A Role for Nuclear Phospholipase Cβ1 in Cell Cycle Control*

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Phosphoinositide signaling resides in the nucleus, and among the enzymes of the cycle, phospholipase C (PLC) appears as the key element both in Saccharomyces cerevisiae and in mammalian cells. The yeast PLC pathway produces multiple inositol polyphosphates that modulate distinct nuclear processes. The mammalian PLCβ1, which localizes in the nucleus, is activated in insulin-like growth factor 1-mediated mitogenesis and undergoes down-regulation during murine erythro-leukemia differentiation. PLCβ1 exists as two polypeptides of 150 and 140 kDa generated from a single gene by alternative RNA splicing, both of them containing in the COOH-terminal tail a cluster of lysine residues responsible for nuclear localization. These clues prompted us to try to establish the critical nuclear target(s) of PLCβ1 subtypes in the control of cell cycle progression. The results reveal that the two subtypes of PLCβ1 that localize in the nucleus induce cell cycle progression in Friend erythroleukemia cells. In fact when they are overexpressed in the nucleus, cyclin D3, along with its kinase (cdk4) but not cyclin E is overexpressed even though cells are serum-starved. As a consequence of this enforced expression, retinoblastoma protein is phosphorylated and E2F-1 transcription factor is activated as well. On the whole the results reveal a direct effect of nuclear PLCβ1 signaling in G1 progression by means of a specific target, i.e. cyclin D3/cdk4.

It is demonstrated that an autonomous intranuclear inositolide cycle exists and that nuclear PLCβ1 is a key enzyme for cell proliferation and differentiation (1). The enzymes of polyphosphoinositide turnover occur in the nucleus of mammalian cells and yeast as well (Ref. references therein), and there is evidence for phosphatidylinositol bisphosphate (PIP2) synthesis and degradation in the nuclear matrix (3). The evidence obtained with confocal and electron microscope analysis indicates that enzymes required for the synthesis and hydrolysis of phosphoinositides are localized at ribonucleoprotein structures of the inner nuclear matrix involved in transcript processing within the interchromatin domains (4). Although phosphatidylinositol cycle is activated only in nuclei from HeLa cells in S phase (5), striking changes occur mainly in PLCβ1 activity a few minutes after growth factor stimulation (1). PLCβ1 is composed of two subtypes, 150-kDa PLCβ1a and 140-kDa PLCβ1b, that are derived from a single gene by alternative RNA splicing (6). The two forms of the PLCβ1 are detectable both in cytosolic and nuclear fractions although PLCβ1b exists almost entirely in the nucleus (7), and the β1a form localizes in equal amount in nuclei and plasma membrane (8). Previous investigations from our group have demonstrated that the nucleus-confined PLCβ1 is directly involved in maintaining the undifferentiated state of Friend erythroleukemia cells even in the presence of inducers of erythroid differentiation, possibly due to a continuous stimulation of the cell cycle (9). With the above in mind, we sought to explore whether PLCβ1 is actually involved in the regulation of cell cycle machinery.

In mammalian cells, proliferation is under the control of factors that regulate the transition between different cell cycle stages at two main checkpoints. The better described checkpoint is at the G1-S phase transition for initiation and completion of DNA replication in S phase. The other checkpoint is at the G2-M phase transition and controls mitosis and cell division (10). The cell cycle is primarily regulated by a family of structurally related serine/threonine protein kinases, which consist of a regulatory subunit, a cyclin, and a catalytic subunit, a cyclin-dependent kinase (cdk). In mammalian cells, cdk4 or cdk6, in combination with the D type cyclins (D1, D2, and D3), and cdk2 in association with cyclin E play key roles in regulating G1 progression (Ref. 11 and references therein).

