BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 122–126 (1997)
ARTICLE NO. RC977778

# Control of Expression of PLC$\beta_1$ by LAC Repressor System: Relationship between Nuclear PLC$\beta_1$ Overexpression and Growth Factor Stimulation

Anna Maria Billi,* Alessandro Matteucci,† Irene Faenza,* Lucia Manzoli,* Silvia Rubbini,* R. Stewart Gilmour,‡ Sue Goo Rhee,§ and Lucio Cocco*,[1]

**Institute of Human Anatomy, University of Bologna, 40126 Bologna, Italy; †CNR Citomorphology Institute and Laboratory of Cell Biology and Electron Microscopy, Istituti Ortopedici Rizzoli, 40136 Bologna, Italy; ‡Department of Molecular Medicine, School of Medicine University of Auckland, New Zealand; and §Laboratory of Cell Signalling, NHLBI, National Institutes of Health, Bethesda, Maryland*

Received October 28, 1997

**Swiss 3T3 cells have a nuclear phosphoinositide signalling system which is under the control of insulin-like growth factor I (IGF-I) and acts separately from that at the plasma membrane. By using the Lac repressor system we were able both to obtain the inducible overexpression of phospholipase C $\beta_1$ (PLC $\beta_1$) and to determine its subcellular localisation and partitioning. Moreover, by comparing the level of expression at the nucleus and the percentage of cells actively incorporating bromodeoxyuridine (BrdU) in S phase it has strengthened the issue of the importance of this PLC in the onset of DNA synthesis mediated by IGF-I. In addition, this system appears to be a very powerful tool for further analysis of the downstream events following the activation of nuclear PLC $\beta_1$.** 

The phosphoinositide-specific phospholipase C in mitogenic signalling has been widely analysed (1). Among the PLC isozymes, PLC$\beta_1$ is of particular interest since localises in the nucleus in addition to its presence at the plasma membrane (2,3). The existence in several cell types of an autonomous intranuclear inositide cycle endowed with conventional lipid kinases, phosphatases and PLC has been documented (4,5,6,7). The increased activity of the nuclear PLC $\beta_1$ within minutes of stimulation of Swiss 3T3 cells by IGF-I (3) has been suggested to be central to the mitotic response since expression of antisense mRNA of PLC $\beta_1$ abolishes the mitogenic effect of IGF-I (8). Here we have attempted to establish stable transfectants overexpressing PLC $\beta_1$ in an inducible system in order to switch on the gene only during the time of interest and maintain the cells with the wild phenotype during the routine passages. For this purpose we have used the Lac repressor system which consists of a vector that expresses Lac repressor targeted to the nucleus, as well as a vector containing Lac operator sequences followed by a cloning site for insertion of the gene of interest, so that the expression of the gene is repressed until an inducer such as IPTG is added to the media. Upon induction, expression of the gene resumes (9). Here we show the establishment of stable transfected Swiss 3T3 cells in which the inducible overexpression of PLC $\beta_1$ localises predominantly in the nucleus and enhances the onset of DNA synthesis driven by IGF-I stimulation.

## MATERIALS AND METHODS

*Construction of vectors, transfection and screening of tranformants.* The vector p3′SSCMV (Stratagene), which permits expression of E.Coli Lac repressor protein, contain the SV40 nuclear localisation signal sequence, which increase transport to the mammalian cell nucleus. The Lac repressor protein is under the control of a mutant polyoma promoter, F9-1, and contains the hygromycin B phosphotransferase gene for selection (9). Swiss 3T3 were transfected with p3′SSCMV at subconfluent state. A mixture of 5 $\mu$g of plasmid DNA and 10 $\mu$l Transfectam (Promega Corporation) was added to $1\times10^5$ cells in one well of six well plate, for 24 hours. Then transformants were selected in medium containing the hygromycin (Sigma Corporation) at a concentration of 250 $\mu$g/ml. Clones were harvested and expanded separately in the presence of hygromycin. The Lac operator construction contains modified lac operator sequences. A NotI cloning site has been included for the insertion of PLC$\beta_1$ gene, which is expressed under the CMV promoter in the eukaryotic expression vector pRc/CMV (Invitrogen Corp.). The neomycin gene is included for G418 selection in cultured cells. Transfection was performed as

[1] To whom correspondence should be addressed at Institute of Human Anatomy, Via Irnerio 48, I-40126 Bologna, Italy. Fax: 39. 51. 251735. E-mail: pietro@biocfarm.unibo.it.

