Identification of Phospholipase C-\(\gamma\)1 as a Mitogen-activated Protein Kinase Substrate*

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The discovery of sequence motifs that mediate protein-protein interactions, coupled with the availability of protein amino acid sequence data, allows for the identification of putative protein binding pairs. The present studies were based on our identification of an amino acid sequence in phosphatidylinositol-specific phospholipase C-\(\gamma\)1 (PLC-\(\gamma\)1) that fits the consensus sequence for a mitogen-activated protein kinase (MAPK) binding site, termed the D-domain. Extracellular signal-regulated kinase 2 (ERK2), an MAPK, and phospho-ERK2 were bound by an immobilized peptide sequence containing the identified PLC-\(\gamma\)1 D-domain. Furthermore, a peptide containing the PLC-\(\gamma\)1 D-domain was able to competitively inhibit the \textit{in vitro} phosphorylation of recombinant PLC-\(\gamma\)1 by recombinant phospho-ERK2, whereas a control peptide derived from a distant region of PLC-\(\gamma\)1 was ineffective. Similarly, the peptide containing the PLC-\(\gamma\)1 D-domain, but not the control peptide, competitively inhibited the \textit{in vitro} phosphorylation of Elk-1 and c-Jun catalyzed by recombinant phospho-ERK2 and phospho-c-Jun N-terminal kinase 3 (phospho-JNK3), another type of MAPK, respectively. Incubation of anti-PLC-\(\gamma\)1 immunocomplexes isolated from rat brain with recombinant phospho-ERK2 opposed the increase in PLC-\(\gamma\)1-catalyzed hydrolysis of phosphatidylinositol 4,5-P\(_2\) (PtdIns(4,5)P\(_2\)), which was produced by a tyrosine kinase associated with the immunocomplexes, whereas \textit{in vitro} phosphorylation of recombinant PLC-\(\gamma\)1 by recombinant phospho-ERK2 did not alter PLC-\(\gamma\)1-catalyzed PtdIns(4,5)P\(_2\) hydrolysis. These studies have uncovered a previously unidentified mechanism for the integration of PLC-\(\gamma\)1- and ERK2-dependent signaling. 

Mitogen-activated protein kinases (MAPK)s\(^1\) are proline-directed, serine/threonine kinases having a minimal consensus substrate sequence of \((S/T)P\), where \(S\), \(T\), and \(P\) represent the amino acids serine, threonine, and proline, respectively (1). The presence of a proline residue in the \(-2\) position is favorable and yields the optimal consensual substrate sequence of \(pX(S/T)P\), where \(X\) is any amino acid (1). Three families of MAPK have been identified: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK, also called stress-activated protein kinase), and p38 stress-activated protein kinase (p38; also called RK/CSBP). Multiple members of each of these MAPK families have been cloned. MAPKs exist in an inactive, unphosphorylated form and an active, phosphorylated form. MAPKs are converted to the active, phosphorylated form by dual-specificity kinases termed MAPK kinases or MAPK/ERK kinases (MEKs), which phosphorylate threonine and tyrosine (Y) residues in the enzymes (2–4).

It has been estimated that \(-90\%\) of all proteins contain a \((S/T)P\) sequence, but not all of these proteins are substrates for MAPKs (5). This indicates that MAPK-dependent phosphorylation of a substrate involves the interaction (docking) of the kinase with a site on the substrate that is distinct from the phosphoacceptor site. Kornfeld and colleagues (5–6) have identified two ERK binding motifs: 1) the FXFP motif and 2) the D-domain motif. In addition to conferring specificity, docking domains may increase the efficiency of substrate phosphorylation (7–11). The location of the MAPK docking site can be either N- or C-terminal to the phosphorylation site (10, 12). The sites on ERKs that are involved in substrate binding have also been identified: the common docking site, which binds the D-domain of the substrate (13), and a distinct hydrophobic pocket for FXFP binding formed between the MAP kinase insert, the \(p\) + 1 site, and an \(a\) helix (14). Phosphatidylinositol-specific phospholipase C (PLC) isoforms catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P\(_2\)), yielding the intracellular messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P\(_3\)) and 1,2-diacylglycerol (1,2-DAG) (15–16). Complementary DNA clones have been isolated for 11 distinct mammalian PLC isoforms (17). Comparison of the predicted amino acid sequences of these clones reveals that PLC isoforms may be grouped into four types: PLC-\(\beta\), PLC-\(\delta\), PLC-\(\gamma\), and PLC-\(\epsilon\); the present studies focus on PLC-\(\gamma\). Growing evidence implicates PLC-\(\gamma\) as an important component of signal transduction processes involved in various cellular processes, including mitogenesis, differentiation, transformation, and neuronal plasticity (18). It is important to note that some PLC-\(\gamma\)-mediated cellular responses have been shown to be independent of PLC-\(\gamma\) lipase activity (19–22), indicating that PLC-\(\gamma\) isoforms function through interactions (direct or indirect) with other signaling molecules, in addition to functioning via Ins(1,4,5)P\(_3\) and 1,2-DAG-dependent signaling pathways. Cross-talk between the MAPK signaling cascade and PLC-\(\gamma\) has been demonstrated in many studies. For example,
Morrison et al. (23) showed that the MEK, Raf, co-immunoprecipitates with, as well as phosphorylates, PLC-γ1. Yong et al. (24) reported that activation of the Raf/MEK/PLC pathway in PC12 cells by nerve growth factor requires PLC-γ1 enzyme activity. We demonstrate that ERK2 and phospho-ERK2 interact with PLC-γ1 both in vitro and within rat brain and that this interaction has functional significance.

