Phospholipase C-γ1 (PLC-γ1) is rapidly activated in response to growth factor stimulation and plays an important role in regulating cell proliferation and differentiation through the generation of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, leading to the activation of protein kinase C (PKC) and increased levels of intracellular calcium, respectively. Given the existing overlap between signaling pathways leading to the activation of protein kinase C (PKC) and differentiation through the generation of the second messengers, PLC-γ1 (PLC-γ1) is rapidly activated in response to oxidative stress. Treatment of normal mouse embryonic fibroblasts (MEF) with H₂O₂ resulted in time- and concentration-dependent tyrosine phosphorylation of PLC-γ1. Phosphorylation could be blocked by pharmacological inhibitors of Src family tyrosine kinases or the epidermal growth factor receptor tyrosine kinase, but not by inhibitors of the platelet-derived growth factor receptor or phosphatidylinositol 3-kinase. To investigate the physiologic relevance of H₂O₂-induced tyrosine phosphorylation of PLC-γ1, we compared survival of normal MEF and PLC-γ1-deficient MEF following exposure to H₂O₂. Treatment of PLC-γ1-deficient MEF with H₂O₂ resulted in rapid cell death, whereas normal MEF were resistant to the stress. Pretreatment of normal MEF with a selective pharmacological inhibitor of PLC-γ1, or inhibitors of inositol trisphosphate receptors and PKC, increased their sensitivity to H₂O₂, whereas treatment of PLC-γ1-deficient MEF with agents capable of directly activating PKC and enhancing calcium mobilization significantly improved their survival. Finally, reconstitution of PLC-γ1 protein expression in PLC-γ1-deficient MEF restored cell survival following H₂O₂ treatment. These findings suggest an important protective function for PLC-γ1 activation during the cellular response to oxidative stress.

As a consequence of our aerobic environment, we are continually exposed to reactive oxygen species. The deleterious and cumulative effects of oxidant injury to macromolecules appear to contribute to the development of a wide variety of disease processes, including diabetes, cancer, and Alzheimer’s disease, and are believed to be a major factor in aging (1). For these reasons our laboratory has long been interested in the signaling pathways that mediate the effects of oxidative stress on cellular physiology.

Oxidants activate many signaling pathways, and we and others have demonstrated that growth factor receptors play an important role in initiating cellular responses to oxidative stress (2–6). Following exposure to H₂O₂, both the epidermal growth factor receptor (EGFR)¹ and the platelet-derived growth factor receptor (PDGFR) are activated, leading to their dimerization and autophosphorylation. Furthermore, we have demonstrated that certain proliferation-associated signaling pathways, including those leading to activation of extracellular signal-regulated kinase (ERK) and Akt, are activated by oxidative stress in a growth factor receptor-dependent manner and in such instances promote cell survival (2). Thus, it appears that at least some signaling pathways involved in regulating proliferation also participate in the cellular response to oxidative stress.

Phospholipase C γ1 (PLC-γ1) is an enzyme that is recruited to the membrane following activation of growth factor receptor tyrosine kinases (7–10). As a result of its interaction with these signaling molecules, PLC-γ1 is activated by a mechanism that relies on tyrosine phosphorylation. Activated PLC-γ1 cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate generating two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). The former functions to activate protein kinase C (PKC); the latter stimulates the release of Ca²⁺ from internal stores. Through the pleiotropic actions of IP₃ and DAG, PLC-γ1 participates in the regulation of cellular proliferation and differentiation (8).

Several laboratories have demonstrated that PLC-γ1 undergoes tyrosine phosphorylation in response to oxidative exposure, but the mechanisms leading to this activation are not well understood (11–13). In addition, the physiologic consequences of PLC-γ1 activation by stress are unclear. Based on our prior studies demonstrating the importance of certain proliferation-
associated signaling pathways in protecting the cell against oxidative stress (2, 14, 15), we hypothesized that PLC-γ1 might also be important for transducing survival signals resulting from oxidant exposure. To investigate this possibility, we have employed normal mouse embryo fibroblasts (wt MEF), MEF that have been rendered deficient for PLC-γ1 by targeted disruption of both plc-γ1 alleles (Plcg1 null), and Plcg1 null MEF in which PLC-γ1 function has been reconstituted through stable ectopic expression of PLC-γ1 (Plexg1 null+). Our findings reported herein demonstrate that H2O2 is a strong and specific activator of PLC-γ1, and further implicate PLC-γ1 and its downstream effectors in conferring protection against oxidative stress.

