Phospholipase C-γ1 Is Required for the Induction of Immediate Early Genes by Platelet-derived Growth Factor*

Received for publication, January 19, 2001
Published, JBC Papers in Press, January 30, 2001, DOI 10.1074/jbc.C100030200

Hong-Jun Liao, Qun-Sheng Ji‡§, and Graham Carpenter‡¶
From the Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

To explore the functional role of phospholipase C-γ1 (PLC-γ1) in the induction of immediate early genes (IEGs), we have examined the influence of Plcg1 gene disruption on the expression of multiple IEG mRNAs induced by platelet-derived growth factor (PDGF). Plcg1-null embryos were used to produce immortalized fibroblasts genetically deficient in PLC-γ1 (Null cells), and retroviral infection of those cells was used to derive PLC-γ1 re-expressing cells (Null+ cells). In terms of PDGF activation of PDGF receptor tyrosine phosphorylation as well as the mitogen-activated protein kinases Erk1 and Erk2, Null and Null+ cells responded equivalently. However, the PDGF-dependent expression of all IEG mRNAs was diminished in cells lacking PLC-γ1. The expression of Fos, COX-2, Kc, Je, and c-fos mRNAs were most strongly compromised, as the stimulation of these genes was reduced by more than 90% in cells lacking PLC-γ1. The combination of PMA and ionomycin, downstream analogs of PLC activation, did provoke expression of mRNAs for these IEGs in the Null cells. We conclude that PLC-γ1 is necessary for the maximal expression of many PDGF-induced IEGs and is essential for significant induction of at least five IEGs.

Receptor-tyrosine kinases (RTKs) transduce extracellular signals to the nucleus provoking gene expression and thereby orchestrating cell responses, such as mitogenesis. Growth factor receptor signaling rapidly results in the transcription of IEGs, which in many cases direct the synthesis of proteins that participate in signaling pathways (1, 2). RTKs mediate these processes by activating, following ligand binding, a variety of intracellular signaling pathways through their intrinsic kinase activity (3). PLC-γ1 is a direct tyrosine kinase substrate for most RTKs (4, 5). Following ligand binding, RTKs dimerize and autophosphorylate tyrosine residues, which then serve as association sites for PLC-γ1, facilitating its tyrosine phosphorylation and activation (3–5). Growth factors are not known to activate PLC-β or PLC-δ isoforms.

PLC-γ1 activity catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce the second messenger molecules inositol 1,4,5-trisphosphate and diacylglycerol (6, 7). The former provokes a transient increase in intracellular free Ca2+ ([Ca2+]i), whereas the latter serves as a direct activator of protein kinase C. However, the mechanism by which PLC-γ1 activity participates in the mitogenic response of cells to growth factors is not understood (4, 5). Based on a variety of experimental approaches, some reports have concluded that in cell culture systems, PLC-γ1 activation is not essential for growth factor induced mitogenesis, whereas other approaches have produced the opposite conclusion (5). In the mouse knockout of Plcg1, the null phenotype is embryonic lethal (E9.0), indicative of an essential role in embryonic cell proliferation (8). PLC-γ1 is expressed in many tissues and cell lines, whereas the PLC-γ2 isoform is specifically expressed in hematopoietic tissues (9). The Plcg2 mouse knockout does not have a lethal phenotype (10), indicating that these two PLC-γ isoforms do not have a redundant function in vivo. Drosophila PLC-γ has been identified, and mutational analysis indicates that it regulates wing vein development and may function as a negative regulator of RAS (11).

The biological significance of PLC-γ1 in T cells activated by antigen is much clearer. In these cells PLC-γ1 function is essential for the induction of IL-2 plus other IEGs and antigen-dependent T cell proliferation (12, 13). To explore the functional role of PLC-γ1 in the transcription of IEGs in growth factor-dependent fibroblast proliferation, we have examined the influence of Plcg1 disruption on the expression of multiple IEGs induced by PDGF in mouse embryo fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM) containing t-glutamine and high glucose was purchased from Life Technologies, Inc. Recombinant rat PDGF-BB was obtained from R&D Systems, Inc, whereas [α-32P]dATP was purchased from New England Life Science Products. Antibodies to PDGF-β receptor and dual phosphorylated Erk1 and 2 were from Santa Cruz Biotechnology, Inc. Antibody to phosphotyrosine was from Zymed Laboratories Inc. Phorbol-12-Myristate-13-Acetate (PMA) and ionomycin were purchased from Sigma. Kc and Je cDNAs were purchased from the American Type Culture Collection. COX-2/TISS 10 cDNA was a gift from Dr. Harvey Hershman, University of California at Los Angeles. FIC cDNA was a gift from Dr. Rolf-Peter Byestek, Bristol-Myers Squibb Pharmaceutical Research Institute. Gly96, Nup475, mTF, MKP-1, Cyrl61, JunB, Pip92, Nur77, and Zip285 cDNAs were gifts from Dr. Lester F. Lau, University of Illinois at Chicago College of Medicine. cDNAs for c-fos and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were gifts from Dr. Ronald Wisdom, Vanderbilt University, whereas cyclophilin cDNA was a gift from Dr. Chuanming Hao, Vanderbilt University.