EXPERIMENTAL PROCEDURES

Animals—Female Sprague-Dawley rats (150–200 days old) were maintained as described in Weeber et al. (25). The rats were given unlimited access to standard rat chow and tap water. All procedures employed for housing, handling, and sacrificing of rats were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Peptide Synthesis—All peptides and peptide columns were commercially synthesized by BIOSOURCE International (Hopkinton, MA). Both of the peptides used in the kinase inhibition assays demonstrated the same solubility in water. Neither peptide altered the pH of the reaction buffers in these assays.

PLC-γ1 D-domain Peptide Pull-down Assay—Peptides containing the sequence of the PLC-γ1 D-domain ( paramselslvyyv, rat PLC-γ1 amino acids 945–960) or a control sequence form PLC-γ1 (fletnltrglpirl, rat PLC-γ1 amino acids 229–247) were synthesized at an 80% purity level and coupled to thiol agarose (1 mg of peptide per ml of resin). Binding of recombinant ERK2 (unactive) and phospho-ERK2 (active) to the peptide-coupled agarose was performed employing a procedure adapted from Zhang et al. (26). Briefly, 5 ng of ERK2, or 50 ng of phospho-ERK2, per 100 μl of binding buffer (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 320 mM sucrose, 75 mM NaCl, 75 mM KCl, and 1 mM (1%, v/v) Triton X-100) were incubated with 50 μl (packed volume) of D-domain or control peptide agarose at 4 °C for 2 h with mixing. The agarose was collected by centrifugation (14,000 × g, 4 min, room temperature) and washed twice with 1 ml of binding buffer. Bound proteins were eluted by boiling in 2× SDS-PAGE loading buffer, separated by SDS-PAGE (7.5% w/v polyacrylamide gels), transferred to polyvinylidene fluoride (PVDF) membrane, and probed for anti-ERK2 or anti-phospho-ERK2 immunoreactivity as described below.