EXPERIMENTAL PROCEDURES

Materials—Hydrogen peroxide (H2O2) was purchased from Sigma. Thapsigargin, G6983, xestospongin C, TPA, ionomycin, FP2, FP3, wortmannin, U73122, AG1478, compound 56, AG1296, and Thapsigargin, Go6983, xestospongin C, TPA, ionomycin, PP2, PP3, wortmannin, U73122, AG1478, compound 56, AG1296, and N-acetyl-cysteine were all obtained from Calbiochem (San Diego, CA). The JNK1 and PLC-γ1 polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology, Inc. (Lake Placid, NY). The phospho-specific ERK rabbit polyclonal antibody was from Promega (Madison, WI). The phospho-specific p38 and phospho-specific Akt polyclonal antibodies were purchased from New England Biolabs, Inc. (Beverly, MA).

Cell Culture, Treatment, and Survival Assays—Establishment of spontaneously immortalized wt MEF and Plcg1 null, derived from targeting vector 1 (TV-1), has been described previously (16). PLC-γ1 was stably re-expressed in plcg1 null MEF using a retroviral expression vector to produce plcg1 null+ MEF (47). Although plcg1 null do not mobilize calcium in response to growth factors, plcg1 null+ do. HeLa and T98G cells were obtained from American Type Culture Collection (Manassas, VA). All cell lines were grown in Dulbecco’s modified Eagle’s medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and were maintained in 5% CO2.

For treatments with H2O2, an 8 mM stock solution was diluted to a working concentration in deionized water and was added immediately to the culture medium (containing serum). For cell survival assays, cells were plated in 60-mm dishes and cultured overnight prior to treatment with H2O2 or other agents. Following treatment, cells were harvested and stained with trypan blue, and live cells were counted using a hemocytometer. The percentage of viable cells in the treatment groups was determined from cell counts in treatment groups divided by cell counts of untreated controls. Thus, a reduction in the number of viable cells in treatment groups reflects cell death and/or inhibition of proliferation.

DAPI Staining—DAPI staining was performed as described previously (18). In brief, prior to staining, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then washed with phosphate-buffered saline. DAPI was added to the fixed cells for 1 h, after which they were examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei.

Immunoprecipitation and Immunoblot Analysis—After stimulation, cells were washed in ice-cold phosphate-buffered saline, then harvested in 1 ml of lysis buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM Na3VO4, 5 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin). Equal amounts of protein were incubated with 5 μg of PLC-γ1 antibody and 35 μl of 50% slurry of protein A-Sepharose for 4 h at 4 °C. Immune complexes were washed four times with the same lysis buffer and were resuspended in 2×...
sample buffer. For immunoblot analysis, the precipitated proteins were resolved on 4–12% NuPAGE BisTris gels (Novex, San Diego, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 10 mM Tris- HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, containing 5% milk, and were then probed with different antibodies. Proteins were detected by using enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech).

JNK Kinase Assays—JNK activity was measured by an immunocomplex kinase assay as described previously (15). In brief, the cells were lysed in 1 ml of lysis buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM β-glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 5 mM NaF, 10 μg/mL leupeptin, and 10 μg/ml aprotonin). Equal amounts of protein samples were precipitated at 4°C for 4 h with 5 μl of a JNK1 antibody with the addition of 35 μl of 50% slurry of protein A-Sepharose (Amersham Pharmacia Biotech). The protein A-Sepharose beads were washed three times each in lysis buffer and kinase assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl2, 1% dithiothreitol, and 0.1% Triton X-100). JNK kinase assays were performed using GST-c-Jun (1-135) as a substrate. Samples were separated on a 12% SDS-polyacrylamide gel and, after drying, were subjected to autoradiography.