Cells and PDGF Treatment—Spontaneously immortalized TV-1 Plcg1-null fibroblasts were established from Plcg1 knockout embryos (8), and PLC-γ1 was subsequently re-expressed in these cells by retroviral infection as previously described (14) to derive Plcg1 Null+ cells. Null+ and Null cells were cultured in DMEM containing 10% fetal calf serum. Subconfluent cells were incubated for 36 h in DMEM plus 0.5% fetal calf serum prior to stimulation by PDGF-BB (25 ng/ml) or other agonists for the indicated times.

Western Blotting—After treatment without or with PDGF, cells were lysed in TGH buffer (1% Triton X-100, 10% glycerol, 50 mM HEPEs, pH 7.2) supplemented with 100 mM NaCl, protease inhibitor mixture tab-

This paper is available on line at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
The probes were labeled with a following immortalization, retroviral infection was employed to growth factors (8, 14) and are referred to herein as Null cells. In the Null and Null activated to similar levels by the addition of PDGF. Also, we showed that for each mRNA, the absence of PLC-γ1 had an attenuating influence that ranged from ~50% (Zif268/egr-1, Nur77, Pip92) to over 90% (FIC, COX-2, JE, KC, c-fos). These data indicate that PLC-γ1 has a role of varying magnitude in the PDGF-dependent induction of many IEGs.

The data in Table I are derived from one set of Null and Null+ cell lines, termed TV-1 (8). To substantiate that the observed differences do represent the absence of PLC-γ1, a second independent set of Null and Null+ cells, known as TV-II (8), were assayed for the PDGF-dependent induction of five IEGs. These genes and the level of induction in the absence of PLC-γ1 are as follows: COX-2 (1%), KC (6%), c-fos (4%), JunB (35%), and Zif268 (47%). Comparison of these results to those reported in Table I for TV-I-derived cells indicates quantitatively similar data were obtained for both TV-I and TV-II Null and Null+ cell lines.

Those genes whose mRNA expression is compromised most by the absence of PLC-γ1 were further studied by determining whether the addition of PMA and ionomycin, which represent downstream pharmacologic mimetics of PLC function (6, 7), to Null cells could induce the accumulation of these IEG mRNAs. The data in Fig. 2 show Northern blot analyses of the mRNA induction time course for FIC, COX-2, c-fos, KC, and JE in PDGF-treated Null and Null+ cells, as well as PMA plus ionomycin-treated Null cells. In each case, the addition of PMA and ionomycin restored mRNA accumulation in the Null cells, indicating that these genes are fully inducible in the Null cells by pharmacologic replacement of PLC function. In most cases, the time course of mRNA accumulation in the presence of PMA and ionomycin was slightly delayed relative to that produced by PDGF. In the case of COX-2, mRNA levels accumulated to a greater extent following the addition of PMA and ionomycin compared with PDGF. In contrast, KC mRNA accumulation following treatment with PMA and ionomycin was less than that achieved by PDGF. Hence, both the time course and quantity of mRNA accumulation were somewhat different for PDGF compared with PMA plus ionomycin induction of these genes.

Lastly, we have assessed the individual capacities of PMA and ionomycin to induce the accumulation of these five IEG mRNAs in Null cells. The results are presented in Table II. In the case of JE mRNA, PMA or ionomycin separately produced significant levels of mRNA accumulation and the combined treatment with both agents was approximately the sum of the individual treatments. In the remaining cases, PMA and ionomycin gave a synergistic response compared with mRNA accumulation provoked by PMA or ionomycin alone. For JE, COX-2, and KC mRNAs, PMA induction was significantly greater than achieved by ionomycin, whereas c-fos and FIC mRNA accumulation was small, but approximately equivalent for either agent alone.