In Vitro Phosphorylation of PLC-γ1—Recombinant PLC-γ1 and PLC-γ2 were prepared essentially as described in Park et al. (27). One microgram of recombinant PLC-γ1 or PLC-γ2 preparation was incubated (30 °C, 20 min, and 25-μl final volume) in kinase reaction buffer (for phospho-ERK2: assay dilution buffer I (20 mM MOPS, pH 7.2, 55 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM diithiothreitol, and 0.01% (v/v) Brij-35); for phospho-JNK3: 75 mM Tris–HCl, pH 7.5, 3.6 mM MOPS, pH 7.2, 4.5 mM β-glycerophosphate, 1.05 mM EDTA, 3.2 mM diithiothreitol, 0.18 mM sodium orthovanadate, 0.5 mM MgCl2, 0.015% (v/v) Brij-35; and for phospho-p38: 30 mM Tris–HCl, 0.03 mM EDTA, 0.1 mM MgSO4, and 0.62 mM diithiothreitol; 13.5 mM MgCl2, and 90 μM [γ-32P]ATP (15 μCi) in the absence or presence of recombinant phospho-ERK2 (100 ng), phospho-JNK3 (100 ng), or phospho-p38 (250 ng). Control reactions were incubated in the absence of protein kinase. Peptide competition studies were performed by preincubating (10 min, 30 °C) 50 ng phospho-ERK2 in the presence of 100 μM PLC-γ1 D-domain (MEMRRKIAESELSELVYYVCRPPFDF, rat PLC-γ1 amino acids 943–966) or PLC-γ1 Control (YSAQKTDMLPFLENTNLTRGLPERIL, rat PLC-γ1 amino acids 229–246) peptide in kinase reaction buffer/MgCl2/ATP solution (see above). Control reactions were performed in the absence of phospho-ERK2 and peptide. Reactions were initiated by addition of recombinant PLC-γ1 (1 μg/reaction) and continues for 30°C. Reactions were terminated by the addition of 0.5 μl of 4× SDS-PAGE sample buffer followed by heating (100 °C, 6 min). The entire mixture was subjected to SDS-PAGE (7.5% w/v polyacrylamide gels) then transferred to PVDF, and [32P]-labeled PLC-γ1 was detected by autoradiography. In addition, the membrane was stained with Coomassie Brilliant Blue R-250 to measure protein loading. PVDF membranes were immersed in a 0.25% (w/v) Brilliant Blue R-250 solution containing 40% (v/v) methanol for 5 min at room temperature, then destained for 15 min in 40% (v/v) methanol.

Phosphorylation of c-Jun and Elk-1—MAPK-dependent phosphorylation of c-Jun and Elk-1 was performed employing a procedure based on a method of Hsiao et al. (29). Phosphorylation of Elk-1 was performed as follows. His-tagged phospho-ERK2 (12.5 ng) was incubated (20 min, 30 °C, 25-μl reaction volume) with 1 μg of recombinant fusion protein of GST-tagged Elk-1, residues 301–428, in phospho-ERK2 kinase reaction buffer (see above), 13.5 mM MgCl2, and 90 μM [γ-32P]ATP (15 μCi) in the absence or presence of the PLC-γ1 D-domain peptide (in a 1:2 dilution series starting at 100 μM and ending at 12.5 μM) or 100 μM PLC-γ1 control peptide. The phospho-ERK2 was preincubated with the appropriate peptide for 10 min at 30 °C prior to adding the substrate to initiate the kinase reaction. The peptide sequences for the PLC-γ1 D-domain and Control peptides were the same as used in the PLC-γ1 in vitro phosphorylation studies. Reactions were terminated by the appropriate peptide for 10 min at 30 °C prior to adding the substrate. Reactions were incubated for 10 min at 30 °C and analyzed as described above.

Tissue Preparation and Subcellular Fractionation—Preparation of Triton X-100 extracts of rat whole brain postnuclear (S1) fraction and hippocampal formation postnuclear membrane (P2) fraction were performed as described in Buckley and Caldwell (29) except buffers also contained 20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, and 10 mM sodium fluoride.

Affinity Capture of Anti-PLC-γ1 Immune Complexes—Anti-PLC-γ1 immunocomplexes were isolated as follows. For determinations of anti-PLC-γ1 immunoreactivities, whole rat brain S1 fraction (100 μg of protein) was incubated with 5 μl of rat anti-PLC-γ1 antibody (in a 1:10 dilution series starting at 100 μl of antibody volume) and with mixing at 4 °C. The immunocomplexes were recovered with protein A-Sepharose beads and washed twice with 1 ml of extraction buffer supplemented with 20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, and 10 mM sodium fluoride, as described in Weeber et al. (25). For GST pull-down assays, whole rat brain S1 fraction (100 μg of protein) was incubated with glutathione-agarose precomplexed with a GST fusion protein of the PLC-γ1 SH2-SH3 domains or glutathione-agarose control beads for 4 h at 4 °C. The beads were collected by centrifugation, and the supernatant was decanted and incubated with 6 μl of rabbit anti-PLC antibodies or non-immune rabbit IgG. The immunocomplexes and controls were processed as described above. For in vitro PLC activity measurements, anti-PLC-γ1 immunocomplexes were immobilized on 96-well plates, as described elsewhere (29).

