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Expression of Mammalian Protein Kinase C in *Schizosaccharomyces pombe*: Isotype-specific Induction of Growth Arrest, Vesicle Formation, and Endocytosis

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Mammalian protein kinase C (PKC) isotypes elicit a number of effects on expression in *Schizosaccharomyces pombe*. A small decrease in growth rate results from PKC-γ expression, and treatment of these cells with phorbol esters leads to marked growth inhibition and vesicle formation. PKC-δ and -η expression causes growth inhibition and vesiculation, and the magnitude of both of these effects is increased by phorbol esters. In contrast, PKC-ϵ expression produces growth inhibition but no vesicle accumulation, and this effect is not responsive to phorbol ester. Finally, PKC-ζ has no observable effect. Thus, isotype-specific biological effects are observed. The accumulation of vesicles correlates with phorbol ester–dependent growth inhibition and occurs only with expression of those isotypes that down-regulate in response to phorbol esters in these cells. Antibodies against mammalian clathrin light chain 1a identified clathrin-coated vesicles and up-regulation of clathrin expression in those cells where vesicles accumulate; the increased vesicular traffic includes an element of endocytosis. Thus expression of specific mammalian PKC isotypes up-regulates endocytosis in *S. pombe*, providing a likely explanation for PKC-mediated receptor internalization in higher eukaryotes.

INTRODUCTION

The protein kinase C (PKC)[1] gene family comprises at least nine mammalian genes encoding 10 or more polypeptides (reviewed in Stabel and Parker, 1991; Nishizuka, 1992). Relatives of this family have been identified in many lower eukaryotic species including *Xenopus*, *Drosophila*, nematodes, and yeast (see Stabel and Parker, 1991 and references therein). An understanding of the function of these proteins has come from a limited genetic analysis in *Drosophila* (Schaeffer *et al.*, 1989; Choi *et al.*, 1991; Smith *et al.*, 1991) but for the most part through the use of broad specificity agonists, such as the tumor-promoting phorbol esters, and through inhibition with broad specificity inhibitors (e.g., staurosporine). These types of analyses have provided circumstantial evidence for PKC action in many cellular processes including secretion, mitogenesis, differentiation, and cell-cell communication (see Nishizuka, 1988). In addition such studies have revealed PKC substrates such as the 80K MARCKS protein, pleckstrin, and F1/GAP43 (see Stabel and Parker, 1991).

Although involvement of PKC is implicated in various processes, the actual contribution of individual PKC isotypes is largely unknown. Overexpression of specific PKC isotypes in mammalian cells has been shown to confer properties usually associated with increased sensitivity to phorbol ester exposure (e.g., Housey *et al.*, 1988). However there is only limited data on the relative ability of PKC isotypes to confer particular properties.

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Abbreviations used: LCa, clathrin light chain a; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

To define rigorously the potential function of individual isotypes, it would be of benefit to be able to categorize genetic complementation groups for the mammalian genes. An attempt to establish this was made possible after the cloning of two PKC homologues from *Schizosaccharomyces pombe* (Mazzei *et al.*, 1993; Toda *et al.*, 1993). These experiments led us to conclude that none of the PKC isotypes tested (α, β_1, γ, δ, ϵ, ζ, or η) could complement the staurosporine supersensitivity of pck1$^-$ or pck2$^-$ *S. pombe* (Toda, Goode, and Parker, unpublished data). However, it was observed that expression of mammalian isotypes in *S. pombe* induced specific and, in some instances, marked phenotypes. The characterization of mammalian PKC expression and of the phenotypes is described here.

MATERIALS AND METHODS

Cell Strain and Culture

The yeast strain *h$^-$ade6-704, leu1-32, ura4-D18* was routinely cultured at 32°C in rich YE medium or in synthetic Edinburgh minimal medium supplemented with 2% (wt/vol) glucose, 100 μg/ml adenine, and 50 μg/ml uracil and free of leucine (minimal selective medium) to select for the stable proragation of the expression plasmids (Moreno *et al.*, 1991). Transformation was by electroporation using the manufacturer's instructions (BioRad Pulse Controller, Richmond, CA) and transformants were spread onto minimal selective plates containing 1 M sorbitol and 1 μM thiamine. Selected individual colonies were plated onto minimal selective plates containing 100 ng/ml 12-*O*-tetracanoylphorbol 13-acetate (TPA) or 1 μM thiamine to assay for a growth phenotype. Stable cell lines were made by diluting selected transformed clones 1000-fold sequentially three times in rich YE medium and then plating onto minimal selective plates with 1 μM thiamine; stable lines maintained the plasmid and therefore grew under selection. Cultures were routinely started by picking a colony into minimal selective medium containing thiamine and culturing overnight until late log phase. Cells were then extensively washed to remove thiamine, rediluted into fresh minimal selective medium with the appropriate additive (thiamine or TPA), and cultured for a further 24–48 h as described in the text. Saturated phase was avoided by redilution if necessary. Growth status was assessed by measuring the OD_{595} (0.4 units = 10^7 cells/ml).

Expression Plasmids

The pREP3X expression plasmid is a derivative of pREP1 (Maundrell, 1990) except that the *Nde* I site in the multiple cloning site has been replaced by an *Xho* I site, so that initiation is from the ATG of the insert. Expression in this plasmid is driven by the nmt1 promoter that expresses at eightfold the level of the alcohol dehydrogenase promoter and is repressed 80-fold by thiamine (Maundrell, 1990; Basi *et al.*, 1992).

All subcloning methods used were as described (Sambrook *et al.*, 1989). The cDNA for PKC-α was excised from the sp64-PKC-α plasmid (Parker *et al.*, 1986) in overlapping *Nco* I-*Nco* I and *Bam*HI-*Stu* I fragments. After filling the recessed ends of the *Nco* I fragment with the Klenow fragment of DNA polymerase I, this fragment was ligated into a filled *Sal* I site in the vector. The resulting plasmid was digested with *Sma* I and then *Bam*HI, and the other fragment of PKC-α was inserted. The cDNA for PKC-β1 was excised from the sp64-PKC-β1 plasmid (Coussens *et al.*, 1986) as a *Sal* I fragment and ligated into *Sal* I-digested vector. The PKC-γ cDNA was subcloned from sp64-PKC-γ (Patel and Stabel, 1989) as a *Bam*HI-filled *Hin*dIII fragment into *Bam*HI-*Sma* I–digested vector. The PKC-δ sequence was excised from pBluescript-PKC-δ (Olivier and Parker, 1991) as a *Pfi*MI-*Nde* I fragment and, after removing the protruding terminus with T4 DNA polymerase, was inserted into a filled *Sal* I site in the vector. PKC-$\delta\Delta$ was constructed by excising the 464-base pair (bp) *Nco* I-*Nco* I fragment from pREP3X-PKC-δ, which contains the whole of the C3 and part of the C4 domain of the kinase, filling the recessed termini and religating the plasmid. This procedure creates an *Nsi* I site that was used to verify the deletion and that the coding frame was maintained (this was also confirmed by the preservation of the C-terminal epitope for the PKC-δ antibody). PKC-ϵ was subcloned from pMT2-PKC-ϵ (Schaap *et al.*, 1989) as an *Xho* I fragment into the *Sal* I site of the vector. Three hundred twenty bp of 5′ noncoding sequence of the PKC-ϵ cDNA was subsequently removed by digesting with *Sac* II and *Nco* I, polishing the ends with T4 DNA polymerase, and religating the plasmid. The PKC-ζ cDNA was excised from pBluescript-PKC-ζ (Ways *et al.*, 1992) with *Xba* I and, after filling the recessed ends, was subcloned into a filled *Sal* I site in the vector. The *Xho* I fragment, containing the cDNA for PKC-η, was excised from pBluescript-PKC-η (Dekker *et al.*, 1992) and inserted into the *Sal* I site of the vector.

Protein Extracts and PKC Assay

Denatured protein extracts were made from equivalent numbers of cells as described (Moreno *et al.*, 1991), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as elsewhere (Olivier and Parker, 1992). Polyclonal rabbit isotype-specific PKC antibodies, directed against the carboxy termini (Marais and Parker, 1989; Dekker *et al.*, 1992; Olivier and Parker, 1992), were used followed by donkey anti rabbit horse radish peroxidase-conjugated antibodies and the electrochemiluminesence detection system (Amersham International, Amersham, United Kingdom). The PKC-η antibody was affinity purified using the epitope peptide attached to Actigel (Sterogene, Arcadia, CA) according to the manufacturer's recommended procedures.