Abbreviations used: PLC, phospholipase C; PtdInsP, phosphatidylinositol 4-phosphate; PtdInsP$_2$, phosphatidylinositol 4,5-bisphosphate; IGF-I, insulin growth factor I; IPTG, isopropyl-$\beta$-D-thiogalactoside; PDGF, platelet-derived growth factor; BrdU, 5-bromodeoxyuridine; NLS, nuclear localisation signal.

0006-291X/97 $25.00

previously described and the transformants were selected in the presence of hygromycin and geneticin (500 $\mu$g/ml). Clones were harvested and expanded separately. The concentration of IPTG, used to relieve the repression, was 10 mM and this was incubated with the cells for 24 hours.

*Cell fractionation and Western Blot analysis.* Nuclei were obtained exactly as described previously (3) and the supernatants from nuclear pellet were spun down at 105,000 x g in order to have a plasma membrane enriched fraction. Proteins (30 $\mu$g) from whole cells, plasma membrane and purified nuclei were separated in 8 or 10% polyacrylamide gels and transferred to nitrocellulose paper. The presence of the Lac repressor protein was detected with the anti-LacI polyclonal sera (Stratagene) and PLC isoforms were detected by PLC $\beta_1$ and $\gamma_1$ antibodies as described previously (10).

*Growth factor stimulation and flow cytometric analysis of the cell cycle.* Swiss 3T3 cells grown to complete confluence in D-MEM containing 10% FCS, were washed twice with serum-free medium containing 1% bovine serum albumin and then incubated in the same medium containing 20 ng/ml IGF-I or 1 $\mu$M bombesin or 10 ng/ml PDGF for 20 hours and then labelled for 60 mins. with 100 $\mu$M BrdU. Cells were trypsinised, washed twice with phosphate-buffered saline, and resuspended in 100 $\mu$l of the same buffer. To this was added 900 $\mu$l of ice-cold 70% ethanol, washed in the same buffer, incubated for 30 mins. in 4 N HCl, washed once more and treated with Triton X-100 for 3 mins. For analysing the samples by fluorescence-activated cell sorting, the cells were washed in phosphate-buffered saline (3× 1ml), resuspended in the same buffer and incubated with fluorescein isothiocyanate-conjugated anti-BrdU MoAb (Becton Dickinson) for 30 mins. at 4°C and counterstained with propidium iodide (5 $\mu$g/ml). The flow-cytometric analysis was then carried out with a FACStar Plus flow-cytometer (11).

