB inhibitor Akti-1/2. No inhibition was observed, however; even at inhibitor concentrations (170 nM) of almost three times that employed usually (58 nM) (Fig. 1A) (manufacturer's instructions) (DeFeo-Jones et al., 2005).

PKC activity has also been shown to be required for vesicle trafficking (Tisdale, 2000; Westermann et al., 1996). Therefore, we investigated whether PKC is required for PCTV budding by using calphostin C, an inhibitor of all known PKC isoforms (Kobayashi et al., 1989a; Kobayashi et al., 1989b). When ER membranes and cytosol were incubated with calphostin C for 20 minutes prior to carrying out a budding assay, significant inhibition of PCTV budding was observed in a concentration dependent manner (Fig. 1B). The highest degree of inhibition (88%) was observed at a final calphostin C concentration of 10 μg (Fig. 1B). The concentration of calphostin C used in our experiments was threefold higher on a 'per microgram protein basis' (IC_{50}<6 nM/μg protein vs IC_{50}<2 nM/μg protein respectively) when compared to that of experiments investigating the inhibition of the export of vesicular stomatitis virus glycoprotein (VSV-G) from the ER of NRK cells (Fabbri et al., 1994). However, this comparison is not exact because the amount of NRK cell protein was based on protein measurements that used relatively impure semi-permeable whole cells as compared with our system, in which the amount of protein used was based on purified ER preparations. Further, the budding machinery used by the two cargoes is different (Fabbri et al., 1994; Siddiqi et al., 2003). To exclude a non-specific effect of calphostin C, we compared the effects of active calphostin C that had been exposed to light, with inactive calphostin C that had not been exposed to light (Bruns et al., 1991). Accordingly, ER membranes and cytosol were incubated with calphostin C in the dark for 20 minutes, followed by a budding assay performed in the dark. In the absence of light activation, PCTV budding proceeded normally (Fig. 1C, Dark). When calphostin C was exposed to light (active calphostin C), however, PCTV budding was severely (82%) attenuated (Fig. 1C, Light). These results reduce the possibility of non-specific inhibition of PCTV-budding activity by calphostin C.

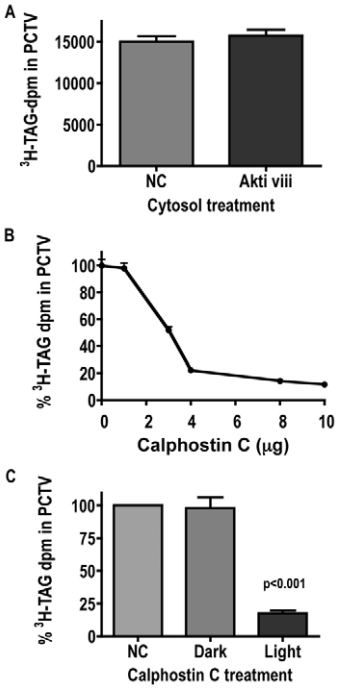

Fig. 1. Inhibitors of PKC but not PKB activity block budding of PCTVs. (A) PCTV-budding activity using native cytosol (NC) or 170 nM the PKB inhibitor Akti-1/2 (Akti viii). (B) PCTV-budding activity in response to increasing doses of calphostin C as indicated. PCTV-budding activity was determined after pre-incubation of the cytosol used with the indicated doses of calphostin C (μg) in the light. The data are shown as a percentage of the activity obtained on incubation of intestinal ER with native cytosol. (C) PCTV-budding activity as a percentage of ER incubated using native cytosol (NC). ER was incubated using cytosol pre-incubated with calphostin C (4 μg) in the dark (Dark) or calphostin C (4 μg) exposed to light (Light). *P* values indicate differences between calphostin C incubated in the light vs incubation in either the dark or cytosol without calphostin C (NC). Data are the mean ± s.e.m., *n*=4.

Although these data support the conclusion that PKC has an important role in the release of PCTVs from the ER, they do not establish which PKC isoform is involved. We have shown previously that PCTVs continued to bud in the absence of Ca^{2+} (Siddiqi et al., 2003), suggesting that cPKC isoforms , which require Ca^{2+} and DAG for activation, are not associated with PCTV-budding activity. To confirm these preliminary findings and because the DAG analogue, phorbol-12-myristate-13-acetate (PMA) has been shown to enhance the release of protein vesicles from the ER (Westermann et al., 1996), we sought to determine the effect of PMA on PCTV budding. No increase in PCTV budding was found (Fig. 2A), supporting the conclusion that a cPKC isoform is not involved in the budding reaction. These results suggest that the nPKC isoforms, which require DAG but not Ca^{2+} for activation, are also not involved in promoting PCTV-budding activity. To further support this conclusion, we used the cPKC inhibitor Ro-31-8220, which had modest but not statistically significant effect on PCTV generation, even at a concentration as high as 50 nM (Fig. 2B). Since neither cPKC nor nPKC isoforms were shown to be involved in PCTV budding, an aPKC isoform was considered.

To test this possibility, we first employed the PKC inhibitor Gö 6983, which inhibits various PKC isoforms in a concentration-dependent manner (7 nM for α, β1, β2; 6 nM for γ; 10 nM for δ; 60 nM for ζ) (Gschwendt et al., 1996). No significant inhibition was observed until 60 nM of the inhibitor were added (Fig. 2C), suggesting that the aPKC isoform PKCζ is required for PCTV budding.

Role of PKCζ in PCTV budding

To support a role for PKCζ in the formation of PCTVs, we first determined whether PKCζ is present in rat intestinal ER, cytosol and the Golgi complex, by using a specific antibody. The anti-PKCζ antibody recognized two protein bands (76 kDa and 50 kDa) in all sub-cellular fractions. However, both bands were more concentrated in cytosol (Fig. 3A), consistent with other reports (Goodnight et al., 1995; Westermann et al., 1996). The 50-kDa band might be

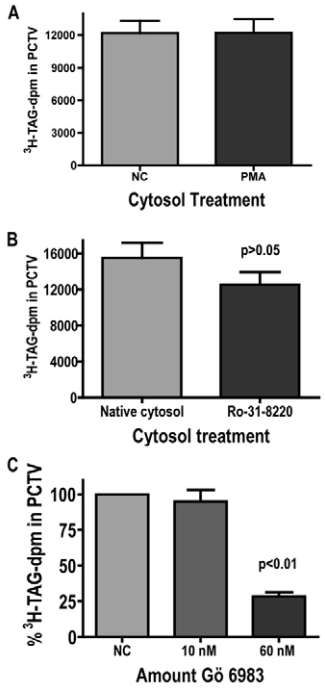

Fig. 2. Activators or inhibitors of cPKC and nPKC isoforms do not affect PCTV-budding activity. The effect of PMA and Ro-31-8220 on PCTV-budding activity. (A) PCTVs were allowed to bud from native intestinal ER using native cytosol (NC) or ER treated with PMA (100 nM). PCTV-budding activity is shown. (B) PCTV-budding activity using native cytosol (Native cytosol) or cytosol treated with 50 nM Ro-31-8220 (Ro-31-8220). (C) The PKCζ isoform is important for PCTV-budding activity as suggested by the concentration of the PKC inhibitor Gö 6983 that is required to inhibit budding. PCTV-budding activity using native cytosol (NC) or cytosol treated with the indicated amount of Gö 6983. Data are expressed as a percentage of dpm of [^{3}H]TAG in PCTV budded from the ER using native cytosol as 100%. *P* values indicate differences between the means, or between 60 nM Gö 6983 and either native cytosol or cytosol treated with 10 nM Gö 6983. Data are the mean ± s.e.m., *n*=4.

protein kinase Mζ or degraded PKCζ (Hirai and Chida, 2003). To further examine the involvement of PKCζ in PCTV budding, we incubated intestinal cytosol and ER with either anti-PKCζ antibodies or pre-immune IgG at 4°C for 1 hour, removed unbound antibodies by washing with cold PBS, and tested the treated cytosol and ER for PCTV-budding activity. The antibody treatment greatly diminished PCTV budding (Fig. 3B, PKCζ Ab) whereas pre-immune IgG had no effect (Fig. 3B, NC).