Immunoblotting—Anti-ERK2 immunoreactivity associated with anti-PLC-γ1 immunocomplexes or bound by immobilized D-domain peptides was measured as follows. Fifty microliters of 2× SDS-PAGE sample buffer was added to the sample, and the mixture was boiled for 5 min. Eluted proteins were separated using 7.5% (w/v) SDS-PAGE gels prior to transfer to PVDF membranes. Membranes were blocked in 5% (w/v) fat-free milk in Tris-buffered saline and blotted with mouse anti-ERK2 antibody (1:1000) followed by anti-mouse horseradish peroxidase-conjugated antibody (1:3000). The immunoreactive proteins were detected using enhanced chemiluminescence.

In Vitro Treatment of Anti-PLC-γ1 Immunocomplexes with Phospho-ERK2—Anti-PLC-γ1 immunocomplexes were captured from 20 μg of rat hippocampal P2 preparation, then incubated (20 min, 35 °C) in the presence of one of the following buffers: A) Assay Dilution Buffer I plus 20 μM protein kinase C (PKC) inhibitor peptide, 2 μM protein kinase A (PKA) inhibitor peptide, and 20 μM Compound R24571; B) Buffer A containing 0.4 unit of recombinant phospho-ERK2; C) Buffer A supplemented with 13.5 mM MgCl2 and 90 μM ATP; or D) Buffer C with 0.4 unit of recombinant phospho-ERK2. The wells were washed three times (5 min each) with 1.25× PLC assay buffer (43.75 mM sodium phosphate, pH 6.8, 87.5 mM KCl, 1.0 mM EDTA, 1.0 mM CaCl2) prior to quantification of PLC activity (see below). The in vitro treatment of recombinant PLC-γ1 with phospho-ERK2 was performed as described for the anti-PLC-γ1 immunocomplexes captured from rat hippocampal P2 preparation. The 100 ng of recombinant PLC-γ1 was first captured onto the microtiter plate prior to the in vitro treatment, or not, with recombinant phospho-ERK2. In addition, incubation of recombinant PLC-γ1 with Buffer C was omitted due to the failure of detecting [γ-32P]phosphate incorporation in the absence of kinase (Fig. 3A). Reconstituted PLC-γ1 lipase activity was then quantified as described below.

Immunocomplex PLC Activity Measurement—PLC activity associated with anti-PLC-γ1 immunocomplexes was quantified as described in Buckley and Caldwell (29). Activity was calculated as nanomoles of Ins(1,4,5)P3 product formed/min/mg of protein present in the sample from which the enzyme was affinity captured.
Identification of a Putative MAPK Docking Motif in PLC-γ1—

We searched the primary structure of PLC-γ1 (Fig. 1A) for one or more amino acid motifs that fit the consensus sequences of ERK docking sites, as identified by Kornfeld and colleagues (5–6): 1) the FXFP motif, having the consensus sequence of FXFP and 2) the D-domain, having two possible consensus sequences: (K/R)(L/I)P and (K/R)(K/R)(K/R)(L/I)P. We did not find an FXFP sequence but did identify overlapping sequences that conform to the two consensus D-domain sequences: ^545RRKKIAL(EL). In addition, we searched for potential (S/T)P and optimal (PXXS/T)P MAPK phosphorylation sites in the primary sequence of PLC-γ1 (Fig. 1A). Seven (S/T)P sequences were identified, but none fit the optimal sequence for MAPK phosphorylation. We also searched the primary structure of ERK2 (Fig. 1B) for possible PLC-γ1-interacting domains. We identified a sequence 112KLLKTQHLSNDHI that demonstrates homology to a PLC-γ1 binding site (KLLK) and optimal (PXXS/T)P MAPK phosphorylation. We found that anti-ERK2 immunoreactivity is associated with anti-PLC-γ1 immunocomplexes isolated from rat whole brain postnuclear (S1) fractions (Fig. 2A), as well as hippocampal formation postnuclear membrane (P2) preparations (data not shown). We detected only minimal anti-phospho-ERK2 immunoreactivity associated with anti-PLC-γ1 immunocomplexes (data not shown).

We tested the hypothesis that ERK2 found associated with anti-PLC-γ1 immunocomplexes was bound, either directly or indirectly (i.e. mediated by a second, intermediary protein), to the SH2 and/or SH3 domains of PLC-γ1. We performed preliminary experiments in which we incubated rat whole brain S1 fractions (Fig. 2A), as well as hippocampal formation postnuclear membrane (P2) preparations (data not shown). We detected only minimal anti-phospho-ERK2 immunoreactivity associated with anti-PLC-γ1 immunocomplexes (data not shown).