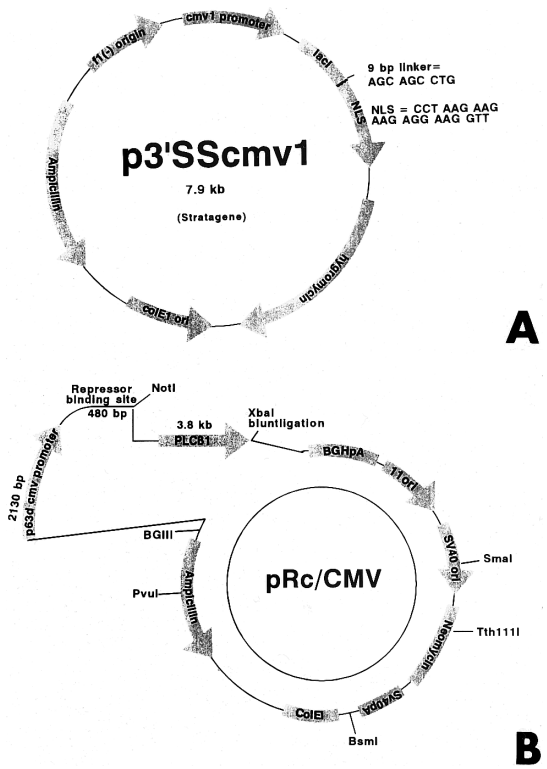



**FIG. 1.** Schematic representation of the eukaryotic vector expressing the Lac repressor with the NLS from SV40 (A) and of pRc/CMV vector in which the full length cDNA for PLC $\beta_1$ has been inserted (B).

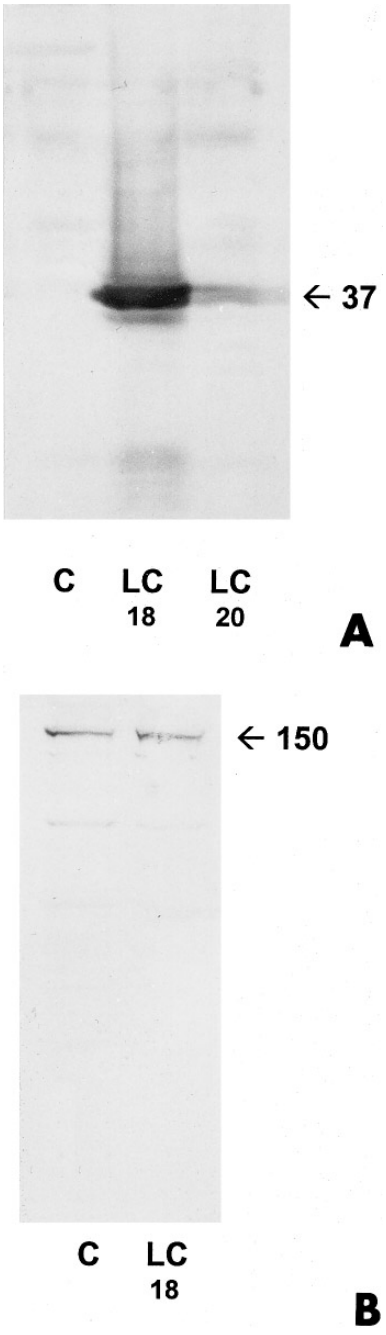
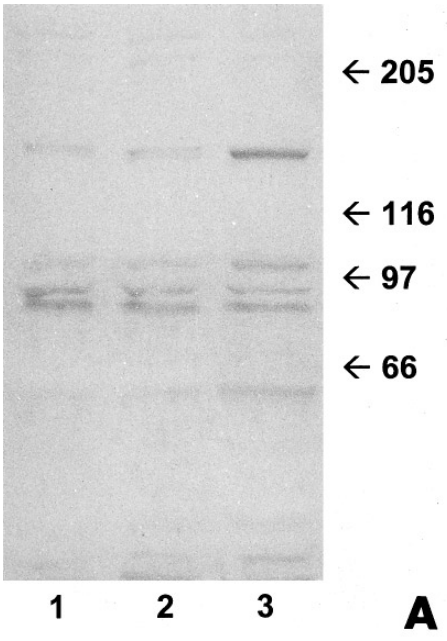



**FIG. 2.** Western Blot analysis of Lac repressor protein and PLC $\beta_1$ in nuclei of Swiss 3T3 cells transfected with the eukaryotic vector expressing the Lac repressor. (A) Nuclear proteins separated in a 10% polyacrylamide gel reacted with anti-Lac repressor protein antibody. (B) Nuclear proteins separated in an 8% polyacrylamide gel reacted with anti-$\beta_1$ PLC antibody. (C) Wild-type 3T3 cells; LC18 and LC20, transfected clones. For each sample 30 $\mu$g proteins was loaded. Arrows indicate $M_r$ (K).