DISCUSSION

The role of PLC-γ1 in the induction of one IEG, c-fos, by PDGF or epidermal growth factor (EGF) receptor-tyrosine kinases has previously been examined in other laboratories using

IEGs. Whereas the total number of IEGs expressed in growth factor-stimulated fibroblasts exceeds 50 (15), this subset was selected for analysis by the availability of probes and was not based on previously published data related to their signaling requirements or promoter elements. At various times after the addition of PDGF to Null or Null+ cells, the mRNA level of each IEG was determined by Northern blotting. The results for all 14 IEGs are presented in Table I. The influence of PLC-γ1 on the level of each mRNA was established by comparing the amount of mRNA at the peak induction times for both cell lines. In no case did the absence of PLC-γ1 alter the time of peak mRNA expression following the addition of PDGF. The results show that for each mRNA, the absence of PLC-γ1 had an attenuating influence that ranged from ~50% (Zif268/egr-1, Nur77, Pip92) to over 90% (FIC, COX-2, JE, KC, c-fos). These data indicate that PLC-γ1 has a role of varying magnitude in the PDGF-dependent induction of many IEGs.

RESULTS

PDGF-dependent Signaling—To examine the potential significance of PLC-γ1 in the stimulation of IEG mRNA levels, we have employed spontaneously immortalized Plcg1−/− fibroblasts that were derived from targeted disruption of the Plcg gene in mice (8). These cells do not increase [Ca2+]i response to growth factors (8, 14) and are referred to herein as Null cells. Following immortalization, retroviral infection was employed to re-express PLC-γ1 in the Null cells (14). The resulting cells, which do mobilize Ca2+ in response to growth factors, are termed Null+ cells. The level of PLC-γ1 expressed in the Null and Null+ cells is shown in Fig. 1A.

To characterize these cells for PDGF-dependent signaling, we first established that Null and Null+ cells express equivalent levels of PDGF β-receptors and that they respond in a comparable manner to the addition of PDGF-BB. The data in Fig. 1B show that both cell lines do express approximately the same amount of PDGF β-receptor and that these receptors are activated to similar levels by the addition of PDGF. Also, we have compared the PDGF-dependent activation of Erk1 and 2 in the Null and Null+ cell lines. As shown in Fig. 1C, these MAP kinase isoforms are activated to similar levels with comparable kinetics regardless of the presence or absence of PLC-γ1. Hence, these studies indicate that PDGF signal transduction is not compromised in unexpected ways by the absence of PLC-γ1.

PDGF-dependent Induction of IEGs—To investigate the induction of IEGs in PDGF-stimulated Null and Null+ cells, we assembled a collection of probes for 14 previously characterized
PLC-γ1 and PDGF Induction of IEGs

### TABLE I

| Gene      | GenBank/EBI accession no. | Functional classification | Peak time | Peak induction of PLC-γ1 mRNA | Induction in absence of PLC-γ1 |
|-----------|---------------------------|---------------------------|-----------|-------------------------------|-------------------------------|
| FIC       | L04694                    | Secreted cytokine         | 60        | 25.0                          | 0.4                           |
| COX-2/TIS-10 | M88242                  | Regulatory enzyme         | 60        | 18.3                          | 0.1                           |
| JE/MCP-1  | M19681                    | Secreted cytokine         | 60        | 30.5                          | 0.2                           |
| KC/GRO    | J04596                    | Secreted cytokine         | 30        | 20.7                          | 0.6                           |
| c-fos     | V00727                    | Transcription factor      | 30        | 12.1                          | 0.9                           |
| Gly96     | X67644                    | Transcription factor      | 30        | 13.5                          | 1.6                           |
| Nup475    | M58691                    | Transcription factor      | 30        | 30.9                          | 4.5                           |
| mTF       | M26071                    | Cell surface receptor     | 60        | 21.8                          | 6.4                           |
| MKP-1     | X61940                    | Regulatory enzyme         | 30        | 20.2                          | 7.0                           |
| Cyrl1     | M32490                    | Secreted growth factor    | 30        | 15.9                          | 5.7                           |
| JunB      | J03236                    | Cytoplasmic protein       | 30        | 31.1                          | 13.4                          |
| Pip92     | M58821                    | Cytoplasmic protein       | 30        | 31.3                          | 14.4                          |
| Nur77     | J04113                    | Orphan steroid receptor   | 60        | 24.2                          | 12.9                          |
| Zif268/erg-1 | M22326                | Transcription factor      | 30        | 20.2                          | 11.1                          |

### TABLE II

| Gene      | Peak time | Relative induction of PMA and/or ionomycin on the induction of immediate early genes in Null cells |
|-----------|-----------|----------------------------------------------------------|
| JE/MCP-1  | 1.5       | 100 67 22                                                  |
| COX-2/TIS-10 | 3.0       | 100 30 3                                                   |
| KC/GRO    | 1.5       | 100 27 10                                                 |
| c-fos     | 0.5       | 100 9 9                                                   |
| FIC       | 1.5       | 100 9 6                                                   |

