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Decline of signal transduction by phospholipase Cγ1 in IMR 90 human diploid fibroblasts at high population doubling levels

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During cellular senescence in vitro, the cells do not respond mitogenically to serum growth factors at high population doubling levels. Phospholipase C activity in low PDL IMR 90 cells showed a 4.7-fold stimulation in response to 10% serum compared to 3.3-fold in high PDL cells when measured in whole cell extracts. Immunoaffinity purified tyrosine phosphorylated protein fraction showed a greater increase (5.2-fold) in phospholipase C activity in low PDL than high PDL cells (2.1-fold) in response to serum. Serum stimulated PLCγ1 activity was diminished in high PDL cells. Immunokinase assay of PLCγ1 immunoprecipitates from serum stimulated IMR 90 fibroblasts suggested that diminished enzymatic activity in high PDL cells is not due to less receptor coupled tyrosine phosphorylated PLCγ1 enzyme. Serum stimulated [^{3}H]thymidine incorporation into DNA declined in parallel with the activity of PLCγ1, suggesting that its activation might play significant roles in this in vitro model for cellular senescence.

Cellular aging; Signal transduction; Tyrosine phosphorylation

1. INTRODUCTION

Human diploid fibroblasts have been used extensively to study cell growth regulation and in vitro cellular senescence [1]. They have a finite life span in vitro where they undergo a limited number of population doublings, then cease to divide despite the presence of growth factors in the medium [2]. Although a number of cell functions have been reported to change with increased population doubling levels (PDL), the exact mechanism(s) leading to cessation of cell division in vitro are not known. At high PDL, human diploid fibroblasts express very low level of the nuclear proto-oncogenes *myc* and *fos* [3,4], whose expression can be increased through stimulation of receptor tyrosine kinases (RTK). It has been proposed that their disappearance plays a role in cellular senescence and loss of growth factor responsiveness [4]. Likewise, the level of the c-*ras* proto-oncogene is diminished in high PDL IMR 90 human diploid fibroblasts [5]. c-*ras* appears to play an important role in growth factor-mediated signal transduction and mitogenesis [6] and decline of its expression at high PDL might also contribute to cellular senescence in vitro.

Several highly mitogenic polypeptide growth factors act through transmembrane receptor tyrosine kinases [7]. Careful investigations with low and high PDL cells have shown that for different RTKs, receptor density in the cell membrane does not change with PDL [8–12]. This suggests a post-receptor defect in signal transduction during cellular senescence. Certain growth factors stimulate the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane by specific phospholipases [13]. Cleavage of PIP_2 yields the intracellular second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) that stimulate intracellular release of Ca^{2+} and protein kinase C respectively [13,14]. Several PIP_2-specific phospholipase C (PLC) isozymes have been purified to homogeneity, and cloned [15,16]. One such isozyme, PLCγ1, appears to be a direct substrate for tyrosine phosphorylation by the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor beta (PDGFRB) [17,18]. Microinjection of antibodies specific for PLCγ1 can inhibit serum and *ras*-mediated cell growth [19], suggesting that receptor tyrosine kinase phosphorylation of PLC may be a key event in mitogenesis. Because of the close association of activation of PLCγ1 with mitogenesis stimulated by receptor tyrosine kinases we have examined their activities in IMR 90 human diploid fibroblasts during in vitro cellular aging. Here we report the decline in activity of the important signal transducing molecule PLCγ1 enzyme in response to serum.

