

PHOSPHATIDYLINOSITOL 4-KINASE III β REGULATES THE TRANSPORT OF CERAMIDE BETWEEN THE ENDOPLASMIC RETICULUM AND GOLGI

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Running Title: PI 4-kinases and ceramide transport

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The recently identified ceramide transfer protein, CERT, is responsible for the bulk of ceramide transport from the ER to the Golgi. CERT has a C-terminal START-domain for ceramide binding and an N-terminal pleckstrin-homology domain that binds phosphatidylinositol 4-phosphate suggesting that phosphatidylinositol 4-kinases are involved in the regulation of CERT-mediated ceramide transport. In the present study fluorescent analogues were used to follow the ER to Golgi transport of ceramide in order to determine which of the four mammalian phosphatidylinositol 4-kinases are involved in this process. Overexpression of pleckstrin-homology domains that bind phosphatidylinositol 4-phosphate strongly inhibited the transport of C5-BODIPY-ceramide to the Golgi. A newly identified PI 3-kinase inhibitor, PIK93 that selectively inhibits the type-III phosphatidylinositol 4-kinase β enzyme, and siRNA-mediated down-regulation of the individual PI4K enzymes, revealed that phosphatidylinositol 4-kinase β has a dominant role in ceramide transport between the ER and the Golgi.

Accordingly, inhibition of phosphatidylinositol 4-kinase III β either by wortmannin or PIK93 inhibited the conversion of [³H]-serine-labeled endogenous ceramide to sphingomyelin. Therefore, phosphatidylinositol 4-kinase β is a key enzyme in the control of sphingomyelin synthesis by controlling the flow of ceramide from the ER to the Golgi compartment.

Sphingomyelin is a critical lipid component of the plasma membrane that together with cholesterol and glycolipids forms a special liquid-ordered microdomain of cellular membranes often referred to as rafts (1). Rafts concentrate many signaling proteins and also contain inositol phospholipids and hence are considered to be active zones in signal transduction (2). The regulation of cholesterol and sphingomyelin metabolism is intimately interrelated but relatively little is known about the regulatory pathways that link them together (3). Efficient synthesis of sphingomyelin in the Golgi has been shown to require the steady supply of ceramide from the ER by a process distinct from the vesicular transport between the two organelles (4).

The mechanism of this transport has been recently revealed by the identification of a lipid transport protein, named CERT¹ by the use of an elegant cloning strategy (5). CERT has a lipid binding START domain at the C-terminus, a PH-domain at the N-terminus and a so-called FFAT (diphenylalanine in an acidic track) domain that binds the ER-localized protein, VAP-A (4). The START domain is both necessary and sufficient for ceramide binding and transport, yet a mutation within the PH domain renders the molecule unable to fulfill its transport function pointing to the PH domain as a critical component for the docking/regulation of the molecule (4). The CERT PH domain shows a high degree of similarity to PH domains that specifically recognize PtdIns4P, such as those of the OSBP, FAPP1 and FAPP2 proteins, and has been shown to localize to the Golgi (6). This finding indicates that PtdIns4P and PI4K enzymes are likely to regulate the transport function of CERT.

There are four PI4K enzymes identified in mammalian genomes that fall into one of two groups. Type III PI4Ks are structural relatives of PI3Ks and are represented by the PI4KIII α and PI4KIII β enzymes that are highly conserved from yeast to men. Their yeast orthologues, *Stt4p* and *Pik1p*, respectively, are essential genes with non-redundant functions (7,8). Type II PI4K enzymes (also in an $-\alpha$ and $-\beta$ form) represent a completely distinct family of kinases (9,10), and their single yeast homologue, *LSB6* is a non-essential gene (11). *Pik1p* and its mammalian homologue, PI4KIII β are Golgi localized peripheral membrane proteins that have been implicated in Golgi to plasma membrane secretion both in yeast and mammalian cells (12-14). However, both the type II α and $-\beta$ PI4K enzymes have been also described in the Golgi/TGN, and shown to regulate vesicular trafficking from the TGN (15-17). The functions attributed to *Stt4p* and its orthologue, PI4KIII α have been linked to the plasma membrane and the ER but not to the Golgi (18-20).

Given the presence of multiple PI4Ks in the Golgi and one in the ER, it was of interest to determine which (if any) of these enzymes are important in supporting the transport of ceramide between the ER and the Golgi. In the present study we used a combination of pharmacological and genetic approaches to address this question and show that PI4KIII β is important in the transport of fluorescent ceramide analogues to the Golgi and that inhibition of PI4KIII β results in the defect of the synthesis of sphingomyelin.

EXPERIMENTAL PROCEDURES

Materials

Wortmannin and PAO were purchased from Sigma-Aldrich (St. Louis, MO), and brefeldin A (BFA) from Epicenter Technologies (Madison, WI). PIK93 was synthesized as described previously (21). [³H]L-serine (20-40Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). The polyclonal antibody against PI4KII α was kindly provided by Dr. Pietro De Camilli. The primary antibody against PI4KIII β was purchased from Transduction Laboratories (San Jose, CA). The affinity purified polyclonal antibody against the PI4KIII α was recently described (20). Secondary antibodies were purchased from KPL (Gaithersburg, MD). Alexa-transferrin, BODIPY® FL C5-ceramide, BODIPY® TR-ceramide and ER-tracker green were obtained from Invitrogen/Molecular Probes (Eugene, OR). The lipofectamine 2000 and oligofectamine reagents were purchased from Invitrogen (Carlsbad, CA). The cDNAs of the HA epitope-tagged K44A mutant dynamin-1 subcloned into the pcDNA3 vector was kindly provided by Dr. K. Nakayama (Tsukuba Science City, Ibaraki, Japan)

DNA constructs and transfections

The EGFP tagged FAPP1- and OSBP-PH domains have been previously described (20). These PH domains were subcloned so that the EGFP was replaced by the monomeric red fluorescent protein (mRFP), kindly provided by Dr. R.Y. Tsien (22). The PH domain of CERT (residues 76–345) has been amplified from a human full-length EST-clone (IMAGE id: 3505746, Open Biosystem, Huntsville, AL) using the primer pairs of: fw: 5'-acatctcgaggcctccatgtcgcgctgcgggctcag-taagtgg-3', rev: 5'-acatgaattccagattc-agtcttgctgttcaatggc-3'. The PH domain was subcloned into the pmRFP-N1 plasmid (fashioned following the pEGFP-N1 plasmid) in frame with the mRFP protein using the XhoI/EcoRI restriction sites. COS-7 cells grown on 25 mm glass cover slips were transfected with 1 μ g plasmid DNAs with the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. For siRNA studies, the same duplexes and procedures were used described in (20). Briefly, COS-7 cells (10^5 cells in 2 ml) were plated in 35-mm culture dishes 1 day before transfection with 20 μ l of 20 μ M siRNA (obtained from Qiagen, Valencia, CA) using Oligofectamine (Invitrogen). After 6 h, the medium was changed to DMEM containing fetal bovine serum (FBS). Transfection with the siRNAs was repeated 1 day later, and live cells were studied by confocal microscopy on the fourth day. The effect of siRNA treatment on the PI4K expression levels was determined in parallel dishes by Western blot analysis.

Confocal Microscopy of the Ceramide Transport

For ceramide transport, COS-7 cells were plated onto 25 mm diameter circular glass cover slips at a density of 3×10^5 cells/dish 1 day before the experiment. On the day of the experiment the coverslip was placed into a chamber and the cells were washed in modified Krebs-Ringer buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl₂, 0.7 mM MgSO₄, 10 mM glucose, 10 mM Na-Hepes, pH 7.4) and incubated on ice for 20

minutes with 0.05 μ M BODIPY® FL C5-ceramide or 0.5 μ M BODIPY® TR ceramide and the excess of the fluorescent dye was removed by washing again with modified Krebs-Ringer buffer. The cover slip was then mounted on a heated stage with the medium temperature (and the objective) kept at 37°C and the distribution of ceramide was analyzed using an inverted Zeiss LSM-510 scanning laser confocal microscope (Thornwood, NY) and an objective heater (Bioptech, Butler, PA).

