Modulation of the Substrate Specificity of the Mammalian Phosphatidylinositol 3-Kinase by Cholesterol Sulfate and Sulfatide

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ABSTRACT: The substrate specificity of the purified, mammalian phosphatidylinositol 3-kinase is subject to modulation by detergents, which are able to switch substrate specificity *in vitro* in favor of $PtdInsP_2$. This effect of the detergents is due to an activation of the phosphatidylinositol biphosphate 3-kinase activity, while the phosphatidylinositol 3-kinase activity is inhibited. The selective inhibition of the phosphatidylinositol 3-kinase activity (p110α/p85α) is shown here also to be observed by employing cholesterol sulfate or sulfatide at low micromolar concentrations, whereas cholesterol and androsterone sulfate fail to inhibit. These naturally occurring sulfated lipids have at these concentrations no effect on the phosphatidylinositol bisphosphate 3-kinase activity but inhibit the manganese-dependent intrinsic protein kinase activity, thus switching substrate specificity toward the more highly phosphorylated inositol lipids. Cholesterol sulfate and sulfatide inhibit the free catalytic subunit p110α but fail to inhibit the homologous phosphatidylinositol 3-kinase from *Saccharomyces cerevisiae* (Vps34p), suggesting that these sulfated lipids act specifically on the mammalian phosphatidylinositol 3-kinase. Consistent with this specificity, the regulatory subunit (p85), which is not conserved in the yeast enzyme, is found to play an important role for the affinity of these inhibitors. The implications for the phosphatidylinositol 3-kinase activity *in vivo* are discussed.

An important component in the mediation of the mitogenic responses of certain growth factor receptors in the PtdIns[1] 3-kinase (Downes & Carter, 1991; Kapeller & Cantley, 1994; Parker & Waterfield, 1992). The mammalian PtdIns 3-kinase is a heterodimer containing a regulatory subunit (p85) and a catalytic subunit (p110). The PtdIns 3-kinase binds to growth factor receptors once they have been activated (Cantley *et al.*, 1991; Fry *et al.*, 1993; Kapeller & Cantley, 1994; Panayotou & Waterfield, 1993). The generation of the PtdIns 3-kinase product, $PtdInsP_3$, is associated with transduction of growth factor signaling in the cell, suggesting that this inositol lipid is actually a second messenger (Downes & Carter, 1991; Kapeller & Cantley, 1994; Parker & Waterfield, 1992; Stephens *et al.*, 1993).

The substrate specificity of the PtdIns 3-kinase determined *in vitro* and *in vivo* is quite different. Whereas the lipid kinase works very well on PtdIns and PtdInsP, $PtdInsP_2$ is a relatively poor substrate (Morgan *et al.*, 1990; Woscholski *et al.*, 1994a). However, *in vivo* metabolic labeling of cells reveals that the lipid kinase actually produces $PtdInsP_3$ (Hawkins *et al.*, 1992; Stephens *et al.*, 1993). This preference for $PtdInsP_2$ can be mimicked by detergents *in vitro* (Woscholski *et al.*, 1994a), suggesting that these detergents are able to alter substrate presentation and/or imitate an unknown regulator of substrate specificity.

Here, we have investigated the substrate specificity of the mammalian PtdIns 3-kinase with respect to lipid substrates and potential modulators. The data reveal that the substrate specificity of the PtdIns 3-kinase can be altered by cholesterol-based detergents (e.g., cholate) and by natural sulfated compounds like cholesterol sulfate or sulfatide. At low micromolar concentrations these natural sulfated lipids selectively inhibit the mammalian PtdIns 3-kinase activity while having negligible effects on the $PtdInsP_2$ 3-kinase activity, consequently altering the substrate specificity.

MATERIAL AND METHODS

Materials and Cells. Inositol lipids and sulfated compounds were obtained from Sigma; [γ-^{32}P]ATP was from Amersham. Maintenance, culture, transformation of yeast (*Schizosaccharomyces pombe*), and the construction of the expression plasmids were as described previously (Kodaki *et al.*, 1994). The free catalytic subunit and the heterodimeric complex of the mammalian PtdIns 3-kinase were expressed in Sf9 cells using the baculovirus system as described earlier (Gout *et al.*, 1992; Hiles *et al.*, 1992).

