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Stimulation by phospholipids of a protein-tyrosine-phosphatase containing two *src* homology 2 domains

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ABSTRACT **PTP1C, a protein-tyrosine-phosphatase (protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) containing two *src* homology 2 domains, is poorly active when assayed with various protein substrates *in vitro*. Its activity is stimulated >1000-fold by anionic phospholipids when myelin basic protein or mitogen-activated protein kinase is used as substrate but reduced in the presence of several other substrates. Data are presented to indicate a direct interaction of the enzyme with phospholipids. Enzyme stimulation directed only toward certain specific substrates is interpreted by assuming that these compounds also bind to the phospholipid vesicles where they will be subjected to rapid enzymatic attack. A possible regulation of PTP1C by its translocation to the cell membrane is hypothesized.**

Protein-tyrosine-phosphatases (PTPs; protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) play a crucial role in cellular processes involving tyrosine phosphorylation (for review, see refs. 1 and 2). PTP1C (also referred to as SH-PTP1, HCP, SHP), a nontransmembrane phosphatase containing two *src* homology 2 (SH2) domains, is predominantly expressed in hematopoietic cells (3–6). It is homologous to the *Drosophila* enzyme corkscrew, identified as a positive transducer of the torso tyrosine kinase signal when acting in concert with the D-*raf* serine/threonine protein kinase (7). Recently, another widely distributed SH2 domain-containing phosphatase designated as SH-PTP2 or PTP2C has been described (8, 9), suggesting the existence of a subfamily of such enzymes. SH2 domains are found in several types of signaling proteins, including a number of intracellular tyrosine kinases (for review, see refs. 10 and 11). They play a major role in signal transduction by allowing the interaction of these proteins with autophosphorylated growth factor receptors.

In an earlier study (12), we reported the purification and characterization of recombinant PTP1C from an adenovirus expression system. Interestingly, the enzyme displayed very low specific activity toward several protein substrates as compared to other activated PTPs (<100 units/mg vs. >10,000 units/mg). The possibility that this low level of activity was due to an autoinhibitory reaction was supported by the observation that the enzyme was activated 20-fold by limited proteolysis (12). A search was therefore undertaken for effectors that might bring about a similar activation without cleavage of the peptide chain. This manuscript reports that PTP1C is activated >1000-fold by selected phospholipids but only when specific proteins are used as substrates.

MATERIALS AND METHODS

Materials. Phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), diacylglycerol (DAG), sphingomyelin (Sph), arachidonic acid, *p*-nitrophenylphosphate (*p*-NPP), rabbit muscle enolase, trypsin, and soybean trypsin inhibitor were from Sigma. PTP1C was purified from an adenovirus expression system (12) and the soluble kinase domain of epidermal growth factor receptor (sEGFR) was from a baculovirus expression system (13). The baculovirus-expressed kinase domain of the insulin receptor (BIRK) was kindly provided by Nicholas Tonks (Cold Spring Harbor Laboratory, Plainview, NY); immunoprecipitated c-*src* was provided by Adam Kashishian (Fred Hutchinson Cancer Research Center, Seattle); glutathione *S*-transferase fusion protein containing the SH2 domains of phospholipase C-γ (PLC-γ-SH2) was provided by Guenter Daum (University of Washington, Seattle); and recombinant extracellular signal-regulated kinase 2 (ERK2) and rabbit muscle mitogen-activated protein kinase (MAP kinase) kinase were provided by Lee Graves (University of Washington, Seattle). Tyrosine phosphorylation of proteins and peptide with [γ-^{32}P]ATP was obtained as follows: reduced, carboxamidomethylated, and maleylated lysozyme (RCM-lysozyme) and myelin basic protein (MBP) by BIRK (14); the nonapeptide ENDYINASL by sEGFR (15); sEGFR and BIRK by autophosphorylation (13, 16); PLC-γ-SH2 by sEGFR in the presence of 10 mM $MnCl_2$; enolase by c-src (17); ERK2 by MAP kinase kinase (18) and essentially freed from ATP by gel filtration on a Sephadex G-25 column. The phosphorylation of PTP1C (0.25 mg/ml) by BIRK (0.02 mg/ml) was performed for 5 min in a buffer containing 25 mM imidazole·HCl (pH 7.0), 1.0 mM dithiothreitol, 10 mM $MgCl_2$, and 3 mM $MnCl_2$, and autodephosphorylation was started by 15 mM EDTA with or without addition of phospholipids.