Furthermore, overexpression of E or D type cyclins is able to accelerate the transition through the G1 phase of the cell cycle (12). Several lines of evidence suggest that the primarily function of D-type cyclins is to stimulate progression through G1, rather than to promote the G1/S transition. In addition it appears that in hematopoietic cell lines, overexpression of cyclin D2 or D3 prevents granulocyte differentiation, whereas overexpression of cyclin D1, which is normally not expressed in those cells, does not (13). Both cyclin D in complex with cdk4 or cdk6 and cyclin E-cdk2 phosphorylate the product of the retinoblastoma gene, the retinoblastoma protein (pRb), a well known tumor suppressor. This tumor suppressor activity relies on pRb role in gating S phase entry through its ability to repress genes activated by the E2F family of transcription factors. In fact phosphorylated pRb (ppRb) releases members of the E2F family that play an integral role in cell cycle progression by inducing the expression of gene required for S phase progression.

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The abbreviations used are: PLC, phospholipase C; IPs, inositol polyphosphates; IGF-1, insulin-like growth factor 1; PIP2, phosphatidylinositol bisphosphate; cdk, cyclin-dependent kinase; Rb, retinoblastoma; pRb, phosphorylated retinoblastoma protein; DTT, dithiothreitol; wt, wild type.
entry, including those involved in DNA synthesis such as S phase regulatory factors cyclin E, cyclin A, and cdk2 (14, 15). All five E2F family members bind the same DNA sequence, but additional levels of regulation have been observed as follows: E2F-1, E2F-2, and E2F-3 associate with pRB (p10); E2F-pRb complexes are found primarily in G1; E2F-4 and E2F-5 preferentially bind to p107 and p130. Enforced expression by micro-injection of E2F-1 induces starved cells to progress through G1 and enter S phase (16). In order to understand the mechanism that couples nuclear PLCβ1 and cell cycle machinery, we carried out a detailed analysis of the expression of the proteins involved in the regulation of the cell cycle. For this purpose we have overexpressed PLCβ1a and -b in the nuclear compartment and the mutant M2b, which lacks the nuclear localization sequence (8, 9), in the cytoplasmic compartment in order to find out how nuclear localization of PLCβ1 acts on cell cycle progression and on key signaling events that regulate the passage through G1 phase.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors and Transfection**—The full-length cDNA for rat PLCβ1a (17), the full-length cDNA for rat PLCβ1b (6), and the mutant for nuclear localization site-directed mutagenesis lysine residues 1056, 1063, and 1070 in region 2 of the COOH terminus were substituted with isoleucine (8), were cloned (6), and the mutant for nuclear localization sequence (8, 9), in the cytoplasmic compartment in order to find out how nuclear localization of PLCβ1a acts on cell cycle progression and on key signaling events that regulate the passage through G1 phase.

**Cell Culture**—Murine erythroleukemia cells (Friend cells, clone 707) were grown in RPMI 1640 supplemented with 10% fetal calf serum. For experimental procedures cells were serum-deprived for 48 h and then stimulated with fresh serum for the indicated hours (figure legends).

**Isolation of Nuclei**—A hypotonic shock combined with non-ionic detergent, essentially described by Martelli et al. (19), has been used. In addition to 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 1 mM EGTA, 10 μg/ml leupeptin, 0.3 μM aprotinin, 15 μg/ml calf heart phosphatase, and 7.5 μg/ml calpain II inhibitor, the buffers were also added to the buffers. Nuclear purity was assessed by detection of m-tubulin in Western blot analysis separated on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography.