Purification of the Mammalian PtdIns 3-Kinase. The PtdIns 3-kinase activity expressed in Sf9 cells as either the free catalytic subunit p110α or the heterodimeric complex (p110α/p85α) was purified as described (Woscholski *et al.*, 1994a). The purified heterodimeric complex preparations did have specific activities of about 110−240 milliunits of $PtdInsP_2$ 3-kinase activity/mg of protein when assayed in the presence of cholate (about 50−90% pure as judged by

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[1] Abbreviations: PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol phosphate; $PtdInsP_2$, phosphatidylinositol biphosphate; $PtdInsP_3$, phosphatidylinositol triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IC-50, inhibitor concentration where 50% inhibition occurs.

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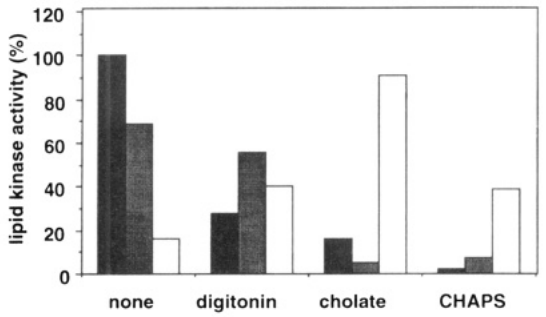

FIGURE 1: Detergent influence of the substrate specificity of the lipid kinase activity. The lipid kinase activity (given as a percentage of the PtdIns lipid kinase activity) of the pure heterodimeric complex (p110α/p85α) was determined using either PtdIns (black bars), PtdInsP (gray bars), or $PtdInsP_2$ (white bars) as a substrate. The assay was performed in the presence of the indicated detergents at their optimal concentration for activating the $PtdInsP_2$ lipid kinase activity (digitonin, 0.2 mM; CHAPS, 1 mM; cholate, 12 mM) using 2.0 mM magnesium. The specific activity of the preparation employed was about 240 milliunits of $PtdInsP_2$ 3-kinase/mg of protein (assayed in the presence of cholate). The data presented represent one of three similar experiments. However, the ratio of ptdInsP and $PtdInsP_2$ 3-kinase activity with respect to its PtdIns activity could vary about 2-fold due to different batches of these lipids and/or different PtdIns 3-kinase preparations employed.

SDS gel electrophoresis) and were stored in 50% (v/v) ethylene glycol at −20 °C prior to use. The free catalytic subunit preparations (about 15−30% pure, 10−20 milliunits of $PtdInsP_2$ 3-kinase/mg of protein when assayed in the presence of cholate) were stored at 4 °C prior to use.

Purification of the PtdIns 3-Kinase from Saccharomyces cerevisiae. The lipid kinase, derived from the VPS34 gene product, Vps34p (Herman & Emr, 1990; Schu *et al.*, 1993), was expressed in the yeast *S. pombe* and the particulate fraction was obtained (Kodaki *et al.*, 1994). The membranes were extracted with 200 mM NaCl and subsequently centrifuged to obtain the partially purified Vps34p/PtdIns 3-kinase. The endogenous PtdIns 3-kinase activity of the *S. pombe* cells is hardly detectable *in vitro*, thus the observed PtdIns 3-kinase activity in the *S. pombe* preparations is due to the overexpressed VPS34p lipid kinase (Kodaki *et al.*, 1994).

Lipid and Protein Kinase Assays. For the Vps34p lipid kinase assay the chloroform-extracted lipids were separated using the borate system (Kodaki *et al.*, 1994) and kinase activity was subsequently quantified using a Phosphorimager (Molecular Dynamics). The lipid and protein kinase assays for the purified mammalian PtdIns 3-kinase preparations was performed as described earlier (Woscholski *et al.*, 1994b). Protein kinase assays were performed using 1 mM manganese. All IC-50 values were determined from double-reciprocal plots.