Preparation of Phospholipid Vesicles. Chloroform solution of a single phospholipid or its mixture with others was dried in a speed vacuum concentrator and then suspended in a buffer containing 20 mM imidazole·HCl (pH 7.0), 1.0 mM EDTA, and 1.0 mM dithiothreitol. Lipid stock solution at a concentration of 1.0 mg/ml was made by brief probe sonication for 0.5–2 min until it became clear.

PTP Activity Assays. Assays were performed at pH 7.0 for protein substrates and at pH 5.0 for ENDYINASL peptide or *p*-NPP as described (12, 15). Reactions were started by addition of substrates after a short preincubation (0.5–2 min) of the PTPs with the lipids. The incubation time for the assays was 5 min unless otherwise noted. One unit of activity is

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Abbreviations: PTP, protein-tyrosine-phosphatase; SH2, *src* homology 2; *p*-NPP, *p*-nitrophenylphosphate; RCM-lysozyme, reduced, carboxamidomethylated, and maleylated lysozyme; MBP, myelin basic protein; sEGFR, soluble kinase domain of epidermal growth factor receptor; PLC-γ-SH2, glutathione *S*-transferase fusion protein containing the SH2 domains of phospholipase C-γ; BIRK, baculovirus-expressed kinase domain of insulin receptor; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; DAG, diacylglycerol; Sph, sphingomyelin.

defined as the amount of enzyme causing the release of 1.0 nmol of phosphate per min.

Limited Trypsin Digestion of PTP1C. Proteolysis was carried out essentially as described (12). Briefly, PTP1C at a final concentration of 0.4 mg/ml was preincubated for 2 min with 0.2 mg of the various phospholipids per ml or buffer alone and then treated with 0.2 μg of trypsin per ml for different periods of time. The reaction was stopped by addition of 1 μg of soybean trypsin inhibitor per ml. Phosphatase activity was assayed as described above. The proteolysis products were analyzed on an SDS/15% polyacrylamide gel.

RESULTS

Effects of Various Phospholipids on the Activity of PTP1C. Fig. 1 illustrates the effects of PS on the activity of PTP1C. With MBP as substrate, enzymatic activity was increased at least 1000-fold at a lipid concentration of 25 μg/ml. By contrast, the activity toward the commonly used RCM-lysozyme, the nonapeptide ENDYINASL, or *p*-NPP was considerably reduced if not totally abolished.

A classic example of the regulation of an enzyme by phospholipids is the activation of protein kinase C (PKC) (19, 20). Similar to the kinase, PTP1C was activated by anionic phospholipids including PA, PS, CL, PG, and PI but not by cationic phospholipids such as PC, PE, and Sph (Table 1). Arachidonic acid, an activator of PKC, was also stimulatory, while DAG, the most powerful activator of PKC, was without effect on the phosphatase. Among the lipids tested, PA was the most effective activator, followed, in decreasing order, by PS, CL, PI, PG, and arachidonic acid. The maximum specific activity obtained (≈10,000 units/mg) is comparable to that reported for other PTPs toward the same substrate—namely, MBP. Furthermore, as observed for PS, all the anionic phospholipids inhibited the activity of PTP1C toward RCM-lysozyme, ENDYINASL, or *p*-NPP, while PC, PE, Sph, and DAG had no effect (data not shown). As shown in Table 1, addition of a second stimulatory phospholipid to PA or PS either slightly enhanced the activation or was without effect. PE, which was not stimulatory by itself, also enhanced the level of activation induced by PA or PS, while PC, Sph, and DAG remained ineffective.