**Preparation of Cytoplasmic Fraction**—The cytoplasmic fraction was obtained by homogenizing cells with 20 strokes in a Dounce homogenizer in 10 mM Tris-Cl, pH 7.8, 2 mM MgCl2, plus protease inhibitors as above. The homogenate, pelleting the nuclei by centrifugation at 14,000 rpm for 10 min, was washed in cold phosphate-buffered saline and fixed in cold 70% ethanol with 10% fetal calf serum for 48 h and then starved for 24 h. Cells were washed in cold phosphate-buffered saline and fixed in cold 70% ethanol for 30 min. The pellet was resuspended in a solution containing 50 μg/ml propidium iodide, 1 mg/ml sodium citrate, 1 μg/ml Triton X-100 and 5 μg/ml RNase A and analyzed by a FACStar Plus flow cytometer (Becton Dickinson).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared by resuspending the cells in 400 μl of lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl), followed by incubation on ice for 10 min. Next the lysates were vortexed for 10 s before centrifugation for 1 min at 14,000 rpm at 4 °C. The pellet nuclei were resuspended in 50 μl of lysis buffer (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA) and left on ice for 20 min with occasional mixing. Subsequently, the lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was stored at ~70 °C. All buffers were supplemented with 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride. A synthetic oligonucleotide containing the consensus binding site for E2F-1, 5'-ATTTAATGGTTGGGGCCTTTCCAA-3' (Santa Cruz Biotechnology), was used as a probe for the assay. The probe was labeled with [γ-32P]ATP using T4 polynucleotide Kinase (Promega) and then purified over G50-pellicon. Where appropriate, the extract was preincubated for 10 min at room temperature with 5 μl unlabeled E2F-1 oligonucleotide and 5 μl unspecific competitor. For antibody perturbation supershift experiments, 0.25 μg of anti-pRB antibody (Santa Cruz Biotechnology) was added to the incubation 10 min prior to addition of oligonucleotide probe and incubated at room temperature. Subsequently, binding to 1 μl of monomeric active E2F-2 oligo was carried out in a reaction mixture containing 1 μg of poly(dI-dC), 20 mM Hepes, pH 7.6, 70 mM KCl, 5 mM MgCl2, 0.05% Nonidet P-40, 12% glycerol, 1 mg/ml bovine serum albumin, 0.5 μM G418 for 30 min at room temperature. DNA-protein complexes were separated on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography.

**Immunochemical Analysis**—Immunoblot analysis of 500 μg of nuclear and cytoplasmic fractions of wt were immunoprecipitated with 2.5 μg of anti-PLCβ1a + b bisorom (Transduction Laboratories) for 2 h at 4 °C with constant agitation, followed by 1 h of incubation with 50 μl of protein A-Sepharose (10 w/v). Pellets were washed three times in phosphate-buffered saline containing 1% Nonidet P-40, twice in 0.1 mM Tris, 0.5 mM MgCl2, and 0.5 mM EDTA, TNE (0.1 M Tris, NaCl, EDTA (TNE)), 50 mM NaF were removed by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose membrane for subsequent immunodetection with the same antibody, followed by detection using ECL (Amer sham Pharma Biotech). For Western blot analysis, proteins from nuclear extract (20 μg) were separated on an SDS-0.1% polyacrylamide gel and transferred onto nitrocellulose paper. The following antibodies were used: polyclonal antibodies to cyclin D3, cyclin E, cdk2, and cdk4 from Santa Cruz Biotechnology; polyclonal antibodies to pRB and to ppRb for specific phosphorylation sites Ser-795, Ser-780, and Ser-807/811, from New England Biolabs.

**Immune Complex Kinase Assay**—Cells were suspended in lysis buffer containing 50 mM Hepes, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 5 μg of aprotinin per ml, 10 mM [3H]phosphatidylinositol bisphosphate and then analyzing the samples by electrophoresis by nuclear debris that is present in the crude supernatant from cell culture.