Quantification of the distribution of BODIPY® FL C5-ceramide between the Golgi and the ER

The transport of the fluorescent ceramide was quantified post-acquisition using the linescan function of the Metamorph software. A line was drawn through the cells in the confocal pictures taken in different time points and the fluorescent intensity was measured in the cytoplasm and over the Golgi complex of the cells. The "uptake ratio" was calculated from the fluorescent light intensity in the Golgi divided by the intensity measured in the cytoplasm. The maximum uptake ratio was measured in control cells after 25 min incubation on 37°C and this value was taken as 100 percent. The quantification was made from the data of three independent experiments in which confocal pictures were taken in twelve different time points and in each time points 7 cells were analyzed.

Labeling of endogenous sphingolipids with [³H]serine

Metabolic labeling of sphingolipids using [³H]L-serine was performed by the modified protocol of Ridgway et al. (23) and Bodenec et al. (24). Briefly, COS-7 cells were seeded on 12 well plates at a density of 3×10^5 cells/well. After 1 day in culture, cells were incubated in a serine-free medium for 4 h before labeling with 100 μ Ci/ml [³H]serine for the indicated times. When inhibitors were used, they were added 10 minutes prior to labeling and they were present throughout the

labeling period. Incubations were terminated by removal of the labeling medium and the addition of ice-cold 5% PCA. Cells were then scraped and centrifuged, and the pellets sonicated for 10 sec in 2 ml methanol/water (5:4, v/v), followed by the addition of 5 ml chloroform/methanol (1:2, v/v). After vigorous shaking phase separation was achieved by adding 4 ml of 0.58% NaCl followed by centrifugation (2000 x g, 5min). The lower phase was washed 3 times with 2 ml of methanol/0.58% NaCl/chloroform (45:47:3, v/v) and dried under nitrogen. The dried samples were subjected to alkaline methanolysis to remove glycerolipids by adding 2 ml of chloroform/0.6 N NaOH in methanol (1:1, v/v) and incubated at room temperature for 1 h. After hydrolysis, 0.8 ml 0.6N HCl was added to the samples, with vortexing. The two phases were separated by centrifugation (2000g, 5min) and the lower phase was extracted again using chloroform/methanol/50mM TRIS pH7.4 (3:48:47, v/v). After drying under N₂, sphingolipids were separated by TLC on Silica-G plates using chloroform/methanol/water (65:25:4, v/v). Lipids were identified by fluorography and the radioactive spots were cut out and counted in a scintillation counter.

RESULTS

Fluorescent ceramide analogues differ in their ability to report on ceramide transport

Fluorescent ceramide analogues have been widely used to follow the transport of ceramide in living cells (25,26). Two of these analogues, BODIPY® FL C5-ceramide (green) and BODIPY® TR ceramide (red) were used in the present study (Figs. 1 and 2). These fluorescent analogues were exogenously added to COS-7 cells for 20 minutes at 4°C and after washing, their transport was followed at 37°C. In order to minimize the simple diffusion of the analogues due to their partial water-solubility, FL-Cer was used in very low concentration (0.05 µM, which

is 100-fold less than recommended by the manufacturer). BODIPY® FL C5-ceramide (FL-Cer) was present in the plasma membrane (PM) immediately after loading but rapidly disappeared from the PM after warming to 37°C showing diffuse cytoplasmic distribution consistent with ER localization. This analogue did not remain in the ER in high amounts but quickly appeared in the Golgi where it continued to accumulate throughout the 30 min period examined (Fig. 1A). The Golgi localized FL-Cer slowly redistributed to the ER when the cells were treated with Brefeldin A, suggesting that FL-Cer is probably bound to a Golgi localized protein (Fig. 1B).

Surprisingly, BODIPY® TR ceramide (TR-Cer) showed a distinct transport pattern. This analogue had to be used at 10-fold higher concentration than FL-Cer (this was still 10-fold less than recommended by the manufacturer) to be reliably detected in our microscope system. Under these conditions this analogue, too, was localized to the plasma membrane upon loading and moved rapidly to the ER during the 37°C incubation. In contrast to its green counterpart, however, TR-Cer was only very slowly transported to the Golgi and accumulated in the ER as shown by its colocalization with the endoplasmic reticulum marker ER-tracker even after a 25 minute incubation at 37°C (Fig 2A). The different transport rates of the two ceramide analogues was best seen in experiments in which the two were added simultaneously (Fig 2B). Here, the Golgi localization of FL-Cer is quite prominent while TR-Cer remains largely in the ER. This indicates that not only is TR-Cer a poor substrate of CERT, but it also is a poor inhibitor of the transport of FL-Cer. Based on these comparisons, FL-Cer was used in all subsequent studies.

To obtain a numerical measure of the transport to the Golgi, we determined the relative fluorescent intensities over the Golgi and the ER using the linescan function of the Metamorph software (Fig. 3). From the fluorescent intensity profiles

obtained along lines drawn across the Golgi, a ratio was formed using the peak fluorescent intensity measured at the Golgi divided by that of the cytoplasm (ER). (see Experimental Procedures for details) The value of this ratio started out around 1 in control cells immediately after starting the 37°C incubation of the loaded cells and reached a maximum value of around 8 by 10 minutes incubation at 37°C. In kinetic analysis, the ratio value found at 25 minutes after incubation at 37°C in control cells was taken as 100 percent in each experiment and the values were expressed as a percent of this value.

Effects of the overexpression of the PH-domains of FAPP1 and OSBP on ceramide transport

Since CERT contains a PH-domain that is essential for its ability to transport ceramide from the ER to Golgi and this PH-domain recognizes PtdIns4P, our goal was to explore the role of PI 4-kinases and their product PtdIns4P in the ER-Golgi transport of ceramide. To investigate the extent to which Golgi PtdIns4P is important in the CERT-mediated ceramide transport, first the effects of overexpression of PH-domains with PtdIns4P binding on FL-Cer accumulation was examined. COS-7 cells were transfected with either the FAPP1-PH-mRFP or the OSBP-PH-mRFP constructs, and their FL-Cer transport was followed by confocal microscopy. As shown in Fig. 4, overexpression of the PH-domains greatly inhibited the transport of FL-Cer to the Golgi and decreased the ability of the Golgi to accumulate fluorescence. It is important to note that expression of the PH domains at low levels did not interfere with the transport and in fact showed colocalization with FL-Cer over the Golgi. This observation showed that the PtdIns4P pool of the Golgi is necessary for efficient ER to Golgi transport of ceramide.

Which PI4K is involved in CERT-mediated ceramide transport?

Since the Golgi/TGN contains several PI4Ks (see above), next we wanted to

determine which PI4K is important for this process. First, the effects of different PI4K inhibitors were examined on the transport of FL-Cer to the Golgi. Wortmannin (Wm), a widely used PI3K inhibitor that also inhibits type III (but not type II PI4Ks) at μ molar concentrations (27), was used to determine which class of PI4K is involved in this process. As shown in Fig. 5, FL-Cer accumulation in the Golgi was significantly inhibited in cells pretreated with 10 μ M Wm indicating the involvement of the type III PI4Ks (Fig 5). Lower concentration of Wm (100 nM) that only inhibits PI3Ks did not have an effect on ceramide transport (not shown).

To discriminate between PI4KIII α and PI4KIII β that have almost identical Wm-sensitivities, we took advantage of our recent identification of a PI3K inhibitor, PIK93 that can discriminate between the two PI4K enzymes (21). While PI4KIII β is inhibited by \sim 90% at 250nM concentration of PIK93, PI4KIII α requires $>10\mu$ M to be inhibited by 50%, and type II PI4Ks are completely resistant to the inhibitor. (These values were obtained with purified enzymes tested *in vitro* in the presence of 100 μ M ATP, Balla A. et al. manuscript in preparation). As shown in Fig. 5, 250 nM of PIK93 almost completely eliminated the Golgi localization of the CERT-PH domain within 5 min treatment, and inhibited FL-Cer accumulation in the Golgi to the same extent as Wm. These data suggested that PI4KIII β is very important for this process. The effects of phenylarsine oxide (PAO) was also tested since this compound is often used as a PI4K inhibitor (28,29). However, pretreatment of the cells with 10 μ M PAO, a concentration that is expected to primarily inhibit the type III α enzyme had multiple effects. It partially inhibited the transport of FL-Cer to the Golgi but it also caused the appearance of small cytoplasmic puncta that was not observed with any of the other inhibitors. PAO treatment also decreased the overall amount of FL-Cer taken up by the cells (data not shown). These data suggested that PAO probably

interferes with FL-Cer transport at multiple steps, and therefore, its effects were not examined in detail.