RESULTS

Cholesterol-Based Detergents Alter the Substrate Specificity of the Mammalian PtdIns 3-Kinase. The substrate specificity of the lipid kinase activity is characterized by a substantial difference with respect to *in vitro* and *in vivo* conditions. Whereas in the living cell $PtdInsP_2$ is the preferred substrate (Kodaki *et al.*, 1994; Stephens *et al.*, 1993), purified PtdIns 3-kinase phosphorylates the less-phosphorylated inositol lipids more efficiently (Figure 1). Under *in vitro* conditions the $PtdInsP_2$ lipid kinase activity can be dramatically increased when the assay is performed in the presence of cholate (Woscholski *et al.*, 1994a). We therefore investigated lipid and protein kinase activities in the presence of differently charged but structurally related detergents (Figure 1).

Table 1: Effect of Compounds on Lipid and Protein Kinase Activity[a]

compound	IC-50 (mM)	p85α phosphorylation [% (mM)]
none	none	100
digitonin	0.04	168 (0.8)
cholate	5.0	103 (14)
CHAPS	0.06	140 (4.8)
cholesterol sulfate	0.01	61 (0.02)
androsterone sulfate	none	98 (0.02)
sulfatide	0.02	39 (0.02)
cholesterol	none	116 (0.02)

[a] The inhibitory concentration where 50% of the PtdIns 3-kinase activity is inhibited (IC-50) was determined for each indicated compound using double-reciprocal plots (not shown). The protein and lipid kinase assays were performed as described in Materials and Methods. The effect of the compounds on the protein kinase activity, given as the percentage of uninhibited p85α phosphorylation, was determined using either detergents at their critical micellar concentration (see *A Guide to the Uses of Detergents in Biology and Biochemistry* from Calbiochem, San Diego, CA, and references within) or the other compounds at 20 μM, as indicated in parentheses in the table. Androsterone sulfate showed up to 1 mM no significant inhibition. Cholesterol was tested up to 125 μM without showing any significant inhibition of either lipid or protein kinase activity.

All detergents employed inhibited the lipid kinase activity when used with PtdIns or PtdInsP as a substrate and activated the lipid kinase activity when used with $PtdInsP_2$ as a substrate. The lipid kinase activity therefore acted on PtdIns and PtdInsP in a similar fashion regardless of the detergent employed, while the activity on $PtdInsP_2$ was activated by the detergents, particularly by the anionic detergent cholate. The presence of digitonin provides a context wherein all substrates are employed almost equally, while cholate and CHAPS caused a change in the substrate specificity in favor of $PtdInsP_2$.

All tested detergents inhibited the PtdIns 3-kinase activity below their critical micelle concentrations (Table 1). However, whereas digitonin and CHAPS were quite potent inhibitors for the PtdIns 3-kinase activity (IC-50, 40−60 μM), cholate was about 100 times weaker as an inhibitor. Since the PtdIns 3-kinase is a dual-specificity kinase, which also phosphorylates its regulatory subunit, p85 (Carpenter *et al.*, 1993; Dhand *et al.*, 1994; Woscholski *et al.*, 1994b), we investigated as well the effect of the detergents on the Mn-dependent protein kinase activity. Employing the detergents at their critical micelle concentrations (3−10 times higher than the corresponding IC-50 concentration for the lipid kinase inhibition) revealed that none of the detergents inhibited the p85 phosphorylation. CHAPS and digitonin actually slightly activated the protein kinase activity.

Cholesterol Sulfate and Sulfatide Inhibit Selectively the PtdIns 3-Kinase. Searching for a natural lipid reflecting the structural properties of CHAPS and cholate led us to employ cholesterol sulfate and sulfatide. We therefore investigated the effects of the naturally occurring cholesterol sulfate. As shown in Figure 2 (top panel), cholesterol sulfate at low micromolar concentrations specifically inhibited the lipid kinase activity when PtdIns was employed as a substrate. A