**RESULTS**

The subcellular distribution of the two subtypes of the PLCβ1a and -b was evaluated by Western blot analysis in wild type erythroleukemic Friend cells (Fig. 1B). Although the PLCβ1a is almost entirely localized in the nucleus, the PLCβ1b is nearly equally distributed among the two compartments. The Western blot analysis of the clones overexpressing the PLCβ1a, PLCβ1b, and the mutant for the nuclear localization sequence M2b, respectively, show that whereas the M2b mutant has lost the capacity for nuclear localization, the clones expressing the two subtypes a, b, and a, b, having the PLC in the nucleus, the b form being entirely nuclear and the a form distributed in both nucleus and cytoplasm, even though in the latter compartment it is less expressed (Fig. 1A). The expression of PLCβ1 forms in wild type cells is very low since the immunoprecipitated PLC from 500 μg of both nuclear and...
cytoplasmic protein gave rise to a signal even lower than the one obtained with 10 μg of protein from nuclei or cytoplasm of transfected cells. The PLC activity of the clones overexpressing PLCβ1a and M2b mutant as well is the same reported in Ref. 9 (i.e. PLCβ1a 38.0 ± 1.5, M2b 9.3 ± 0.7 nmol of IP3/mg of protein/30 min of incubation). These values compared with nuclear PLC activity in wild type cells (9.8 ± 0.6 nmol of IP3/mg protein/30 min of incubation) show a nearly 3-fold increase of the nuclear activity in the case of PLCβ1a and PLCβ1b (32.7 ± 2.0 nmol of IP3/mg of protein/30 min of incubation) with no changes in the case of PLC M2b, which conversely is 3-fold higher in the cytoplasm.

These features characterize all the clones we have selected for each type of transfectant and show a complete agreement between the enzymatic activity and the level of expression of the PLCβ1 forms. To determine whether PLCβ1 is involved in cell cycle progression, we examined cyclin D3, cyclin E, cdk2, and cdk4 protein expression in serum-starved cells and after stimulation with serum. In erythroleukemic Friend cells cultured without inducer, the protein levels of cyclin D3 and D2 fluctuate during the cell cycle, whereas the level of cyclin D2 changes little. Cyclin D1 protein is not detected in these cells. Cyclin D3 increases as cells progress through G1 phase and reaches a maximum level in S phase. Cyclin E peaks at the transition from G1 to S phase (21). Fig. 2 shows that in nuclear extracts of Friend cells, cyclin D3 with its related cdk4 and cyclin E were expressed at barely detectable levels in serum-starved wild type cells and accumulated after stimulation with serum, whereas the level of cdk2 protein, whose activity is low in G1 phase and increases during S phase (21), is constant after serum stimulation. On the contrary, the clones overexpressing in the nucleus both PLCβ1a and PLCβ1b show high levels of cyclin D3 and cdk4 in serum-starved cells, whereas M2b mutant clone and wild type cells as well do not. Since the expression of cyclin E does not increase in serum-starved transfectants overexpressing PLCβ1a or PLCβ1b, we thought that overexpression of enzymatically active forms of PLCβ1 in the nucleus could exert a specific effect, via cyclin D3/cdk4 complex formation, in sustaining cell cycle progression even in the absence of serum. Therefore, we have evaluated the activity of the cyclin D3/cdk4 complex in vitro in order to establish whether the complex is enzymatically active. Cyclin D3 and cdk4 were immunoprecipitated from total cell lysates, and immune complex was then used for in vitro kinase assay by using a bacterially produced glutathione S-transferase-Rb fusion protein as a substrate. Fig. 2C shows that the activity of cyclin D3/cdk4 complex, which is almost absent in serum-starved wild type cells and M2b clone, increases after serum stimulation. On the contrary, in the clones overexpressing PLCβ1a and -b in the nucleus the activity of the complex is present even after serum starvation, and its level is similar to that of both wild type cells and M2b clone after serum stimulation. This in vitro feature prompted us to analyze the in vivo phosphorylation status of pRb. We have evaluated pRb phosphorylation by using specific antibodies that detect specific phosphorylated sites as follows: Ser-780, Ser-795, and Ser-807/811 as physiologically occurs (Fig. 3).

FIG. 1. Analysis of PLCβ1 phenotype in stable transfectants and Friend cells wt. A, immunoblot analysis of 10 μg of nuclear and cytoplasmic lysate of the stable transfectants as follows: Ov1a, cells transfected with PLCβ1a; Ov1b, cells transfected with PLCβ1b; M2b, cells transfected with the mutant lacking the nuclear localization sequence. B, immunoblot analysis of 500 μg of nuclear (N) and cytoplasmic (C) fractions of wt immunoprecipitated (IP) with 2.5 μg of anti-PLCβ1 (a and b) (Signal Transduction). The Western blots (WB) and PLC activities of stable transfectants reported here are representative of 5 other clones, which behave exactly as the ones in the figure.