Native proteins for PKC assays were isolated essentially as described (Moreno *et al.*, 1991) except that the extraction and resuspension buffers contained 20 mM tris(hydroxymethyl)aminomethan pH 7.5, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 2 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 10 mM benzamidine, and 0.5% (vol/vol) Triton-X100, and the extracts were precipitated with 45% (wt/vol) ammonium sulphate. After equilibrating for the amount of protein in the extracts by Bradford assay (BioRad), PKC assays were performed as described elsewhere (Dekker *et al.*, 1992). All assays contained 0.75 mM $CaCl_2$, and substrates were assayed at 250 μg/ml. Phosphatidylserine (PS) and TPA were used at 1.25 mg/ml and 250 ng/ml, respectively.

Growth Curves

Freshly saturated cultures (grown in thiamine) were extensively washed in minimal selective medium and diluted 1 to 100 into fresh minimal selective medium. After 24 h, these cultures were rediluted to give a final OD_{595} of 0.02 and incubated in selective minimal medium with no additives, with 100 ng/ml TPA or with 1 μM thiamine. At subsequent intervals, quadruplicate aliquots of the cultures were transferred to 96-well dishes, and the OD_{595} was determined using a 96-well plate reader (Molecular Devices, Menlo Park, CA).

Electron Microscopy

Twenty milliliters of log phase cells, cultured for 44 h in minimal selective medium with the indicated additive, were washed and fixed with 1% monomeric glutaraldehyde in 0.1 M Sorensens phosphate buffer (pH 7.4) for 1 h at room temperature. After fixation, the cells were treated with 0.5 M NH_4Cl in phosphate buffer for 4 h and washed in buffer overnight at 4°C. The cells were dehydrated with a graded series of methanol at progressively lower temperature and infiltrated with Lowicryl HM20 resin at −50°C. The resin was polymerized by UV light for 48 h at −50°C. Ultrathin sections were mounted on

carbon coated grids and air dried. After adding contrast with uranyl acetate and lead citrate, the sections were examined with a Jeol 1200 FX electron microscope (Cranford, NJ).

For immunogold studies, ultrathin sections were mounted on carbon-coated Ni grids and labeled as follows. After 5 min on a drop of phosphate-buffered saline (PBS) and 1 h preincubation on immuno blocking solution (IBS) (5% normal goat serum, 5% ovalbumin, and 5% bovine serum albumin in PBS), the grids were transferred onto drops of IBS containing antibody and blocking peptide as indicated in the text and incubated at 4°C overnight. The sections were then washed three times over a 15-min period with IBS and then incubated with the appropriate immunogold conjugate for 2 h (goat anti-rabbit or goat anti-mouse IgG, 10 nm gold, 1/50 dilution with IBS). After 3 × 5 min rinses in PBS and 3 × 5 min in distilled water, the sections were air dried and then contrasted and examined as described above. A variety of controls were performed to determine antibody specificity including omitting the primary antibody and treating the section with an inappropriate antibody raised in the same host species. The PKC-η affinity-purified antibody was concentrated fivefold in a speedvac before use at a dilution of 1/10, and the epitope peptide was added to a concentration of 2.5 mM for determination of specificity. The PKC-δ antibody was diluted 1/2. The CON.1 and pep1a antibodies were diluted 1/2 and 1/10, respectively. Peptide 1a, shown to be 85% pure by high-performance liquid chromatography, was added to the pep1a antibody at concentrations between 0 and 0.63 mM to determine specific binding. Quantification of immunogold labeling was performed on random photographs of calibrated magnification using a personal computer-connected digitizing unit. The number of gold particles over vesicles was related to the surface area occupied by vesicles and was expressed as the number of particles per μm^2 of vesicles. Ten electron micrographs were examined for each treatment, and the results are expressed as means ± SD.

Lucifer Yellow Uptake

Twenty milliliters of cells prepared as described for electron microscopy were washed twice with ice-cold minimal medium and resuspended in 1 ml of ice-cold minimal selective medium containing 5 mg/ml of lucifer yellow carbonyl hydrazine (Sigma, St. Louis, MO) and 1 mM NaN_3. Five hundred microliters of each culture were incubated at 32°C, whereas the other 500 μl was kept on ice. After 90 min, all cultures were washed five times with 2 ml of ice-cold minimal medium, and, after the final wash, the pellets were vortexed for 1 min with 1 ml of acid-washed glass beads (400–600 μm diameter, Sigma). Two milliliters of medium were added to the beads, and the liquid was transferred to a fresh tube and cleared by centrifugation at 50 000 × *g* for 15 min. The fluorescence (excitation 426 nm, emission 550 nm) was determined using a Perkin-Elmer fluorimeter (Norwalk, CT) and quantified by comparison to a standard curve of lucifer yellow.

RESULTS

Expression of Mammalian PKCs in S. pombe

The mammalian PKCs were subcloned into pREP3X (a variant of the pREP1 vector) (Maundrell, 1990). The nmt1 promoter, which drives expression in this plasmid, is efficiently repressed by thiamine (Maundrell, 1990; Basi *et al.*, 1992).

The *S. pombe* strain was transformed with the pREP3X, pREP3X-PKC-α, -β_1, -γ, -δ, -ϵ, -ζ, and -η plasmids by electroporation, and the transformants were spread onto minimal selective plates containing 1 M sorbitol and 1 μM thiamine. Resultant colonies were picked into minimal selective medium, and denatured cell extracts, made after 30 h of culture in the absence of thiamine, were separated by SDS-PAGE and screened for expression of the relevant PKC isotype by Western blotting. A colony expressing each of PKC-γ, -δ, -ϵ, -ζ, and η as well as a control transformant was diluted into rich medium giving a final dilution of 10^9 and then spread onto minimal selective plates containing 1 μM thiamine. A number of the resulting stable cell lines were checked for expression of the relevant PKC, and a representative clone of each was selected for further experiments. No PKC protein was detected in the PKC-α and -β_1 transformants, and no stable cell lines were created.

Expression of PKCs was confirmed by isolation of PKC activity from the stable cell lines. Using protamine sulphate as substrate, which is phosphorylated equally by most PKC isotypes in a cofactor-independent manner (Bazzi and Nelsestuen 1987), PKC-γ, -ϵ, and -η activity is detectable in extracts from the relevant cells cultured in the absence of thiamine but not in extracts from parallel cultures with thiamine (Figure 1A). Low but reproducible activity was also seen in PKC-δ–induced cells. An insignificant amount of activity is associated with PKC-ζ expression (Figure 1A) that agrees with previous results that protamine sulphate is a poor substrate for this isotype (Nakanishi and Exton, 1992). A peptide based upon the sequence of the pseudosubstrate site of PKC-δ can be used to assay for cofactor dependent PKC activity (Olivier and Parker, 1991). Extracts from cells cultured in the absence of thiamine were assayed in the presence or absence of the PKC activators, PS and TPA. PKC-γ, -δ, -ϵ, and -η phosphorylate this peptide substrate in a cofactor-dependent manner (Figure 1B). The level of activity extracted from the PKC-expressing *S. pombe* cells was in the region of 0.3–1 U per mg of protein, depending upon the particular isotype. The high relative level of cofactor-independent activity in the PKC-δ and -η extracts (Figure 1B) correlates with extensive proteolytic degradation of these isotypes that can occur during extraction of native protein. However, chromatographic separation of extracts containing PKC-δ and -η yields full length proteins that are clearly dependent on cofactors for activity (Figure 1C) (Goode and Parker, 1994). PKC-ζ shows no kinase activity with this substrate (Figure 1B); this is expected because the assay conditions are not optimized for PKC-ζ (see Goode and Parker, 1994).

The pREP series of vectors use the nmt1 promoter that is repressed by thiamine. The isolated stable clones express the relevant mammalian PKC, and expression is repressed by thiamine (Figure 2A). PKC-γ and -δ show concentration-dependent repression, and other isotypes display a complete repression between 100 and 1000 nM thiamine.