### FIG. 2

Induction of FIC, COX-2, c-fos, KC, and JE mRNAs by PDGF or PMA plus ionomycin. Quiescent cells were prepared as previously described. After the addition of PDGF (25 ng/ml) to Null+ and Null cells, or PMA (100 ng/ml) plus ionomycin (2 μM) addition to Null cells for the indicated times, equal aliquots (5 μg) of total RNA were electrophoresed, transferred to membranes, and probed with a labeled cDNA fragment for each IEG. A probe for cyclophilin (cyl) or GAPDH (GA) was used as internal control.

strategies that involve either a dominant-negative fusion protein/antibody inhibition approach or an approach that exploits the specificity of Tyr-1021 of the PDGF β-receptor for PLC-γ1 association and activation. Using microinjection of either a fusion protein of glutathione S-transferase with one SH2 domain from PLC-γ1 or antibody to PLC-γ1, Roche et al. (16) reported that expression of a β-galactosidase reporter containing the c-fos promoter was decreased by ~90% in PDGF-stimulated NIH 3T3 cells. They also demonstrated that these reagents blocked PDGF-dependent induction of DNA synthesis. Analogously, Wang et al. (17) microinjected a fusion protein of glutathione S-transferase with both SH2 domains plus the SH3 domain of PLC-γ1 into NIH 3T3 cells and reported that expression of a c-fos serum response element reporter construct was blocked in PDGF-treated cells. Similar results were reported with these reagents in EGF-treated MDCK cells. This group also reported that the EGF-dependent or PDGF-dependent initiation of DNA synthesis in these cells was blocked by microinjection of the dominant-negative protein. These papers, therefore, indicate that PLC-γ1 is necessary for PDGF- or EGF-dependent expression of the c-fos promoter and for the induction of DNA synthesis in quiescent cells.

Mutagenesis of Tyr-1021 in the PDGF-β receptor abrogates PLC-γ1 association with the receptor, and this prevents tyrosine phosphorylation and activation of this signaling protein (18–21). However, PDGF-induced mitogenesis is either not impaired (18) or is slightly decreased (21) by this receptor mutation, leading to the conclusion that PLC-γ1 is dispensable for PDGF-dependent mitogenesis. However, this receptor mutant (Y1021F) has not been employed to assess the role of PLC-γ1 in the induction of any IEG. In a related approach, termed an “add back” strategy (22), the data were interpreted to indicate that the PLC-γ1 pathway in the absence of several other, but not all, PDGF receptor-dependent signaling pathways can support the induction of several IEG mRNAs, including c-fos, KC, and JunB (23). However, another study of these PDGF receptor add back mutants concluded that the PLC-γ1 specific Tyr-1021 site, could support the expression of KC mRNA but not other IEG mRNAs (24). There are limitations to interpretations in the approaches described above, which mainly revolve around the issue of specificity for the inhibition of PLC-γ1 function and/or the extent to which apparent PLC-γ1 regulation of reporter constructs accurately reflects regulation of endogenous mRNA expression. Also, differences in cell lines may account for some of the contradictory results obtained with various experimental approaches. The interpretation of add back mutant data are complex, as the strategy is meant to reveal what downstream
responses can be activated through PLC-γ1 in the absence of other potentially supportive pathways.

Therefore, we have used cell lines in which the Plcg1 gene has been selectively disrupted and have then re-expressed PLC-γ1 in these cells to compare a closely related set of cells in which PLC-γ1 is either absent or present and in which other PDGF-dependent signaling pathways have not been altered. A major finding is that the absence of PLC-γ1 attenuates to varying extents the PDGF-dependent accumulation of all IEGs studied. This suggests a wide role for PLC-γ1 in IEG signaling downstream of the PDGF receptor. Whereas the data presented in this manuscript argue for a significant role of PLC-γ1 downstream of the PDGF receptor. Whereas the data presented in this manuscript argue for a significant role of PLC-γ1 in the induction of multiple IEG mRNAs, it is highly likely that other signaling pathways independent of PLC-function, such as the Ras/MAP kinase pathway, also participate in the maximal induction of these IEGs.

In antigen-activated T cells, PLC-γ isoforms are thought to be essential for the downstream activation of multiple IEGs, such as IL-2 (12, 13). However, that information is somewhat indirect as it relies on the capacity of immunosuppressive agents, such as cyclosporin, to block the Ca²⁺-sensitive phosphatase calcineurin. Recently it has been reported that immunosuppressive agents, including cyclosporin, also interfere with T cell receptor-dependent activation of Jun kinase and p38 pathways in a calcineurin-independent manner (24). This illustrates the difficulty of specifically interrupting signal transduction pathways with pharmacologic inhibitors.