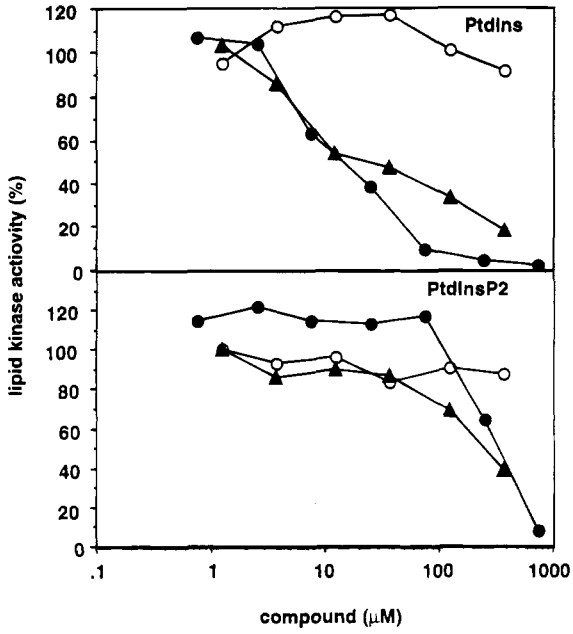

FIGURE 2: Specific inhibition of the PtdIns lipid kinase activity by sulfated lipids. The lipid kinase activity of the heterodimeric complex (p110α/p85α) was determined using either PtdIns (top panel) or $PtdInsP_2$ (bottom panel) as a substrate (2 mM magnesium) in the presence of the indicated concentrations of androsterone sulfate (open circles), cholesterol sulfate (closed circles), or cerebroside sulfatide (closed triangles). The activity is given as the percentage of the activity in the absence of any of these compounds present (100% activity represents 30 milliunits of PtdIns 3-kinase/mL and 6 milliunits of $PtdInsP_2$ 3-kinase/mL), employing the same 3-kinase preparation as described in Figure 1. The data presented represent one of three similar experiments.

control compound, androsterone sulfate, showed only negligible effects on the lipid kinase activity.

For comparison, we tested the sulfated cerebroside lipid, sulfatide, which indeed inhibited the PtdIns lipid kinase activity, although slightly higher concentrations were needed and the inhibitory effect of $PtdInsP_2$ phosphorylation was stronger when compared to cholesterol sulfate (Figure 2, bottom panel). Although no substantial activation of the $PtdInsP_2$ lipid kinase activity was observed (Figure 2, bottom panel), these naturally occurring lipids inhibited selectively the PtdIns kinase activity in striking similarity to the tested cholesterol-type detergents (see above and Figure 1). Interestingly, the apparently affinity for cholesterol sulfate and sulfatide is in the range of 10−20 μM (corresponding to 4−8 mol %), which is 2−4-fold lower compared to digitonin or CHAPS (Table 1). However, both compounds, cholesterol sulfate and sulfatide, altered the substrate specificity in favor of polyphosphoinositides. Both sulfated compounds inhibited also the intrinsic protein kinase activity of the PtdIns 3-kinase, while cholesterol itself and androsterone sulfate did not have any significant effect on either lipid or protein kinase activity (Table 1). The effects on the p85 phosphorylation are in contrast to the observed effects of the detergents.

Sulfatide and Cholesterol Sulfate Do Not Affect the Dependence on ATP or Lipid. The data presented demonstrate that cholesterol sulfate and sulfatide alter the substrate specificity in favor of $PtdInsP_2$, an effect which was also observed when structurally related detergents were employed (see above). It has been shown recently that cholate alters the substrate specificity in favor of $PtdInsP_2$ by increasing