To further analyze the role of the PI4K enzymes, the proteins were down-regulated by siRNA treatment. These studies showed that knock-down of PI4KIII β caused a strong inhibition of ceramide transport compared to cells treated with a control siRNA (Fig. 6A and B) and this effect was very comparable to those evoked by the inhibitors. Down-regulation of the other PI4Ks, PI4KII α and III α caused a small but consistent inhibition of FL-Cer accumulation in the Golgi, but did not inhibit the rate by which this equilibrium was reached (Fig. 6C). We also noted that the cells in which PI4KIII α was knocked down accumulated less FL-Cer within the cell (not shown). Importantly, knock-down of the PI4KII α or PI4KIII α had no effect on the expression level of PI4KIII β (Fig. 6), hence the small alterations in ceramide transport in these latter cases cannot be attributed to changes in PI4KIII β levels. Treatment with PI4KIII β si RNA failed to show any effect on FL-Cer uptake or transport (not shown), but we could not detect the endogenous protein in COS-7 cells with antibodies available for us, so the role of this enzyme in ceramide transport (if any) needs further investigations.

Since pharmacological inhibition or knock down of PI4KIII β caused only a partial inhibition of ceramide transport compared to the effects of overexpressed PH domains, we examined whether there is an additive effect when PI4Ks are downregulated in combination. However, we were unable to show any additive effects with the combined down-regulation of more than one enzyme (for example the type II α or type III α in addition to type III β) or by the addition of type III β inhibitors to cells in which type II α was down-regulated (data not shown).

Inhibition of PI4KIII β affects endogenous sphingomyelin synthesis

To determine whether the observed effects of PI4KIII β inhibition on FL-ceramide transport reflect a similar defect in endogenous ceramide movements, we examined the effects of Wm and PIK93 on the labeling of endogenous sphingolipids in [3 H]serine-labeled cells. Pretreatment of COS-7 cells with 250 nM PIK93 had a significant inhibitory effect on the incorporation of [3 H]serine into sphingomyelin without any effect on the labeling of ceramide (Fig. 7). A slight but consistent effect on glycosyl-ceramide (GlcCer) synthesis was also observed but this was minor compared to the effects on sphingomyelin labeling (Fig. 7). A similar effect was observed when 10 μ M Wm was used instead of PIK93. It is important to note that sphingomyelin synthesis was not completely blocked even though at these concentrations both inhibitors completely inhibit the PI4KIII β enzyme.

DISCUSSION

Several yeast studies indicate a relationship between lipid transport and PI 4-kinases. The connection between the yeast PI/PC transfer protein, Sec14p and PtdIns4P production is well established (13,30,31), and so is the importance of Stt4p in PS to PE conversion in extramitochondrial sites (32). A recent synthetic lethality screen also identified genetic connections between the Stt4p PI4K and several effectors linked to sphingolipid metabolism (33). The presence of PH domains with specific recognition of PtdIns4P in proteins, such as the OSBP CERT and FAPP2, all of which have lipid transfer functions also places PtdIns4P and PI4Ks in the center of interest concerning lipid homeostasis. In the present study, we used the CERT-mediated transport of ceramide between the ER and the Golgi to determine which of the four PI4Ks are important for this process since all four mammalian PI4Ks are localized to either the Golgi or the ER (34).

The present data – based on both pharmacological and genetic evidence – clearly suggest that PI4KIII β has a prominent role in facilitating ceramide transport between these organelles. Since the PH domain of CERT binds PtdIns4P and when expressed in isolation, it also localizes to the Golgi (6) it is feasible to assume that PI4KIII β regulates the release of ceramide from CERT and its transfer to the Golgi membrane. The selective inhibition of sphingomyelin synthesis from [³H]serine labeled ceramide by PI4KIII β inhibitors is also consistent with the need for PtdIns4P formation at the Golgi, since sphingomyelin is synthesized in the luminal side of the Golgi and it requires CERT for ceramide transfer from the ER (35). As reported earlier (4), CERT is not required for the synthesis of GlcCer and indeed, we found only a slight effect of PI4KIII β inhibition on GlcCer labeling. The PI4K requirement of CERT-mediated ceramide transport, however, is also not absolute, as both the transport of FL-Cer to the Golgi and the incorporation of [³H]serine to sphingomyelin was only partially inhibited by PI4KIII β inhibition. This could either mean that other PI4Ks can provide PtdIns4P for this process or alternatively, the process is only partially dependent on PtdIns4P production.

We reported recently that the Golgi localization of the PH domains of FAPP1 and OSBP depended on both PI4K type III β and type II α (20). Given the similarity between the former PH domains and that of CERT, the role of PI4KII α could have been expected. However, we could not demonstrate any involvement of either this or the type II β PI4K enzyme in this process in knock-down experiments. Similarly, no additivity was found between the effects of combined down-regulation or inhibition of the PI4Ks. The only effect of PI4KII α depletion was a slightly reduced accumulation of FL-Cer in the Golgi, but the kinetic of the uptake was not different from that of control cells. A similar effect was also seen after the knock-down of PI4KIII α , an enzyme primarily localized to the ER but also

implicated in PtdIns4P production at the plasma membrane (20). While we cannot completely rule out that these other PI4Ks also contribute to the regulation of ceramide transport between the ER and the Golgi, their role – if any – is far less prominent than that of the PI4KIII β enzyme.

The more potent inhibition of ceramide transport by the overexpressed OSBP and FAPP1 PH domains can be due to the ability of these PH domains to bind and sequester Arf1 in addition to binding PtdIns4P (6,36), hence, interfering with the interaction of the CERT-PH domain with the Golgi more efficiently. Moreover, at this level of overexpression the PH domains already alter the Golgi structure (20,36) and this may also explain their more efficient disruption of the transport process. Altered Golgi morphology could also contribute to the smaller Golgi accumulation of FL-Cer in PI4KIII β -depleted cells, although we did not observe major alterations in Golgi morphology in such cells using immunostaining with gm130 antibody (not shown).

The molecular details of how PtdIns4P could regulate the transport function of the CERT protein is not clear at present. However, there are several recent observations that could provide some clues to this process. Expression of the full-length CERT protein is localized to the Golgi only at low expression levels (37) (and B. Toth and T. Balla unpublished observations), while the isolated PH domain localizes to the Golgi even when expressed at higher levels (6) and (B. Toth and T. Balla unpublished observations). These observations imply that the PH domain is at least partially masked within the CERT molecule and that the localization of the CERT molecule also requires additional molecules that are in limited amounts relative to the expressed CERT protein. A recent report identified OSBP as a protein that is required for the interaction of CERT with the ER-bound VAP-A protein (37), thereby establishing a molecular link

between the transport of cholesterol metabolites and ceramide. Since OSBP also has a PH domain that recognizes PtdIns4P, and the structure of the lipid binding domain of the yeast OSBP homologue, Kes1p suggest that charged lipids could regulate its lipid binding (38,39), the PI4K-mediated regulation of these transport processes deserves further attention.

Lastly, this is the first report to use the recently identified inhibitor PIK93 to discriminate between the two forms of type III PI4Ks. This inhibitor, while still potently inhibits some of the PI3Ks, is quite effective against PI4KIII β at concentrations (200-300 nM) that do not inhibit the PI4KIII α enzyme or the type II PI4Ks. The present study demonstrates that this inhibitor will be an extremely

valuable tool to determine the multiple roles of PI4KIII β in the Golgi and perhaps in other cellular compartments.

In summary, the present studies establish PI4KIII β as the key PI4K enzyme that contributes to the regulation of CERT-mediated transport of ceramide between the ER and the Golgi. This finding further supports the central role of PI4KIII β in regulating Golgi functions, namely the vesicular trafficking from the Golgi to the plasma membrane as shown in both yeast and mammalian cells. It will be the task of future studies to determine whether the transport of ceramide to the Golgi and perhaps its conversion to sphingomyelin is part of the regulatory process also contributing to vesicle budding and fission.