Statistical Analysis—An unpaired Student’s t test was used to assess statistical significance of differences between normal MEF and Plcg1 null MEF. Differences were considered significant for p values of 0.05 or less.

RESULTS

H2O2 Stimulates Tyrosine Phosphorylation of PLC-γ1—PLC-γ1 is a tyrosine kinase substrate, and it is well established that tyrosine phosphorylation of PLC-γ1 is necessary for its activation (9, 10). To examine the effect of H2O2 treatment on PLC-γ1 phosphorylation, wt MEF were exposed to various doses of the oxidant for 15 min and tyrosine phosphorylation of PLC-γ1 was examined by precipitating PLC-γ1 with a PLC-γ1 antibody, followed by Western blotting analysis with a phosphotyrosine-specific antibody. Western blot analysis with a PLC-γ1 antibody was used to verify that equal amounts of PLC-γ1 protein were present in the immunoprecipitates. As shown in Fig. 1A, H2O2 treatment resulted in PLC-γ1 tyrosine phosphorylation in a dose- and time-dependent manner. A significant increase in PLC-γ1 phosphorylation was detected with H2O2 concentrations as low as 200 μM and activation was rapid, occurring within 5 min of treatment. Maximum levels of PLC-γ1 phosphorylation were recorded 10–20 min after the addition of H2O2. It is important to note that, in these experiments, H2O2 was added directly to the serum-containing culture medium, as opposed to cells being treated with H2O2 while in phosphate-buffered saline. The fact that such low doses of H2O2 increase PLC-γ1 tyrosine phosphorylation, despite the presence of anti-oxidants in the supplemented medium, supports the physiologic relevance of our observations. This response was not unique to MEF as H2O2 treatment also led to phosphorylation of PLC-γ1 in two human cell lines, HeLa and T98G glioblastoma cells (Fig. 1C). To confirm the involvement of reactive oxygen species in PLC-γ1 phosphorylation, the ability of N-acetylcysteine (NAC), a scavenger of reactive oxygen species and precursor of glutathione (19), to block phosphorylation was assessed. As shown in Fig. 1B, NAC acted in a dose-dependent manner to inhibit PLC-γ1 phosphorylation in wt MEF (Fig. 1D).

Upstream Mediators of PLC-γ1 Phosphorylation by H2O2 Treatment—We and others have previously provided evidence that growth factor receptors, including EGFR and PDGFR, play an important role in mediating the activation of ERK and Akt in response to oxidant injury (2, 3, 5). Since PLC-γ1 is also strongly activated by growth factor stimulation of these receptors, we investigated their involvement in mediating PLC-γ1 activation following H2O2 treatment. As shown in Fig. 2A, AG1478 and compound 56, selective inhibitors of EGFR tyrosine kinase activity (20, 21), prevented PLC-γ1 tyrosine phosphorylation in response to H2O2 treatment. In contrast, AG1296, a specific inhibitor of PDGFR tyrosine kinase activity, had no effect on PLC-γ1 phosphorylation, although these cells have been shown to express functional PDGFRs (17). These findings suggest a role for EGFR, but not PDGFR in mediating PLC-γ1 phosphorylation by oxidative stress. Previous studies have shown that Src family tyrosine kinases are involved in signaling events stimulated by reactive oxygen species (22–24) and Src kinase family members have been also demonstrated to phosphorylate PLC-γ1 and PLC-γ2 in vitro (25, 26). To address the role of Src family kinases in H2O2-mediated phosphorylation of PLC-γ1, we utilized the selective Src family tyrosine kinase inhibitor PP2. Phosphorylation of PLC-γ1 by H2O2 was completely blocked by the presence of 10 μM PP2. Similar treatment with the same concentration of the inactive analog PP9 had no effect on PLC-γ1 phosphorylation by H2O2, indicating that the effect of PP2 was selective.