FIG. 2. Effect of overexpression of PLCβ1 subtypes (a and b) and the mutant M2b on both the expression and the activity of cell cycle proteins. A, changes in the level of expression of cyclin D3 and cdk4 in the presence or absence of serum. B, changes in the level of expression of cyclin E and cdk2 in the presence or absence of serum. C, changes in the activity of cyclin D3/cdk4 complex in the presence or absence of serum. Cells transfected with PLCβ1a (Ov1a), cells transfected with PLCβ1b (Ov1b), cells transfected with the mutant lacking the nuclear localization sequence (M2b), and wild type cells (Wt) were serum-deprived for 48 h and then stimulated with fresh serum (10%) for the indicated time. The Western blots and the activity assay of stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.
Ser-795, even during serum starvation, whereas this does not occur at all in both the M2b mutant and the wild type cells (Fig. 3). Indeed cells overexpressing PLC\(_{\beta1}\) in the nucleus show phosphorylated pRb (Ser-795) after serum starvation for 48 h. The two antibodies against phosphorylation sites Ser-780 and Ser-807/811 did not reveal any phosphorylation in serum-starved clones overexpressing PLC\(_{\beta1}\) in the nucleus. The phosphorylation in starved clones, overexpressing PLC\(_{\beta1}\) in the nucleus, is restricted to Ser-795 possibly indicating a specific effect linked to the two subtypes of PLC. It is concluded that the overexpression of PLC\(_{\beta1}\)a and -b in the nucleus up-regulates both the expression and the activity of the cyclin D3/CDK4 complex and affects pRb phosphorylation. The absence of these two events in cells overexpressing the same PLC in the cytoplasmic compartment indicates a very specific effect linked to the subcellular localization of this signaling PLC.

The Rb proteins bind to a number of cellular proteins like E2F that are involved in the regulation of transcription of genes relevant for the control of the cell cycle. E2F transcription factors regulate the transition from the G1 to S phase, and its activity is regulated by members of pRb family (22) and appears to be a downstream step of the signaling cascade that takes place in G1 phase. To assess whether overexpression of the two subtypes of PLC\(_{\beta1}\) could affect functional changes in E2F activity, we evaluated E2F DNA binding activity in resting cells and in cells after stimulation with serum. Fig. 4 shows the effect of the overexpression of PLC\(_{\beta1}\)a and -b on E2F-1 binding (19). As expected, in serum-starved clones overexpressing the two nuclear subtypes of PLC\(_{\beta1}\) the binding activity was higher than in wild type cells and M2b mutant cells, suggesting that PLC\(_{\beta1}\)a and -b when localized in the nucleus are indeed involved in the regulation of proteins responsible for the progression of cell cycle. To address whether, after serum starvation, pRb is bound to E2F-1 in both wild type cells and M2b clone and not in serum-starved cells expressing PLC\(_{\beta1}\)a and -b in the nucleus, we have added anti-pRb antibody in the binding assay. Fig. 4 shows a change in the mobility of the E2F-1 complex only in serum-starved wild type cells and M2b mutant clone. These observations indicate that the E2F-1 transcription factor is already released from pRb in serum-starved Friend cells overexpressing PLC\(_{\beta1}\)a or -b in the nucleus. Given that E2F transcription factors play an integral role in cell cycle progression by inducing the expression of genes required for S phase entry, we checked the effect of the overexpression of PLC\(_{\beta1}\)a and -b and M2b mutant on the behavior of the cell cycle in these transfectants by means of flow cytometry. In the presence of serum the clones overexpressing PLC\(_{\beta1}\)a and -b show a cell cycle profile characterized by a higher S phase compared with wt and the M2b mutant (Fig. 5). Moreover, when the clones overexpressing PLC\(_{\beta1}\)a and -b in the nucleus are serum-starved, they still maintain a higher S phase compared with wt and M2b cells, the flow cytometric analysis of which shows an almost complete block in G1 phase. Therefore, cells overexpressing the two subtypes of PLC\(_{\beta1}\) could at least in part effectively replace the requirement for serum as a mitogenic signal.