FOOTNOTES:

¹ The abbreviations used are: CERT, ceramide transfer protein; FAPP, four phosphate adaptor protein; FL-Cer, BODIPY® FL C5-ceramide; OSBP, oxysterol binding protein; PAO, phenylarsine oxide; PH, pleckstrin-homology; PI4K, phosphatidylinositol 4 kinase; PtdIns4P, phosphatidylinositol 4-phosphate; TR-Cer, BODIPY® TR ceramide; Wm, wortmannin;

ACKNOWLEDGEMENTS

We would like to thank Drs. Jun Guo and Pietro De Camilli for the anti-PI4KIII α antibody, and Dr. Roger Y. Tsien for the monomeric red fluorescent protein. The confocal microscopy work was performed at the Microscopy & Imaging Core (National Institute of Child Health and Development, NIH) with the kind assistance of Drs. Vincent Schram and James T. Russell. The research of BT, AB, HM and TB was supported by the Intramural Research Program of the National Institute of Child Health and Human Development of the National Institutes of Health. ZK and KS wish to thank NIH (AI-44009) for support.

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LEGEND TO FIGURES

Fig. 1. Transport of fluorescent ceramide to the Golgi. COS-7 cells were incubated for 20 min on ice in modified Krebs-Ringer solution containing 0.05 μ M FL-Cer (complexed to BSA). After washing, cells were warmed to 37°C and the transport of FL-Cer was followed by confocal microscopy. After loading, FL-Cer is localized to the plasma membrane and then is rapidly transported to the ER and finally to the Golgi, where it accumulates after 10 minutes (A). Brefeldin A (5 μ g/ml) treatment causes a slow redistribution of the Golgi-accumulated FL-Cer back to the ER (B).

Fig. 2. Differential transport of different fluorescent ceramide analogues. (A) COS-7 cells were incubated for 20 minutes on ice in modified Krebs-Ringer solution containing 0.5 μ M TR-Cer (complexed to BSA). After washing, cells were warmed to 37°C and the transport of TR-Cer was followed by confocal microscopy. On 37°C, TR-Cer is transported from the PM to the ER and it accumulates there even after 30 min on 37°C. The ER was labeled using 1 μ M ER-tracker. (B) COS-7 cells were loaded simultaneously

with 0.05 μM FL-Cer and 0.5 μM TR-Cer as described above. The transport and localization of the two fluorescent analogues were followed by confocal microscopy at 37°C. The two fluorescent ceramide analogues show distinct localization. (C) Chemical structures of the two ceramide analogues.

Fig. 3. Quantification of the transfer of FL-Cer to the Golgi. The linescan function of the Metamorph software was used to measuring pixel intensities along the line drawn through the cell. An “uptake ratio” was calculated as a ratio between the light intensity measured in the Golgi divided by that measured in the cytoplasm/ER. Examples are shown in a control cell at 0 min and 15 min incubation on 37°C. (see Experimental Procedures for further details).

Fig. 4. The effect of overexpressed PH-domains capable of PtdIns4P binding on FL-Cer transport. (A) COS-7 cells were transfected with the mRFP-fused PH-domains of FAPP1 and OSBP proteins. Transfected cells were loaded with FL-Cer on ice and the transport was followed at 37 °C as described in the legend to Fig. 1. Both PH-domains inhibit the transport of FL-Cer to the Golgi. (B) Time course of the FL-Cer accumulation in the Golgi as measured by linescan analysis as described in the legend to Fig. 3. Data points were calculated from three independent experiments in which at each time point 6-8 cells were analyzed. The curves were fitted using the single exponential fit of the Prism software. Error bars (usually less than 10%) were omitted for better clarity.

Fig. 5. Effect of PI4K inhibitors on FL-Cer transport. (A) COS-7 cells were transfected with the mRFP-tagged PH-domain of CERT and studied by confocal microscopy. The PH-domain shows Golgi localization in cells expressing moderately low level of the protein as described in (6). Treatment with 250 nM PIK93 for 10 minutes causes the loss of the Golgi localization of the CERT-PH domain. (B) COS-7 cells were pretreated for 10 minutes with wortmannin (10 μM) or PIK93 (250 nM) and FL-Cer transport was studied as described in the legend to Fig. 1. Both inhibitors inhibited FL-Cer transport to the Golgi. (C) The time course of the FL-Cer transport was analyzed as described in the legend to Fig. 3. Data points were calculated from three independent experiments in which at each time point 6-8 cells were analyzed. The curves were fitted using the single exponential fit of the Prism software. Error bars (usually less than 10%) were omitted for better clarity.

Fig. 6. Effect of siRNA-induced down-regulation of distinct PI4Ks on FL-Cer transport. COS-7 cells were transfected with siRNAs directed against the different PI4Ks as detailed under the *Experimental Procedures*. On the 4th day of treatment, FL-Cer transport was studied as described in the legend to Fig. 1. (A) Representative pictures taken at 15 minutes incubation at 37 °C. (B) The efficiency of the siRNA knock down is shown by Western-blot analysis. (C) The time course of FL-Cer transport was analyzed as described in the legend to Fig. 3. The knock down of PI4KIII β inhibited FL-Cer transport to the Golgi to a similar extent as observed with PI4KIII β inhibition. Down-regulation of PI4KII α and PI4KIII α caused a small but consistent inhibition of FL-Cer accumulation but not the rate of uptake, while Control siRNA-treatment caused no alteration in FL-Cer accumulation. Data points were calculated from three independent experiments in which at each time point 6-8 cells were analyzed. The curves were fitted using the single exponential fit of the Prism software. Error bars (usually less than 10%) were omitted for better clarity.

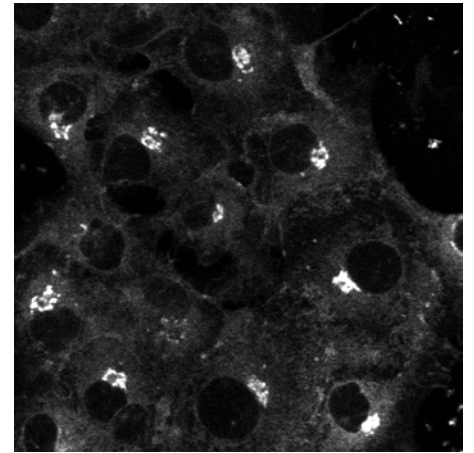
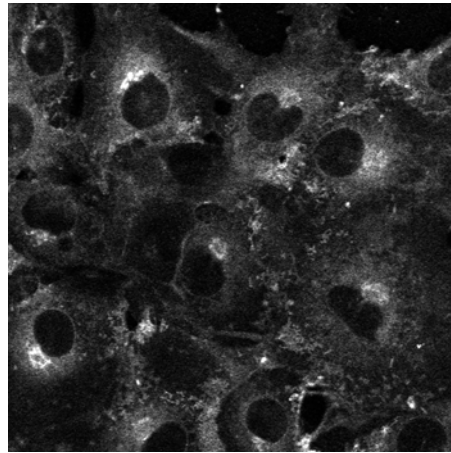
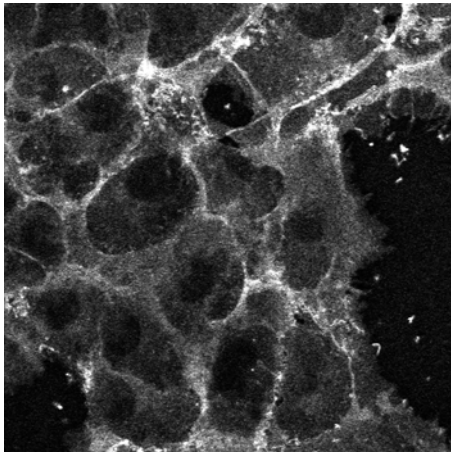
Fig. 7. Effects of PI4KIII β inhibition on [3 H]serine-labeling of endogenous sphingolipids. COS-7 cells were incubated in serine-free medium for 4 h before labeling with 100 μ Ci/ml [3 H]serine and analyzing the labeled sphingolipids as described under *Experimental Procedures*. (A) Time course of [3 H]serine incorporation into ceramide, glycosyl-ceramides (GlcCer) and sphingomyelin (SM) in control cells (filled circles) and after a 10 minutes pretreatment with 250 nM PIK93 (open circles). Means \pm S.E.M (or range) of two (15 and 30 min) and four (60 min) separate experiments each performed in duplicates. The control (100%) incorporation values were (in cpm): 20,506 \pm 2,500 for ceramide, 5,419 \pm 720 for GlcCer and 6,350 \pm 923 for SM (means \pm SEM, n=4) (B) The effects of wortmannin (10 μ M) and PIK93 (250 nM) on the [3 H]serine-labeling of sphingolipids. Means \pm S.E.M of 3 experiments performed in duplicates. (C) TLC analysis of sphingolipids from a representative experiment.