We considered the possibility that the antibody inhibition is due to non-specific effects. As a first approach to this question, we incubated anti-PKCζ antibodies with immunogenic peptide at 4°C for 2 hours prior to treating the cytosol and ER aliquots with the pre-incubated antibody. The blocking effect of the antibody was completely abrogated by this treatment (Fig. 3B, PKCζ Ab+An).

To further support a role for PKCζ, we immunodepleted PKCζ from the cytosol (Fig. 4A, compare Cyto with PKCζ-dep Cyto) and washed the ER membranes with 2 M urea to remove PKCζ (Fig. 4B, compare ER with 2M Urea washed ER). Using the PKCζ-depleted cytosol and ER aliquots, only minimal PCTV-budding activity was observed (Fig. 5A, –PKCζ). By contrast, IgG-treated cytosol and ER had robust activity as expected (Fig. 5A, NC). To reduce the potential of non-specific effects of the antibody depletion, the 2 M urea ER wash, or the possibility that the anti-PKCζ antibody

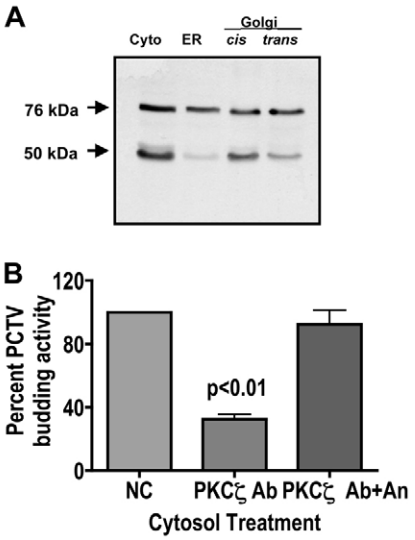

Fig. 3. (A) PKCζ is present in intestinal sub-cellular fractions as identified by immunoblotting, and antibodies against PKCζ inhibit PCTV budding. Enterocytes were isolated, homogenized and separated into sub-cellular fractions (Materials and Methods). 30 µg protein from each fraction was separated by 12% SDS-PAGE, the proteins transblotted to nitrocellulose membranes and PKCζ identified by anti-PKCζ antibodies using ECL. The different sub-cellular fractions whose proteins are separated on the gel are shown above each lane. (B) The effect of anti-PKCζ antibodies on PCTV-budding activity as a percentage of budding activity using cytosol treated with IgG. Cytosol and ER were treated either with IgG (NC), or anti-PKCζ antibodies (10 µl) (PKCζ Ab) or anti-PKCζ antibodies (10 µl) previously treated with PKCζ antigen (20 µg) (PKCζ Ab+An). Excess antibodies and antigen were removed from the ER by washing, and from the cytosol using anti-IgG bound to beads. Data are the mean ± s.e.m., *n*=4. *P* values indicate differences between the mean of PKCζ Ab and either NC or PKCζ Ab+An.

immunoprecipitated protein(s) associated with PKCζ that were active in the budding process (Hirai and Chida, 2003; Puls et al., 1997), we attempted to restore PCTV-budding activity by adding recombinant PKCζ (rPKCζ) to the PKCζ-depleted reaction. On addition of 2.5 µg rPKCζ to PKCζ-depleted cytosol, budding activity was completely re-established (Fig. 5A, +rPKCζ) suggesting that our observations on PKCζ depletion were not the result of non-specific effects. The greater reduction in activity in the immunodepletion experiment vs the anti-PKCζ-antibody

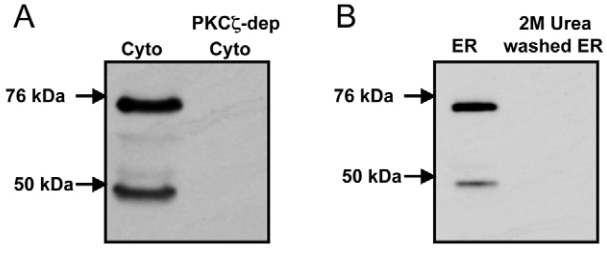

Fig. 4. Successful removal of PKCζ from cytosol and ER. (A) Cytosol was twice treated either with bead bound IgG (Cyto) or bead bound anti-PKCζ antibodies (PKCζ-dep Cyto). 30 µg of treated cytosol protein were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and PKCζ identified by immunoblot (ECL). (B) ER was treated either with cold HEPES (ER) or cold HEPES plus 2 M urea (2M Urea washed ER). The proteins were separated by 12% SDS-PAGE. PKCζ was detected by immunoblotting (ECL).

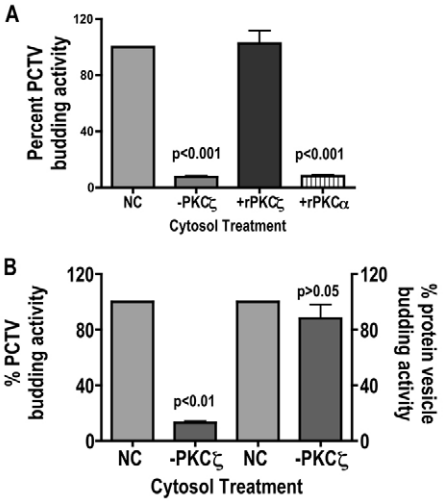

Fig. 5. Cytosol immunodepleted of PKCζ does not support PCTV budding but does support protein-vesicle budding. (A) PCTVs were budded from ER using native cytosol and ER treated with IgG (NC), cytosol immunodepleted of PKCζ (–PKCζ), or cytosol immunodepleted of PKCζ to which either 2.5 μg recombinant PKCζ (+rPKCζ) or 2.5 μg PKCα (+rPKCα) was added. For NC, ER treated with 10 mM HEPES, pH 7.2 was used. When PKCζ-depleted cytosol was used, the accompanying ER was treated with 2 M urea. Data are the mean ± s.e.m., n=4. P values indicate differences between the means of –PKCζ and NC or +rPKCζ, or the difference between +rPKCα and NC or +rPKCζ. (B) PCTV and protein vesicles were budded from [^{14}C]TAG and [^{3}H]protein loaded ER using cytosol treated with IgG (NC) or cytosol immunodepleted of PKCζ (–PKCζ). After incubation (Materials and Methods), PCTV and protein vesicles were separated on a continuous sucrose gradient. The gradient was resolved into 20 fractions of 0.5 ml each. The first three fractions were considered to be PCTV and fractions 8 to 10 were considered to be protein vesicles. The TAG was extracted from the PCTV-containing fractions and the proteins collected from the protein-vesicle fractions after TCA precipitation. PCTV-budding activity is shown on the left and protein-vesicle budding activity on the right.

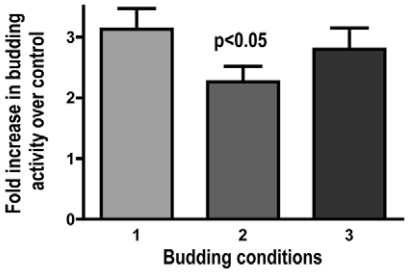

Fig. 6. Pre-phosphorylating intestinal ER with rPKCζ and ATP supports PCTV budding. Bar 1 describes the results obtained when [^{3}H]TAG pre-loaded intestinal ER (500 μg) and cytosol (1 mg) were incubated with ATP and PCTV-budding activity was determined. Bar 2 reports the PCTV-budding activity obtained when pre-phosphorylated and [^{3}H]TAG pre-loaded ER (500 μg) (Materials and Methods) were incubated with PKCζ-depleted cytosol (1 mg). Bar 3 shows the PCTV-budding activity obtained when pre-phosphorylated and [^{3}H]TAG pre-loaded ER (500 μg) was incubated with recombinant L-FABP (40 μg).

addition experiment (Fig. 3B, PKCζ Ab) might be due to the incomplete blocking of PKCζ activity under the latter conditions, because it required two rounds of immunodepletion to remove all the PKCζ. rPKCζ (at 2.5 μg and 5 μg) did not initiate PCTV budding when incubated with ER and an ATP-regenerating system in the absence of cytosol (data not shown). These data support our prior observation that liver fatty-acid-binding protein (L-FABP) can generate PCTV-budding activity in the absence of cytosol (Neeli et al., 2007). It is possible that, following immunodepletion of the cytosol of PKCζ, we also depleted enough L-FABP to account for the reduced PCTV-budding activity. This is unlikely because the addition of rPKCζ to PKCζ-depleted cytosol completely restored PCTV-budding activity that would not have occurred if significant amounts of L-FABP had been removed on PKCζ immunodepletion. The large amount of L-FABP in intestinal cytosol (Bass et al., 1985) as compared with the quantity of PKCζ supports this conclusion. In contrast to the robust budding activity obtained when rPKCζ was added to the PKCζ-depleted system, when a different PKC (PKCα) was added, little budding activity was observed (Fig. 5A, +rPKCα). In sumary, these data support the specificity of the recombinant protein added to the PKCζ-depleted ER and cytosol to restore budding activity.