**DISCUSSION**

Nuclear PLC\(_{\beta1}\) signaling was suggested to be one of the earliest events following both exposure of human osteosarcoma Saos cells to interleukin-1\(\alpha\) (23) and murine 3T3 cells to IGF-1 (18). A decrease in nuclear PIP\(_2\) hydrolysis and down-regulation of nuclear PLC\(_{\beta1}\) have been detected during differentiation of erythroleukemia cells (24). Moreover, we have shown that nuclear PLC\(_{\beta1}\) is involved in maintaining the undifferen-
tated state of Friend erythroleukemia cells, possibly by opposing the inhibition of the cell cycle progression necessary for erythroid differentiation (9). Thus this isoform is a good candidate as a key element for the signaling cascade involved in cell growth and proliferation. The contention that subcellular localization of the inositol lipid cycle is central to the function of the cycle itself in controlling the signaling events has been strengthened by the discovery that in *Saccharomyces cerevisiae* a nuclear PLC, called PLC1, homologous in function to the mammalian PLCβ1, and two inositol polyphosphate kinases constitute a signaling pathway that affects transcriptional control (25). Our study was intended to find out how nuclear PLCβ1 is involved in the regulation of cellular proliferation. To address this issue we have tried to identify the targets of nuclear PLCβ1 in the nuclear compartment. Therefore we first examined the expression of cyclins and cdks associated with G1-S phase transition. Our evidence suggests that the overexpression of PLCβ1 in the nucleus is directly responsible for the overexpression and activation of cyclin D3-cdk4 complex, which is known to stimulate progression through G1 rather than to promote the G1-S transition (11). This is indeed a specific target, given that the other cyclin, cyclin E, which is responsible for G1-S transition (26) is not affected at all by nuclear PLCβ1. The two splicing products of this isoform behave in the same fashion, even though the 1b is more specific for nuclear localization and 1a localizes also in the cytoplasmic compartment. This suggests that once PLCβ1 is in the nucleus its role is different from its counterpart located at the plasma membrane. This is supported by the absence of an effect following overexpression of the M2b mutant in which PLC localizes exclusively to the cytosolic compartment (8, 9). In this case the effects on both cyclin D3 and cdk4 as well are totally absent. Only the clones overexpressing both PLCβ1a and -b, but not the M2b mutant, show a higher S phase compared with wild type and M2b-transfected clones both in cycling and serum-starved cells. This implies that, since cyclin E is not affected, both the overexpression and the activation of the cyclin D3-cdk4 complex are actually responsible for maintaining the cell cycle progression. Downstream events such as the phosphorylation of pRb and the subsequent release of E2F-1 transcription factor are also only observed in PLCβ1a and -b clones after serum starvation.

It should be noted that the overexpression of PLCβ1 in the nucleus gives rise to enzymatically functional PLC whose activity is 3-fold higher than the one of wt cells. This enhanced signaling capacity generates second messengers such as diacylglycerol, whose role in nuclear protein kinase C activation has been widely explored (19, 27, 28) and inositol trisphosphate, whose nuclear kinases, by producing the other inositol polyphosphates, are involved in mRNA export and transcription in yeast (25). Thus, data reported here appear to shed new light on the lipid-mediated signaling machinery residing in the nucleus, implicating nuclear PLCβ1 as a key element for the specific regulation of the cyclin D3-cdk4 complex and its downstream targets such as pRb and E2F in facilitating cell cycle progression.