A

0 min

5 min

20 min



FL-Cer

B

FL-Cer 20 min 37 °C

+ BFA 10 min

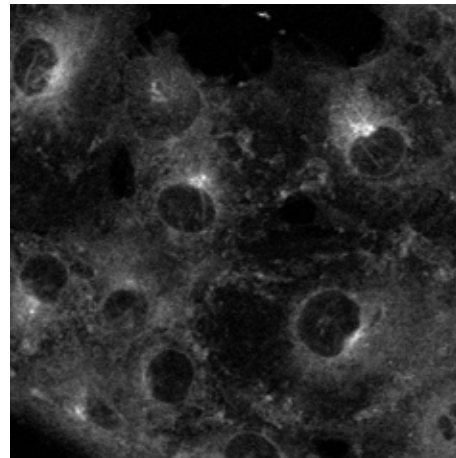
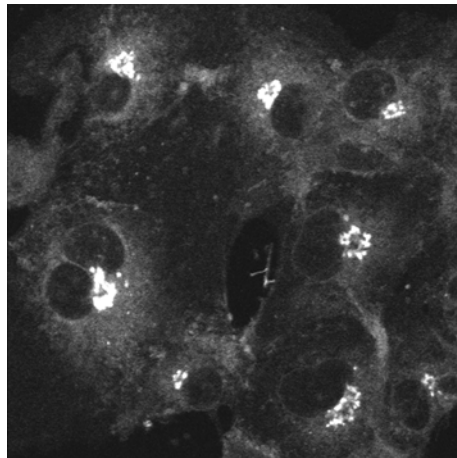


Figure 1

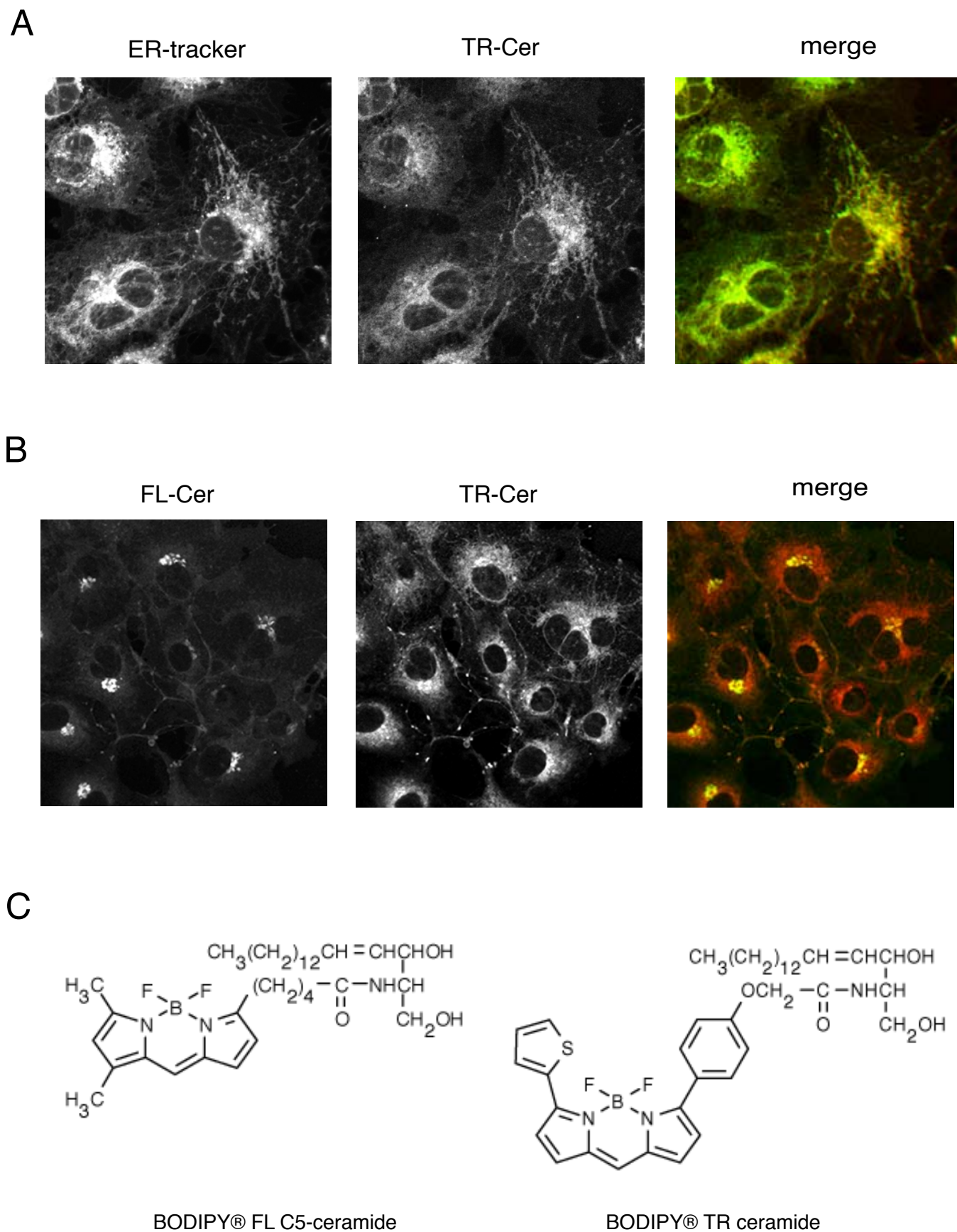


Figure 2

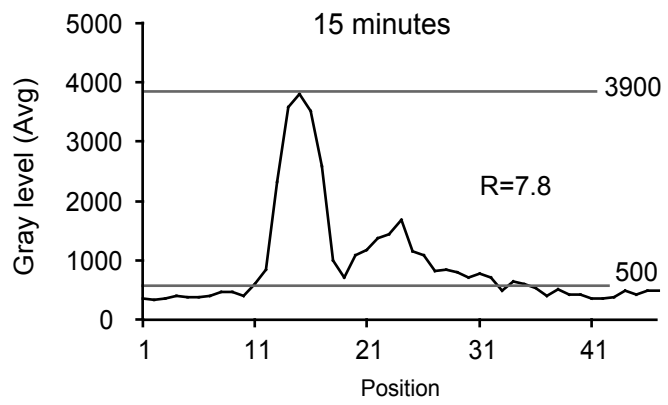
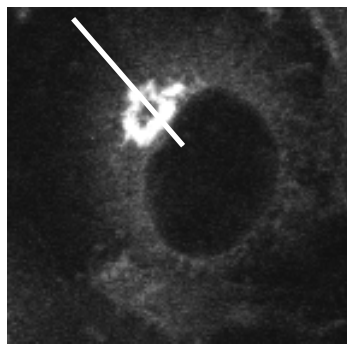
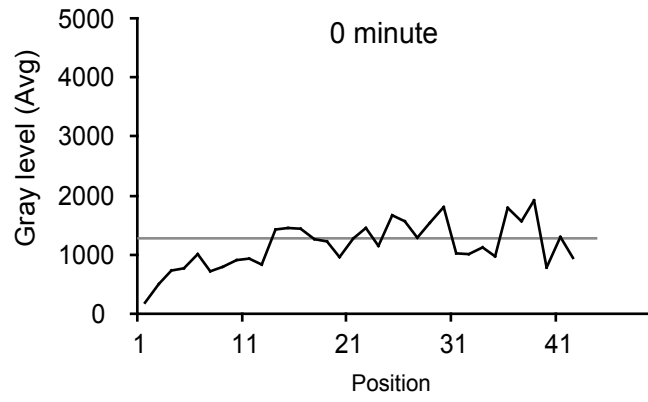
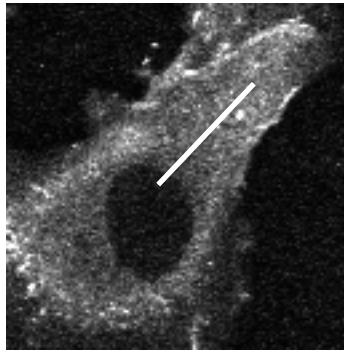