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2. MATERIALS AND METHODS

2.1. *Cell culture and preparation of cell extract*

Human fetal lung diploid fibroblast cell strain IMR 90 was obtained

from the Institute for Medical Research, Camden, NJ. The cells were used between population doubling levels 20 and 60 (29–88% of lifespan completed). Maximal PDL attained in our laboratory was PDL 68. The cells were routinely grown in EMEM with 10% fetal bovine serum in the presence of 50 U/ml penicillin and 50 μg/ml of streptomycin at 37°C in an atmosphere of 5% CO_2 in air. For experiments, confluent monolayers in a 10 cm dish were washed with 5.0 ml of PBS four times. Then fresh EMEM containing 0.2% fetal bovine serum was added and the cells were starved for 40 h. The cells were then stimulated with 10% serum containing EMEM for 30 min. The cell monolayer was washed 3 times with PBS and lysed in situ with extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, and 0.25% aprotinin) for 30 min at 4°C. The lysed cells were scraped off the plates and the cell lysate was centrifuged at 10 000 $\times g$ for 30 min at 4°C. The supernatant was used for each experiment as cell extract after protein determination.

2.2. *Phospholipase assay*

There is a significant difference in radiolabel uptake between low and high PDL fibroblasts [20]. Consequently we did not attempt to estimate the in vivo production of IP_3 after [^{3}H]inositol labeling as a measure of PLC activation. However, equal amounts of protein from serum stimulated low and high PDL IMR 90 cells were assayed for PLC activity using [^{3}H]PIP_2 (L-3-phosphatidylinositol([^{3}H]2-inositol)4,5-bisphosphate) as substrate as described before [21,22]. Briefly, in a 100 μl reaction mixture 10 μl of [^{3}H]PIP_2 was added along with PLC assay buffer (100 mM NaCl, 0.6% Na deoxycholate, 2 mM Ca^{2+}, 4 mM EGTA, 5 mM 2-mercaptoethanol and 20 mM Tris-maleate pH 6.0) and cell extract. The reaction was incubated at 37°C. Preliminary experiments suggested that under this assay condition, the reaction is linear for 20 min, and at 30 min the reaction plateaus. Therefore, all the PLC reactions were carried out for 30 min. The enzymatic reaction was stopped by adding 0.5 ml of $CHCl_3/CH_3OH$/11.6 N HCl (100:100:0.6). 0.15 ml of 1.0 N HCl was added. After extraction, 0.4 ml of the aqueous layer was counted and converted to picomols using the initial specific activity of the substrate. All PLC assay experiments were repeated at least 3 times with similar results, and one representative experiment has been shown.

2.3. *Immunoaffinity purification of tyrosine phosphorylated proteins*

Cell extracts containing equal amounts of protein from serum stimulated and unstimulated cells were allowed to bind to 50 μl of agarose-linked phosphotyrosine monoclonal antibody at a ratio of 1 mg of protein to 100 μl of packed bead matrix for 2 h at 4°C [23,24]. After absorption of the proteins, the antibody matrix was washed twice with 1 ml of extraction buffer and once with 1 ml of cold water. The antibody bound proteins were eluted with 150 μl of 2 mM phenylphosphate in PLC assay buffer at 4°C for 30 minutes. Equal portions of this eluate were assayed for PLC activity using ^{3}H-PIP_2 as substrate.

2.4. *Immunoprecipitation and immunokinase assay*

Equal amounts of cell extract from control and serum stimulated low and high PDL IMR 90 cells were immunoprecipitated with PLCγ1 antipeptide antibody (a kind gift of Dr Graham Carpenter, Vanderbilt University). The immunobeads were assayed for tyrosine kinase activity in the presence of [γ-^{32}P]ATP [25] and the labeled proteins were separated on 7.5% polyacrylamide gel as we described before [25].

2.5. *DNA synthesis assay*

IMR 90 cells were split into 96-well plates at a dilution of 600 cells/well in EMEM plus 10% fetal bovine serum and allowed to grow for 24 h at 37°C. The cells were washed once with PBS, and starved in EMEM plus 0.2% fetal bovine serum for an additional 24 h. The media was then changed with fresh EMEM containing either 0.2% (unstimulated) or 20% fetal bovine serum (stimulated). Each well was given a 4 h pulse of 5.0 μCi/ml [^{3}H]thymidine (0.5 μCi/well) at 16–20 h following serum stimulation. The plates were processed using an automatic cell harvester and the [^{3}H]thymidine incorporation was measured in the presence of scintillation cocktail. Data shown in Table I represent the means and standard deviations of 6 determinations.