Several studies have implicated PI3-K in the activation of PLC-γ1 (27–29), but others have not (30). As we have demonstrated previously that the PI3-K/Akt signaling pathway is activated in response to H2O2 treatment (2), we used the PI3-K inhibitor wortmannin to test the possibility that PI3-K is involved in mediating PLC-γ1 phosphorylation in response to H2O2. As shown in Fig. 2C, inhibition of PI3-K had no effect on the tyrosine phosphorylation of PLC-γ1 by H2O2. Consistent

![Fig. 2. Upstream mediators of PLC-γ1 phosphorylation by H2O2 treatment](http://www.jbc.org/)

**A**
- IP: PLC-γ1
- IB: p-Tyr
- IP: PLC-γ1
- IB: p-Tyr
- IP: PLC-γ1
- IB: PLC-γ1

**B**
- 5 μM H2O2
- 10 μM H2O2
- + Wortmannin
- - + Wortmannin
- - + Wortmannin
- - + Wortmannin

**C**
- IP: PLC-γ1
- IB: p-Tyr
- IP: PLC-γ1
- IB: PLC-γ1

**Fig. 2.** Upstream mediators of PLC-γ1 phosphorylation by H2O2 treatment. A, inhibition of H2O2-induced PLC-γ1 tyrosine phosphorylation by pretreatment with EGFR inhibitors. Cells were pretreated with various inhibitors or a solvent control for 30 min prior to treatment with H2O2 (600 μM, 15 min). PLC-γ1 phosphorylation was assayed by Western blotting. B, wt MEF were pretreated with the indicated doses of PP2 or its inactive analog PP3 for 30 min prior to H2O2 (600 μM) treatment. Lysates were prepared from cells 15 min after treatment with H2O2 and were subjected to immunoprecipitation (IP) with a PLC-γ1 antibody. Precipitates were then analyzed by immunoblotting (IB) using antibodies specific for either phosphotyrosine or PLC-γ1. C, inhibition of PI3-K has no effect on tyrosine phosphorylation of PLC-γ1 by H2O2. wt MEF were treated with the PI3-K inhibitor wortmannin (200 nM) for 30 min prior to treatment with H2O2 (600 μM), and cell lysates were prepared 15 min later. Phosphorylation of PLC-γ1 was assayed using immunoprecipitation and immunoblotting techniques, as described above. All experiments were repeated three times, and data from one representative experiment are shown.
with this, wortmannin does not affect calcium mobilization in response to H$_2$O$_2$ (data not shown).

Influence of PLC-γ1 Status on Cell Survival following Oxidant Challenge—H$_2$O$_2$ acts in a concentration-dependent manner to induce apoptosis in a variety of cell types (15, 31–33), and a number of different signaling pathways have been shown to be involved in controlling cellular sensitivity to oxidant injury. To explore the role of PLC-γ1 in influencing cell survival following H$_2$O$_2$ treatment, we compared the sensitivity of wt MEF and Pleg1 null MEF to various concentrations of the oxidant. Trypan blue exclusion was employed to assess viability 24 h after treatment. As shown in Fig. 3, treatment with 300 μM H$_2$O$_2$ inhibited the growth of both wt and Pleg1 null MEF, resulting in a reduction in the number of viable cells relative to untreated (control) cultures. At higher concentrations (450–600 μM), H$_2$O$_2$ became markedly cytotoxic for Pleg1 null MEF, with less than 1% of cells surviving treatment with 600 μM H$_2$O$_2$. However, little evidence of cytotoxicity was observed in wt MEF (Fig. 3, bottom panel; see also Fig. 4B and Fig. 7C). This differential sensitivity of wt and Pleg1 null MEF to H$_2$O$_2$ appears to be specific for oxidative stress as wt and Pleg1 null MEF did not differ in their susceptibility to another stressor, thapsigargin. Thapsigargin disrupts calcium homeostasis by inhibiting calcium ATPases on the membrane of the endoplasmic reticulum, which triggers a unique signaling cascade referred to as the unfolded protein response (34). Depending on the cell type, thapsigargin induces growth arrest and/or cell death. Thapsigargin had the same effect on wt and Pleg1 null MEF; although some growth arrest was observed, the treatment was toxic and resulted in significant cell death in both cell lines (Fig. 3, bottom panel). Consistent with the lack of a role for PLC-γ1 in influencing the responsiveness of MEF to thapsigargin treatment, no PLC-γ1 phosphorylation was evident in wt MEF following treatment with the agent (Fig. 3, top panel).