To examine further the substrate specificity of the phospholipid-modulated PTP1C, several tyrosine-phosphorylated proteins of potential physiological relevance were tested as shown in Fig. 2. In the absence of phospholipids, PTP1C displayed very low, if any, activity (<20 units/mg) toward sEGFR, PLC-γ-SH2, enolase, BIRK, and ERK2. These low levels of activity were either unaffected (e.g., PLC-γ-SH2 and enolase) or further reduced (e.g., sEGFR and BIRK) in the presence of phospholipids, except for ERK2. In the latter instance, addition of a mixture of PA and PS caused rapid and total tyrosine dephosphorylation of the substrate. Further studies with 0.3 μM purified phosphorylated ERK2 following a standard assay procedure as used for RCM-lysozyme and MBP revealed that PA, PG, PI, and PS (each at 50 μg/ml) increased the activity of the enzyme from 0.03 unit/mg to 50, 28, 36, and 22 units/mg, respectively. These values are 20–50 times higher than that obtained when CD45 alone was used to dephosphorylate ERK2 (0.9 unit/mg). PC and PE remained ineffective.

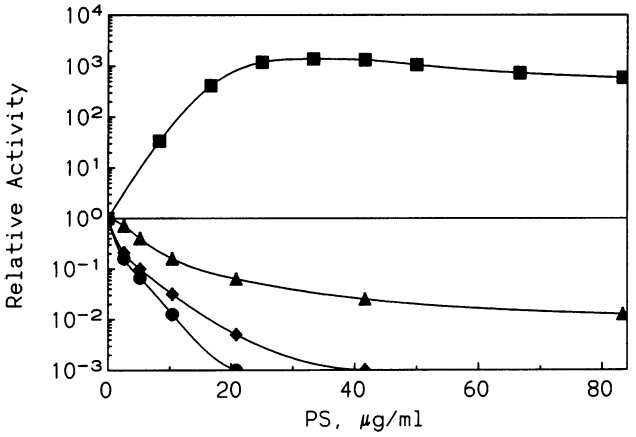

FIG. 1. Effects of PS on the activity of PTP1C toward 2 μM MBP (■), 2 μM RCM-lysozyme (▲), 3.5 μM ENDYINASL (◆), or 10 mM *p*-NPP (●).

Table 1. Effects of various membrane phospholipids on the activity of PTP1C

Lipid	Alone	+ PA	+ PS
Control	5	7,500	5,000
PA	7500	—	8,000
PS	5000	8,000	—
PC	5	7,500	5,000
PE	7	11,000	8,500
PG	2100	9,000	8,500
PI	2200	11,000	10,000
CL	4300	9,500	8,500
DAG	5	7,500	6,000
Sph	5	7,500	5,500
AA	750	8,000	7,000

Assays were performed with 2 μM MBP in the presence of each phospholipid alone (25 μg/ml) or its mixture with either PA or PS at 25 μg/ml. Data represent specific activity in units/mg. AA, arachidonic acid.

As illustrated in Fig. 2 (three rightmost lanes), PTP1C is readily phosphorylated by BIRK and undergoes slow self-dephosphorylation. Unexpectedly, no enhancement of the autodephosphorylation reaction was brought about by the phospholipids; if anything, the reaction appeared to be inhibited.

Factors Affecting the Activation of PTP1C. Table 2 illustrates the factors affecting the activity of PTP1C. The phospholipids did not shift the pH optimum of the enzyme, which remained between pH 6.5 and 7.0. The activity of phospholipid-stimulated PTP1C increased with increasing concentrations of MBP but decreased after that. High concentrations of nonphosphorylated MBP reduced the level of activation even more. However, no such effect was observed in the absence of phospholipids. This may be indicative of the competition between MBP and the enzyme for phospholipid binding (21). Bovine serum albumin, a carrier protein routinely included in the assay mixture, also appeared to interfere somewhat with the activation process since, when its concentration was reduced to 0.15 mg/ml, the stimulation was increased by nearly half. The activation did not require metal ions as the analyses were carried out in the presence of 1 mM EDTA. Excess Ca^{2+} and Mg^{2+} enhanced by 2-fold the activation brought about by PS but inhibited by nearly 80% the stimulation afforded by PA. Mn^{2+} and Zn^{2+} were inhibitory, and so was spermine. The basal activity of PTP1C toward MBP appeared to be insensitive to the inhibitory action of the classical inhibitors vanadate and heparin, but this conclusion could be misleading in view of the very low levels of activity displayed (12). The lipid-enhanced PTP1C activity was reduced by either inhibitor. Intact phospholipid vesicles were much more effective than detergent-mixed micelles. Addition of Triton X-100 almost abolished the stimulatory effects of the phospholipids.