3. RESULTS AND DISCUSSION

Phospholipase C catalyzed hydrolysis of PIP_2 to produce IP_3 and DAG is considered to be an initial triggering event in signal transduction induced by several growth factors [13]. PLC activity was measured first in an in vitro assay of IMR 90 cell extracts prepared from serum stimulated cells. Quiescent confluent monolayers of low and high PLD IMR 90 cells were stimulated with 10% fetal bovine serum for 30 min. Aliquots of cell extracts containing equal amounts of protein were used in an vitro PIP_2 hydrolysis assay [21–23], which measures the cleavage of exogenous, radiolabeled PIP_2, to yield DAG and radiolabeled IP_3. The labeled IP_3 accumulates in the aqueous phase after organic extraction of the reaction and is measured by scintillation spectrometry. The results are shown in Fig. 1. When serum stimulated PIP_2 hydrolysis in IMR 90 cells was compared, the fold increase over basal levels was significantly less at PDL 57 (3.3-fold) than at PDL 21 (4.7-fold).

A specific phospholipase, PLCγ1, becomes phosphorylated at tyrosine residue(s) in response to the growth factors EGF and PDGF [17,18] and can be detected in the tyrosine phosphorylated proteins from stimulated cell extracts [23,26]. Quiescent low and high PDL IMR 90 cells were stimulated with 10% fetal bovine serum for 30 min. Cell extracts were prepared and were allowed to bind to an antiphosphotyrosine monoclonal antibody immobilized on agarose beads. The bound tyrosine phosphorylated proteins were then eluted with 2 mM phenylphosphate. The eluant was used to assay PLC activity using radiolabeled PIP_2 as substrate. The fold stimulation of serum induced PIP_2 hydrolysis in the tyrosine phosphorylated protein fraction was 2.1-fold in

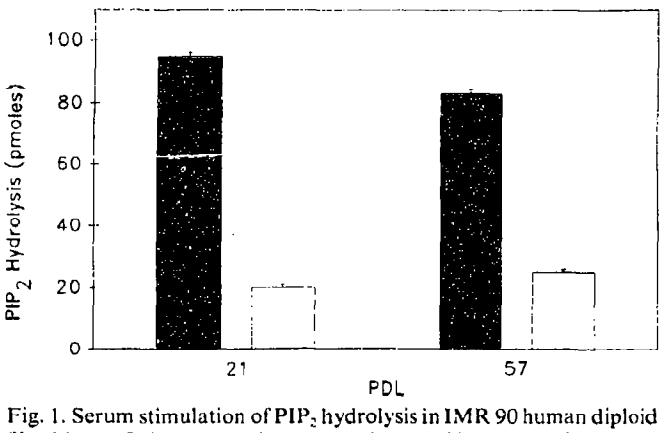

Fig. 1. Serum stimulation of PIP_2 hydrolysis in IMR 90 human diploid fibroblasts. Quiescent confluent monolayers of IMR 90 cells at PDL 21 and 57 were stimulated with 10% fetal bovine serum for 30 min at 37°C. 50 μg of cell extract were used in an in vitro PLC assay using [^{3}H]PIP_2 as substrate, as described in section 2. The data represent the pmols of IP_3 produced ± SD (n = 3). Dark and open bars are in the presence and absence of serum, respectively.