In mammalian cells phorbol esters activate PKC, producing responses known to be PKC mediated (Nishizuka, 1992). A characteristic of phorbol ester treatment is PKC down-regulation upon prolonged incubation

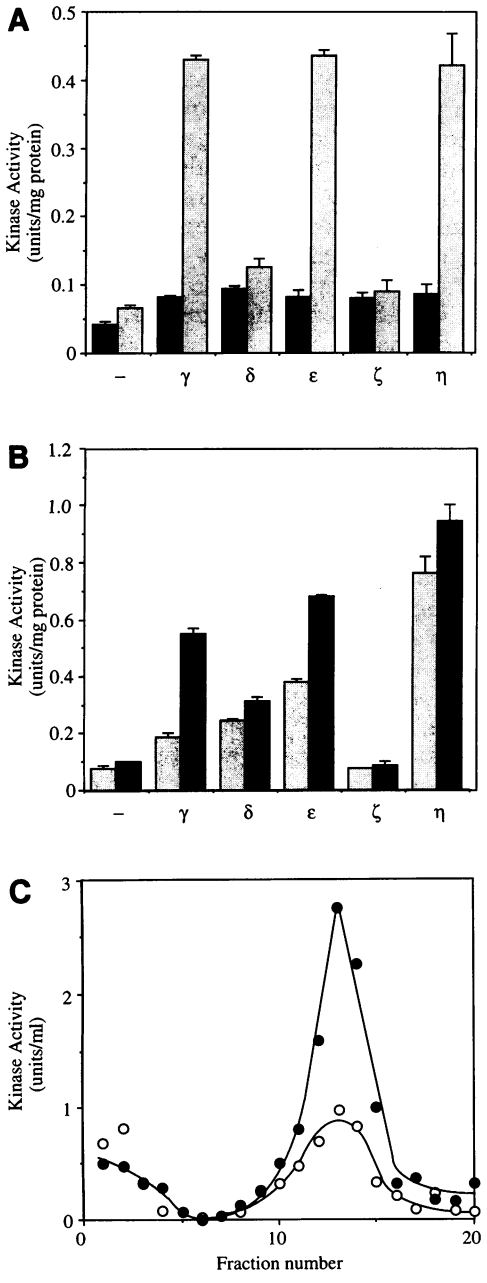

Figure 1. PKC activity extracted from stable *S. pombe* cell lines. (A) PKC expression in the presence or absence of thiamine. Equivalent amounts of protein extracted from cells grown in the absence (▨) or presence (■) of 1 μM thiamine for 30 h were assayed for the ability to phosphorylate protamine sulphate. (B) Extracted PKC activity is cofactor dependent. The same extracts from cells grown in the absence of thiamine were assayed for cofactor independent (▨, −PS −TPA) and dependent (■, +PS +TPA) kinase activity using the serine containing PKC-δ pseudosubstrate site peptide (Olivier and Parker, 1991) as substrate. Results are the mean and range of duplicate samples. (C) Partial purification of PKC-η. Extracts from cells grown in the absence of thiamine (to induce PKC-η) were fractionated on a MonoQ 5/5 column. Proteins were eluted on a linear NaCl gradient from 0 to 0.6 M. The activity was determined in the absence (O-O) or presence (●-●) of TPA and PS (see MATERIALS AND METHODS), and the peak elutes at 0.4 M.

(Rodriguez-Pena and Rozengurt, 1984; Young *et al.*, 1987). The PKC-expressing *S. pombe* strains were treated with TPA to determine if mammalian PKCs remained phorbol ester responsive when expressed in this organism. TPA at 10–100 ng/ml causes down-regulation of PKC-γ, -δ, and -η and does not affect the levels of PKC-ζ even up to 1 μg/ml (Figure 2B). This result agrees with previous demonstrations that PKC-ζ does not respond to phorbol esters in vitro (Nakanishi and Exton, 1992) or in mammalian cells (Gschwendt *et al.*, 1992; Ways *et al.*, 1992). Surprisingly, PKC-ε levels were not down-regulated (TPA does cause the down-regulation of PKC-ε in mammalian cells, albeit at a rate that can be slower than for PKC-δ) (Olivier and Parker, 1992). This lack of PKC-ε down-regulation was observed in a number of different isolates (see below). The expressed PKC isotypes thus divide into two groups on the basis of phorbol ester sensitivity, i.e., PKC-ζ and -ε are not affected, whereas PKC-γ, -δ, and -η protein levels are down-regulated.

PKC Isotype-specific Growth Inhibition

Inclusion of thiamine in the minimal selective medium leads to an increased growth rate for all strains, including vector controls (Figure 3A). Clones stably transformed with PKC-γ and -ζ vectors grew at approximately the same rate as vector control cells when plated on selective minimal plates in the absence of thiamine (Figure 3A). However, cells transformed with the PKC-δ, -ε, and -η vectors showed a marked growth inhibition when cultured in the absence of thiamine and PKC-η–transformed cells show a detectable growth inhibition even in the presence of thiamine, because of slight leakiness of the promoter (Figure 3A). Thus, the growth inhibition correlates with expression of PKC-δ, -ε, and -η. PKC-γ and -ζ are expressed but no growth effect is noticeable compared to vector alone. We have been unable to detect expression of PKC-α or -β_1 or a phenotype after transformation with these isotypes even though these cDNAs are readily expressed in mammalian cells (Pears *et al.*, 1990). To check for errors introduced in subcloning, expression constructs were reengineered using different strategies and with the PKC-α and -β_1 cDNAs from different shuttle vectors. However, again no phenotype or expression was seen, and these isotypes have not been investigated further here.

In light of the TPA responsiveness of certain PKC isotypes expressed in *S. pombe* (see Figure 2B), we determined the effect of TPA on the growth of the PKC-*S. pombe* lines. TPA up to a concentration of 1 μg/ml had no effect on the growth of vector or PKC-ζ–transformed cells (Figure 3A). In contrast, treatment of PKC-γ expressing *S. pombe* with as little as 10 ng/ml TPA led to growth inhibition (Figure 3A). Cells cultured with TPA in the presence of thiamine showed no growth phenotype.

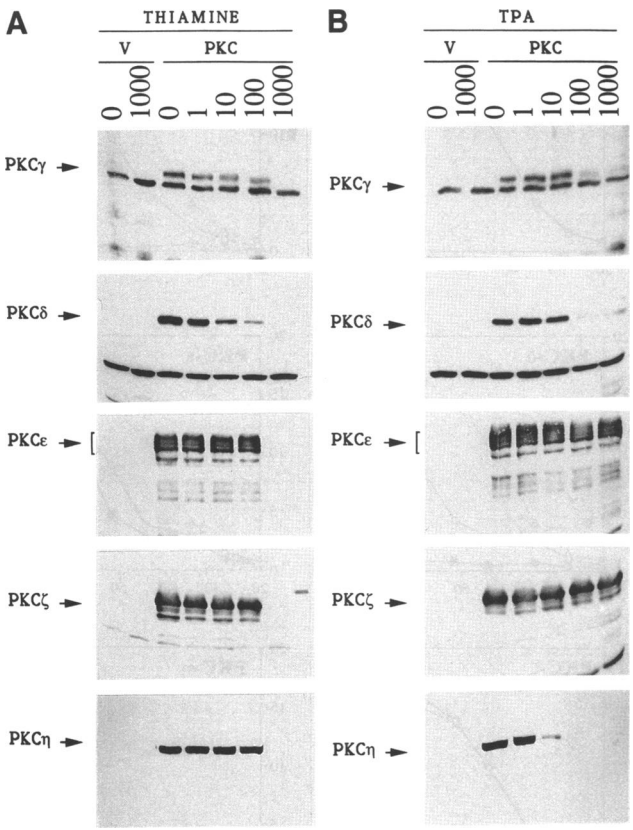

Figure 2. (A) PKC expression is repressed by thiamine. Denatured extracts collected from vector control (V) and PKC-γ, -δ, -ε, -ζ, and -η (PKC) stable cells cultured for 30 h in minimal selective medium containing no additive or containing 1, 10, 100, or 1000 nM thiamine as indicated were analyzed by Western blotting using isotype-specific PKC antibodies. (B) PKC-γ, -δ, and -η down-regulate in response to TPA. Extracts from parallel cell cultures containing no additive or 1, 10, 100, or 1000 ng/ml TPA were analyzed as for A. Positions of the PKCs are indicated by arrows. PKC-ε migrates as multiple bands at ~90 kDa, whereas the other isotypes have a molecular mass of 76–80 kDa.