## RESULTS AND DISCUSSION

Fig. 1 shows the cloning strategy of both Lac repressor and PLC $\beta_1$. By using this strategy we have ob-



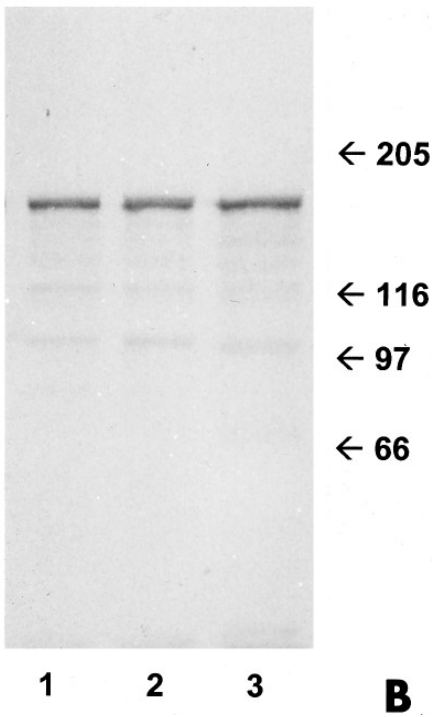




**FIG. 3.** Western Blot analysis of PLC $\beta_1$ in 3T3 cells (clone LC18) transfected with both vectors (see Fig. 1). (A) Whole cell proteins reacted with anti-$\beta_1$ PLC antibody; (B) Nuclear proteins reacted with anti-$\gamma_1$ PLC antibody. For each panel, lane 1 refers to wild 3T3 cells, lane 2 overexpressing clone-IPTG, and lane 3 overexpressing clone +IPTG. For each lane 30 $\mu$g protein was loaded. Arrows indicate $M_r$ (K).

tained several stable transfectants carrying the Lac repressor. The actual localisation of Lac repressor protein in the nucleus has been checked and over several

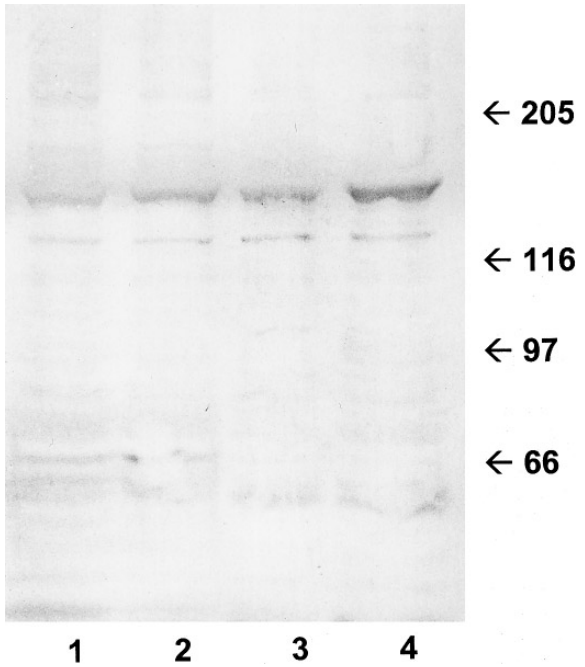



**FIG. 4.** Western Blot analysis of overexpressed PLC $\beta_1$ in membrane (lanes 1 and 2) and nuclei (lanes 3 and 4) of transfected Swiss 3T3 cells. Lanes 1 and 3, -IPTG, lanes 2 and 4, +IPTG. For each lane 30 $\mu$g protein was loaded. Arrows indicate $M_r$ (K).

clones selected by hygromycin resistance only clones LC18 and LC20 showed Lac repressor to localise in the nucleus and namely the LC18 to a greater extended (Fig. 2A). Before transfection with the second vector carrying the PLC $\beta_1$ full length cDNA (pRc/CMV PLC $\beta_1$) LC18 clone was checked for the expression of PLC $\beta_1$ in order to ascertain whether the insertion of Lac repressor would affect PLC $\beta_1$. This is not the case, since as shown in Fig. 2B the level of expression of this phospholipase remains constant respect to wild type cells.