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C-terminal SH2 beads following pre-clearing of the extracts with the C-terminal SH2 beads (50 μg), even though a significant amount of ERK2 was still present within the sample (data not shown). These studies demonstrated that rat brain ERK2 indirectly binds to the PLC-γ1 C-terminal SH2 domain in vitro. However, pre-clearing of rat whole brain S1 preparations (100 μg) with an excess (50 μg) of agarose-coupled GST-SH2-SH2-SH3 domains of PLC-γ1 prior to isolation of anti-PLC-γ1 immunocomplexes did not dissociate the ERK2-PLC-γ1 complex (Fig. 2B). This approach is analogous to a procedure described by Snyder and colleagues (32), who reported that in the presence of 10 μg of GST-PLC-γ1 SH3 domain co-immunoprecipitation of PLC-γ1 and phosphoinositide 3-kinase enhancer is blocked. Thus, these studies demonstrate that, although ERK2 can bind indirectly to the PLC-γ1 C-terminal SH2 domain in vitro, the PLC-γ1 SH2 and SH3 domains do not significantly contribute to the binding of ERK2 within anti-PLC-γ1 immunocomplexes isolated from rat brain.

Phospho-ERK2 Directly Interacts with and Phosphorylates PLC-γ1 in Vitro—The identification of a putative ERK2 docking site in PLC-γ1 and co-immunoprecipitation of PLC-γ1 and ERK2 indicated that the two proteins may directly bind to each other. To test this hypothesis and to determine whether PLC-γ1 is a substrate for phospho-ERK2-catalyzed phosphorylation, we incubated recombinant PLC-γ1 and PLC-γ2 with recombinant phospho-ERK2 in the presence of [γ-32P]ATP. We also sought to determine whether other MAPKs, specifically p38 and JNK3, were able to phosphorylate PLC-γ1. Fig. 3A shows that PLC-γ1 serves as an in vitro substrate for phospho-ERK2, as well as phospho-p38y and phospho-JNK3, whereas negligible phosphorylation of PLC-γ2 was detected in the presence of these MAPKs (Fig. 3B). Control reactions incubated in the absence of recombinant MAPK demonstrated that the phosphorylation of PLC-γ1 was catalyzed by the recombinant MAPK.

Recombinant ERK2 and Phospho-ERK2 Bind to Immobilized PLC-γ1 D-domain Peptide in Vitro—To determine whether the identified PLC-γ1 D-domain sequence is capable of binding ERK2, the sequence of the PLC-γ1 D-domain (Fig. 4A) and a control peptide sequence outside of the PLC-γ1 D-domain (Fig. 4A) were coupled to thiol-agarose and incubated with recombinant ERK2. Similarly, we incubated the immobilized peptides with recombinant phospho-ERK2. Both recombinant ERK2 (Fig. 4B) and recombinant phospho-ERK2 (Fig. 4C) were capable of binding to the immobilized D-domain peptide. In contrast, neither ERK2 nor phospho-ERK2 was bound by the PLC-γ1 control peptide, confirming that the binding was specific for the D-domain.

The PLC-γ1 D-domain Inhibits the MAPK-catalyzed Phosphorylation of MAPK Substrates—To substantiate the claim that the peptide sequence that we identified in PLC-γ1 functions as a putative D-domain, we assessed the ability of a peptide containing the PLC-γ1 D-domain (Fig. 5A) to competitively inhibit the phosphorylation of a known phospho-ERK2 substrate, Elk-1, and a known JNK substrate, c-Jun. Several investigators (e.g. Refs. 8–10) have demonstrated that D-domain peptides competitively inhibit the binding of MAPKs to their substrates in vitro. The PLC-γ1 D-domain peptide inhibited both c-Jun phosphorylation by active JNK-3 (Fig. 5B) and Elk-1 phosphorylation by active ERK2 (Fig. 5C) in a concentration-dependent manner, whereas the PLC-γ1 control peptide did not inhibit either Elk-1 or c-Jun phosphorylation by the appropriate kinase. The Elk-1 substrate contains multiple MAPK phosphorylation sites, accounting for the appearance of three phosphorylated bands in the autoradiogram. The PLC-γ1 D-domain peptide inhibited the phosphorylation of the upper two bands, whereas it did not alter labeling of the lower, least phosphorylated band, suggesting that it may result from phos-
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Fig. 4. Immobilized PLC-γ1 D-domain binds recombinant ERK2 and recombinant phospho-ERK2. A, amino acid sequences of synthetic peptides corresponding to the putative PLC-γ1 D-domain or a PLC-γ1-control peptide which contain a C-terminal cysteine for coupling to agarose were incubated with unphosphorylated ERK2 (B) or phospho-ERK2 (C). The agarose was collected by centrifugation and washed with buffer. Bound protein was eluted with SDS-PAGE loading buffer, separated by SDS-PAGE, and transferred to PVDF membrane, and the blot was probed for anti-ERK2 or anti-phospho-ERK2 immunoreactivity as described under “Experimental Procedures.” Each result is representative of at least three separate experiments.