To quantify the growth inhibitory effects of PKC expression and TPA treatment, growth studies in liquid culture were performed. All strains, including controls, grow faster in the presence compared with the absence of thiamine. PKC-δ, -ε, and -η cells, and to a lesser extent PKC-γ cells, grow significantly slower than vector controls and PKC-ζ cells in the absence of thiamine (saturation is delayed by ~20 h) (Figure 3B), in agreement with the growth characteristics observed on solid medium (Figure 3A). TPA totally inhibited the growth of PKC-γ, -δ, and -η cells whereas no effect was seen on the other cells (Figure 3B). PKC-γ, -δ, -ε, and -η are known to be TPA responsive in mammalian cells (PKC-ζ is not). That TPA potentiates the growth inhibitory effect of PKC-γ, -δ, and -η is expected. The lack of effect on PKC-ε cells, however, would not have been predicted but does correlate with the failure of TPA to induce down-regulation of PKC-ε in these cell lines (Figure 2B). It should be noted however that PKC-ε isolated from this *S. pombe* strain displays typical TPA/phospholipid-dependent kinase activity in vitro (see above).

Expression of PKC isotypes in *S. pombe* generates four distinct phenotypes. PKC-ζ has no effect in its own right and is unaffected by phorbol esters. PKC-γ expression has little effect unless activated by phorbol esters, which correlates with TPA-induced activation and down-regulation of PKC-γ. PKC-δ and -η produce a marked growth inhibition that is amplified by phorbol esters, also in agreement with the TPA-induced down-regulation of these isotypes. Finally, PKC-ε expression produces a phenotype that is not affected by phorbol esters, which is consistent with the lack of down-regulation of PKC-ε protein levels in response to TPA (see Table 1).

The Phenotype Is a Consequence of Kinase Function

Convenient restriction sites in the PKC-δ sequence were used to remove the C3 region that includes the ATP binding site of the kinase domain leaving intact the entire regulatory domain and carboxy terminus. *S. pombe* cells transformed with this kinase-defective PKC-δ construct (PKC-δΔ) exhibited no phenotype even after TPA treatment, whereas full length PKC-δ consistently produced a marked growth inhibitory effect in the same experiment (Figure 4A). Both the kinase-defective and full length PKC-δ proteins were expressed as detected by Western blotting (Figure 4B). This result indicates that the phenotype results from expression of kinase activity of the introduced PKC and is not a result of, for example, a nonproductive sequestration of effector in competition with endogenous protein kinases.

PKC Expression Induces Vesicle Accumulation

The *S. pombe*-PKC strains were cultured in the presence or absence of thiamine for 44 h and then processed for transmission electron microscopy. Expression of PKC-δ was accompanied by a massive accumulation of vesicles as shown in Figure 5, B and C. They were fairly homogeneous in size, ranging from 70 to 90 nm in diameter, and were distributed throughout the cell cytoplasm. The precise number varied but in the most extreme cases there were ≤720 vesicles in a cell section. Their presence depended on the expression of PKC-δ because there were few if any vesicles in the presence of thiamine (Figure 5A), after expression of PKC-δΔ, or in vector control cells (Table 1). A massive accumulation of vesicles was also observed in cells expressing PKC-η, and some vesicles were even seen when these cells were cultured in the presence of thiamine (Table 1) in agreement with the slight growth inhibition of PKC-η cells cultured with thiamine.

Vesicles did not accumulate in cells expressing PKC-γ unless growth inhibition was triggered by addition of

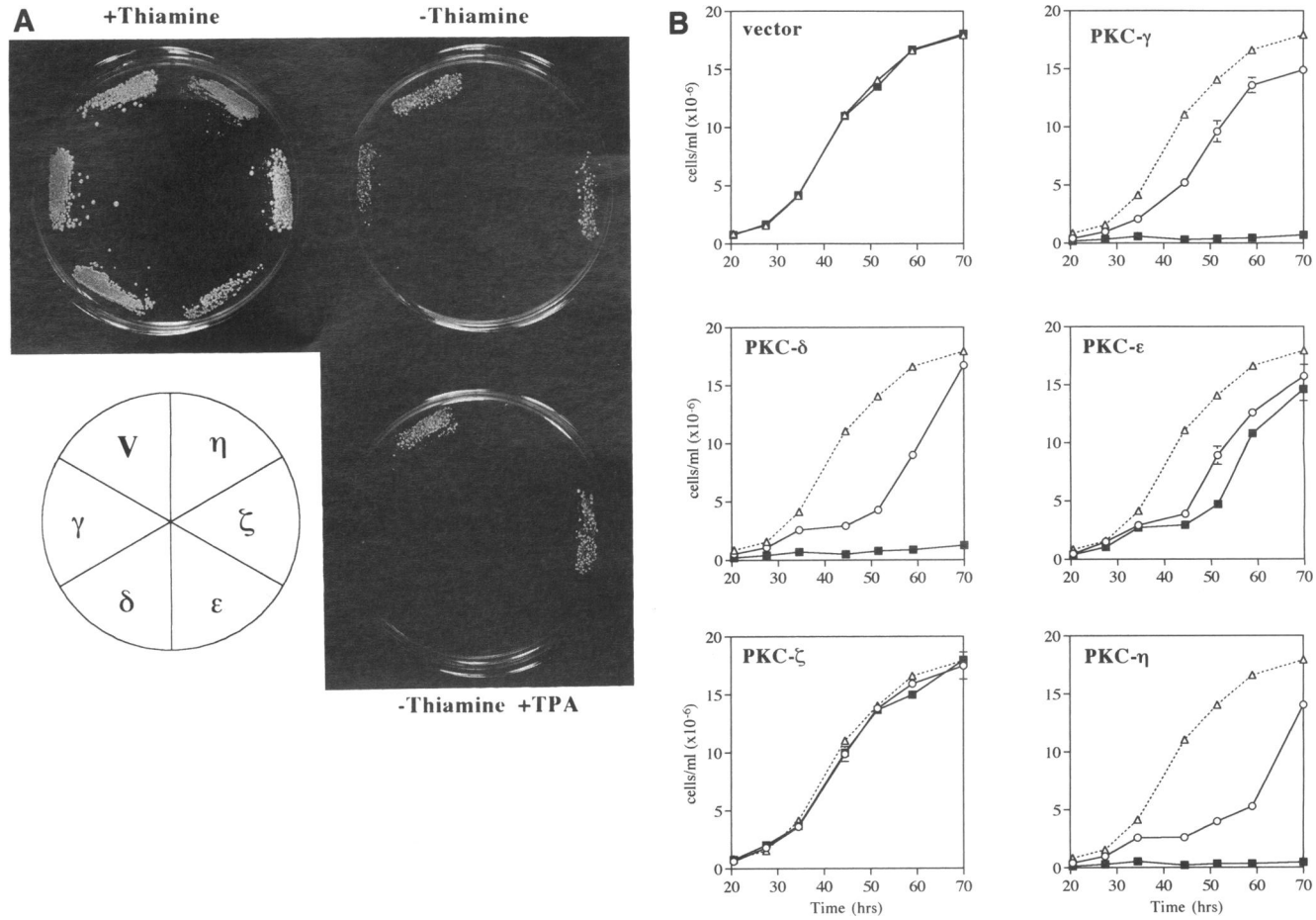

Figure 3. Growth phenotype and effect of TPA. (A) Solid medium. Fresh saturated cultures grown in minimal selective medium with 1 μM thiamine were washed in minimal medium and then streaked onto selective minimal plates with no additives, 1 μM thiamine, or 10 ng/ml TPA as indicated. Plates were incubated at 32°C for 3 d. (B) Liquid culture. Vector control and PKC-γ, -δ, -ϵ, -ζ, and -η cells, precultured in minimal selective medium without thiamine for 24 h, were diluted to 5×10^5 cells/ml in minimal selective medium with no additives (open symbols) or with 100 ng/ml TPA (closed symbols). The cell density was determined after the indicated intervals of culture at 32°C. Results are means and SD of quadruplicate samples. The graphs for the PKC strains include the result of the vector control cultured without thiamine (– –Δ– –) for comparison.

TPA when large numbers of vesicles amassed (Table 1). Even this treatment, however, failed to cause accumulation in vector controls and in cells expressing PKC-ϵ or -ζ (Table 1). Because PKC-ϵ expression was associated with growth suppression but not with vesicle accumulation, there is no correlate between these two events (the reasons for the induced growth defects are unclear).

Functionally, PKC isotypes can again be divided into the same four separate categories, the properties of which are summarized in Table 1. Each condition leading to accumulation of vesicles also led to a TPA-responsive growth arrest.