Figure 3

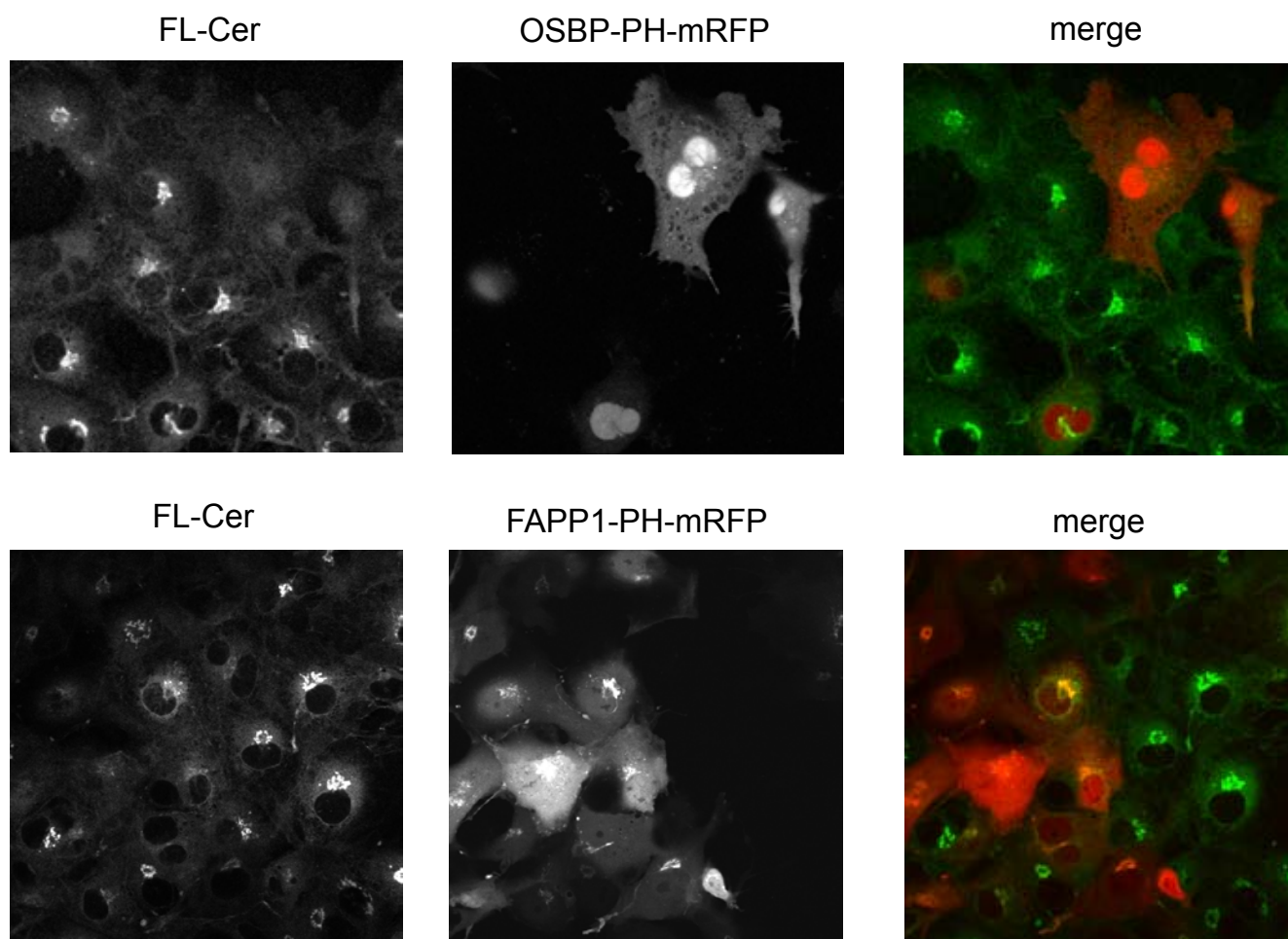
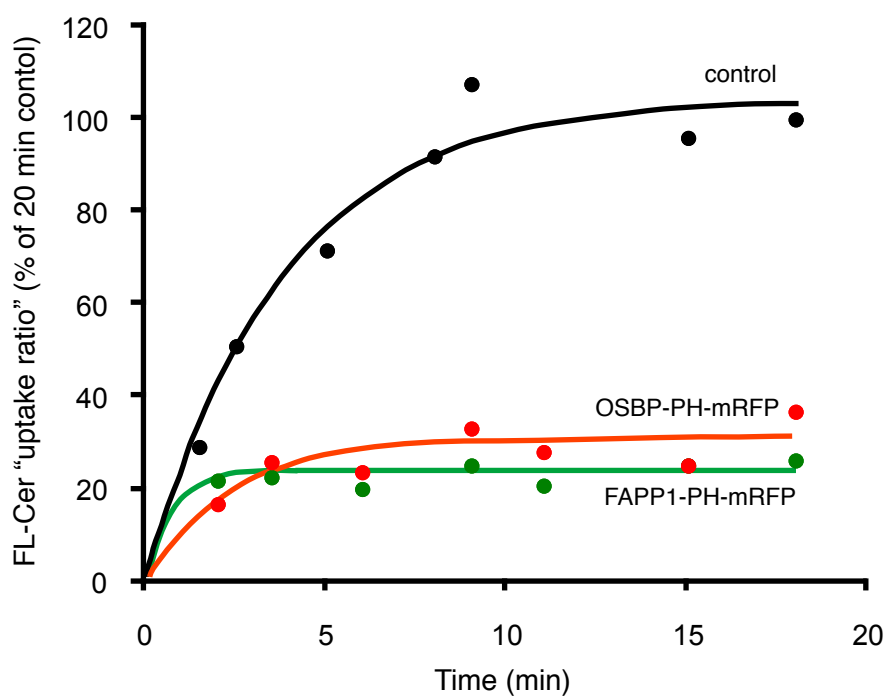
A**B**