Although PKCζ-depleted cytosol did not support PCTV-budding activity, we wondered whether it would support protein-vesicle budding. To answer this question, we incubated 2 M urea washed ER pre-loaded with [^{14}C]TAG to mark newly synthesized TAG and [^{3}H]protein to mark newly synthesized protein with cytosol. The cytosol had been treated with IgG (Fig. 5B, NC) or cytosol immunodepleted of PKCζ (Fig. 5B, –PKCζ). Consistent with the data shown in Fig. 5A, PKCζ-depleted cytosol did not support PCTV budding (Fig. 5B; two bars on the left, –PKCζ vs NC). By contrast, the PKCζ-depleted cytosol continued to show robust protein-vesicle budding (Fig. 5B; two bars on the right, –PKCζ vs NC), suggesting that PKCζ activity is not required to generate protein vesicles from intestinal ER, which is consistent with the data of Fabbri and colleagues in cell culture systems (Fabbri et al., 1994). These data also support the conclusion that the process for immunodepleting the cytosol of PKCζ or of washing the ER did not result in non-specific effects on ER budding.

It is possible that either the PKCζ activity occurs prior to the recruitment of the PCTV-budding machinery or that its action is necessary during the entire budding process. To discriminate between these two possibilities, we incubated ER membranes with rPKCζ and [γ-^{32}P]ATP, washed the membranes (pre-incubated ER), and then attempted to bud PCTV from these membranes using PKCζ-depleted cytosol. When native ER (not incubated with rPKCζ and ATP) and cytosol was used, (Fig. 6, bar 1), ~3.1-fold more PCTVs were generated than under negative control conditions. When ER, pre-incubated with rPKCζ and ATP and then washed, was used with PKCζ-depleted cytosol (Fig. 6, bar 2), PCTV formation was significantly but modestly reduced (~2.3-fold vs control, 28% reduction; P<0.05), suggesting that continual activity by PKCζ is not required for the majority of PCTV budding to occur. This speculation is supported by the finding that, during incubation, the disintegrations per minute (dpm) of ^{32}P of the pre-incubated ER was reduced by 17%, despite the presence of NaF to inhibit phosphatase activity. When L-FABP was added to pre-incubated ER (Fig. 6, bar 3), PCTV-budding activity was nearly the same as that in native cytosol and ER (~2.8-fold control values) but not significantly different from the activity of PKCζ-depleted cytosol. In summary, these data suggest that the PCTV-budding machinery, whose assembly requires the activity of PKCζ, is competent to bud PCTVs without additional kinase activity.

As a final test of PKCζ activity in our system, we investigated the effect of a specific PKCζ inhibitor, a myristoylated 13 amino acid pseudosubstrate (Krotova et al., 2006), on PCTV generation.

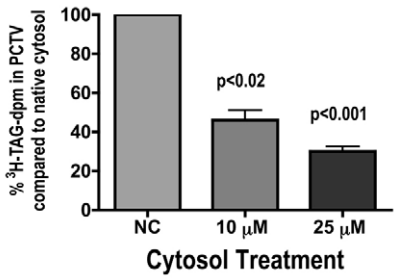

Fig. 7. PKCζ pseudo-substrate inhibits PCTV-budding activity. PCTV-budding activity was assayed using native ER (500 μg) and cytosol (1 mg) (NC) or native cytosol (1 mg) and ER (500 μg) treated with 10 μM or 25 μM PKCζ pseudo-substrate as indicated. The budding activity using pseudo-substrate is expressed as a percentage of the budding activity using native cytosol. Data are the mean ± s.e.m., *n*=4. *P* values indicate differences between the means of the budding activity using the pseudo-substrate and the activity using native cytosol.

The pseudosubstrate was incubated with intestinal cytosol and ER for 3 hours at 4°C at two different final concentrations (10 μM and 25 μM). This treatment reduced PCTV formation in a concentration-dependent manner (Fig. 7). Please notice the much higher concentration of pseudosubstrate required to inhibit 50% of the insulin stimulation of rat adipocytes (100 μM) when the adipocytes were incubated with pseudosubstrate for 45 minutes prior to stimulation (Standaert et al., 1997).

To increase our confidence in the specificity of our observations with respect to PKCζ, we wished to test the effects of the removal of another PKC isoform (PKCα) – that is used in caveolar endocytosis (Sharma et al., 2004) – on PCTV budding. To this end, we immunodepleted intestinal cytosol of PKCα (Fig. 8A, lane 1) and washed intestinal ER membranes with 2 M urea to remove any remaining PKCα (Fig. 8A, lane 2). The treated cytosol and ER were then used in a PCTV-budding assay. No reduction in activity was found when compared with IgG-treated native cytosol (Fig. 8B). These results support the specificity of our findings related to PKCζ. Taken together, the data strongly suggest that PKCζ is required for the budding of PCTVs from intestinal ER membranes.

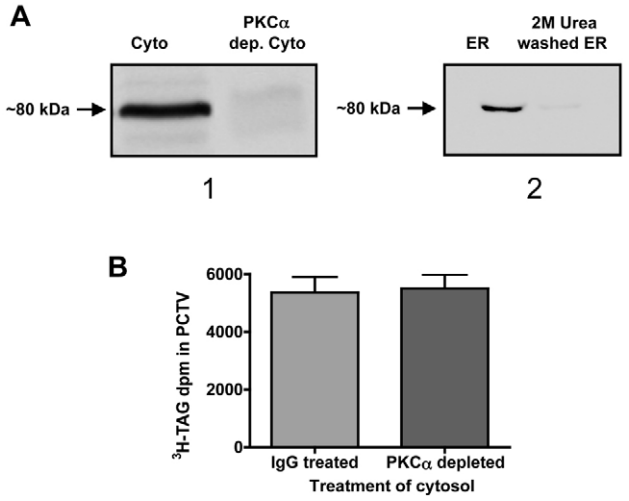

Fig. 8. PKCα-depleted ER and cytosol has no effect on PCTV budding. (A1) Cytosol was twice treated either with bead-bound IgG (Cyto) or bead-bound anti-PKCα antibodies (PKCα-dep Cyto). 30 μg of treated cytosol protein were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and PKCα was identified by immunoblotting (ECL). (A2) ER was treated either with cold HEPES (ER) or cold HEPES plus 2 M urea (2 M Urea washed ER). PKCα was detected by immunoblot (ECL). (B) ER was washed with 10 mM HEPESand cytosol was treated with bead-bound IgG (IgG treated). PKCα was removed from cytosol by immunodepletion (Fig. 8A1) and ER by 2 M urea wash (Fig. 8A2). Treated ER (500 μg) and cytosol (1 mg, PKCα depleted) were then used in a PCTV-budding assay and the results shown. Data are the mean ± s.e.m.

Phosphorylation of proteins during vesicle budding

Having established that PKCζ activity is associated with PCTV budding, our second major goal was to identify the PKCζ protein substrate, likely to be present on intestinal ER membranes. To this end, we first determined whether protein(s) were phosphorylated under appropriate conditions for PCTV budding from intestinal ER membranes by using [γ-^{32}P]ATP. After the budding reaction, the proteins were solubilized, separated by SDS-PAGE, and ^{32}P-labeled proteins were identified by autoradiography. Under the conditions employed, several phosphoproteins were detected; the most prominent was a band at ~9 kDa (Fig. 9A).