Endocytosis Contributes to Vesicle Accumulation

Morphological observations suggested that certain PKC isotypes were associated with an exaggeration of membranes on the endocytic, and not the exocytic, pathway.

At the cell surface, there were frequent images of what appeared to be budding vesicles (Figure 5C) with the same shape and size as clathrin-coated vesicles. There were also occasional images that suggested the presence of a coat similar to clathrin. These were not seen in the absence of PKC expression. There was also an increased number of vacuoles, the final delivery site for endocytosed material.

In contrast, there were few observable changes in the exocytic pathway. Golgi stacks were readily apparent (Figure 5, A and C), and the endoplasmic reticulum (ER) had the same distribution both surrounding the nucleus and lying just beneath the plasma membrane. There was no evidence of any membrane accumulation; neither organelle was swollen, and the amounts appeared normal.

The origin of the vesicles was confirmed by labeling Lowicryl sections with a variety of antibodies to known

Table 1. PKC-induced phenotypes

	Growth inhibition		Down-regulation	Vesicle accumulation	
Culture condition	−thiamine	−thiamine +TPA	−thiamine +TPA	−thiamine	−thiamine +TPA
Vector	−	−	NA	−	−
PKC-γ	±	++	+	−	+
PKC-δ	+	++	+	+	ND
PKC-ε	+	+	−	−	−
PKC-ζ	−	−	−	−	−
PKC-η	+	++	+	+	ND
PKC-δΔ	−	−	ND	−	ND

The effect of PKC isotype expression on growth status and vesicle accumulation is compared with the ability of TPA to cause down-regulation.

NA, not applicable; ND, not determined.

components of clathrin-coated vesicles. Two antibodies were used successfully, both against clathrin light chain a (LCa). LCa and LCb, together with the clathrin heavy chain, form triskelions, the subunits of the clathrin coat (Pearse and Crowther 1987). Polyclonal antibody pep1a (against the N-terminal region of LCa) (DeLuca-Flaherty *et al.*, 1990) labeled accumulated vesicles in cells expressing PKC-η (Figures 6A and 9A) or PKC-δ. Labelling was specific for these vesicles and for parts of the plasma membrane, which could represent the sites of budding vesicles (there was also occasional labeling of the vacuoles that might represent delivery sites for the endocytic vesicles). There was no labeling of Golgi or ER (including the nuclear envelope). The level of nonspecific labeling was low as shown by the absence of labeling over the nuclear matrix and mitochondria. Specific labeling was absent in cells containing the control vector and in PKC-δ cells cultured with thiamine. Concurrent addition of the pep1a epitope peptide (0.63 mM) with the antibody reduced labeling by 94% (Figure 6B), as calculated by a reduction in the density of gold particles over vesicles from 113 ± 37 to 7.3 ± 4.6 particles per μm^2 of vesicles. Thus binding was specific.

The pep1a antibody detects an immunoreactive protein of a similar molecular mass to purified porcine brain clathrin triskelions (38kDa) (Figure 7). The amount of this protein appears to be up-regulated in the PKC-δ and PKC-η expressing cells, correlating with vesicle accumulation (Figure 7). Binding to this 38-kDa protein is decreased when the pep1a antibody is preincubated with the epitope peptide. Additional minor proteins are detected with this antibody, some of which are up-regulated in cells accumulating vesicles (Figure 7). Therefore, to confirm the identity of this 38-kDa protein, parallel Western blots were probed with the monoclonal CON.1 antibody (Nathke *et al.*, 1992) (directed against a conserved epitope at the amino terminus of clathrin light chains) that identified a single protein (Figure 7) that comigrated with the specific protein identified by the pep1a antibody. Thus both antibodies identify a protein of similar molecular mass to mammalian LCa and this binding is specifically competed. The low level of this protein in cells expressing PKC-ε is interesting and does correlate with a lack of vesicle accumulation in these cells. However, this observation has not been investigated here.

The water soluble, membrane impermeable, fluorescent dye, lucifer yellow, has been used previously to determine rates of endocytosis (Riezman, 1985). When PKC-η cells, cultured in the absence of thiamine for 44 h, were compared with parallel cultures of vector controls for the uptake of lucifer yellow over a 90-min period, specific staining was significantly increased (Figure 8A). Furthermore, lucifer yellow uptake was increased when PKC-γ cells were cultured with phorbol esters for 44 h (Figure 8B), correlating with TPA-induced vesicle formation in these cells.

PKC Locates on Vesicles

As an assessment of the mechanism of PKC action, the intracellular location of PKC-δ and -η was determined by immunogold staining using isotype-specific PKC antibodies. Although the levels of binding were low (28% of the level obtained with pep1a), both PKC-η (Figure 9B) and PKC-δ specifically located on vesicles. The observed pattern of labeling with the PKC-η antibody is similar to that with the pep1a antibody (Figures 9A and 6A). Gold particles were associated mainly with membranes (57 ± 9% on vesicles, 20 ± 8% on the plasma membrane, n = 33) with little staining of the nucleus, nuclear envelope, vacuoles, Golgi, ER, or mitochondria. Addition of 2.5 mM of antigen with the PKC-η antibody markedly decreased binding (the number of gold particles on vesicles and plasma membrane was reduced by 86 and 80%, respectively). Binding of neither of the

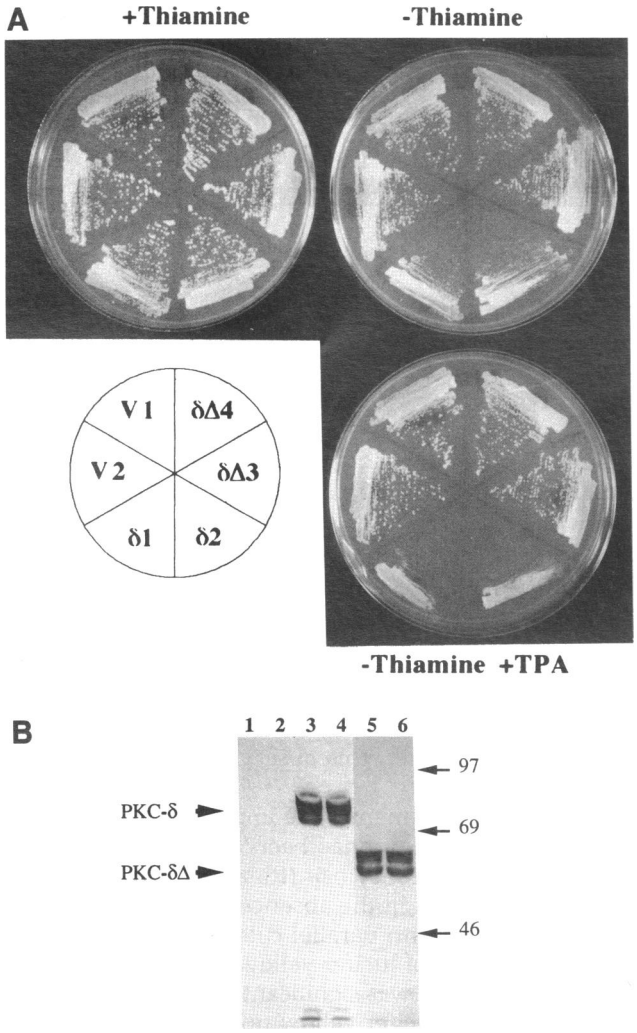

Figure 4. PKC-δ growth inhibition requires kinase activity. Cells transformed with vector or with pREP3X-PKC-δ or -δΔ were expanded in minimal selective medium containing thiamine and then washed to remove thiamine. (A) Cells were streaked onto minimal selective plates containing 1 μM thiamine, no additives, or 100 ng/ml TPA as indicated. (B) The same washed cells were also expanded for 30 h to mid-log phase in minimal selective medium without thiamine and then harvested and examined by Western blot for PKC-δ or -δΔ expression. Lanes are the same representative examples of vector controls (1 and 2), PKC-δ (3 and 4), and PKC-δΔ expressors (5 and 6) as in A. The positions of molecular mass size markers (kDa), and the PKCs are indicated. PKC-δΔ has a molecular mass of 57 kDa.

PKC antibodies was seen in vector control cells or in PKC-δ cells cultured in the presence of thiamine. The PKC-η antibody also did not bind to vesicles produced in PKC-δ cells.