Figure 4

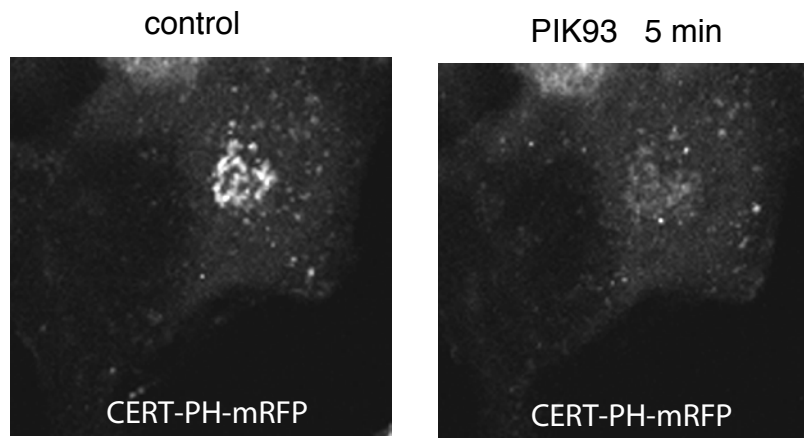
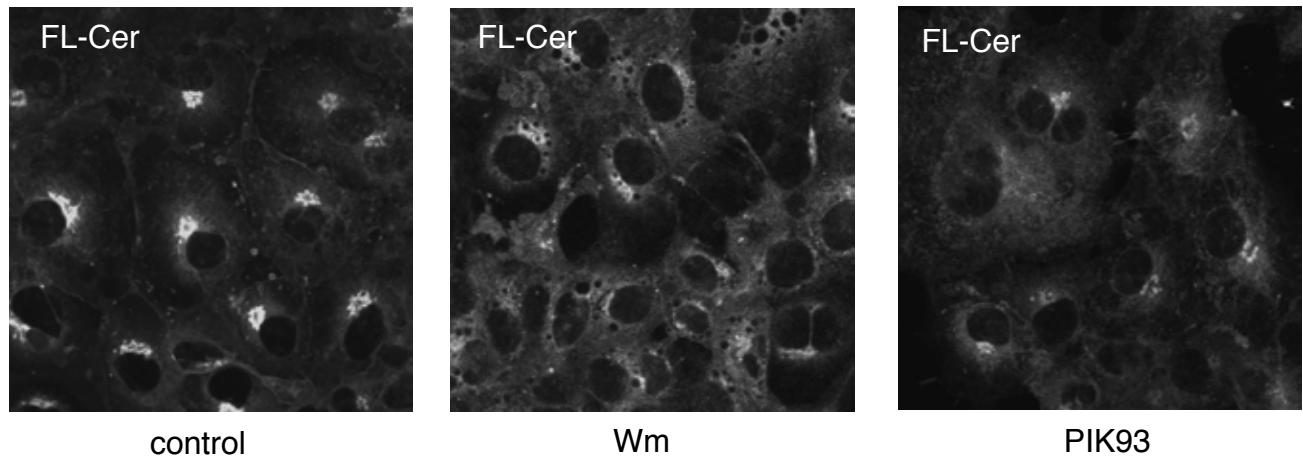
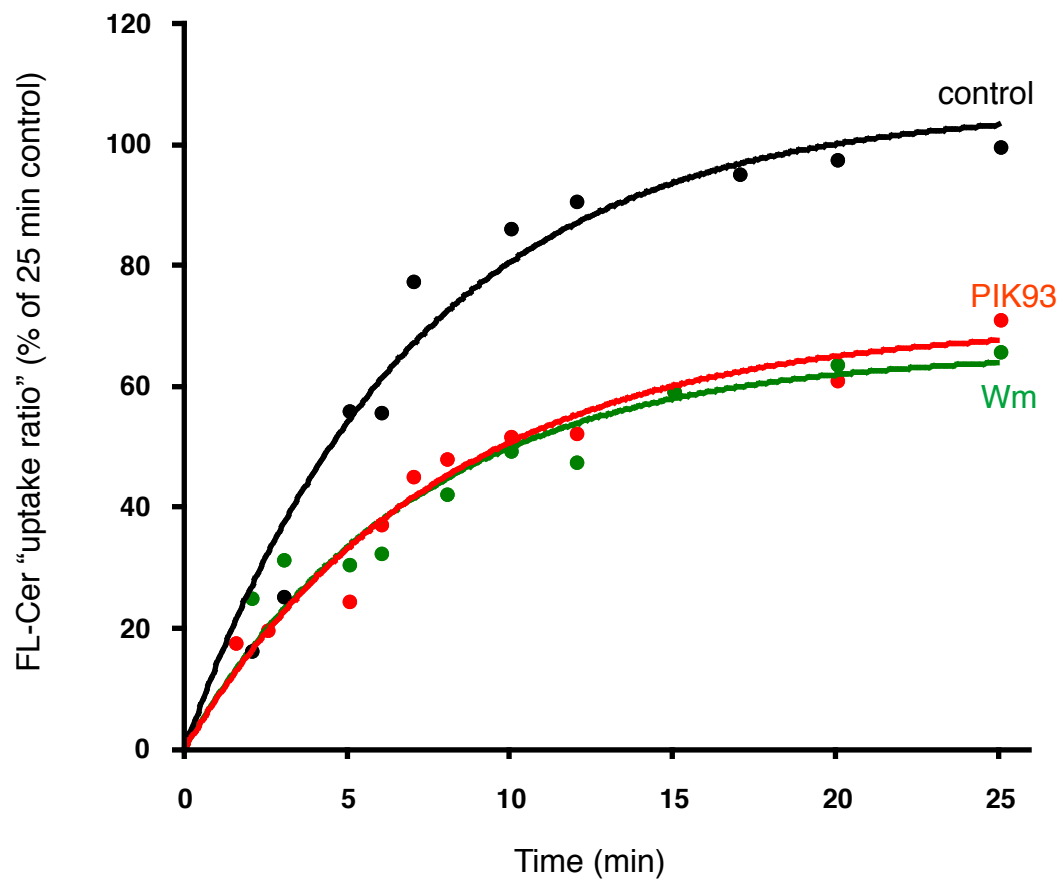
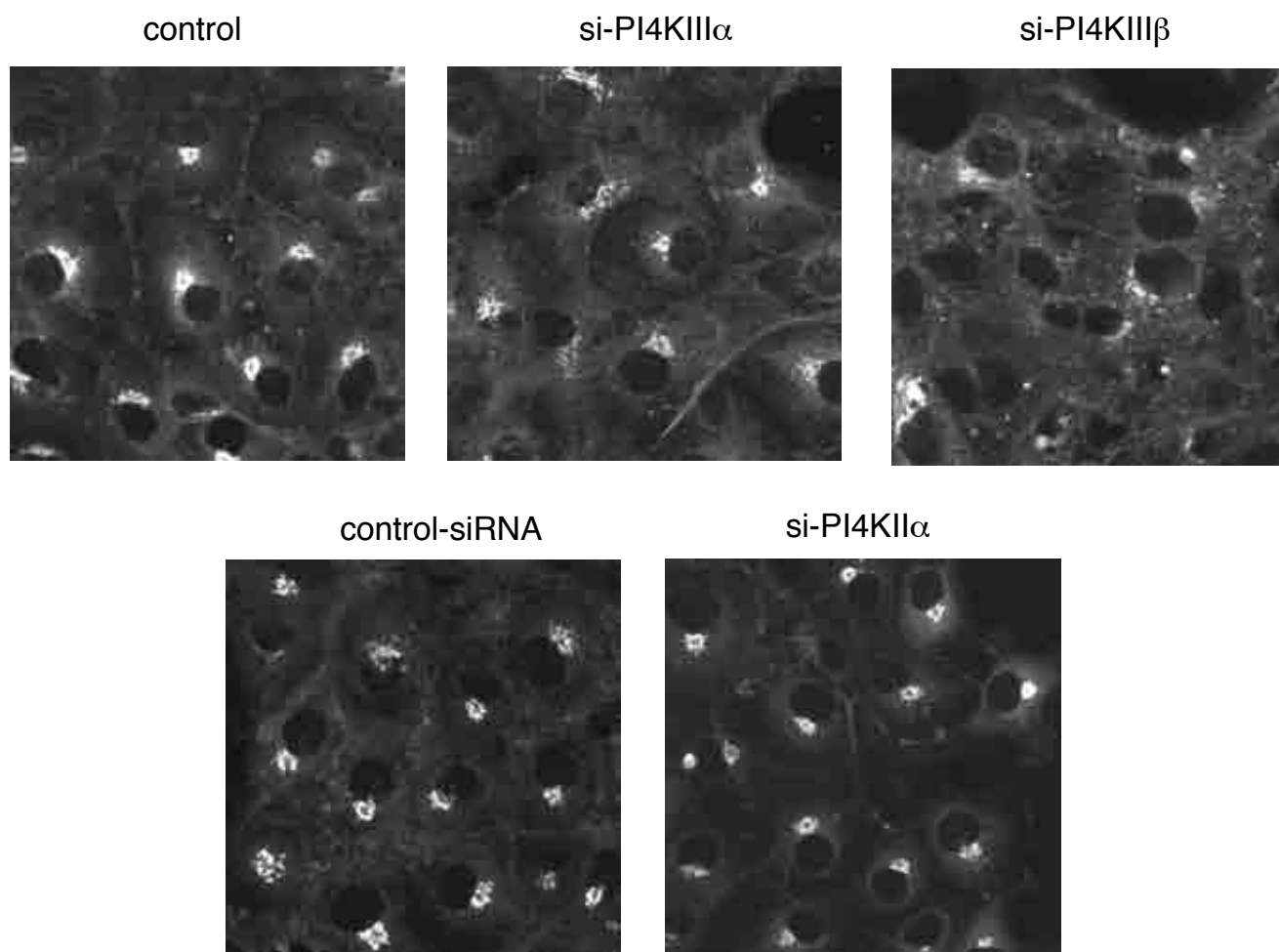
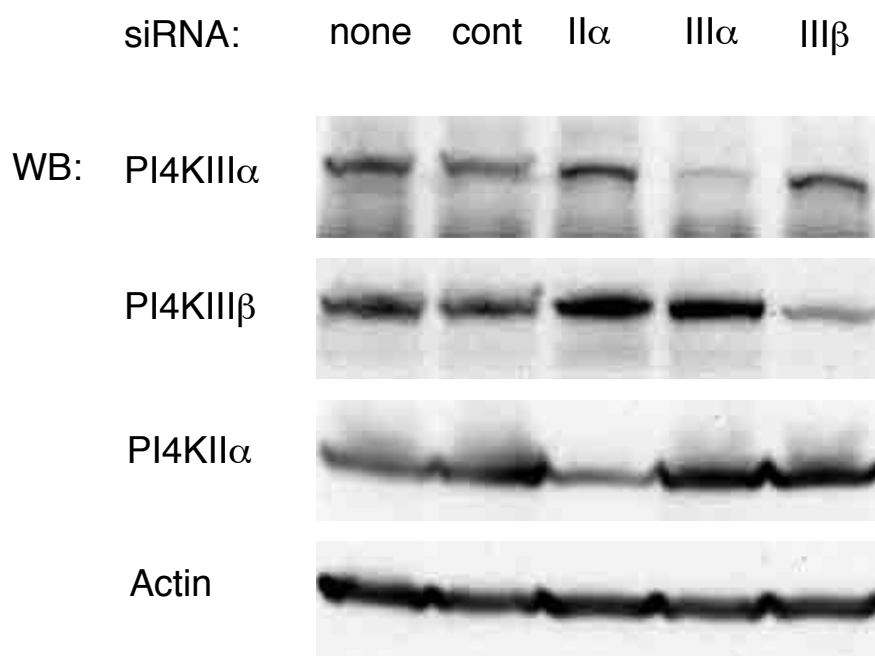
A**B****C**