The phosphorylated proteins could be either associated with the active formation of PCTV or they could be related to other events in the incubation system, including the formation of protein vesicles. We postulated that, if active vesicle formation was the cause, protein phosphorylation should be reduced if the vesicle-budding reaction were blocked. Accordingly, we carried out ER-budding reactions both at 4°C (Fig. 9B) or without cytosol (Fig. 9C), conditions in which no PCTV or protein-vesicle budding would be expected (Siddiqi et al., 2003; Rexach and Schekman, 1991). When the incubation was carried out under either condition, little to no protein phosphorylation was observed. We also considered the possibility that the incubation of cytosol alone, without ER, is associated with protein phosphorylation. Under the above conditions (Fig. 9D), we found two bands of phosphorylated proteins following autoradiography (25 kDa and 37 kDa), showing that cytosolic proteins are being phosphorylated in the absence of a vesicle budding event. Importantly, the major 9-kDa band, as well as other less-prominent bands, were not identified under these conditions.

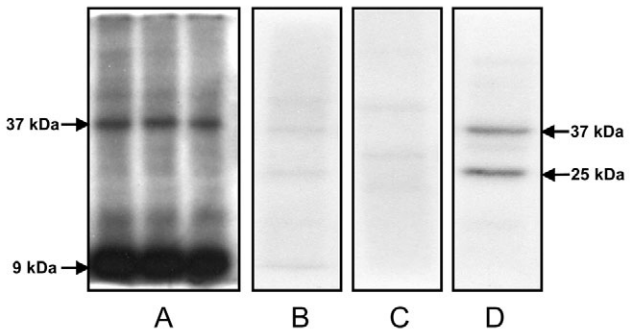

Fig. 9. Autoradiogram of intestinal ER under conditions favoring PCTV budding identifies a 9-kDa phosphorylated protein. Intestinal ER (500 μg) was incubated for 30 minutes at 37°C with native intestinal cytosol (1 mg) and an ATP-regenerating system including 20 μCi [γ-^{32}P]ATP. The reaction was stopped by placing the incubation tube on ice and adding cold 10 mM HEPES buffer (pH 7.2). 20 μl were solubilized in Laemmeli buffer and the proteins separated by 15% SDS-PAGE. The gel was dried and autoradiography performed at –70°C for 10 days. (A) Complete reaction, incubated at 37°C in triplicate. (B) Complete reaction incubated at 4°C. (C) PCTV-budding reaction without cytosol incubated at 37°C. (D) PCTV-budding reaction without ER incubated at 37°C.

These results are consistent with ER vesicular budding being associated with protein phosphorylation. However, they do not distinguish between PCTV and protein vesicles, nor do they rule out a role for the 9-kDa protein in other ER functions.

PKCζ phosphorylates a 9-kDa protein in association with PCTV budding

We next wished to determine whether PKCζ phosphorylated the 9-kDa protein in association with PCTV budding. As before, a 9-kDa protein was heavily phosphorylated when intestinal ER was incubated with native cytosol in a PCTV-budding reaction (Fig. 10A, lane 1). However, when PKCζ-depleted cytosol and ER that had been washed with 2 M urea were used, phosphorylation of the 9-kDa band was greatly attenuated (Fig. 10A, lane 2). Similarly, when either the non-specific PKC inhibitor calphostin C (Fig. 10A, lane 3) or the specific PKCζ inhibitor pseudosubstrate (Fig. 10A, lane 4) was added to the reaction, the phosphorylation signal was greatly attenuated. These results strongly support the hypothesis that PKCζ phosphorylates an unknown 9-kDa protein whose phosphorylation is associated with the budding of PCTVs from ER membranes.

The phosphorylated 9-kDa protein is an ER protein

The blots shown in Fig. 10 do not reveal whether the 9-kDa protein is an ER or a cytosolic protein nor whether it is selected for inclusion in PCTV. To answer this question, we performed a budding assay to which [γ-^{32}P]ATP was added and after the reaction, separated the components on a continuous sucrose gradient, and performed autoradiography using the same amount of protein in each lane. As shown in Fig. 10B, lane 1, the ER gave a strong signal whereas the cytosol gave a small signal (Fig. 10B, lane 2) and PCTV gave a modest signal (Fig. 10B, lane 3). These data indicate that the 9-kDa protein is an ER-associated protein that is included in PCTV in a non-specific manner. If the protein were selected for transport in PCTV, a signal stronger than that seen in the ER would be expected because PCTV proteins are a selected subset of total ER proteins.

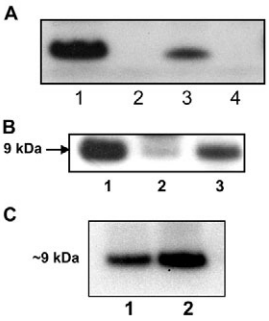

Fig. 10. The 9-kDa protein is an ER resident protein and its phosphorylation is reduced by PKCζ removal or inhibition. (A) Lane 1: PCTV-budding reaction including 20 μCi [^{32}P-γP] ATP (see Fig. 1A) was performed using native cytosol. Lane 2: a similar PCTV-budding reaction was performed using PKCζ-immunodepleted cytosol and ER washed with 2 M urea. Lane 3: PCTV-budding assay was performed similar to that shown in lane 1, except that 4 μg of calphostin C were pre-incubated with the cytosol. Lane 4: PCTV-budding assay was performed as that shown in lane1 using cytosol treated with 50 μM pseudosubstrate. The reaction was stopped by placing the incubation tubes on ice and ading cold HEPES buffer (10 mM, pH 7.2). Of the reaction 30 μl were solubilized in Laemmli buffer and the proteins separated on an 8%-16% gradient SDS-PAGE. Autoradiography was performed on the separated proteins (10 days at –70°C). Only the band at 9 kDa is shown. (B) A PCTV-budding reaction supplemented with 50 μCi [γ–^{32}P]ATP was performed and the reaction components were separated on a continuous sucrose gradient. PCTV were collected from the top of the gradient, ER from the bottom and the remaining fractions were taken as cytosol. 30 μg protein from each fraction (cytosolic proteins were solubilized from a TCA precipitate) were separated by 8%-16% gradient SDS-PAGE and autoradiography was performed on the separated proteins (30 hours at –70°C). Lane 1, ER; lane 2, cytosol; lane 3, PCTV. Only the band at 9 kDa is shown. (C) Lane 1: result from intestinal ER (500 μg) and cytosol (1 mg) incubated with [γ-^{32}P]ATP and 2 mM NaF as in Fig. 9A and an autoradiogram was performed after 40 μg of the proteins had been separated by 8%-16% SDS-PAGE. Lane 2: ER proteins (2 mg) were incubated with rPKCζ (5 μg) and [γ-^{32}P]ATP for 30 minutes at 37°C in the presence of 2 mM NaF. The ER was isolated, washed and 40 μg of the proteins were separated by 8%-16% SDS-PAGE. The gel was dried and the phosphorylation of ER proteins determined by autoradiography.

PKCζ phosphorylates the 9-kDa ER protein

We next wished to show that PKCζ specifically phosphorylates the 9-kDa protein in the ER. To this end, intestinal ER was incubated with [γ-^{32}P]ATP and either whole cytosol or rPKCζ. After the incubation, proteins were solubilized, separated by SDS-PAGE, and the resulting gel autoradiographed (Fig. 10C). Although the 9-kDa band gave a strong signal under both conditions (Fig. 10C, lanes 1 and 2), the reduced signal seen when native cytosol was used to mediate phosphorylation (lane 1) compared with that seen when recombinant PKCζ was used (lane 2) is explained by the reduced proportion of ER proteins when the incubation included whole cytosol as compared with rPKCζ. We therefore conclude that PKCζ can specifically phosphorylate the 9-kDa protein.