Thus, PKC-δ and -η expression, and PKC-γ when stimulated with TPA, cause an increased rate of formation and an accumulation of endocytic vesicles in *S. pombe*. PKC proteins (δ and η) locate on these vesicles.

DISCUSSION

Expression of mammalian PKCs in *S. pombe* has provided information on several aspects of the potential biology of these enzymes. First, it has shown that isotype-specific functions exist in this context and that these distinctions occur in part at the different levels of activation (response to phorbol esters) and of effect (phenotype). Second, the potential to suppress the phenotypes will allow screening for effectors and inhibitors of PKC function, whether gene products or small molecules. Third, and of most immediate relevance, it appears that certain PKC isotypes can stimulate the endocytic pathway; this effect may provide the mechanistic basis of PKC-induced receptor internalization in higher eukaryotes.

Sequence homologies have led to the division of PKCs into three classes (see Nishizuka, 1992). PKC-α, -β_1, -β_2, and -γ are classical (cPKCs), PKC-δ, -ε, -η, and -θ have been termed novel (nPKCs), and PKC-ζ and -λ are atypical (aPKCs). Although these divisions are based on structural criteria, it is likely that functional differences will divide along similar lines. For example, the nPKCs that lack the C2 domain and are therefore calcium-independent for activity can be activated in the absence of a calcium signal, whereas the cPKCs that are calcium-dependent for activity are not necessarily activated under these circumstances (Ha and Exton, 1993; Olivier and Parker, 1994). In addition to differences in activation, the isotypes may be functionally distinct in terms of signal output, and recent evidence supports this suggestion. For example, isotypes show differences in substrate site sequence preferences (Marais *et al.*, 1990) and in their ability to phosphorylate certain potentially physiological substrates (e.g., Ido *et al.*, 1987; Sheu *et al.*, 1990; Goode *et al.*, 1992).

Thus the existence of multiple isotypes may be justified by distinct functions, in addition to selective activation, and indeed the phenotypes resulting from expression of mammalian PKCs in *S. pombe* demonstrate that PKC isotypes subdivide into functional classes in a whole cell assay. Four clear phenotypes are produced after expression of five PKC isotypes in *S. pombe*. PKC-ζ produces no effect, and PKC-γ produces growth inhibition and vesicle accumulation only after activation by exogenously added phorbol ester. Expression of PKC-δ and -η produce similar phenotypes suggesting that these isotypes are at least partially activated in yeast in the absence of added activators. Full activation after TPA treatment results in a more pronounced phenotype. Finally PKC-ε expression results in growth inhibition, but no vesicles are seen and TPA does not alter the phenotype, suggesting that PKC-ε activity cannot be enhanced by phorbol esters in *S. pombe* (even though the extracted enzyme can be activated by TPA).

The functional effects generally correlate with the class distinctions based on sequence comparisons with

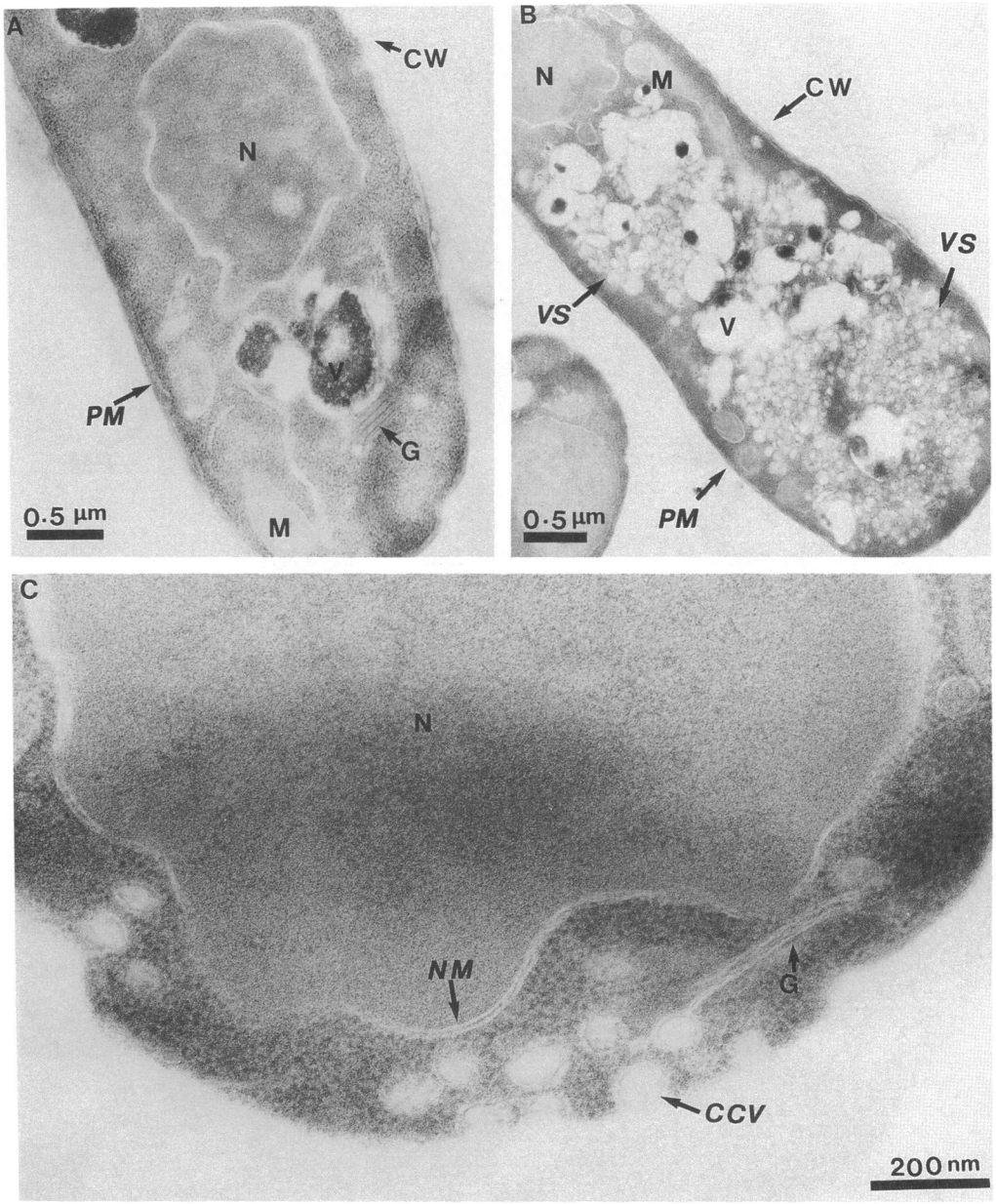

Figure 5. PKC-δ induces vesicle accumulation. Mid-log-phase PKC-δ–transformed cells cultured in minimal selective medium with (A) or without (B and C) 1 μM thiamine for 44 h were examined by electron microscopy as described in MATERIALS AND METHODS. PKC-δ induces a large accumulation of vesicles (B) and active invagination from the membrane (C). CCV, putative budding clathrin-coated vesicle; CW, cell wall; G, Golgi apparatus; M, mitochondrion; N, nucleus; NM, nuclear membrane; PM, plasma membrane; V, vacuole; VS, vesicle.

one additional division being identified. For example, PKC-γ, the only cPKC expressed, produced a phenotype distinct from other isotypes in terms of activation. In contrast, expression of the aPKC, PKC-ζ led to no phenotype under any condition tested. This aPKC contains only a single cysteine repeat region in the C1 domain (Ono *et al.*, 1989) and does not bind phorbol esters (Nakanishi and Exton, 1992; Ways *et al.*, 1992; Kochs *et al.*, 1993); the lack of response to phorbol esters is expected. This isotype can also be activated by different lipids when compared with other PKC isotypes (Nakanishi *et al.*, 1993), although this may not be an exclusive property (Singh *et al.*, 1993). PKC-ζ may be functionally distinct from the other tested PKCs and/or its specific activator may not be produced in *S. pombe*. Support for the latter possibility arises from experiments in which an active form of PKC-ζ does cause growth inhibition in *S. pombe* (Goode and Parker, 1994). The former suggestion, however, is not discounted because the exact nature of the phenotype induced by the active form of PKC-ζ has not been determined. Expression of the nPKCs led to growth inhibition, but these isotypes divide into two classes on the basis of the nature of the phenotype produced. PKC-δ and -η expression induce growth inhibition and vesicle accumulation, both of which were accentuated by TPA treatment. On the other