Evidence for a Direct Interaction of PTP1C with Anionic Phospholipids. As indicated above, phospholipid activation of PTP1C was observed only when MBP and MAP kinase were used as substrates; enzymatic activity was greatly

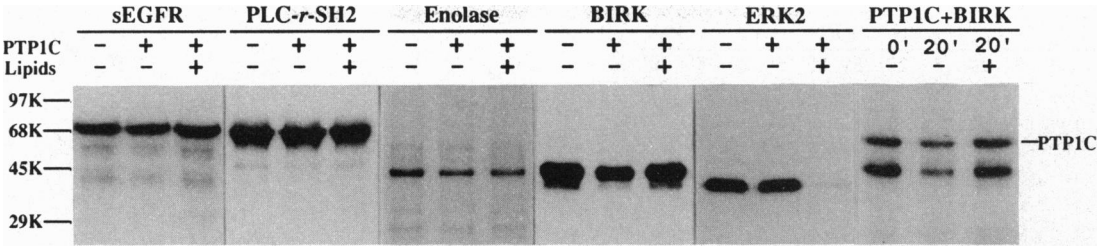

FIG. 2. Dephosphorylation of sEGFR, PLC-γ-SH2, enolase, BIRK, ERK2, and PTP1C. Reactions were carried out for 20 min in the presence or absence of a lipid mixture containing PA and PS (25 μg/ml each). The concentration of PTP1C used was 1.6 μg/ml for ERK2 and 8 μg/ml for the other substrates. ERK2 was phosphorylated predominantly on tyrosine residues. For autodephosphorylation of PTP1C, excess EDTA was added to stop the phosphorylation reaction, and the reaction mixture was further incubated for 0 or 20 min with or without the addition of phospholipids. The molecular sizes of marker proteins (kDa) and the position of PTP1C are shown on the left and right, respectively.

decreased when assays were carried out in the presence of RCM-lysozyme, the ENDYINASL peptide, *p*-NPP, or several other protein substrates. These data could have suggested at first that the activation process was simply due to an interaction of the phospholipids with the substrates rather than with the enzyme. However, this is unlikely for the following reasons. First, the effects were specific for PTP1C and were not observed with any of the other phosphatases tested, including CD45, receptor PTPα, and wild-type human T-cell enzyme (TC-PTP) and its truncated form TCΔC11 PTP (data not shown). Second, when activity measurements were performed for 30 sec instead of the usual 5 min, 8 times higher values were obtained when the enzyme rather than the substrate was preincubated with the phospholipids (Fig. 3). Third, phospholipids greatly altered the proteolytic digestion pattern of the enzyme. As previously reported (12), a section containing 41 amino acids can be cleaved from the C terminus of PTP1C, leaving a trypsin-resistant core representing 93% of the molecule. This truncation was accompanied by a 20-fold increase in activity when the enzyme was assayed in the presence of protein substrates. As shown in Fig. 4, tryptic attack in the presence of PA, PG, PI, or PS resulted in a rapid and extensive degradation of the phosphatase with total loss of enzymatic activity. As expected, PC that had no stimulatory effect on PTP1C activity did not increase the susceptibility of the enzyme to tryptic attack. Likewise, phospholipids did not affect the rate of degradation of TC-PTP, another phosphatase activated by limited trypsinolysis (data not shown). These data suggest that those anionic phospholipids that activate PTP1C must do so by causing a significant change in the conformation of the enzyme.

While limited trypsinolysis by itself caused a 20-fold activation of PTP1C, the truncated enzyme could be further activated by phospholipids to the same level as observed with the intact molecule, although an ≈5 times higher concentration of lipid was required (Fig. 5; see also Fig. 1). This indicates that the C-terminal segment of the enzyme does not play a key role in the phospholipid-induced activation process.