Phosphorylation of the 9-kDa protein is specific to PCTV budding

To differentiate between protein phosphorylation events that are related to PCTV or protein-vesicle budding, we took advantage of our prior observations that PCTV budding does not require the COPII component Sar1, a GTPase that is necessary for protein-vesicle budding (Siddiqi et al., 2003; Barlowe et al., 1993; Kuge et al., 1994). To remove Sar1 from the preparation, cytosol

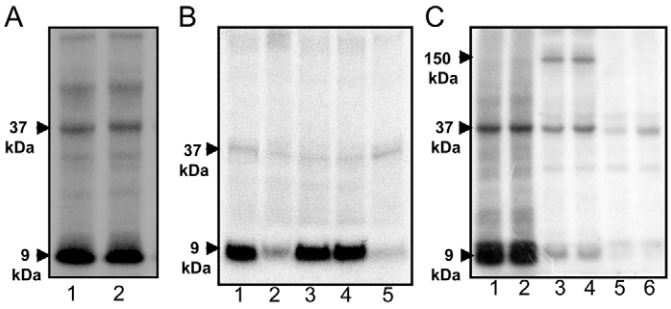

Fig. 11. Autoradiograms of PCTV-budding reactions showing the 9-kDa band to be phosphorylated only during active PCTV budding. The 9-kDa band is absent in liver and kidney ER. PCTV-budding reactions were performed as described for Fig. 1; proteins were separated by 15% SDS-PAGE and autoradiographed. (A) Complete reaction except that the cytosol was either mock immunodepleted using IgG (lane 1) or immunodepleted of Sar1 (lane 2). The ER had been washed with 2 M urea (Materials and Methods). (B) PCTV-budding reaction using ER treated with the indicated antibodies (lane 1, IgG; lane 2, anti-apoB48; lane 3, anti-rBet1; lane 4, anti-Sec22b; lane 5, anti-VAMP7 antibodies). Excess antibodies were removed by washing. Post incubation proteins were separated by 8%-16% SDS-PAGE and autoradiographed. (C) ER and cytosol from intestine (lanea1, 2), liver (lanes 3, 4) and kidney (lanes 5, 6) were incubated with the complete budding reaction mix as described for Fig. 9. In each lane, 30 μg of protein were separated by 15% SDS-PAGE and autoradiographed.

immunodepleted of Sar1 and ER washed with 2 M urea (Siddiqi et al., 2003) were used in our in vitro ER-budding assay supplemented with [γ^{32}-P]ATP. The results showed that those proteins phosphorylated using native cytosol and ER (Fig. 9A) were also phosphorylated under Sar1-depleted conditions (Fig. 11A, lane 2) supporting the thesis that the phosphorylated 9-kDa protein was probably associated with PCTV budding and not with the generation of protein vesicles. The results obtained using mock (IgG-depleted) cytosol (Fig. 11A, lane 1), supported the results obtained in Fig. 9A and were similar to Sar1-depleted cytosol.

To more clearly tie the observed phosphorylation of the 9-kDa protein to PCTV budding, we next tested protein phosphorylation under conditions in which PCTV budding was inhibited but protein-vesicle budding was allowed to continue. Our prior studies have shown that vesicle-associated membrane protein 7 (VAMP7) is present in intestinal ER membranes and serves as a v-SNARE on PCTV. Further, anti-VAMP7 antibody treatment of intestinal ER greatly inhibits PCTV delivery of TAG to the Golgi complex but newly synthesized protein continues to be delivered normally (Siddiqi et al., 2006a). Additional data (not shown) determined that the inhibitory step was in part at the level of PCTV budding. To supplement the VAMP7 data, we next tested whether PCTV-budding inhibition could be obtained by using anti-apoB48 antibodies. We incubated anti-apoB48 antibodies with ER membranes and found that these antibodies severely attenuated PCTV-budding activity (97%, Table 1). However, these antibody-inhibition studies could be influenced by non-specific effects of the antibodies, such as stearic hindrance. To address this possibility, ER membranes were treated with antibodies against two ER-resident SNARE proteins, rBet1 and Sec22b, that do not operate as vSNAREs for PCTV (Siddiqi et al., 2003; Siddiqi et al., 2006b). In both cases, no effect on PCTV budding was observed as compared with the PCTV-budding activity of native ER (Table 1), supporting the specificity of the inhibitory effect of the apoB48 antibody.

Knowing that antibodies against both VAMP7 and apoB48 inhibited PCTV budding, we then tested whether blocking the generation of PCTVs by treating intestinal ER with these antibodies also blocks the phosphorylation of the 9-kDa protein. Accordingly, ER membranes were treated with anti-apoB48 antibodies and the excess antibody was removed. When the treated ER membranes were used in the PCTV-budding reaction, the phosphorylation signal of the 9-kDa band was severely attenuated (90%) (Fig. 11B, compare lane 1 with lane 2). Significant reduction in the signal strength of the 9-kDa band was also observed when the ER membranes were treated with anti-VAMP7 antibodies (Fig. 11B, lane 5). By contrast, when the ER was treated with antibodies against SNAREs, present in the ER but not required for PCTV budding (Siddiqi et al., 2003), phosphorylation of the 9-kDa protein was unaffected [Fig. 11B, lane 3 (anti-rBet1); and lane 4 (anti-Sec22b)]. These studies suggest that stearic hindrance or other non-specific effects of the antibodies used were not the cause of the inhibition of the phosphorylation of the 9-kDa protein when antibodies against apoB48 or VAMP7 were employed. Phosphorylation of other substrates was less affected by antibody treatment; the 37-kDa band, whose signal is also attenuated in the absence of PCTV budding, appears to be related to a cytosolic protein that is phosphorylated in the absence of ER or ER budding (Fig. 9D). Collectively these results support the hypothesis that the observed phosphorylation of the 9-kDa protein correlates specifically with PCTV budding and not the generation of protein-vesicles from ER membranes.

Table 1. Effect of antibody treatment of rat intestinal ER prior to PCTV budding

Antibody treatment	^{3}[H]TAG dpm in PCTVs
IgG	8440±861
Anti-apoB48	448±49*
Anti-rBet1	8495±893
Anti-Sec22b	8710±902

Intestinal ER (500 μg protein) was treated with the indicated antibodies or IgG for 60 minutes at 4°C, the ER washed to remove excess antibody, and then used in a PCTV budding assay as described (Materials and Methods). PCTV were collected from the top of a continuous sucrose gradient and its ^{3}[H]TAG-dpm determined. Data are the mean ± s.e.m., n=4. *P<0.001, indicating a significant difference between the mean of ER treated with anti-apoB48 as compared with the means of ER treated with IgG, anti-rBet1 and anti-Sec22b.

Interactions of the 9-kDa protein with PCTV proteins, and the effect of protein phosphorylation on the ER binding of L-FABP

Although we have shown a requirement for PKCζ in PCTV budding and have presented data that suggest that PKCζ phosphorylates a 9-kDa protein that is associated with the budding of this vesicle, the question of how these two processes are linked has not been addressed. One way to investigate this is to determine whether proteins that are known to be important for PCTV budding become associated with the 9-kDa protein when it is phosphorylated. To this end, we performed a series of co-immunoprecipitation experiments under conditions where the 9-kDa protein is or is not phosphorylated.

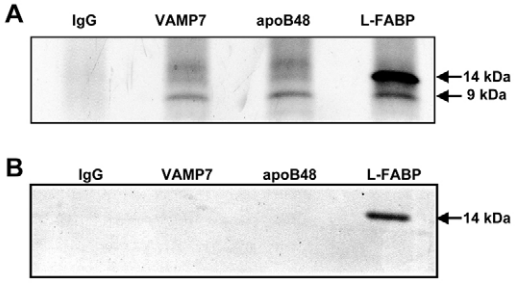

Fig. 12. The 9-kDa protein interacts with VAMP7, apoB48 and L-FABP in a phosphorylation-dependent manner. (A) Intestinal ER (500 μg) was incubated for 30 minutes at 37°C with native intestinal cytosol (1 mg) and an ATP-regenerating system. The reaction was stopped by placing the incubation tube on ice and adding cold HEPES buffer (10 mM, pH 7.2). After incubation, the ER was isolated, solubilized in cold PBS containing 2% Triton X-100 and incubated with 20 μl primary antibodies (anti-rabbit IgG, anti-VAMP7, anti-apoB48 or anti-L-FABP) for 2 hours at 4°C. Appropriate secondary antibodies bound to agarose beads were added and allowed to incubate overnight at 4°C. Immune complexes bound to the beads were isolated, washed ten times, and proteins separated on 8%-16% SDS-PAGE. The gel was stained with SimplyBlue^{TM} SafeStain (Invitrogen) to identify the proteins. Only the low-molecular-weight bands are shown. (B) A reaction similar to that shown in A was performed, with the exception that PKCζ-immunodepleted intestinal cytosol and 2 M urea-washed intestinal ER was used with an ATP-regenerating system. Similar to A, the reaction was stopped post incubation, the ER isolated and solubilized ER membranes were subjected to immunoprecipitation using 20 μl primary antibodies (anti-rabbit IgG, anti-VAMP7, anti-apoB48 or anti-L-FABP). The bead-bound immune complexes were isolated and proteins separated on 8%-16% SDS-PAGE. The proteins were identified by staining with SimplyBlue SafeStain. Only the low-molecular-weight bands are shown. The primary antibodies used are shown above each lane.

