Phospholipids Can Switch the GTPase Substrate Preference of a GTPase-activating Protein*

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The major cellular inhibitors of the small GTPases of the Ras superfamily are the GTPase-activating proteins (GAPs), which stimulate the intrinsic GTP hydrolyzing activity of GTPases, thereby inactivating them. The catalytic activity of several GAPs is reportedly inhibited or stimulated by various phospholipids and fatty acids in vitro, indicating a likely physiological role for lipids in the regulation of small GTPases. We find that the p190 RhoGAP, a potent GAP for the Rho and Rac GTPases, is similarly sensitive to phospholipids. Interestingly, however, several of the tested phospholipids were found to effectively inhibit the RhoGAP activity of p190 but stimulate its RacGAP activity. Thus, phospholipids have the ability to "switch" the GTPase substrate preference of a GAP, thereby providing a novel regulatory mechanism for the small GTPases.

GTase-activating proteins (GAPs) for the small GTPases of the Ras superfamily are potent stimulators of intrinsic GTP hydrolyzing activity and are the major down-modulators of the Ras superfamily are potent stimulators of intrinsic GTP hydrolysis, thereby inactivating them. The catalytic function of the RacGAP, n-chimaerin, is inhibited by some phospholipids and stimulated by others (7). Several GAPs for the Arf GTPases depend on phosphoinositides for GAP activity (8–10).

Here, we report that phospholipids strongly influence the GAP activity of the p190 RhoGAPs (p190A and p190B), which regulate both Rho and Rac GTPases (11, 12). Interestingly, some of the phospholipids are potent inhibitors of p190 RhoGAP activity but are stimulators of its RacGAP activity. This finding indicates that phospholipids have the potential to "switch" the GAP substrate preference for a GAP, thereby providing a novel regulatory mechanism for determining signaling specificity in vivo.

EXPERIMENTAL PROCEDURES

Materials—t-α-Phosphatidylethanolamine (PE), t-α-phosphatidylcholine (PC), t-α-phosphatidylserine (PS), L-phosphatidate (PA), and PIP2 were obtained from Sigma. Radioactive nucleotides ([γ-32P]GTP and [α-32P]GTP) were from PerkinElmer Life Sciences. Nitrocellulose filters were from Schleicher & Schuell.

Preparation of Recombinant Proteins—Hexa-histidine-tagged full-length p190A and p190B proteins and the GAP domain-containing some of the phospholipids are potent inhibitors of p190 RhoGAP activity but are stimulators of its RacGAP activity. This finding indicates that phospholipids have the potential to “switch” the GAP substrate preference for a GAP, thereby providing a novel regulatory mechanism for determining signaling specificity in vivo.

The in vivo regulation of GAPs is poorly understood, but it catalytic activity of several GAPs is reportedly inhibited or stimulated by various phospholipids and fatty acids in vitro, indicating a likely physiological role for lipids in the regulation of small GTPases. We find that the p190 RhoGAP, a potent GAP for the Rho and Rac GTPases, is similarly sensitive to phospholipids. Interestingly, however, several of the tested phospholipids were found to effectively inhibit the RhoGAP activity of p190 but stimulate its RacGAP activity. Thus, phospholipids have the ability to “switch” the GTPase substrate preference of a GAP, thereby providing a novel regulatory mechanism for the small GTPases.
others, intrinsic GTP hydrolysis rates differ significantly for the RhoA and Rac1 GTPases. In a typical experiment, 50% of RhoA-bound $^{32}$P-GTP is hydrolyzed in 15 min at 30 °C, whereas with Rac1, 30% of GTP hydrolysis occurs in 5 min at 20 °C (Fig. 1A). Notably, the intrinsic enzymatic activity of neither of the small GTPases is influenced by PS.

The purified p190A and p190B proteins exhibit a comparable catalytic GTPase stimulating activity on prenylated RhoA and Rac1 GTPases. When p190 GAP activity is assayed in the presence of PS, the RhoGAP activity of both p190 proteins is substantially inhibited. In a typical experiment, the amount of GTP-bound Rho remaining after incubation with p190A or p190B increases from 50% to 80 and 90%, respectively, in the presence of PS (Fig. 1A). Strikingly, when Rac1 is used as substrate, the presence of PS in the GAP assay results in a considerable decrease in GTP-bound Rac, when compared with reactions without PS (Fig. 1, B and C). This effect of PS is seen both with p190A and p190B and indicates that PS stimulates the RacGAP activity of the p190 proteins.

The RhoGAP-inhibiting and RacGAP-promoting effect of PS is also seen with an isolated C-terminal fragment of p190. GTP hydrolysis by prenylated RhoA and Rac1 was measured in A, B, E, and F by filter binding assay and in C and D by charcoal precipitation. The concentration of the C-terminal domain of p190A was varied between 0.24 and 24 nM; PS was present at 0.1 mg/ml. In A–D, the result of one typical experiment of six (A and B) or three (C and D) similar ones is shown. The results of 29 and 61 measurements are represented statistically in E and F, respectively.