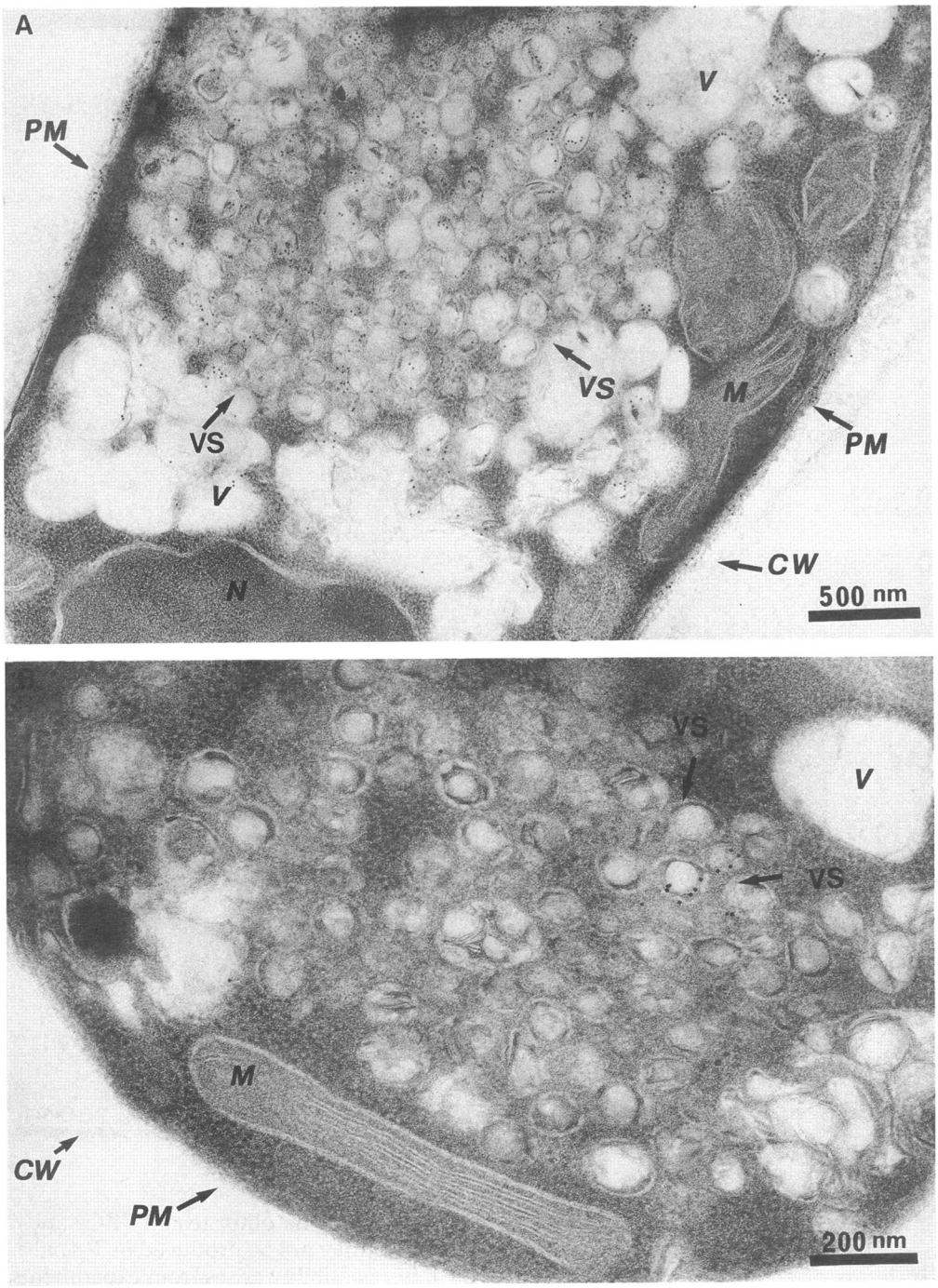

Figure 6. (A) Clathrin LCa localizes on vesicles and the plasma membrane. PKC-η expressing cells were cultured for 44 h in the absence of thiamine and then prepared for immuno electron microscopy (as described in MATERIALS AND METHODS). Ultrathin sections were incubated with the rabbit polyclonal pep1a anti clathrin LCa activity followed by gold-conjugated goat anti rabbit IgG. The gold particles are seen primarily on 70–90-nm vesicles and the plasma membrane but not over the nucleus, mitochondria, or cytoplasm. (B) pep1a epitope peptide decreases labelling. Peptide 1a (0.63 mM) was added to sections concurrently with the antibody. The density of labeling was decreased by 93% as described in the text. CW, cell wall; M, mitochondrion; N, nucleus; PM, plasma membrane; V, vacuole; VS, vesicle.

hand, PKC-ϵ expression caused no noticeable vesicle accumulation, and the growth inhibitory phenotype was not responsive to TPA treatment. The unresponsiveness to TPA is confirmed by the lack of PKC-ϵ down-regulation in response to TPA. Sixteen different clonal isolates of *S. pombe* expressing PKC-ϵ from three independent transformations have been examined during the course of these studies, and neither TPA-induced down-regulation nor TPA-dependent effects on growth in any of these lines has been observed. Even though PKC-ϵ is activated by TPA in vitro, it does not induce the same effects as PKC-δ or -η in vivo; a clear isotype specificity has been identified.

Mammalian PKC-α (Riedel *et al.*, 1993b, c) and -β_1 (Riedel *et al.*, 1993a) have been expressed in *Saccharomyces cerevisiae*, and phenotypes have been demon-

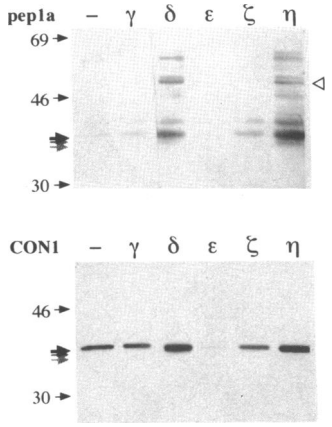

Figure 7. PKC-δ and -η expression up-regulates clathrin LCa expression. Vector controls (–) and PKC-γ, -δ, -ε, -ζ, and -η cells were cultured for 30 h in minimal selective medium without thiamine, and denatured extracts were examined by Western blotting using the pep1a and CON.1 anti clathrin LCa antibodies. →, protein of the same molecular mass detected by both antibodies that was specifically competed with peptide 1a. ◁, protein whose binding to pep1a antibody was also competed with this peptide. The positions of two alternatively spliced forms of LCa found in porcine brain (DeLuca-Flaherty *et al.*, 1990) are indicated by shaded arrows. The positions of the molecular mass size markers (kDa) are also shown.

strated that have been characterized mainly as growth inhibition. We were unable to see expression or effects of these particular isotypes in *S. pombe* and therefore cannot compare the phenotypes in these two distinct yeast species. The growth inhibition induced by expression of PKC-α and -β_1 in *S. cerevisiae* is dependent upon catalytic activity as judged by mutational analysis (Riedel *et al.*, 1993a, c). Likewise, in *S. pombe* the observed phenotypes involve catalytic functions, as shown by two independent observations. First, a mutant PKC-δ that lacks the C3 domain does not produce the effects despite being expressed at equivalent levels to full length PKC-δ. Second, PKC-γ elicits a phenotype only after the addition of the potent PKC activator TPA and furthermore the phenotypes in PKC-δ and -η cells are potentiated by TPA. The phenotypes produced are therefore a consequence of the kinase function of the introduced PKC.

It has been suggested that PKC may play a role in the control of vesicle traffic in mammalian cells, especially of secretory processes (see Nishizuka, 1988; Nishizaki *et al.*, 1992). Therefore we determined the nature of the vesicles seen in the *S. pombe*-PKC cells. Vesicles include some that appear to be budding from the plasma membrane, suggesting an endocytic process (Figure 5C). Mammalian clathrin LCa antibody (pep1a) bound vesicles on electron microscope sections, and this binding was shown to be specific. Thus clathrin-coated vesicles are present. This antibody specifically detected a protein of a molecular mass similar to porcine LCa in extracts from the yeast cells, and the level of this protein appeared to be up-regulated in cells where vesicles also accumulated. Another LCa antibody (CON.1) detected a protein of the same mass in the cell extracts. Furthermore, a monoclonal antibody directed against human βadaptin (B1M6 antibody) (Robinson, 1987) stains PKC-δ expressers more intensely than vector controls, as detected by fluorescent immunostaining (Ayscough, unpublished data). Taken together, these data strongly suggest that endocytic vesicles form a significant part of the up-regulation of vesiculation seen in *S. pombe* after PKC expression. Further evidence arises from the lucifer yellow experiments in which the uptake of this membrane-impermeable dye increased in cells where vesicles are present.