Examination of the kinetics of the response to 600 μM H$_2$O$_2$ treatment revealed that the onset of death occurred rapidly in Pleg1 null MEF (Fig. 4A). Within 4 h, these cells began to shrink, round up, and detach from the plate, and by 8 h less than 1% of the cells remained viable. The wt MEF, on the other hand, remained attached to the plate, but took on a more flattened, spread-out appearance. Thus, the growth inhibitory effects of H$_2$O$_2$ were already apparent at early time points. Pleg1 null MEF stained with DAPI displayed features typical of apoptosis including condensation and fragmentation of nuclei (Fig. 4B). In contrast, nuclei from H$_2$O$_2$-treated wt MEF remained homogeneously stained with little evidence of fragmentation (Fig. 4B).

H$_2$O$_2$-induced Activation of Mitogen-activated Protein Kinases (MAPK) and Akt Do Not Rely on PLC-γ1—We have previously demonstrated that both the MAPK (including ERK, JNK, and p38 members) and PI3-K/Akt signaling pathways are activated in response to H$_2$O$_2$ treatment and influence cell survival (2, 15). To rule out the possibility that the differential sensitivity of wt MEF and Pleg1 null MEF to H$_2$O$_2$ might be attributed to effects of PLC-γ1 on one or more of these pathways, we examined whether the PLC-γ1 status affected activation of these kinases in response to oxidant treatment. wt MEF and Pleg1 null MEF were harvested at various times after exposure to 600 μM H$_2$O$_2$, and examined for ERK, p38, and Akt activation by Western blot analysis using phosphospecific ERK, p38, and Akt antibodies. JNK activity was assessed by an immunocomplex kinase assay using GST-c-Jun as a substrate. As shown in Fig. 5, no significant differences in the levels of ERK, JNK, or Akt phosphorylation were noted between wt MEF and Pleg1 null MEF. Although p38 activation was actually higher in the Pleg1 null MEF relative to wt MEF, neither SB202190 nor SB203580 (pharmacologic inhibitors of p38) affected MEF cell survival in response to H$_2$O$_2$ treatment (data not shown). Thus, the influence of PLC-γ1 on cell survival appears to be independent of MAPK and PI3-K/Akt signaling pathways.

PLC-γ1 Function Is Required for Its Pro-survival Role in Response to H$_2$O$_2$—Our results thus far suggest that PLC-γ1 serves a pro-survival function during H$_2$O$_2$ treatment. If so,
then inhibition of PLC-γ1 activity would be expected to increase the sensitivity of wt MEF cells to H2O2. To test this possibility, wt MEF were treated with U73122, a selective inhibitor of phosphoinositide-specific PLCγ function, prior to challenge with H2O2. As shown in Fig. 6A, 30 min of pretreatment with 5 μM U73122 did significantly enhance the sensitivity of wt MEF to H2O2 (Fig. 6A). U73122 treatment in the absence of H2O2 had no effect on the cells (data not shown). The inactive analog of U73122, known as U73433, could not be included in these survival assays because it was toxic to both the wt and Plcg1 null MEF.

PLC-γ1 catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate the second messenger molecules IP3 and DAG. The former provokes a transient increase in intracellular free Ca2+, whereas the latter serves as a direct activator of PKC. It is through these second messengers that PLC-γ1 exerts its influence on proliferation. To examine their importance in influencing cell survival in response to oxidative stress, two complementary approaches were employed. First, we pretreated wt MEF with a combination of XeC (an IP3 receptor blocker) and Go6983 (a PKC inhibitor) to abrogate the PLC-γ1-mediated increases in intracellular Ca2+ and PKC activity. Cell survival was assessed 24 h later by trypan blue dye exclusion. As expected, the addition of XeC and Go6983 partially reduced the survival of wt MEF following exposure to H2O2 (Fig. 6B), although treatment with XeC and Go6983 in the absence of H2O2 had no effect on the cells (data not shown). In the second approach, Plcg1 null MEF were treated with pharmacologic agents that act downstream of PLC-γ1 to increase intracellular Ca2+ (ionomycin) and activate PKC (TPA). Consistent with the hypothesis that these PLC-γ1 effector functions are important in promoting cell survival during oxidant injury, the combination of ionomycin and TPA markedly improved survival of H2O2-treated Plcg1 null MEF.