The RhoGAP-inhibiting and RacGAP-promoting effect of PS on p190 GAP activity was highly significant, and the findings were reproducible using several independent preparations of the GAP and the GTPases. On average, in the presence of PS, the level of GTP-bound RhoA following p190 incubation was increased from 29.2 ± 6.0% to 65.8 ± 6.9%, whereas the level of GTP-bound Rac decreased from 62.6 ± 3.75% to 37.1 ± 3.9% (Fig. 2, E and F). Enhancement of RacGAP activity by PS is also seen at 30 °C (decreasing the reaction time to 90 s), indicating that the opposing effect of PS on substrate specificity is not due to the temperature difference in the RhoGAP and RacGAP assays.

The filter binding assay of GTPase activity can potentially be
Phospholipids Regulate p190 GAP Specificity

A notable difference between these findings is that in our studies, the effect of phospholipids is to switch the GAP catalytic activity of the p190 GAPs. The observation that several of the tested lipids inhibit the p190 GAP activity but stimulate its RacGAP activity is provocative, suggesting that phospholipids can alter the catalytic substrate specificity of a GAP. Although phospholipids have previously been shown to inhibit or stimulate catalytic activity for some GAPs, this is the first demonstration that a phospholipid can exert an opposing regulatory effect on a GAP depending on the provided GAP catalytic region. Interestingly, phosphorylation of a Rac/Cdc42 GAP, called MgcRacGAP, allows it to function as a RacGAP as well as a RhoGAP (4). Thus, phosphorylation may be another mechanism by which the substrate preference of GAPs is regulated. A notable difference between these findings is that in our studies, the effect of phospholipids is to switch the GAP catalytic preference of the GAP, whereas MgcRacGAP phosphorylation...
results in the acquisition of an additional substrate interaction. The fact that the phospholipid effect on p190 depends on GTPase prenylation raises a general consideration for the analysis of GAP specificity. Notably, in every previously reported case of a phospholipid-mediated regulation of GAP activity, the GTPases used for analysis were prepared in bacteria and, therefore, lacked prenylation. Thus, analogous assays with prenylated GTPases could reveal phospholipid effects on substrate preference by other GAPs similar to those described here. Our findings with p50 RhoGAP, however, indicate that not all GAPs are regulated in this manner, even with prenylated GTPases. The absence of detectable phospholipid regulation of p50 RhoGAP also indicates that the observed effects of phospholipids are not simply due to effects on the GTPases that alter their sensitivity to all GAPs but, rather, are specific effects on the interaction between p190 and the Rho and Rac GTPases.

The fact that the C-terminal fragment of p190 RhoGAP is sensitive to phospholipids suggests that a direct interaction of the phospholipids within or near the catalytic region is probably required for regulation. We imagine that a lipid-mediated subtle conformational change is a likely mechanism for altering substrate preference, and future structural studies would be required to formally test this hypothesis. For some GAPs, pleckstrin homology domains mediate lipid binding (8, 17); however, p190 proteins do not have pleckstrin homology domains. Notably, the difference in the effect of PA versus PI, PS, and PIP2 on p190 GAP activity is reminiscent of findings with the ArfGAPs, where two separate phospholipid regulatory sites were identified (16). The requirement for a prenylated C terminus of the GTPases in phospholipid sensitivity is consistent with our previous finding that prenylation plays a role in the GAP-GTPase interaction (15), suggesting that prenylation can be an important determinant of GAP-specific recognition by GAPs.

The physiological significance of phospholipid-mediated GAP regulation has been difficult to establish and remains a challenge to the GTPase field. Significant issues include the fact that dozens of cellular GAPs can potentially regulate a particular GTPase, and so it is difficult to study the regulation of a GTPase by any one GAP in “isolation” in vivo. There is also the fundamental problem that the extraction of GAPS from cellular membranes, their frequent site of action, requires the use of detergents that may disrupt the interaction of the GAPS with regulatory lipids.

Although the physiological role of these phospholipid effects is difficult to assess, the ability of PI, PS, and PIP2 to switch the substrate preference for p190 RhoGAP from Rho to Rac suggests some potential scenarios for in vivo regulation. The non-random distribution of PI and PS within various cellular membranes raises the possibility that the p190 substrate preference could be influenced by p190 localization to a particular membrane “subdomain.” In addition, the signaling role of PIP2, as a product of phospholipase C activation, makes it possible for p190 substrate preference to be regulated by various extracellular signals that impinge on phospholipase C.

In summary, we find that the GTPase substrate preference for the p190 GAPs can be regulated by several physiological phospholipids. The fact that these lipids can effectively convert p190 to either a RhoGAP or a RacGAP provides a novel regulatory mechanism by which GTPase-mediated signaling can be regulated in a context-dependent manner.

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TABLE I

Effect of various phospholipids on the RhoGAP and RacGAP activities of full-length p50RhoGAP or of its isolated GAP catalytic domain.

| PL     | Full-length p50RhoGAP | GAP domain of p50RhoGAP |
|--------|-----------------------|-------------------------|
|        | RhoA                  | Rac1                    |
|        | RhoA                  | Rac1                    |
| No PL  | 50.3 ± 8.3            | 44.4 ± 6.3              |
| PS     | 49.3 ± 11.6           | 46.9 ± 9.4              |
| PA     | 54.9 ± 11.2           | 43.8 ± 7.5              |
| PC     | 52.4 ± 11.1           | 48.4 ± 10.1             |
| PE     | 48                    | 45.2                    |

The indicated phospholipids (PLs) were provided at a concentration of 0.1 mg/ml. The results of 2–8 independent experiments carried out in duplicate are shown with standard deviations. ND, not determined.