Cell transfected with the full length sense cDNA for PLC $\beta_1$ after IPTG induction show an increased expression of PLC $\beta_1$ in Western Blot analysis (Fig. 3A). As a control we checked the expression of the other isozyme detected in wild-type 3T3 cells, i.e. the

**TABLE I**

Percentage Distribution of PLC$\beta_1$ between Membrane and Nuclei of Swiss 3T3 Cells Overexpressing the Enzyme after IPTG Induction

| Conditions | Membrane | Nuclei |
|---|---|---|
| −IPTG | 44 | 56 |
| +IPTG | 29 | 71 |

*Note.* Values obtained by a densitometric analysis of Western Blots are the averages of 5 separate experiments.

**TABLE II**

Flow Cytometric Analysis of the Cell Cycle of Swiss 3T3 Cells

| Addition | WT | −IPTG | +IPTG |
|---|---|---|---|
| None | 2 | 2 | 3 |
| Bombesin | 20 | 21 | 26 |
| PDGF | 16 | 18 | 18 |
| IGF-I | 12 | 13 | 31 |

*Note.* Percentage of cells in S phase after BrdU incorporation in wild type cells (WT) and stable transfectants with (+) or without (−) addition of IPTG. Values are the averages of 3 separate experiments.

PLC $\gamma_1$ isoform (12). In whole cell homogenates the amount of this PLC is unaffected (Fig. 3B), neither its localisation changes (not shown) remaining completely cytoplasmatic as occurs in wild 3T3 and several other cells (3,6,12,13).

We have then analysed the partitioning of PLC $\beta_1$ in nuclei and plasma membrane. The Western blot in Fig. 4 shows that after IPTG treatment the overexpressed PLC localises mainly in nuclei but also in a good extent in the plasma membrane. The densitometric analysis in Table I gives rise to a quantitative evaluation of this phenomenon. We have subsequently analysed in this new system of overexpression of PLC $\beta_1$ the response to some growth factors which act differently on nuclear inositol lipid cycle.

Previous study showed that whilst with IGF-I the hydrolysis of nuclear PtdInsP and $PtdInsP_2$ is increased and there is a rapid and transient 100% activation of PLC $\beta_1$ (3,14), with bombesin there is no affect on both nuclear inositol lipid cycle (14) and PLC $\beta_1$ (8). This is true also for PDGF (8). The most dramatic increase of mitogenicity is achieved in PLC $\beta_1$ overexpressing cells after IPTG induction by IGF-I which does not activate PLC at the plasma membrane whilst does it at the nucleus (1, 2, 3). On the contrary PDGF, which acts trough plasma membrane PLC $\gamma_1$ and not trough nuclear PLC $\beta_1$ (15), does not affect, in cells overexpressing nuclear PLC $\beta_1$, the entering in S phase. Bombesin, well know to activate membrane PLC $\beta$ family, increases the percentage of cells in S-phase to a lower extent respect to IGF-I according to the amount of PLC $\beta_1$ which localises in the plasma membrane of our stable transfectans after binding of IPTG to the Lac repressor (Table II).