Table 2. Factors affecting activity of PTP1C

Factor	No lipid	PA (25 μg/ml)	PS (25 μg/ml)
pH			
6.25	0.9	1080	800
6.5	1.0	1550	1050
6.75	1.1	1630	1110
7.0	1.0	1500	1000
7.25	0.8	1010	690
7.5	0.5	670	390
Effector			
Control (2.0 μM MBP)	1.0	1500	1000
MBP (3.0 μM)	1.6	2500	1700
MBP (6.0 μM)	3.0	1600	1100
Nonphosphorylated MBP (10 μM)	1.0	250	160
BSA (0.15 mg/ml)	0.7	2600	1400
Ca^{2+} (1 mM)	0.8	270	1800
Mg^{2+} (1 mM)	0.9	290	2000
Mn^{2+} (1 mM)	0.7	30	80
Zn^{2+} (1 mM)	0.1	60	30
Spermine (0.5 mM)	0.8	150	50
Heparin (2 μg/ml)	3.0	240	290
Na_3VO_4 (0.2 mM)	1.7	30	10
Triton X-100 (0.1%)	1.0	100	70

Data represent relative activities with the value obtained under standard conditions [2 μM MBP, 1.0 mM EDTA, and 1.0 mg of bovine serum albumin (BSA) per ml at pH 7.0] being defined as 1.0. Concentration of metal ions is in excess of that of EDTA.

DISCUSSION

The activation of PTP1C by phospholipids is too specific to be ascribed simply to a distortion of the molecule that would unmask the catalytic site. If this were the case, enzyme stimulation would be observed with all substrates tested. That the activation is restricted to the substrates MBP and MAP kinase can be interpreted by assuming that these two substrates also bind to phospholipid vesicles; their positioning in close proximity to enzyme would render them highly susceptible to enzymatic attack. The interaction of MBP with membrane phospholipids has been well documented (21). The decreased PTP1C activity toward other substrates could simply be attributed to their inability to gain access to the phospholipid-associated enzyme. If this were the case,

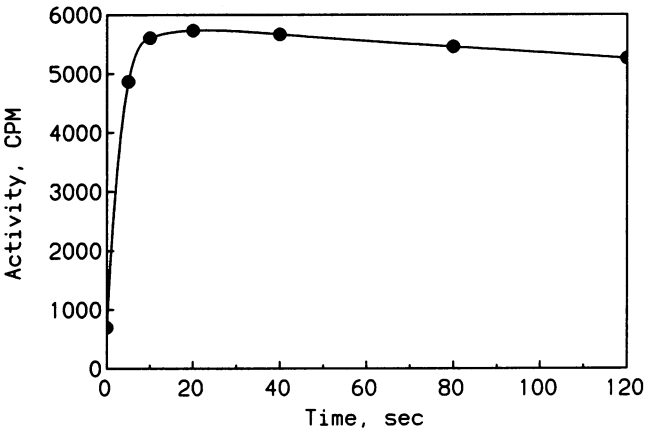

FIG. 3. Time course of activation of PTP1C by PS. The enzyme was preincubated with the phospholipids for the time indicated before the start of the reaction; assays were carried out with 2 μM MBP for 30 sec. For the zero time point, MBP was preincubated with PS for 1 min.

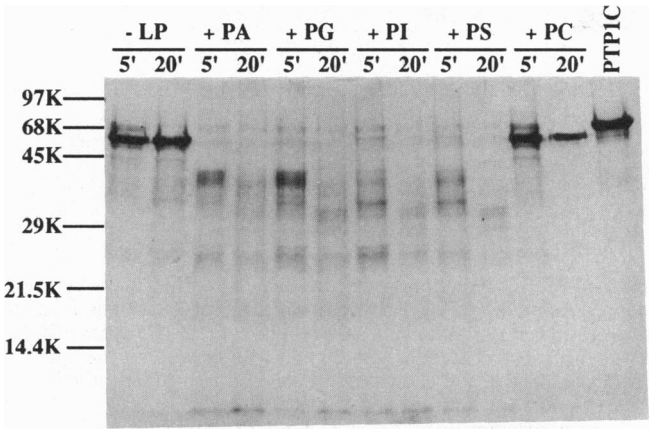

FIG. 4. Limited trypsin digestion (for 5 and 20 min) of PTP1C in the absence (−LP lanes) or in the presence (+ lanes) of various phospholipids as indicated. Lane PTP1C represents intact enzyme. Molecular sizes of standard marker proteins (kDa) are shown on the left.