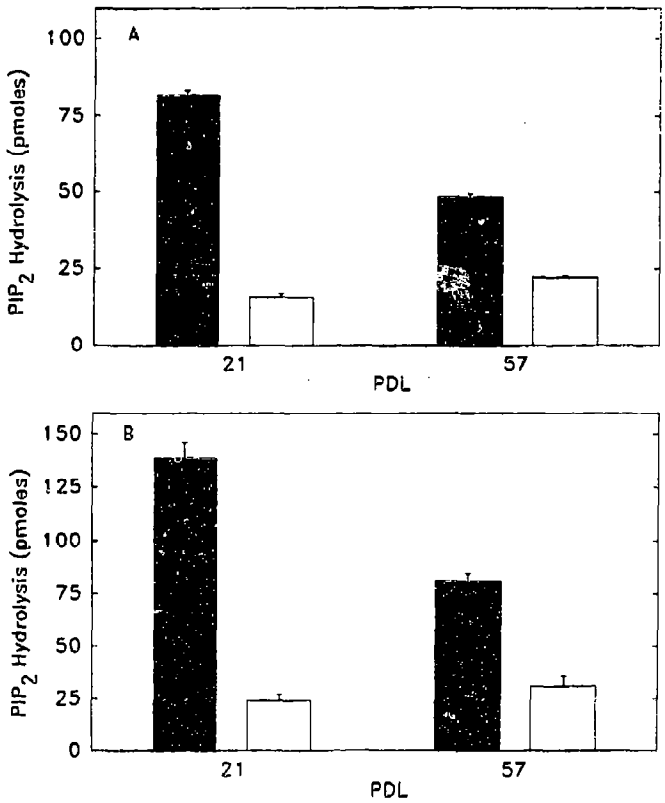

Fig. 2. Serum stimulation of PIP_2 hydrolysis in affinity purified tyrosine phosphorylated protein fraction and PLCγ1 immunoprecipitates. (A) Quiescent confluent monolayers of IMR 90 cells were stimulated with 10% fetal bovine serum for 30 min at 37°C. Equal amounts of cleared cell lysate were allowed to bind to antiphosphotyrosine monoclonal antibody agarose bead matrix for 2 h, the bound proteins were eluted in 2 mM phenylphosphate and the eluates were assayed for PLC activity as described in section 2. The data represent pmols of IP_3 produced ± SD (n = 3). Dark and open bars are in the presence and absence of serum, respectively. (B) Equal amounts of cleared cell lysate were immunoprecipitated with PLCγ1 antipeptide antibody and the washed immunobeads were assayed for PLC activity as described in section 2. The data represent pmols of IP_3 produced ± SD (n = 3). The dark and open bars are in the presence and absence of serum, respectively. The PDL of the IMR 90 cells used are shown.