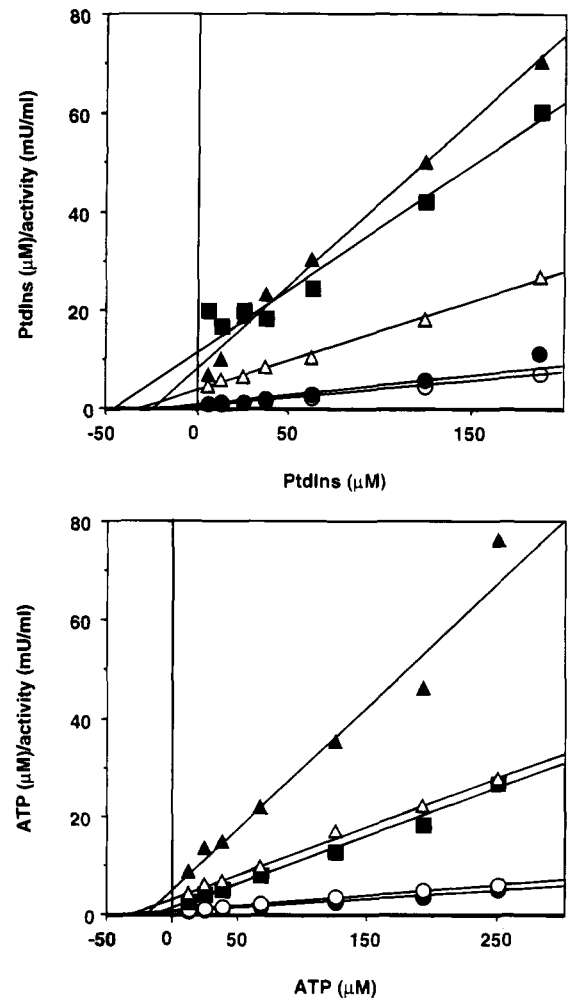

FIGURE 3: Characterization of the inhibition by the sulfated lipids with respect to ATP and PtdIns. The PtdIns kinase activity of the heterodimeric complex (110 milliunits of PtdIns 3-kinase activity/mg of protein; 50% purity) was determined in the presence of 0.125 mM androsterone sulfate (open circles), cholesterol sulfate (closed squares), sulfatide (open triangles), or 12 mM cholate (closed triangles) with varying concentrations of either ATP (bottom panel) or PtdIns (top panel) while the corresponding other substrate was kept constant (ATP, 0.125 mM; PtdIns, 0.25 mM). The PtdIns 3-kinase was tested as well in the absence of any compounds as a control (closed circles). The data are presented as a single-reciprocal plot (Hanes plot). The analysis by linear regression resulted in correlation coefficients higher than 0.98 for all shown data. The data presented represent one of two similar experiments.

the affinity for ATP with this specific lipid substrate (Woscholski *et al.*, 1994a). We therefore investigated the effects of the three natural sulfated lipids and cholate (for comparison) on the K_m values for the substrates PtdIns and ATP.

As shown in Figure 3, all three sulfated compounds had only slight effects on the apparent affinity of ATP (the K_m value was actually 3-fold decreased for cholesterol sulfate and 2-fold decreased for cholate), but with the exception of the control compound androsterone sulfate, all reduced substantially the V_{max} of the enzyme. Cholesterol sulfate (K_m for PtdIns, 44 μM) and sulfatide (K_m for PtdIns, 31 μM) increased 2−3-fold the K_m values for PtdIns, while androsterone sulfate (K_m for PtdIns, 11 μM) did not change significantly the apparent affinity for PtdIns (K_m for PtdIns, 14 μM). Although both sulfated compounds produced an approximately 3-fold decrease in the apparent PtdIns affinity,

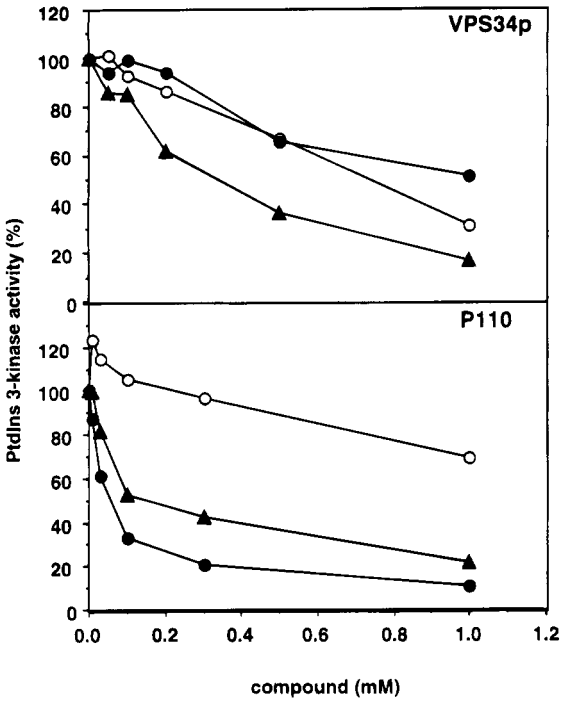

FIGURE 4: Effect of sulfated lipids on the free catalytic subunit p110α and the structurally related Vps34p/PtdIns 3-kinase. The PtdIns 3-kinase activity (43 microunits of PtdIns 3-kinase activity/mg of protein) of the partially purified VPS34 kinase from the yeast *S. cerevisiae* (top panel) and purified free catalytic subunit (20 milliunits of $PtdInsP_2$ 3-kinase activity/mg of protein; assayed in the presence of cholate) of the mammalian PtdIns 3-kinase (bottom panel) were tested in the presence of the indicated concentration of sulfated lipids (for further information see Figure 2). Only PtdIns was employed as a substrate. The data presented represent one of two similar experiments. However, the activating effect of low concentrations of androsterone sulfate is not always as evident.