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REFERENCES

1. Coco, L., Capitanii, S., Maraldi, N. M., Mazzotti, G., Barnabei, O., Rizzioli, R., Gilmour, R. S., Wirtz, R. W. A., Rhee, S. G., and Manzoli, F. A. (1998) *Adv. Enzyme Regul.* 38, 351–363
2. Chi, T. H., and Crabtree, G. R. (2000) *Science* 281, 1937–1939
3. Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A. J., and van Bergen en Henegouwen, P. M. P. (1992) *J. Biol. Chem.* 267, 5078–5084
4. Maraldi, N. M., Zini, N., Santi, S., and Manzoli, F. A. (1999) *J. Cell. Physiol.* 181, 203–217
5. York, J. D., and Majerus, P. W. (1994) *J. Biol. Chem.* 269, 7847–7850
6. Bakh, Y. Y., Lee, Y. H., Lee, T. G., See, J., Ryu, S. H., and Suh, P. G. (1994) *J. Biol. Chem.* 269, 8240–8245
7. Bakh, Y. Y., Song, H., Baek, S. H., Park, B. Y., Kim, H., Ryu, S. H., and Suh, P. G. (1998) *Biochim. Biophys. Acta* 1389, 76–80
8. Kim, C. G., Park, D., and Rhee, S. G. (1996) *J. Biol. Chem.* 271, 21187–21192
9. Matteucci, A., Faenza, I., Gilmour, R. S., Manzoli, L., Billi, A. M., Peruzzi, D., Ravelloni, A., Rhee, S. G., and Cocco, L. (1998) *Cancer Res.* 58, 5057–5060
10. Murray, A. (1994) *Curr. Opin. Cell Biol.* 6, 872–876
11. Sherr, C. J. (1994) *Cell* 78, 551–555
12. Obitsubo, M., and Roberts, J. (1993) *Science* 259, 1908–1912
13. Kato, J., and Sherr, C. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 11513–11517
14. Herwig, S., and Strauss, M. (1997) *Eur. J. Biochem.* 246, 581–601
15. Lam, E. F. W., and La Thangue, C. N. B. (1994) *Curr. Opin. Cell Biol.* 6, 859–866
16. Lukas, J., Bartkova, J., and Bartek, J. (1996) *Mol. Cell. Biol.* 16, 6917–6925
17. Suh, P. G., Ryu, S. H., Moon, K. H., Suh, H. W., and Rhee, S. G. (1988) *Cell* 54, 161–169
18. Manzoli, L., Billi, A. M., Rubbini, S., Bavelloni, A., Faenza, I., Gilmour, R. S., Rhee, S. G., and Cocco, L. (1997) *Cancer Lett.* 115, 871–876
19. Martelli, A. M., Tabellini, G., Bortol, R., Manzoli, L., Bareggi, R., Baldini, G., Grill, V., Zweyer, M., Narducci, P., and Cocco, L. (2000) *Cancer Lett.* 158, 815–821
20. Martelli, A. M., Gilmour, R. S., Bertagnolo, V., Neri, L. M., Manzoli, L., and Cocco, L. (1992) *Nature* 358, 242–245
21. Marks, P. A., Bichon, V. M., Yokawa, H. K., and Rifkind, R. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 10522–10524
22. Harbour, J. W. (2000) *Nat. Cell Biol.* 2, E65–E67
23. Marmiroli, S., Ognibene, A., Bavelloni, A., Cinti, C., Cocco, L., and Maraldi, N. M. (1994) *J. Biol. Chem.* 269, 13–16
24. Coco, L., Martelli, A. B., Capitanii, S., Maraldi, N. M., Mazzotti, G., Barnabei, O., Gilmour, R. S., and Manzoli, F. A. (1995) *Adv. Enzyme Regul.* 35, 23–33
25. Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) *Science* 281, 2026–2029
26. Harbour, J. W., Luo, R. X., Dei Santiu, A., Postigo, A. A., and Dean, D. C. (1999) *Cell* 98, 859–869
27. Topham, M. K. (1998) *Nature* 394, 697–700
28. Driouch, N. (1998) *Nature* 394, 619–620