The up-regulation of endocytosis offers a possible explanation for certain PKC-mediated events at the cell membrane. PKC activity plays a role in growth factor–induced receptor internalization and recycling/down-regulation (see Schlessinger, 1988). However, a mechanism for this effect had not been found. PKC phosphorylates the epidermal growth factor receptor (Cochet *et al.*, 1984; Davis and Czech, 1985; Downward *et al.*, 1985), but this event does not affect the rate of receptor internalization (the kinetics of internalization are not affected when the PKC sub-

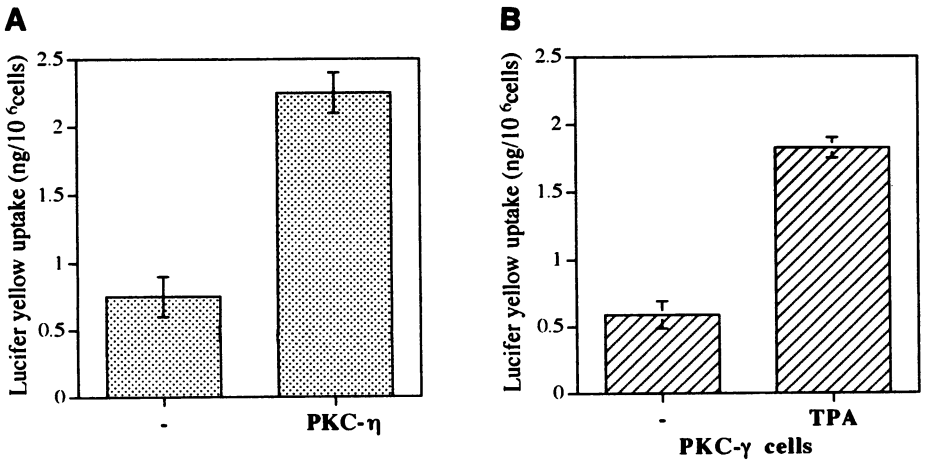

Figure 8. Lucifer yellow uptake. (A) Vector controls and PKC-η cells were incubated for 44 h in minimal selective medium. Samples, treated as described in MATERIALS AND METHODS, were incubated for 90 min with lucifer yellow either on ice or at 32°C. The difference between these two values is plotted and indicates the specific rate of dye uptake and thus endocytosis. (B) PKC-γ–expressing cells were incubated for 44 h in minimal selective medium with no additive or with 100 ng/ml TPA. Cells were analyzed as for A, and the values plotted are likewise the uptake at 32°C less than at 0°C. Results are the means and ranges of duplicates.

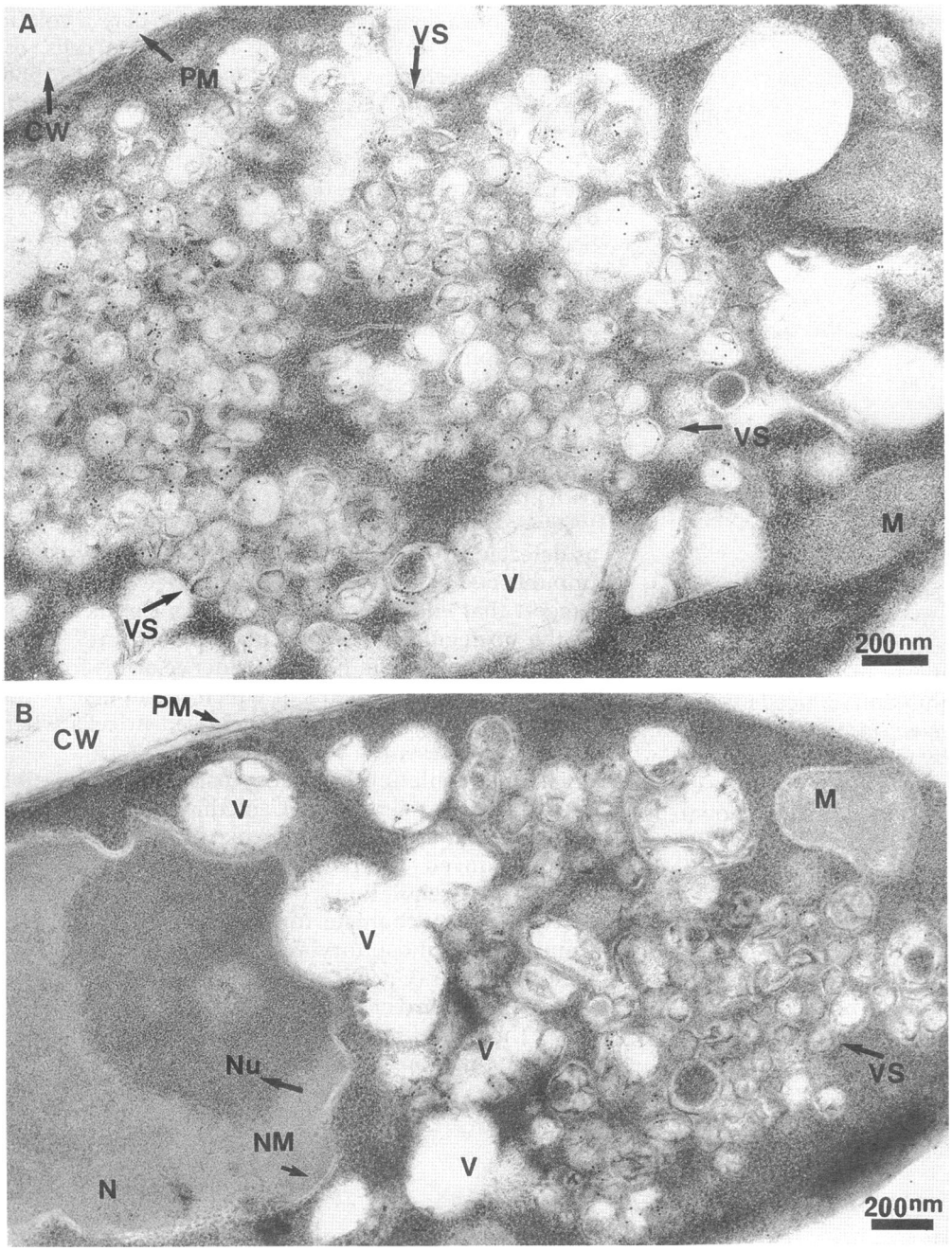

Figure 9. PKC-η antibody binds sections with a similar pattern as pep1a. PKC-η–expressing cells incubated in minimal selective medium without thiamine for 44 h and prepared as for Figure 6A were incubated with the pep1a (A) or PKC-η (B) specific antibodies followed by gold-conjugated second antibodies. Gold particles are seen with a similar pattern and primarily over vesicles and the plasma membrane. CV, coated vesicle; CW, cell wall; G, Golgi apparatus; M, mitochondrion; N, nucleus; NM, nuclear membrane; Nu, nucleolus; PM, plasma membrane; V, vacuole; VS, vesicle.

strate site is mutated) (Countaway *et al.*, 1990; Heisermann *et al.*, 1990). The transferrin receptor is also internalized in a PKC-dependent manner and is phosphorylated by PKC (May *et al.*, 1985), but again direct phosphorylation is not responsible for PKC-dependent internalization (Eichholtz *et al.*, 1992). The results in this paper show that certain PKC isotypes up-regulate endocytic processes; this effect would cause internalization and perhaps down-regulation of proteins at the cell membrane, including cell surface receptors. Because PKC is activated in response to stimulation of various receptors, this pathway would contribute to both homologous and heterologous desensitization. The precise mechanism of the PKC-induced effect remains obscure.

In conclusion, various mammalian PKC isotypes induce growth inhibition in *S. pombe*. In some but not all instances this is associated with increased vesicle formation, a component of which is endocytic. Whereas the growth phenotype may well prove useful

in screening for modifiers of PKC function, the up-regulation of endocytosis is reminiscent of TPA effects in mammalian cells suggesting a direct role for PKC and providing a route for the elucidation of PKC function in this context.

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