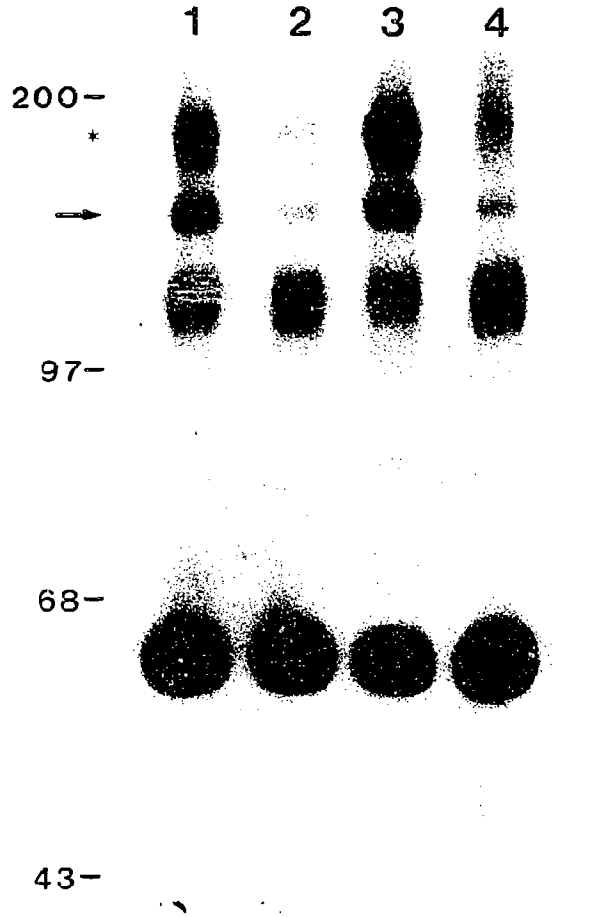

Fig. 3. Immunokinase assay of PLCγ1 immunoprecipitates. Quiescent confluent monolayers of IMR 90 cells (PDL 21 and 59) were stimulated with 10% fetal bovine serum for 30 min. Equal amounts of cell lysate were immunoprecipitated with PLCγ1 antibody. The washed immunobeads were used in an in vitro kinase assay using [γ^{32}P]ATP. The labeled proteins were analyzed on a SDS polyacrylamide gel. The gel was treated with alkali as we described before [25]. Lanes 1 and 2 are cells of PDL 21; lanes 3 and 4 are cells of PDL 59. Lanes 1 and 3 are in the presence, and lanes 2 and 4 are in the absence of serum. The molecular weight markers are shown in kilodaltons in the left margin. The arrow indicates the phosphorylated PCLγ1 protein and the asterisk indicates the putative PDGFR.

the high PDL cells compared to 5.2-fold in the low PDL cells (Fig. 2A). These data indicate that the PLC activity found in the fraction of the cell extract containing tyrosine phosphorylated proteins was diminished in high PDL IMR 90 cells. To confirm a decline in PLCγ1 activity in high PDL IMR 90 cells, extracts of serum stimulated low and high PDL IMR 90 cells were immunoprecipitated with PLCγ1-specific antipeptide antibody. The washed immunoprecipitated protein was then assayed for PLC activity using [^{3}H]PIP_2 as substrate (Fig. 2B). Serum stimulated PLCγ1 activity was 5.8-fold at low PDL versus 2.6-fold at high PDL. This increment corresponds very well with the antiphosphotyrosine immunopurified PLC activity (Fig. 2A).

Present evidence indicates that PLCγ1 becomes physically associated with ligand activated receptor tyrosine kinases such as EGFR and PDGFR [17,18]. We tested whether tyrosine kinase activity in low and high PDL IMR 90 cells could be detected in anti-PLCγ1 immunoprecipitates. The cells were stimulated with 10% fetal bovine serum and the cell extracts were immunoprecipitated with PLCγ1 antipeptide antibody. The immunobeads were used in an immunokinase assay with [γ-^{32}P]ATP, and the reaction products were analyzed on an SDS polyacrylamide gel (Fig. 3). The data suggest that only after serum stimulation, the 145 kDa PLCγ1 protein (indicated by arrow) coupled to a receptor tyrosine kinase, which we speculate is the 185 kDa PDGFR (indicated by an asterisk). These data also suggest that the amount of PLCγ1 protein associated with the receptor is not lower in high PDL cells compared to

Table I

Serum stimulated DNA synthesis in IMR90 human diploid fibroblasts

PDL	Treatment	[^{3}H]TdR incorporation[a] (cpm ±SD)
28	without serum	15 726 ± 1264
	with serum	31 594 ± 734
57	without serum	6 996 ± 261
	with serum	10 101 ± 334

The assay was done as described in section 2.
[a] The values are the mean ±SD for 6 replicates.

low PDL cells suggesting that the lower PLCγ1 activity (Fig. 2B) is not due to diminished coupling of the phospholipase Cγ1 molecule with the receptor protein.

As PLCγ1 has been implicated in growth and mitogenesis [13,19], we wanted to confirm that the mitogenic response of the IMR 90 cells declined during in vitro aging. We therefore measured serum stimulated [^{3}H]thymidine incorporation in IRM 90 cells at PDL 28 and PDL 57 (Table I). At PDL 57 [^{3}H]thymidine incorporation was 1.4-fold, compared to 2-fold at PDL 28. These findings, taken together, provide evidence suggesting that components of the signal transduction machinery distal to plasma membrane receptors undergo alteration during in vitro cell senescence. Inasmuch as PIP_2 represents 0.5% or less of membrane phospholipid, a diminished ability to hydrolyze this substrate after growth factor stimulation could contribute to an attenuated growth response which was reflected in a lower mitogenic response to serum at high PDL.

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