phorylation of phosphoacceptor sites independent of interaction of phospho-ERK2 with the D-domain of Elk-1. Similar results have been reported by Bardwell et al. (10).

The PLC-γ1 D-domain Inhibits the Phospho-ERK2-catalyzed Phosphorylation of PLC-γ1—In the presence of the PLC-γ1 D-domain peptide, the phospho-ERK2-catalyzed phosphorylation of PLC-γ1 was blocked, whereas the PLC-γ1 control peptide did not affect the phosphorylation of PLC-γ1 (Fig. 6A). Coomassie staining of the membrane demonstrated equal loading of the recombinant PLC-γ1 (Fig. 6B).

Treatment of Anti-PLC-γ1 Immunocomplexes with Phospho-ERK2 Reverses the Tyrosine Kinase-dependent Stimulation of Immunocomplex PLC Activity—Phospho-ERK2 phosphorylation of recombinant PLC-γ1 did not alter lipase activity (Fig. 7A). In contrast, phospho-ERK2 exerted significant effects on anti-PLCγ1 immunocomplex PLC activity (Fig. 7B). Anti-PLC-γ1 immunocomplexes were isolated from rat hippocampal formation P2 preparations, then treated with or without ATP and with or without phospho-ERK2. Subsequently, PLC activity was measured. PLC-γ1 enzyme activity was significantly increased by treatment with ATP alone. We have shown previously that this effect of ATP is produced by a tyrosine kinase that is associated with the anti-PLC-γ1 immunocomplex (29). In the absence of ATP, phospho-ERK2 did not significantly alter PLC-γ1 enzyme activity. In the presence of ATP, phospho-ERK2 significantly reduced, but did not completely reverse, the increase in PLC-γ1 enzyme activity that was produced by a tyrosine kinase associated with the immunocomplexes.

DISCUSSION

We have made several important and novel observations in these studies. First, we provide evidence that PLC-γ1 associates with ERK2 in rat brain extracts and that dual phosphorylation of ERK2 in its activation loop is not critical for the association as demonstrated by minimal immunoreactivity of a phospho-specific antibody for ERK1/2 in a Western blot of PLC-γ1 immunoprecipitates. This association, therefore, was probably not mediated via binding to a phosphoacceptor site found within the PLC-γ1 sequence due to conformational constraints imposed on ERK2 by the non-phosphorylated “activation lip” of ERK2. However, the interaction of PLC-γ1 with phospho-ERK2 was direct, because recombinant PLC-γ1 was a substrate for recombinant phospho-ERK2 in vitro. This, in turn, is a second novel finding; i.e. PLC-γ1 is a MAPK substrate. The finding that PLC-γ2 was minimally phosphorylated by MAPKs under the same conditions demonstrated specificity of the kinase reactions. Although our results demonstrate that PLC-γ2 is a poor phospho-MAPK substrate, it is possible that MAPK-dependent phosphorylation of PLC-γ2 may occur under other conditions: e.g. after PLC-γ2 has been phosphorylated by another protein kinase. Third, using both peptide-binding and MAPK substrate phosphorylation assays, we identified a peptide sequence within the primary sequence of PLC-γ1 that conforms to the consensus sequence for an ERK-docking site, a D-domain. A peptide version of the PLC-γ1 D-domain inhibited the in vitro phosphorylation of PLC-γ1 by phospho-ERK2. This result demonstrates that the interaction between PLC-γ1 and phospho-ERK2 is dependent on the common docking domain of phospho-ERK2 and strongly implicates the D-domain of PLC-γ1 as the ERK2-binding site on PLC-γ1. Furthermore, it indicates that interactions between other sites on phospho-ERK2 (e.g. putative PLC-γ1 interaction motifs shown in Fig. 1B) and PLC-γ1 do not play a significant role in PLC-γ1-phospho-ERK2 binding. Finally, phospho-ERK2 treatment of anti-PLC-γ1 immunocomplexes under conditions allowing for substrate phosphorylation revealed that phospho-ERK2 opposes tyrosine kinase-dependent stimulation of PLC-γ1 enzyme activity, whereas phosphorylation of recombinant
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PLC-γ1 by phospho-ERK2 under similar conditions did not affect the lipase activity of PLC-γ1.