Taken together, the experiments described above argue strongly that PLC-γ1 activation exerts a pro-survival influence during the cellular response to oxidant injury. As a final test of this hypothesis, we examined how reconstitution of PLC-γ1 expression in Plcg1 null MEF would affect their response to H2O2 treatment. Plcg1 null cells with restored PLC-γ1 expression (Plcg1 null+) were generated previously and have been shown to exhibit normal PLC-γ1 function in response to proliferative signals (17, 30). As shown in Fig. 7A, the ectopically expressed PLC-γ1 in these cells undergoes tyrosine phosphorylation in response to H2O2 treatment similar to that seen in wt MEF. Next, we compared the sensitivity of wt MEF, Plcg1 null, and Plcg1 null+ to various doses of H2O2. As depicted in Fig. 7B, reconstitution of PLC-γ1 expression in the Plcg1 null MEF rescued them from H2O2-induced cytotoxicity, resulting in cell viability essentially identical to that seen in wt MEF. As seen in Fig. 7C, morphologic examination of H2O2-treated wt MEF, Plcg1 null, and Plcg1 null+ MEF confirmed the viability measurements obtained by trypan blue dye exclusion in Fig. 7B, indicating that PLC-γ1 function is important in mediating resistance to harmful effects of H2O2.
DISCUSSION

PLC-γ1 is known to play an important role in regulating cell proliferation through its interactions with both receptor and non-receptor tyrosine kinases. However, despite the existence of significant overlap between proliferative and stress signaling pathways, a role for PLC-γ1 in mediating cellular responses to stress has not been appreciated. Here we have demonstrated that PLC-γ1 is tyrosine-phosphorylated in response to H$_2$O$_2$ treatment through Src family kinases and/or an EGFR-dependent mechanism. In addition, using Plcg1 null MEF and Plcg1 null MEF in which PLC-γ1 function is restored, we have provided evidence that PLC-γ1 expression supports cell survival following acute oxidant injury. Taken together, these findings implicate PLC-γ1 as an important regulatory molecule in the cellular response to oxidative stress.

That PLC-γ1 undergoes phosphorylation on tyrosine residues in response to H$_2$O$_2$ treatment has been reported by others (11–13). However, most of the previous studies have employed high doses of H$_2$O$_2$ (in excess of 1 mM, and usually between 5 and 10 mM) and/or have required co-treatment with the phosphatase inhibitor pervanadate to observe phosphorylation. Such observations suggest that H$_2$O$_2$ is, at best, a weak activator of PLC-γ1. In addition, the use of such high concentrations of H$_2$O$_2$ raises questions concerning the biological relevance of the response, as they can be markedly cytotoxic for cells, leading to rapid necrosis. Our current studies have em-
ployed much lower concentrations of H$_2$O$_2$, and it is added to cells in the presence of serum, which contains significant anti-oxidant activity. We have demonstrated that, at concentrations as low as 200 μM, treatment with H$_2$O$_2$ results in PLC-γ1 phosphorylation, with concentration-dependent increases occurring over a range of 200–1000 μM in MEF (Fig. 1, A and B). Survival of MEF deficient in PLC-γ1 function is severely compromised over the same dose-response range of H$_2$O$_2$ (Fig. 3), which strongly supports the biologic relevance of PLC-γ1 phosphorylation and the role of PLC-γ1 in protecting cells against H$_2$O$_2$-induced cell death.

A number of questions remain concerning the mechanisms leading to PLC-γ1 phosphorylation in response to oxidant injury, although our studies implicate both the EGFR and Src family kinases in the process. Previously we proposed that oxidants might act to mimic the action of EGF (and perhaps other growth factors) leading to elevated EGFR kinase activity (2, 5). This in turn triggers the activation of other signaling molecules including non-receptor type tyrosine kinases, such as members of the Src family. Indeed, PLC-γ1 phosphorylation occurs rapidly in response to oxidant treatment, over the same time frame as that seen for phosphorylation of EGFR by EGF and H$_2$O$_2$ (2, 35). However, in response to certain stimuli, Src kinase activation has been found to lead to phosphorylation of the EGFR (36). Therefore, it is certainly possible that H$_2$O$_2$ acts in a similar fashion, first activating Src kinases, followed by activation of the EGFR.