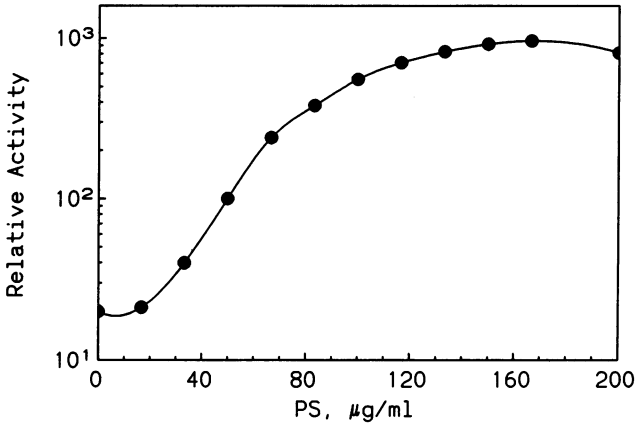

FIG. 5. Activation of PTP1C by PS after limited proteolysis. Enzyme was treated with trypsin for 20 min before addition of trypsin inhibitor. Activity assays were carried out with 2.0 μM MBP. Data represent relative values with the activity of the intact PTP1C in the absence of phospholipids being defined as 1.0.

PTP1C would preferentially dephosphorylate those substrates that also interact with the membrane.

The negative charges provided by the anionic phospholipids on the surface of the artificial membrane vesicles must play a crucial role in the activation of PTP1C. Phospholipids are known to distribute asymmetrically on cell membranes (22). For example, PC and Sph are preferentially located in the outer layer, while PS, PI, and PE are found in the inner layer. Such an asymmetric distribution would allow for regulation of PTP1C, assuming that the enzyme can associate with, or translocate to, the plasma membrane. While the recombinant PTP1C used in this study was purified from the cytosolic fraction of adenovirus-infected 293 cells (12), a significant portion of the enzyme remained attached to the membrane and nuclear fractions. Furthermore, a translocation of endogenous PTP1C to the membrane fraction in HL-60 cells upon phorbol 12-myristate 13-acetate-induced differentiation was observed (unpublished data). Which segment of the molecule participates in the phospholipid interaction and what signal would induce the translocation process are not known. SH2 domains could be directly involved by promoting interaction of the enzyme with tyrosine-phosphorylated growth factor receptors or other membrane-associated proteins.

The effectiveness of PA in activating PTP1C is of distinct interest. While PA constitutes a minor membrane component, it plays a crucial role in the *de novo* biosynthesis of lipids and can serve as a potential second messenger in signal transduction—for instance, through its conversion to DAG for the sustained activation of PKC (for review, see refs. 23–26). After stimulation by calcium-mobilizing agonists or growth factors, it accumulates to a high level from the phospholipase D-catalyzed breakdown of PC in a guanine nucleotide binding protein-regulated reaction. While PA was shown to elicit various cellular responses, including an increase in intracellular Ca^{2+} and in DNA synthesis, its direct targets are still unclear. It would be tempting to surmise that one of its functions would be to activate PTP1C. In fact, one might suggest that this enzyme would serve as one of the binding proteins for this second messenger. PA generated from the ligand-induced turnover of membrane phospholipids and the distribution of anionic compounds such as PS, Cl, PG, and PI on the inner surface of the plasma membrane could bring about the activation of the enzyme. In this way, the activation of PTP1C could be reminiscent of that of PKC, whose interaction with Ca^{2+}, DAG, and other unsaturated fatty acids stimulates its binding to phospholipids and causes its activation (26).

MAP kinase (or ERK2) plays a crucial role in downstream signaling from growth factor receptors. Its activity is regulated by both tyrosine and threonine phosphorylation (27, 28) catalyzed by a MAP kinase kinase, which itself is reported to be controlled by another protein kinase—namely, raf-1 (29, 30). While ERK2 is acted upon and inactivated by other PTPs including CD45, its rather specific dephosphorylation by the phospholipid-stimulated PTP1C could be of distinct physiological significance. The recent identification in *Drosophila* of a homolog of PTP1C (corkscrew) and the participation of this enzyme in signal transduction involving the torso and D-*raf* gene products might help to clarify this point (7).

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