Identification of the PLC-γ1 D-domain sequence was based on consensus sequences defined by Kornfeld and colleagues (5–6). Other investigators (e.g., Refs. 3, 12–13, 33) have identified similar, but different, MAPK docking sequence motifs. In general, MAPK binding motifs consist of peptide sequences of 20 amino acids, or less. Rather than specifying consensus sequences, Sharrocks and colleagues (34) have developed models of MAPK docking domains that identify regions of sequence similarity: specifically, basic, LXl, and hydrophobic motifs. These regions play differing roles in dictating specificity for interactions with members of the ERK, JNK, and p38 stress-activated protein kinase families. The p38 kinases bind proteins that have a basic region and hydrophobic region, whereas the intervening LXl motif is dispensable; ERKs require the presence of all motifs, although the hydrophobic motif is less important than the other two regions (34). The PLC-γ1 D-domain that we identified consists of a basic region and an (L/I)LXl motif; in addition, an adjacent hydrophobic region (Xp38LVV) is readily identifiable. This indicates that, in addition to binding ERKs, the PLC-γ1 D-domain may bind p38 and JNK family members. In support of this proposal, we have found that PLC-γ1 is an in vitro substrate for p38γ and JNK3.

PLC-γ1-ERK2 protein complexes were immunoprecipitated from rat brain lysates, indicating that the observed interactions between recombinant proteins are not simply in vitro artifacts. However, we were able to detect only minimal anti-phospho-ERK2 immunoreactivity associated with anti-PLC-γ1 immunocomplexes. It is worth noting that, similar to our data, Husi et al. (35) reported that the N-methyl-d-aspartic acid receptor is isolated in association with ERK2, but not phospho-ERK2, and Loeb et al. (36) reported that the TrkA receptor is found in association with ERK1, but not phospho-ERK1, or either form of ERK2. Interestingly, PLC-γ1 has been shown to co-immunoprecipitate with an unidentified protein of molecular mass of ~42,000 Da, and the amount of phosphorylated protein (molecular mass ~ 42,000 Da) associated with anti-PLC-γ1 immunoprecipitates is significantly increased following receptor activation (37, 38). Our results indicate that this protein may be ERK2.

Results of the experiments in which we assessed the effects of phospho-ERK2 pretreatment on in vitro PLC activity indicate that PLC-γ1 is regulated, either directly or indirectly, by phospho-ERK2. Phospho-ERK2 treatment opposed stimulation of PLC-γ1 enzyme activity by a tyrosine kinase that co-immunoprecipitated with PLC-γ1. Two possible mechanisms may underlie this effect. Phospho-ERK2-catalyzed phosphorylation

Fig. 6. PLC-γ1 D-domain peptide inhibits phospho-ERK2-catalyzed phosphorylation of recombinant PLC-γ1. A, recombinant PLC-γ1 was incubated in vitro with [γ-32P]ATP and without (lane 1, no kinase) phospho-ERK2 or with recombinant phospho-ERK2 (lanes 2–4, +ERK2) in the absence (lane 4) or presence of the PLC-γ1 D-domain peptide (lane 2) or the PLC-γ1 control peptide (lane 3). Proteins were transferred to PVDF, and radioactivity was detected by autoradiography (A) or protein was stained with (B) Coomassie stain. The results shown are representative of three separate experiments.