this change is unlikely to be responsible for the observed strong inhibition of the catalytic activity at the high inhibitor concentration (125 μM) employed (see Figure 2 for comparison).

Since the PtdIns concentration in the assay is 0.25 mM, which is 5-fold higher than any measured K_m value for PtdIns, any competition by the sulfated compounds for the lipid substrate is unlikely to occur. Furthermore, if the K_m values for PtdIns were analyzed in the presence of 50 μM sulfated compound, no significant decrease in lipid affinity could be observed (data not shown). Thus, the change in the K_m for PtdIns is due to the high inhibitor concentration (IC-50s are about 10−20 μM when assayed in the presence of 250 μM PtdIns) and the low PtdIns concentrations employed (Figure 3). Cholate had no significant effect on the ATP or PtdIns affinity, which as described previously is in contrast to the effect when tested with $PtdInsP_2$ as a substrate (Woscholski *et al.*, 1994a).

Sulfatide and Cholesterol Sulfate Inhibit the Free Catalytic Subunit of the Mammalian PtdIns 3-Kinase but Not the PtdIns 3-Kinase Derived from S. cerevisiae (Vps34p). Since these sulfated compounds affected the catalytic activity of the p85/p110 complex, we investigated whether similar inhibition is observed for the free p110 catalytic subunit and likewise for the homologous PtdIns 3-kinase derived from the yeast *S. cerevisiae*, the product of the VPS34 gene, Vps34p (Herman & Emr, 1990; Schu *et al.*, 1993). As shown in Figure 4, the Vps34p lipid kinase is inhibited at much higher concentrations by both cholesterol sulfate and the control compound androsterone sulfate, which is not effective on mammalian PtdIns 3-kinase, indicating that the VPS34 kinase does not show any comparable high sensitivity to these sulfated compounds (IC-50 values higher than 1 mM). However, sulfatide did inhibit the Vps34p lipid kinase with greater potency than the cholesterol-based compounds (IC-50 value about 0.5 mM).

The free catalytic subunit p110 (Figure 4) was characterized by about a 10-fold lower affinity for the sulfated lipids (IC-50, 80−120 μM) compared to the heterodimeric complex (Table 1) but at least a 10-fold higher affinity as compared to the Vps34p lipid kinase (Figure 4). Whereas the yeast enzyme was equally affected by cholesterol sulfate and androsterone sulfate, the free catalytic subunit p110 was specifically inhibited by cholesterol sulfate and sulfatide. Thus, the PtdIns 3-kinase is affected by these compounds regardless of whether the kinase is presented as the complexed or free catalytic subunit, although the complexed form of the PtdIns 3-kinase is more sensitive to the compounds.

DISCUSSION

The mammalian PtdIns 3-kinase is characterized by a differential substrate specificity *in vivo* and *in vitro* (Kodaki *et al.*, 1994; Stephens *et al.*, 1993; Woscholski *et al.*, 1994a). The *in vivo* substrate $PtdInsP_2$ is *in vitro* a very poor substrate, except when presented in a mixed micellar assay using detergents (Figure 1). Even nonionic detergents (digitonin) are able to activate the kinase activity toward $PtdInsP_2$, which is in contrast to the strong inhibition of the lipid kinase activity (PtdIns and $PtdInsP_2$) by nonionic detergents like Triton X-100 or NP-40 (data not shown). The cholesterol-based detergents not only activate the $PtdInsP_2$ 3-kinase activity but also inhibit strongly the activity toward the phosphoinositides PtdIns and PtdInsP.