The nuclear localisation of PLC $\beta_1$ constitutes and intriguing issue and more insight has been given by the identification in its carboxyl-terminal domain of a region (named region 2) in which lysines 1056, 1063 and 1070 forming a cluster of basic residues are responsible for nuclear association of PLC $\beta_1$ (16). However in order to further understand both the actual partitioning between plasma membrane and nucleus and the effect of its activation on cell cycle progression it was necessary to set up a system in which the expression of this PLC was inducible having, in this way, a reliable internal control as well as an enhanced expression, given the relatively low levels of PLC $\beta_1$ in cells. In Swiss 3T3 cells overexpressing PLC $\beta_1$ this enzyme localised in very high extent in the nucleus even though a certain amount is detected also in the plasma membrane enriched fraction. The analysis of cell cycle hints at a key role of the nuclear PLC $\beta_1$ in IGF-I induced mitogenesis and this patway is unique for this growth factor since the level of overexpression at the nucleus parallels the degree of activation of BrdU incorporation whilst bombesin, which does not activate nuclear polyphosphoinositides hydrolysis as well as PLC $\beta_1$ (8,14), enhances the entering in S phase to a lower extent compatible with the amount of overexpression of PLC $\beta_1$ at the plasma membrane. Therefore the establishment of the inducible overexpression of PLC $\beta_1$ gave us an opportunity of giving more insight into the role of this PLC in nuclear autonomous signalling via inositol lipids and a powerful tool for further investigations on the downstream events at the nucleus following the activation of polyphosphoinositide hydrolysis.

## ACKNOWLEDGMENTS

This work was supported by the Italian Association for Cancer Research (AIRC) and "Funds for Selected Research Topics" from the University of Bologna. We are grateful to Jane Treagus for skilled assistance in the preparation of vectors and to Dr. M. Vitale for flow cytometric analysis.

## REFERENCES

1. Noh, D. Y., Shin, S. H., and Rhee, S. G. (1995) *Biochim. Biophys. Acta* **1242,** 99–114.
2. Berridge, M. J. (1993) *Nature (London)* **361,** 315–325.
3. Martelli, A. M., Gilmour, R. S., Bertagnolo, V., Neri, L. M., Manzoli, L., and Cocco L. (1992) *Nature (London)* **358,** 242–245.
4. Cocco, L., Martelli, A. M., and Gilmour, R. S. (1994) *Cell. Signalling* **6,** 481–485.
5. York, J. D., Saffitz, E., and Majerus, P. W. (1994) *J. Biol. Chem.* **269,** 19992–19999.
6. Divecha, N., Rhee, S. G., Letcher, A. J., and Irvine, R. F. (1993) *Biochem. J.* **289,** 617–620.
7. Martelli, A. M., Billi, A. M., Gilmour, R. S., Neri, L. M., Manzoli, L., Ognibene, A., and Cocco, L. (1994) *Cancer Res.* **54,** 2536–2540.
8. Manzoli, L., Billi, A. M., Rubbini, S., Bavelloni, A., Faenza, I., Gimour, R. S., Rhee, S. G., and Cocco, L. (1997) *Cancer Res.* **57,** 2137–2139.
9. DuCoeur, L. C., Wyborski, D. L., and Short, J. M. (1992) *Stratagies* **5,** 70–72.
10. Manzoli, L., Billi, A. M., Gilmour, R. S., Martelli, A. M., Matteucci, A., Rubbini, S., Weber, G., and Cocco, L. (1995) *Cancer Res.* **55,** 2978–2980.

11. Zamai, L., Falcieri, E., Gobbi, P., Santi, S., Falconi, M., Marhefka, G., and Vitale, M. (1996) *Cytometry* **25,** 324–332.
12. Ryu, S. H., Kim, U. H., Wahl, M. I., Brown, A. B., Carpenter, G., Huang, K. P., and Rhee, S. G. (1990) *J. Biol. Chem.* **29,** 17941–17945.
13. McBride, K., Rhee, S. G., and Jaken, S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7111–7115.
14. Divecha, N., Banfic, H., and Irvine, R. F. (1991) *EMBO J.* **10,** 3207–3214.
15. Rhee, S. G., and Bae, Y. S. (1997) *J. Biol. Chem.* **272,** 15045–15048.
16. Kim, C. G., Park, D., and Rhee, S. G. (1996) *J. Biol. Chem.* **271,** 21187–21192.