Fig. 7. Phospho-ERK2 treatment significantly reduces ATP-dependent stimulation of immunocomplex PLC-γ1 catalytic activity. PLC-γ1 was affinity captured as recombinant PLC-γ1 (A), or from rat hippocampal formation postnuclear particulate (P2) fraction (B). Captured recombinant PLC-γ1 was treated with one of the following: buffer, buffer containing recombinant phospho-ERK2 (+pERK2); or buffer containing recombinant phospho-ERK2 and ATP (+pERK2 + ATP). Captured PLC-γ1 from rat hippocampal formation postnuclear particulate (P2) fraction was treated with buffer; buffer containing ATP (+ATP); buffer containing recombinant phospho-ERK2 (+pERK2); or buffer containing recombinant phospho-ERK2 and ATP (+pERK2 + ATP). Subsequently, PLC activity associated with the immunocomplex was determined. PLC activity is calculated as nanomoles of Ins(1,4,5)P3 product formed/min/mg of protein, then expressed within each experiment as a percentage of the mean of the buffer value. Each point is the average ± S.E. (n = 3 preparations) after background subtraction. In B, one-way analysis of variance revealed a significant overall effect of treatment F(3, 11) = 12.61, p < 0.002. Newman-Kuels multiple comparison test revealed p < 0.01 (** ) for buffer versus +ATP and p < 0.05 for +ATP versus +pERK2 + ATP.
of PLC-γ1 may oppose tyrosine kinase-dependent phosphorylation of the enzyme, similar to the mechanism proposed by Rhee and colleagues (39) for the regulation of PLC-γ1 activity by PKA and PKC. Tyrosine phosphorylation consequent to activation of PLC-γ1 may be reduced either as the result of reduced efficiency of PLC-γ1 phosphorylation by one or more tyrosine kinases or increased efficiency of PLC-γ1 dephosphorylation by one or more tyrosine phosphatases. Alternatively, phospho-ERK2 may indirectly decrease PLC-γ1 enzyme activity as the result of inhibiting the catalytic activity of the tyrosine kinase that is responsible for the genistein-inhibited activation of the isozyme. Studies aimed at testing these possibilities are being conducted in our laboratory. Finally, it should be noted that, because PLC-γ1 is commonly positioned early within a signal transduction pathway, relatively small changes in PLC-γ1 enzyme activity become amplified, often producing 2- to 4-fold changes in physiologic responses (e.g. functioning of ion channels, changes in gene expression) (40).

The physiologic significance of associations between PLC-γ1 and MAPKs is currently uncertain. Several possibilities exist. First, MAPKs may regulate the catalytic activity of PLC-γ1. Our results support a model (see Fig. 8) in which phospho-ERK2 acts to oppose tyrosine kinase-dependent activation of PLC-γ1, indicating that MAPKs, at least phospho-ERK2, may act to down-regulate PLC-γ1 signaling. This is in contrast to the reported stimulatory effect of phospho-ERK1/2 on PLC-γ1 (41). A second possible significance of MAPK interactions with PLC-γ1 is that PLC-γ1 may target MAPKs to signaling complexes containing components (e.g. PKC, Raf, and MEK) of pathways that produce MAPK activation, thus increasing the efficiency of transducing signals from PLC-γ1 to MAPKs. Once activated, the phospho-MAPK may, or may not, dissociate from PLC-γ1 and regulate downstream effectors (e.g. transcription factors). In support of this proposal are reports of PLC-γ1 association with PKC-ζ (42) and Raf (23), and the identified roles of PLC-γ1 in the control of cellular processes that are dependent on gene transcription (43–45). Third, association of a MAPK with a PLC isozyme may allow for the MAPK to gain access to, and phosphorylate, a protein (e.g. one bound to the SH2 domains of PLC-γ1) that the MAPK does not itself directly bind to the MAPK. This is analogous to the demonstration that JNK can phosphorylate proteins lacking JNK-binding sites but that are bound to c-Jun (46).

PLC-γ1 has been reported to directly interact with various classes of cellular proteins, including receptor and non-receptor tyrosine kinases (26, 47–48), ion channel-forming receptors (49), phospholipase D2 (50), the synaptic vesicle protein, synaptotagmin (51), the p21Ras-specific guanine nucleotide exchange factor SOS1 (21), and the serine/threonine kinase Raf1 (23). Our studies demonstrate that ERK2 and phospho-ERK2 can be added to this list. Thus, in addition to regulating cellular functions via controlling Ins(1,4,5)P3 and 1,2-DAG production, PLC-γ1 appears to act as a scaffolding protein, which may account for the observations that some PLC-γ1-mediated cellular responses are independent of catalytic activity (19–22). Additional studies are needed to determine whether MAPK-dependent phosphorylation of PLC-γ1 regulates its functioning as a scaffolding protein.

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Identification of Phospholipase C-γ1 as a Mitogen-activated Protein Kinase Substrate
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