Cholesterol sulfate, a naturally occurring sulfated lipid, inhibits the PtdIns 3-kinase activity without affecting the $PtdInsP_2$ 3-kinase activity (Figure 2). This selective inhibition results effectively in a shift of substrate specificity toward $PtdInsP_2$. Similar effects could be observed by employing sulfatide, a sulfated sphingosine-based lipid, whereas androsterone sulfate or cholesterol itself had no effect on the mammalian PtdIns 3-kinase activity. Both sulfated lipids (cholesterol sulfate and sulfatide) are able to inhibit the intrinsic protein kinase activity of the mammalian PtdIns 3-kinase (Table 1). Thus, the sulfated lipids are able to inhibit specifically the protein kinase activity and the PtdIns 3-kinase activity at low micromolar concentrations but tolerate $PtdInsP_2$ as a substrate. They therefore have the ability to shift the catalytic activity toward $PtdInsP_2$ by inhibiting the autophosphorylation (Table 1) and the PtdIns kinase activity (Figure 2).

The affinity of cholesterol sulfate and sulfatide for the hetereodimeric form is about 10−20 μM, whereas the Vps34p lipid kinase seems to be much less sensitive to these sulfated lipids (Figures 2 and 4). Therefore, it seems that the compounds inhibit specifically the mammalian PtdIns 3-kinase. The binding of the inhibitors is probably influenced by the regulatory subunit p85, since the inhibitor potency is lower for the free catalytic subunit p110α (Figure 4). Taking these observations together, it is likely that the catalytic subunit p110α mediates the action of the sulfated

compounds, indicating that this subunit could have a binding site for these compounds. This is consistent with only minor effects of these inhibitors being observed on the affinity for either ATP and PtdIns (Figure 3). Furthermore, both sulfated compounds still inhibit the PtdIns 3-kinase activity if PtdIns is presented as mixed micelles using cholate (data not shown). Thus, the lipid presentation seems not to be important in the inhibitory action of these sulfated compounds.

Since cholesterol sulfate and sulfatides are naturally occurring membrane components, a shift in substrate specificity induced by these compounds may contribute to the observed difference in substrate specificity *in vivo* and *in vitro*. The specific effect of cholesterol sulfate, which is actually quite abundant in epithelial cells (Jetten *et al.*, 1989; Williams & Elias, 1981; Williams *et al.*, 1987), suggest that PtdIns 3-kinase might play a role in the development of certain diseases (e.g., ichthyosis) associated with high levels of cholesterol sulfate in the plasma membrane (Williams & Elias, 1981) and also in the development of epithelial cells (Jetten *et al.*, 1989; Rearick *et al.*, 1987). Cholesterol sulfate is known to activate protein kinase C-η at low micromolar concentrations (Ikuta *et al.*, 1994). A recently cloned protein kinase C-related protein kinase, termed PRK1 (Palmer *et al.*, 1995), is also activated at low micromolar concentrations by cholesterol sulfate (R. Palmer, unpublished observation), suggesting that cholesterol sulfate may act upon a number of lipid-interacting enzymes involved in signal transduction.

Sulfatides are found in the membranes of nervous tissue (Svennerholm *et al.*, 1992), as is PtdIns 3-kinase (Morgan *et al.*, 1990), indicating that in brain PtdIns 3-kinase might be modulated with respect to its substrate specificity and autophosphorylation-dependent regulation (Woscholski *et al.*, 1994a) by sulfatides. Similar to cholesterol sulfate, sulfatides have been characterized as activators of different enzymes (e.g., cyclic nucleotide phosphodiesterase and protein kinase C) involved in signal transduction (Fujiki *et al.*, 1986; Higashi & Yamagata, 1992). Sulfated lipids also appear to play some part in the entry of viruses and monocellular parasites into mammalian cells. Sulfatides have been described to bind to the gp120 glycoprotein of the human immunodeficiency virus (McAlarney *et al.*, 1994). Cholesterol sulfate and sulfatide both bind to the circumsporozoite protein from the malaria plasmodium (Pancake *et al.*, 1992). Since a PtdIns 3-kinase may be associated with membrane vesicle transport (Kodaki *et al.*, 1994; Schu *et al.*, 1993), it is possible that these issues are connected.

In conclusion, it has been shown that the substrate specificity of the PtdIns 3-kinase (p110α/p85α) is modified by two naturally occurring sulfated lipids. Whether these effects are the basis of the distinctive specificity of the PtdIns 3-kinase *in vivo* remains to be established.