In regard to the expression of c-fos mRNA, we have previously reported that EGF induces the mRNA for this IEG in Plcg1+/+ and Plcg1−/− cells (25). This implies that c-fos induction by EGF is not reliant on PLC-γ1. This observation has been confirmed comparing the Null and Null+ cells employed in this study. Therefore, at least for induction of this one IEG, the EGF and PDGF receptors display a distinctly different requirement for PLC-γ1. Also, we have measured the PDGF-dependent incorporation of [³H]thymidine in quiescent serum-starved Null and Null+ cells. The stimulation of thymidine incorporation was approximately equivalent (about 3-fold) for both cell lines. Hence, maximal expression of many IEGs does not seem to be essential for PDGF-dependent entry of these cells into S phase.

Acknowledgment—We thank David Coon for technical assistance.

REFERENCES
1. Herschman, H. R. (1991) *Annu. Rev. Biochem.* 60, 281–319
2. Rollins, B. J., and Stiles, C. D. (1989) *Adv. Cancer Res.* 53, 1–32
3. Schlessinger, J. (2000) *Cell* 103, 211–225
4. Rhee, S. G., and Ban, Y. S. (1997) *J. Biol. Chem.* 272, 15454–15458
5. Carpenter, G., and Ji, Q.-S. (1999) *Exp. Cell. Res.* 253, 15–24
6. Berridge, M. J., and Irvine, R. F. (1984) *Nature* 312, 315–321
7. Nishizuka, Y. (1980) *Science* 205, 305–312
8. Ji, Q.-S., Winnier, G. E., Niswender, K. D., Horstman, D., Wisdom, R., Magnuson, M. A., and Carpenter, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 2999–3003
9. Homma, Y., Takenawa, T., Emori, Y., Sorimachi, H., and Suzuki, K. (1989) *Biochem. Biophys. Res. Commun.* 164, 406–412
10. Wang, D., Peng, J., Wen, R., Marine, J. C., Sangster, M. Y., Purganas, E., Hoffmeyer, A., Jackson, C. W., Cleveland, J. L., Murray, P. J., and Ihle, J. N. (2000) *Immunity* 13, 25–35
11. Thackeray, J. R., Gaines, P. C. W., Ebert, P., and Carlson, J. R. (1998) *Development* 125, 5033–5042
12. Rao, A., Luo, C., and Hogan, P. G. (1997) *Annu. Rev. Immunol.* 15, 707–747
13. Crabtree, G. R., and Clipstone, N. A. (1994) *Annu. Rev. Biochem.* 63, 1045–1083
14. Ji, Q.-S., Chattopadhyay, A., Vecchi, M., and Carpenter, G. (1999) *Mol. Cell. Biol.* 19, 4961–4970
15. Almendral, J. M., Sommer, D., Macdon ald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988) *Mol. Cell. Biol.* 8, 2140–2145
16. Roche, S., McGlade, J., Jones, M., Gish, G. D., Pawson, T., and Courtneidge, S. A. (1996) *EMBO J.* 15, 4940–4948
17. Wang, Z., Gluck, S., Zhang, L., and Moran, M. F. (1998) *Mol. Cell. Biol.* 18, 590–597
18. Rönnestrand, L., Mori, S., Arridsson, A.-K., Eriksson, A., Wernstedt, C., Helman, U., Claesson-Welsh, L., and Heldin, C.-H. (1992) *EMBO J.* 11, 3911–3919
19. Kashishian, A., and Cooper, J. A. (1993) *Mol. Biol. Cell* 4, 49–57
20. Larose, L., Gish, G., Shoelson, S., and Pawson, T. (1993) *Oncogene* 8, 2493–2499
21. Valius, M., Bazenet, C., and Kazlauskas, A. (1993) *Mol. Cell. Biol.* 13, 133–143
22. Valius, M., and Kazlauskas, A. (1993) *Cell* 73, 321–334
23. Montmayeur, J.-P., Valius, M., Vandenheede, J., and Kazlauskas, A. (1997) *J. Biol. Chem.* 272, 32670–32678
24. Fambrough, D., McClure, K., Kazlauskas, A., and Lander, E. S. (1999) *Cell* 97, 727–741
25. Ji, Q.-S., Ermini, S., Baulida, J., Sun, F.-L., and Carpenter, G. (1998) *Mol. Biol. Cell* 9, 749–757

---

**H.-J. Liao and G. Carpenter, unpublished observations.**