In the first series of experiments, we used conditions where the 9-kDa protein would be expected to be phosphorylated, thereby incubating native intestinal ER with native cytosol and an ATP-regenerating system. After the incubation, the ER was isolated, solubilized in 2% Triton X-100, and VAMP7, apoB48, and L-FABP were separately immunoprecipitated using antibodies directed against each protein. The immunoprecipitated proteins were separated by SDS-PAGE and protein bands identified using SimplyBlue™. A protein band at 9-kDa is clearly identified in the immunoprecipitates of each protein except when IgG was used for immunoprecipitation (Fig. 12A, IgG, VAMP7, apoB48, and L-FABP). Consistent with the anticipated results using the antibody against L-FABP, a band at 14 kDa, was also found (Fig. 12A, L-FABP).

In the second series of experiments, we used conditions in which we would expect the 9-kDa protein not to be phosphorylated. Intestinal ER membranes were urea washed to remove PKCζ and incubated with PKCζ depleted cytosol and an ATP regenerating system. After incubation, the ER was isolated, solubilized in 2% Triton X-100, and VAMP7, apoB48 and L-FABP separately immunoprecipitated using their specific antibodies. Protein(s) co-immunoprecipitated by the antibodies were determined by separating the proteins by SDS-PAGE and staining the gel with SimplyBlue™. In the absence of PKCζ, the 9-kDa protein was not associated with any of the immunoprecipitated proteins (Fig. 12B, IgG, VAMP7, apoB48 or L-FABP). A band at 14 kDa, however, was present when anti-L-FABP antibody was used (Fig. 12B, L-FABP). In summary, one interpretation of these results is that the phosphorylation of the 9-kDa protein enables it to bind separately to each of the three proteins shown. An alternative explanation of the data is that phosphorylation of the 9-kDa protein enables the formation of a protein complex that facilitates PCTV budding.

Because L-FABP interacts with the 9-kDa protein (Fig. 12), it is possible that the phosphorylation of the 9-kDa protein increases the amount of L-FABP bound to ER membranes and, therefore, increases the generation of PCTV. To test this potential mechanism, when ER membranes were incubated with L-FABP, without ATP, a more than 2.6-fold increase in L-FABP binding occurred (Fig. 13A2 and 13B, bar 2) over the amount of L-FABP bound to freshly isolated ER membranes (Fig. 13A1 and 13B, bar 1). On the addition of ATP to the ER-L-FABP incubation, only slightly more (more than 2.7-fold increase) L-FABP became bound as compared to when freshly isolated ER was used (Fig. 13A3 and 13B, bar 3). These data suggest that the phosphorylation of the 9-kDa protein does not influence L-FABP binding to ER membranes. For comparison, whole cytosol immunoblotted for L-FABP is shown (Fig. 13A4 and 13B, bar 4).

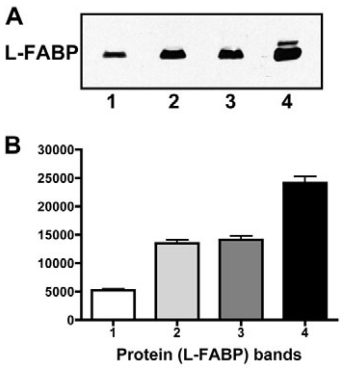

Fig. 13. Binding of L-FABP to intestinal ER membranes does not require ATP. (A) Intestinal ER (500 μg) was incubated with recombinant L-FABP (rL-FABP, 40 μg) in the absence or presence of ATP for 1 hour at 4°C. Post incubation, ER membranes were washed with cold PBS six times. 30 μg protein each of untreated intestinal ER (lane 1), ER treated with rL-FABP in the absence of ATP (lane 2), ER incubated with rL-FABP in the presence of ATP (lane 3) and native intestinal cytosol (lane 4) were separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane, and L-FABP was identified by immunoblotting using ECL reagents for detection. A representative immunoblot is shown. (B) Densitometric analysis of L-FABP. The L-FABP bands shown in Fig. 13A were quantified using Image J software (NIH). Data are presented as arbitrary densitometry units (mean ± s.e.m.; n=4).

To further support the correlation of phosphorylation of the 9-kDa protein with PCTV budding, and to support the specificity of the process, we compared phosphorylation of the 9-kDa protein by intestinal ER and cytosol with that by ER and cytosol isolated from liver and kidney. We chose these organs because the liver exports the TAG-rich very-low-density lipoprotein (VLDL), whereas the kidney represents a non-TAG-rich-lipoprotein-exporting organ. In contrast to the intense band seen when intestinal ER and cytosol were used (Fig. 11C, lanes 1 and 2), phosphorylation of the 9-kDa band occurred only minimally after incubation of ER and cytosol from the liver (Fig. 11C, lanes 3 and 4) or not at all with kidney ER and cytosol (Fig. 11C, lanes 5 and 6) each supplemented with [γ-^{32}P]ATP. These observations further support the hypothesis that the phosphorylation of the 9-kDa protein is specific for the intestine and predominantly related to PCTV budding.

Discussion

The export of chylomicrons from enterocytes into mesenteric lymph has been shown to be to be a function regulated by small intestine (Mansbach, 2nd and Arnold, 1986). An increase of the dietary fat load for 1 week and inclusion of phosphatidylcholine in an intraduodenal infusion of glyceryltrioleate were both associated with an increase in chylomicron output (Mansbach, 2nd and Arnold, 1986) whereas bile diversion has been associated with decreased output (Tso et al., 1981). The intracellular site at which output control occurs was not revealed by these studies but the rate-limiting step in chylomicron transport across the cell was subsequently shown to be its exit from the ER (Mansbach and Dowell, 2000). Because of the presumed importance of this potential site of regulation, we have focused our attention on chylomicron exit from the ER. We have proposed that chylomicrons exit the ER in a transport vesicle, PCTV, which is specifically utilized to transport chylomicrons from their site of formation in the ER to the cis-Golgi (Kumar and Mansbach, 2nd, 1999).

The intracellular transport of proteins and lipids through the secretory pathway is a highly regulated process that is mediated by specialized vesicles. These vesicles are generated from donor membranes and carry cargo to specific destinations. We have shown that the TAG containing chylomicrons are exported from the ER in large vesicles (250 nm) consistent with the size of their cargo (Zilversmit, 1967), by a COPII protein independent mechanism (Siddiqi et al., 2003) in contrast to the COPII dependence of most newly synthesized protein transporting vesicles. Consistent with our findings, vesicles carrying other cargoes such as viruses have also been shown to be generated from ER membranes by a COPII independent mechanism (Husain and Moss, 2003). The COPII independence of PCTV-budding suggests the potential for a different regulatory mechanism compared with that of COPII-dependent protein-transporting vesicles that are known to be regulated by an

ATP-dependent kinase (Aridor and Balch, 2000). Which, if any, protein kinase is associated with PCTV budding has not been previously examined.

Protein phosphorylation is most likely to be caused by protein kinases that have been implicated in various intracellular endocytic (Lin et al., 1986) and exocytic (Buccione et al., 1994; Ozawa et al., 1993; Fabbri et al., 1994) transport events. Protein transport from the ER to the Golgi has been proposed to be regulated by protein kinases, such as the bidirectional traffic of proteins between the ER and Golgi (Lee and Lindstedt, 2000) and the addition of GTP-loaded Sar1 to ER exit sites (Aridor and Balch, 2000). Also, protein export from the ER is inhibited by Cdc2-kinase-mediated disassembly of ER exit sites (Kano et al., 2004). Although PKA has not been described in ER-to-Golgi vesicle traffic, PKB has been shown to be associated with ER to Golgi trafficking of vesicles carrying nascent proteins (Du et al., 2006). However, as we have shown here, PKB is not required for the formation of PCTV. With respect to PKC, it has previously been found not to have a role in the ER-to-Golgi transport of protein vesicles (Fabbri et al., 1994). By contrast, our data suggest that a PKC isoform is required for PCTV-budding, which highlights another difference between the generation of protein vesicles and PCTVs from ER membranes.