REFERENCES

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Soltoff, S. (1991) *Cell 64*, 281–302.

Carpenter, C. L., Auger, K. R., Duckworth, B. C., Hou, W. M., & Schaffhausen, B. (1993) *Mol. Cell. Biol. 13*, 1657–1665.

Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., & Yonezawa, K. (1994) *EMBO J. 13*, 522–533.

Downes, C. P., & Carter, A. N. (1991) *Cell. Signalling 3*, 501–513.

Fry, M. J., Panayotou, G., Booker, G. W., & Waterfield, M. D. (1993) *Protein Sci. 2*, 1785–1797.

Fujiki, H., Yamashita, K., Suganuma, M., Horichi, T., Taniguchi, N., & Makita, A. (1986) *Biochem. Biophys. Res. Commun. 138*, 153–158.

Gout, I., Dhand, R., Panayotou, G., Fry, M. J., Hiles, I., Otsu, M., & Waterfield, M. D. (1992) *Biochem. J. 288*, 395–405.

Hawkins, P. T., Jackson, T. R., & Stephens, L. R. (1992) *Nature 358*, 157–159.

Herman, P. K., & Emr, S. D. (1990) *Mol. Cell. Biol. 10*, 6742–6754.

Higashi, H., & Yamagata, T. (1992) *FEBS Lett. 314*, 53–57.

Hiles, I. D., Otsu, M., Volinia, S., Fry, M. J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsuan, J. J., Courtneidge, S. A., Parker, P. J., & Waterfield, M. D. (1992) *Cell 70*, 419–429.

Ikuta, T., Chida, K., Tajima, O., Matsuura, Y., Iwamori, M., Ueda, Y., Mizuno, K., Ohno, S., & Kuroki, T. (1994) *Cell Growth Differ. 5*, 943–947.

Jetten, A. M., George, M. A., Nervi, C. N., Boone, L. R., & Rearick, J. I. R. (1989) *J. Invest. Dermatol. 92*, 203–209.

Kapeller, R., & Cantley, L. C. (1994) *Bioessays 16*, 565–576.

Kodaki, T., Woscholski, R., Emr, S., Waterfield, M. D., Nurse, P., & Parker, P. J. (1994) *Eur. J. Biochem. 219*, 775–780.

McAlarney, T., Apostolski, S., Lederman, S., & Latov, N. (1994) *J. Neurosci. Res. 37*, 453–460.

Morgan, S. J., Smith, A. D., & Parker, P. J. (1990) *Eur. J. Biochem. 191*, 761–767.

Palmer, R. H., Ridden, J., & Parker, P. J. (1995) *Eur. J. Biochem. 227*, 344–351.

Panayotou, G., & Waterfield, M. D. (1993) *Bioessays 15*, 171–177.

Pancake, S. J., Holt, G. D., Mellouk, S., & Hoffman, S. L. (1992) *J. Cell Biol. 117*, 1351–1357.

Parker, P. J., & Waterfield, M. D. (1992) *Cell Growth Differ. 3*, 747–752.

Rearick, J. I., Albro, P. W., & Jetten, A. M. (1987) *J. Biol. Chem. 262*, 13069–13074.

Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., & Emr, S. D. (1993) *Science 260*, 88–91.

Stephens, L. R., Jackson, T. R., & Hawkins, P. T. (1993) *Biochim. Biophys. Acta 1179*, 27–75.

Svennerholm, L., Bostrom, K., Fredman, P., Jungbjer, B., Mansson, J. E., & Rynmark, B. M. (1992) *Biochim. Biophys. Acta 1128*, 1–7.

Williams, M. L., & Elias, P. M. (1981) *J. Clin. Invest. 68*, 1404–1410.

Williams, M. L., Rutherford, S. L., & Feingold, K. R. (1987) *J. Lipid Res. 28*, 955–967.

Woscholski, R., Dhand, R., Fry, M. J., Waterfield, M. D., & Parker, P. J. (1994a) *J. Biol. Chem. 269*, 25067–25072.

Woscholski, R., Kodaki, T., McKinnon, M., Waterfield, M. D., & Parker, P. J. (1994b) *FEBS Lett. 342*, 109–114.

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