It has been suggested that the increase in tyrosine phosphorylation is due, at least in part, to inactivation of phosphatases by H$_2$O$_2$, a mechanism that assumes a certain basal level of tyrosine kinase activity in the absence of overt stimulation. In support of this view, H$_2$O$_2$ has been shown to reversibly inactivate protein tyrosine phosphatase 1B in cells and contribute to EGFR phosphorylation in response to EGF treatment (37). Direct activation of tyrosine kinases and inactivation of phosphatases by oxidants are not mutually exclusive events. Rather, they are both likely to influence tyrosine phosphatase levels and therefore the activities of upstream kinases that ultimately regulate PLC-γ1 phosphorylation. However, oxidant-mediated inactivation of a phosphatase involved in the regulation of tyrosine phosphorylation on PLC-γ1 is obviously not sufficient to produce dramatic increase in tyrosine phosphorylation of PLC-γ1 that results from H$_2$O$_2$ treatment.

A major conclusion of our studies is that PLC-γ1 has a pro-survival function in the cell's response to acute oxidative stress. Consistent with our observations, overexpression of PLC-γ1 in rat pheochromocytoma PC12 cells was reported to inhibit apoptosis induced by short wave length ultraviolet radiation (UVC) (38). UVC is believed to exert its cellular affects, at least in part, through generation of oxidative stress. However, a subsequent report showed no protective influence of PLC-γ1 overexpression against several different treatments including H$_2$O$_2$ in NIH3T3 cells, although PLC-γ1 overexpression did favor survival (39). Interpretation of the significance of PLC-γ1 expression levels in the previous studies is complicated by the fact that PLC-γ1 was overexpressed in cells with otherwise normal PLC-γ1 activity. Our approach, which utilized MEF derived from embryos in which Plcg1 expression has been eliminated through targeted gene disruption, avoided such complications and further allowed us the opportunity to evaluate how restoration of PLC-γ1 function in the Plcg1 null fibroblasts affected their response to H$_2$O$_2$. We observed that ectopic expression of PLC-γ1 in Plcg1 null MEF conferred resistance to H$_2$O$_2$ equivalent to that seen in wt MEF (Fig. 7, B and C). That PLC-γ1 may have a similar role in other stress circumstances is indicated by the finding that when grown in suspension, Plcg1 null are more sensitive to death than are Plcg1 null +. 

It is not clear how PLC-γ1 protects cells from oxidant injury. PLC-γ1 exhibits its influence on proliferation largely through the generation of the second messengers inositol 1,4,5-triphosphate and diacylglycerol, which in turn provoke the mobilization of Ca$^{2+}$ and activate protein kinase C, respectively. That these downstream events also contribute to PLC-γ1's influence on cell survival following H$_2$O$_2$ treatment was evidenced by the finding that inhibitors of PKC and Ca$^{2+}$ mobilization rendered wt MEF more sensitive to H$_2$O$_2$ treatment, whereas pharmacologic activation of PKC and Ca$^{2+}$ mobilization in Plcg1 null MEF enhanced their survival. Importantly, the pharmacologic agents were less effective modulators of survival in oxidant-treated cells than was manipulation of Plcg1 expression. This may be a reflection of the expected differences in the pharmacologic approach (due to, for example, the degree of inhibition or activation achieved and the specificity of the agents actions). However, it could reflect additional, unrecognized roles of PLC-γ1. In this regard it is also interesting to note that wt and Plcg1 null MEF do not differ in their mitogenic response to EGF stimulation (40). The marked differences in sensitivity to oxidant injury observed in wt MEF as compared with Plcg1 null MEF may thus be attributable to a unique function of PLC-γ1 during stress. Attempts to identify downstream targets of PLC-γ1 activation that are involved in mediating stress resistance are currently under way.

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