The role of different PKC isoforms in the regulation of intracellular transport vesicles distal to the Golgi complex is well-described (Westermann et al., 1996) and supported by data showing that the release of post-Golgi vesicles carrying heparan sulphate proteoglycans (HSPGs) is blocked by the PKC inhibitor calphostin C. Further, the release of post-Golgi vesicles is stimulated by PMA in human hepatoma cells (Westermann et al., 1996). It has been suggested that either PKCα or PKCζ regulates the formation of HSPG-transporting vesicles; stimulation of vesicle formation by PMA supports the role of PKCα, whereas Ca^{2+} independence of HSPG-vesicle budding indicates the involvement of PKCζ (Westermann et al., 1996). PKCι and/or PKCλ are involved in retrograde protein transport from vesicular tubular clusters (VTCs) to the ER. As an example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is transported from the VTC to the ER, mediated by Rab2 – whose activation requires PKCι and/or PKCλ (Tisdale, 2000).

The multiple isoforms of PKC that control vesicle trafficking, have each a specifically defined role in intracellular vesicle transport and different activators, which makes identification of the isoform associated with PCTV budding important. Although the inhibition of PCTV budding by calphostin C first suggested that involvement of PKC, the data gave no information rearging which of the isoforms is used. We exploited the capabilities of Gö 6983 to inhibit different isoforms of PKC depending on the concentration of the inhibitor, to establish which group of PKC isoforms is specifically associated with PCTVs. Since 60 nM was required for significant inhibition of PCTV-budding activity, PKCζ was suggested to be the functional isoform for our system. Guided by this information, we focused our studies on the relationship between PCTV budding and PKCζ.

Confirmation of PKCζ as the important PKC for PCTV-budding activity was obtained both by using antibodies that specifically block PKCζ and by immunodepletion of PKCζ from intestinal cytosol. Both blocked the PCTV-budding process. An alternative explanation for the results of both experiments is that protein(s) important to the budding process other than PKCζ were bound to PKCζ and were either removed or inhibited by the PKCζ antibody. Although PKCζ is known to bind to other proteins (Corbit et al., 2003), this is an unlikely explanation for our observations because no proteins other than PKCζ were immunoprecipitated by the anti-PKCζ antibody following Coomassie Blue staining (data not shown) and, more importantly, the blocking effect upon PKCζ removal was reversed by the addition of recombinant PKCζ.

Activation of PKC isoforms involves its translocation from the cytosol to different sub-cellular membranes that include that of the ER, VTCs, and Golgi (Tisdale, 2000; Nishizuka, 1992; Mochly-Rosen et al., 1991). In concert with this, PMA, which activates cPKCs and nPKCs, leads to the translocation of both classes of PKCs from the cytosol to membranes (Goodnight et al., 1995). However, PKCζ – which is not activated by PMA – is not transported to membranes by PMA, although PKCζ is also found in the cytosol (Fig. 3A) (Brady et al., 2005). However, following appropriate stimulation, PKCζ will bind to mitochondria (Pinton et al., 2004). The binding of PKCζ to ER membranes has not been described previously.

Although PKCζ, activity is associated with chylomicron transport as suggested here, mice whose PKCζ gene has been disrupted appear phenotypically normal (Leitges et al., 2001). This is somewhat surprising because PKCζ has a wide tissue distribution in mice (Leitges et al., 2001). Our data are the first report of its presence in the intestine although PKCζ has been found in the intestinal Caco2 cell line (Banan et al., 2002). With respect to lipid absorption, however, a phenotype would not necessarily be expected because of the low fat content of mouse diets (4% w:w) unless a larger dietary lipid load were used.

Since PKCζ is not required for protein-vesicle budding, it is not surprising that PKCζ has – until now – not been shown to be functional in ER-to-Golgi cargo transport. The specialized function of PKCζ in the transport of chylomicrons from the ER to the Golgi in PCTVs is similar to the unique function of VAMP7 in the intestine in the same process. VAMP7 is normally functional in post-Golgi transport vesicles but in the intestine it is both required for PCTV budding from intestinal ER and used as the vSNARE component of the PCTV-Golgi SNARE complex (Siddiqi et al., 2006a; Siddiqi et al., 2006b).

Because PKCζ is a cytosolic enzyme, it does not require a membranous or micellar structure for activation and lacks DAG and Ca^{2+} requirements, which raises the question of how PKCζ is activated. Thus far, phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] (Hirai and Chida, 2003), phosphatidate (Limatola et al., 1994), 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Balendran et al., 2000) and ceramide (Bourbon et al., 2000; Muller et al., 1995) have been described as activators, and arachidonate as an inhibitor of PKCζ (Muller et al., 1995). Ceramide has a potential role in lipid absorption in that it is produced as a hydrolytic product both during the absorption of sphingomyelin (Nilsson and Duan, 2006) and during sphingomylinase attack of apical membrane sphingomyelin (Hui and Howles, 2002). Supporting this role for ceramide is the fact that both carboxylester-lipase knockout (*Cel*$^{-/-}$) mice and Caco2 cells that lack CEL produce lipoprotein particles that are much smaller than chylomicrons. These smaller TAG-containing particles can be reverted to normal-sized chylomicrons in Caco2 cells by adding CEL to the incubation medium (Kirby et al., 2002). These findings were attributed to the ability of CEL to hydrolyze ceramide that might accumulate in enterocytes in its absence (Kirby et al., 2002). These data might be consistent with our findings because ceramide has a known biphasic effect on the activation of PKCζ (Muller et al., 1995), in that ceramide activates PKCζ at low concentrations but inhibits its activation at higher

concentrations. Thus, the excess ceramide under reduced CEL conditions could interfere with PKCζ activation resulting in impaired chylomicron output by the ER, a result shown here when PKCζ activity is reduced. An alternative explanation for the findings by Hui and Howles is that ceramide might disrupt the Golgi and cause the alteration in lipoprotein output (Hui and Howles, 2002).

Phosphorylation of proteins has been shown to be a regulatory factor in many intracellular transport events including the ER-to-Golgi transport of proteins (Palmer et al., 2005; Du et al., 2006). Our study describes an in vitro assay to monitor protein phosphorylation during the budding of PCTVs from intestinal ER membranes. We observed that, although several proteins were phosphorylated during PCTV budding or following the incubation of cytosol alone, only the phosphorylation of a 9-kDa protein occurred together with the release of PCTV. The link between phosphorylation of the small molecular-weight protein and the generation of PCTVs is supported by the following: (1) When PCTV budding, but not protein-vesicle budding, was inhibited by antibodies against VAMP7 (Siddiqi et al., 2006a), the phosphorylation of the 9-kDa band was severely attenuated. (2) By contrast, when protein-vesicle budding but not PCTV budding was inhibited using Sar1-depleted cytosol (Siddiqi et al., 2003), the 9-kDa protein continued to be phosphorylated at a rate similar to that of native cytosol. (3) When ER and cytosol from liver or kidney were used, a 9-kDa band was not phosphorylated despite the ability of liver to produce the TAG-rich lipoprotein VLDL. In summary, the evidence presented so far is consistent with the hypothesis that generation of PCTVs is specifically associated with the phosphorylation of a 9-kDa protein.

An important question is how the budding activity of L-FABP relates to the activity of PKCζ, because L-FABP by itself can induce PCTV budding without the requirement for PKCζ (Neeli et al., 2007) (our data). Unfortunately, L-FABP itself, which is not phosphorylated during budding, offers no opportunity for regulation except by increasing its expression in response to lipid feeding (Poirier et al., 1997), a process that is too slow for the intermittent nature of dietary fat intake. Additionally, ER protein phosphorylation does not enhance recruitment of L-FABP to ER membranes. Finally, the vesicle produced by L-FABP alone is not competent to fuse with the Golgi complex, indicating a requirement for additional proteins, such as the COPII proteins (Siddiqi et al., 2003), to create a mature Golgi-fusion-competent vesicle. Therefore, the interaction of L-FABP with the 9-kDa protein, as well as the here-shown binding of the 9-kDa protein to ApoB48 and VAMP7 (Fig. 12), offers a potential mechanism by which the generation of chylomicron-transporting vesicles can be regulated. The hypothesis that this regulatory step occurs through phosphorylation of the 9-kDa protein is supported by data that show an interaction of the 9-kDa protein with proteins important for PCTV budding in a phosphorylation-dependent manner (Fig. 12). We propose, therefore, that the PCTV-budding machinery organized by L-FABP is modified and regulated, at least in part, by the phosphorylation of the 9-kDa protein, which – once formed – is relatively stable (Fig. 6).