Figure 5

A**B**

C

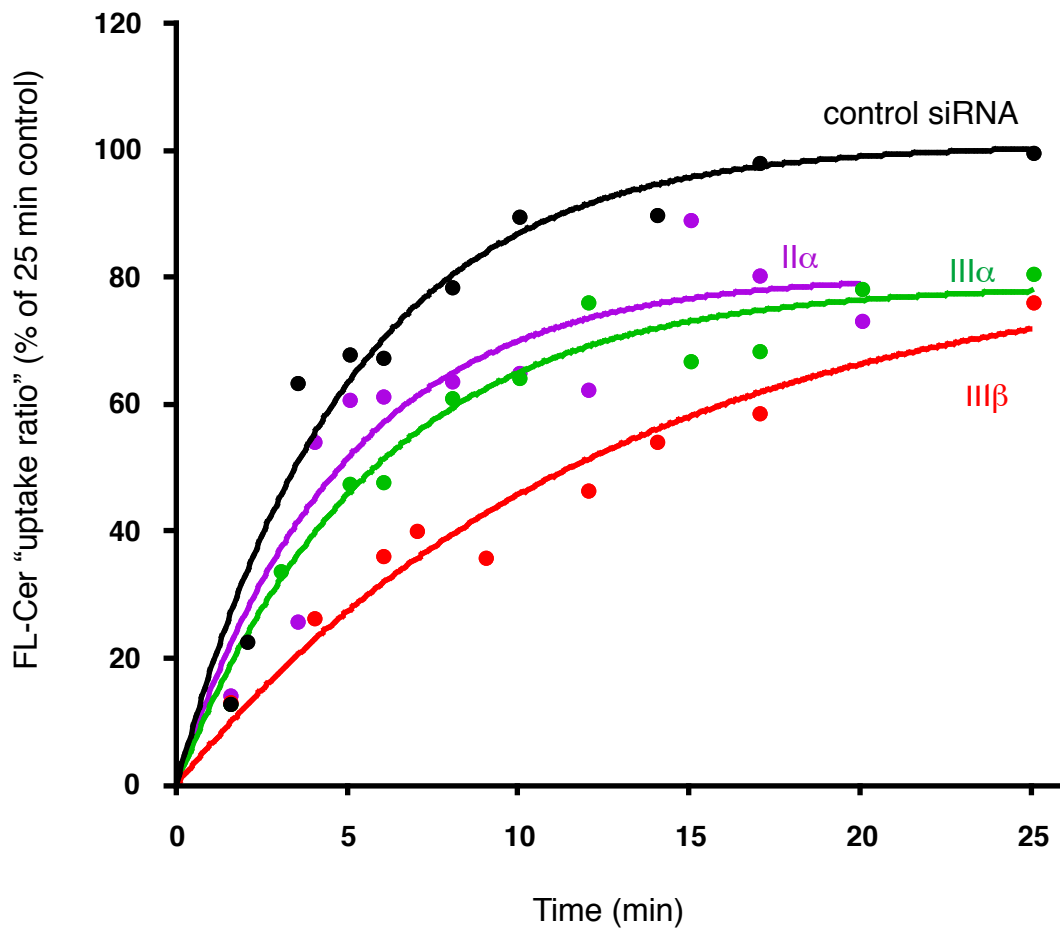
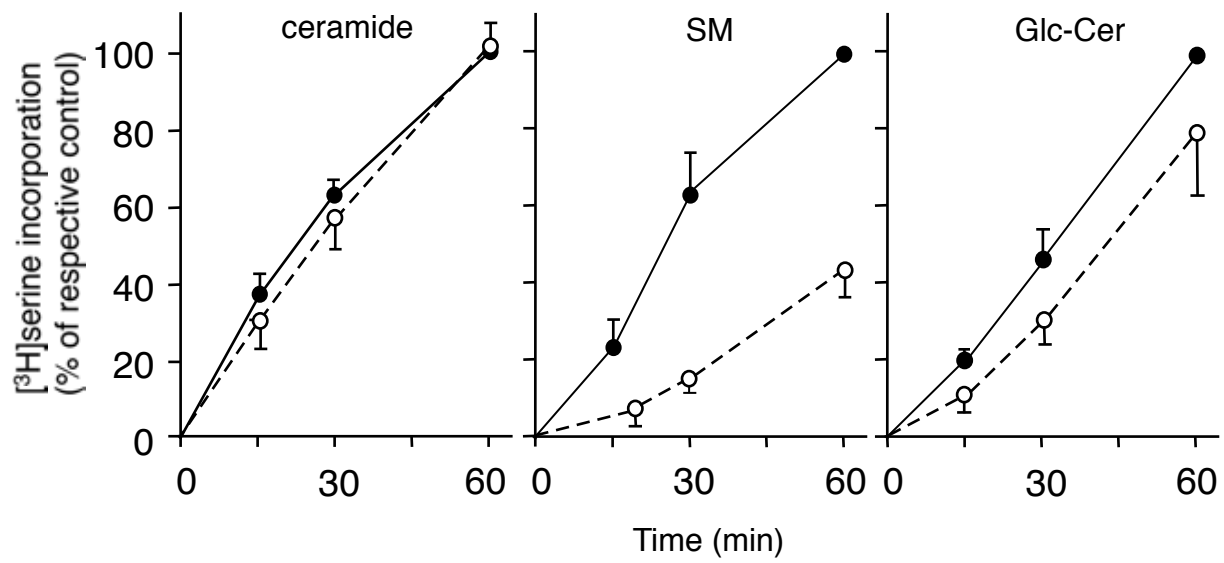
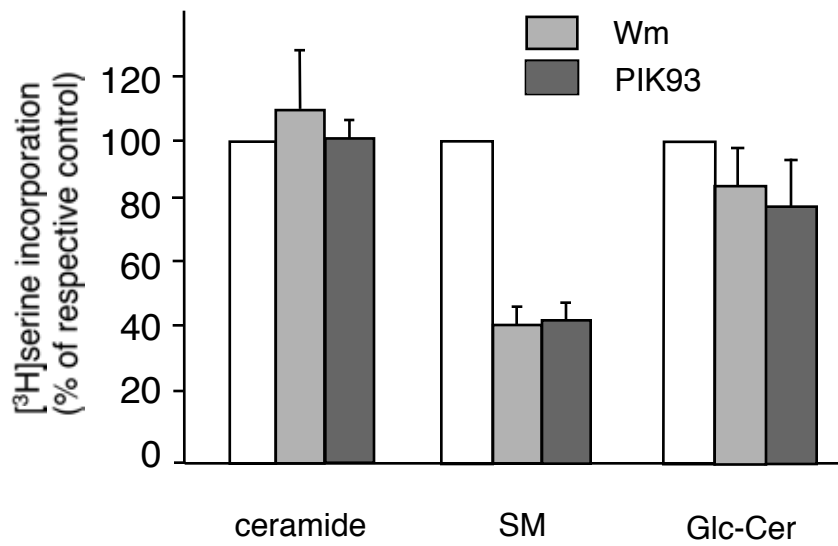


Figure 6C

A**B****C**