In summary, our data suggest that the aPKC isoform PKCζ is required for PCTV budding and that, in association with PCTV budding, an unidentified 9-kDa protein is phosphorylated. Studies on the identification of this small-molecular-weight protein and its functionality in regulating lipid absorption are ongoing in our laboratory.

Materials and Methods

Materials

[^{3}H]Oleic acid (9.2 Ci/mM) and [γ-^{32}P]ATP (6000 Ci/mM) were procured from PerkinElmer Life Sciences (Boston, MA). Inhibitors of PKC isoforms calphostin C, Ro-31-2281 and Gö 6983 were purchased from Sigma Chemical Co. (St Louis, MO). PKC ζ pseudo-substrate inhibitor (Myr-SIYRRGARRWRKL-OH) was a kind gift from Fred Gorelick (Yale University, New Haven, CT). The peptide originated in the laboratory of Steven Pandol and Joseph Reeves (UCLA, Los Angeles, CA). PKB inhibitor VIII (Akti-1/2) was obtained from Calbiochem (San Diego, CA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham International (Piscataway, NJ). Gel-electrophoresis and immunoblot reagents were purchased from Bio-Rad (Hercules, CA). Protease Inhibitor Cocktail tablets were from Boehringer Mannheim (Indianapolis, IN). Other biochemicals used were of analytical grade and purchased from local companies. Sprague Daley rats (150-200 g) were purchased from Harlan (Indianapolis, IN).

Antibodies and recombinant protein

Rabbit polyclonal anti-PKCζ, anti-PKCα and anti-phosphotyrosine antibodies, mouse monoclonal antibodies against phosphothreonine, goat anti-rabbit IgG, goat anti-mouse IgG and goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (St Louis, MO). Rabbit polyclonal antibodies against rat VAMP7 (amino acids 105-123) have been characterized previously (Siddiqi et al., 2006a). These antibodies recognize VAMP7 but do not crossreact with VAMP1 or VAMP2, as judged by immunoblotting (Siddiqi et al., 2006a). Polyclonal antibodies against mammalian Sar1 have been described (Siddiqi et al., 2003). Antibody against liver fatty-acid-binding protein (L-FABP) were a generous gift from Judith Storch (Rutgers University, New Brunswick, NJ). Goat anti-rabbit IgG conjugated with agarose beads was purchased from Sigma Chemical Co. Rabbit polyclonal anti-phosphoserine antibodies, recombinant human PKCζ and recombinant human PKCα were obtained from StressGen Biotechnologies (Victoria, Canada).

Depletion of Sar1, PKCζ or PKCα from the cytosol

Removal of the indicated protein was performed by immunoadsorption (Siddiqi et al., 2003). Briefly, 50 μl of cytosol (1 mg) were incubated with 20 μl of either rabbit polyclonal anti-Sar1, anti-PKCζ or anti-PKCα antibodies at 4°C for 2 hours and then anti-rabbit IgG conjugated with agarose beads was added. The antibody-protein complexes were removed by centrifugation. Successful depletion of Sar1, PKCζ or PKCα from cytosol was obtained by two rounds of immunodepletion and confirmed by immunoblotting.

Removal of Sar1, PKCζ and PKCα from the ER

To remove either Sar1, PKCζ or PKCα from membranes, ER was incubated with 2 M urea for 15 minutes at 4°C (Siddiqi et al., 2003). ER membranes were washed twice with cold phosphate-buffered saline (PBS) and the removal of Sar1, PKCζ or PKCα was determined by immunoblotting.

Metabolic labeling of enterocytes, and isolation of ER and cytosol

Intestinal epithelial cells (enterocytes) were isolated from the proximal half of rat small intestine and the ER was loaded with [^{3}H]TAG (Kumar and Mansbach, 1997). Briefly, enterocytes were released from intestinal villi, incubated with [^{3}H]oleate with carrier bovine serum albumen (BSA), and washed twice with 2% BSA to remove excess [^{3}H]oleate. The cells were homogenized using a Parr bomb (Parr) and the ER and Golgi isolated using a sucrose-step gradient. The ER-enriched fraction was re-centrifuged through the gradient to get highly purified ER. The ER preparation contained no Golgi, endosomes or lysosomes (Siddiqi et al., 2006a). Cytosol was isolated from rat enterocytes (Siddiqi et al., 2003). For studies that required doubly labeled ER, the ER was loaded with [^{14}C]TAG to mark newly synthesized TAG and [^{3}H]protein to mark newly synthesized protein (Siddiqi et al., 2003). The ER used was derived from intesitinal cells incubated with [^{14}C]oleate, BSA and [^{3}H]leucine.

Gel electrophoresis, immunoblotting and autoradiography

Protein samples prepared in Laemmli buffer were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad). After blocking the membrane with 5% Blotto, membranes were incubated with specific primary and then appropriate secondary antibodies conjugated to HRP. Proteins were detected by developing the blots using ECL and exposing the developed blots to Kodak Biomax film (Siddiqi et al., 2003).

For autoradiography, proteins were separated by SDS-PAGE and gels were rinsed three times with distilled water and stained with SimplyBlue^{TM} SafeStain (Invitrogen, Carlsbad, CA). After destaining with distilled water, gels were dried and autoradiographed using Kodak Biomax film at –70°C for 1 week.

In vitro PCTV-budding assay

[^{3}H]TAG-loaded ER (500 μg) was incubated at 35°C for 30 minutes with cytosol (0.8-1 mg) and an ATP-regenerating system in the absence of Golgi acceptor (total volume 500 μl) (Siddiqi et al., 2003). The reaction mixture was resolved on a continuous sucrose (0.1-1.15 M) gradient by ultracentrifugation (Siddiqi et al., 2003).

PCTVs, containing the [^{3}H]TAG, were collected by aspiration from the top of the gradient. When doubly labeled ER was used, the incubation conditions were the same but the reaction was resolved on a continuous sucrose gradient. Disintegrations per minute (dpm) of [^{3}H]protein were determined in each fraction after precipitation with trichloroacetic acid (TCA; 20%) and the TCA was removed by acetone (– 20°C) (Siddiqi et al., 2003). Radiolabled TAG was only found in fractions 1 to 3 and protein in fractions 1 to 3 and 8 to 10 with the majority (81%) of the [^{3}H]protein dpm in fractions 8 to 10.

In vitro phosphorylation assay

To assess the phosphorylation of proteins during PCTV formation, we used conditions similar to those in our in vitro PCTV-budding assay except that the ER (500 μg) contained unlabeled TAG and the incubation medium was supplemented with 2 mM NaF, 20 μCi of [γ-^{32}P]ATP and protease inhibitors cocktail in a total volume of 500 μl. Incubation was for either 5 or 30 minutes at 35°C. The reaction was stopped by placing the tubes on ice and adding cold HEPES buffer (10 mM). To detect the phosphorylated proteins, 50 μg protein of the reaction mix was separated using SDS-PAGE and autoradiographed.

Pre-phosphorylation of ER membranes

Intestinal ER (2 mg) was incubated with 5 μg of recombinant PKCζ (rPKCζ) an ATP-generating system (1 mM ATP, 5 mM phosphocreatine, 5 U creatine phosphokinase), 50 μCi of [γ-^{32}P]ATP, 2 mM NaF, 5 mM Mg^{2+}, 2 mM DTT and 2.5 mM Ca^{2+} for 30 minutes at 37°C. Post incubation, the reaction was stopped by placing the tubes on ice and by adding cold HEPES buffer (10 mM). The ER membranes were washed five times with cold PBS supplemented with 2 mM NaF. Membrane proteins (40 μg) were separated on 8-16% SDS-PAGE and phosphorylation of ER proteins was determined by autoradiography. Another portion of the phosphorylated, washed ER was used in the PCTV-budding assay as indicated.

Measurement of [^{3}H]TAG

The [^{3}H]TAG was extracted and its radioactivity was quantified essentially as described (Kumar and Mansbach, 1997).

Statistical analysis

Comparisons between mean values were carried out using a statistical package supplied by GraphPad Software (Instat, GraphPad Software, Inc., San Diego, CA) using a two-tailed *t*-test. *P* values of <0.05 indicate significant differences.

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