Retinoblastoma protein dephosphorylation is an early event of cellular response to prooxidant conditions

Franca Esposito, Lucia Russo, Tommaso Russo, Filiberto Cimino*

Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, via S. Pansini 5, 80131 Naples, Italy

Received 25 January 2000; received in revised form 24 February 2000

Edited by Guido Tettamanti

Abstract The modification of intracellular redox conditions with diethylmaleate (DEM), a glutathione-depleting agent, induces a p53-independent growth arrest mediated by the accumulation of p21^{waf1} mRNA and protein. The same treatment also induces the retinoblastoma protein (pRb) dephosphorylation. This dephosphorylation (i) is very fast, being observed already 5 min after the exposure of the cells to DEM, (ii) is dependent on the prooxidant effects of DEM, being prevented by the treatment with *N*-acetylcysteine and (iii) is completely reversible, since the rephosphorylation of pRb is promptly obtained upon the removal of the glutathione-depleting agent from the culture medium. The dephosphorylation of pRb is independent of the accumulation of p21^{waf1} induced by DEM; in fact, p21^{waf1} levels start to increase much later after DEM treatment and accordingly cyclin-dependent kinase activities are not yet induced when pRb is already dephosphorylated following DEM treatment. Finally, pRb dephosphorylation is catalyzed by phosphatases activated by DEM treatment.

Key words: Reactive oxygen species; Cell cycle; p21^{waf1}; E2F; Ser–Thr phosphatase; Glutathione

1. Introduction

Reactive oxygen species (ROS) have been studied for a long time only as agents able to inflict damage on DNA, proteins and fatty acids [1–3]. This damage often leads, depending on the length and the extent of the exposure, to cell death or to cell transformation as a consequence of gene mutation. More recently, several results suggest some possible physiologic roles for ROS, namely as mediators of extracellular signal transduction and/or as regulators of cell cycle progression [4,5]. We previously demonstrated that the increase of ROS concentration, due to glutathione (GSH) depletion induced by diethylmaleate (DEM), induces an accumulation of p21^{waf1} [6].

This protein is an inhibitor of several cyclin-dependent kinases (CDK), which regulate cell cycle progression, and is the main target of p53 tumor suppressor. In fact p53, activated by different events including genome damage, induces the transcription of the p21^{waf1} gene and in turn the increase of p21^{waf1} protein concentration [7]. However, our results demonstrated that the ROS-induced p21^{waf1} accumulation is independent of p53 activation [6] which, on the contrary, seems to be inhibited in our experimental conditions [6], whereas the phenomenon that we observed requires the activation of the ras-MAPK pathway and does not depend upon p21^{waf1} gene transcription [8].

As expected, the effect of the observed increase of p21^{waf1} concentration is the accumulation of cells in the G1 phase [6]. In fact, G1 → S transition is based on the release of E2F transcription factors from the complex in which they are bound to the retinoblastoma protein (pRb). This release is the consequence of pRb phosphorylation which is catalyzed by CDKs, therefore the accumulation of p21^{waf1} inhibits pRb phosphorylation, thus blocking E2F release and the G1 → S transition [9,10].

Another possible mechanism affecting pRb phosphorylation could be based on the effect of protein phosphatases. We previously demonstrated that a short exposure of cultured cells to DEM leads to the dephosphorylation of p21^{waf1} as demonstrated by the appearance in Western blots decorated with anti-p21^{waf1} antibody, of a faster migrating band [11]. Several results suggest that this early phenomenon could be due to the activation of protein Ser–Thr phosphatases by ROS [11]. Therefore, it is conceivable to hypothesize that ROS could cause the dephosphorylation of other proteins, through the activation of those protein phosphatases.

Herein we report the results showing that the dephosphorylation of pRb is an early event which follows the modification of intracellular redox conditions induced by DEM, thus indicating that intracellular ROS concentrations affect cell cycle progression through multiple mechanisms which also include the activation of Ser–Thr phosphatases.

2. Materials and methods

2.1. Cell lines and materials

Human pro-myelocytic leukemia cells (HL60) were cultured in RPMI 1640 medium (Gibco-BRL) containing 10% fetal calf serum (FCS, ICN), 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin; COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (ICN), 100 units/ml penicillin and 100 mg/ml streptomycin. Both cell lines were grown at 37°C in a 5% CO_2 atmosphere.

Anti-p21^{waf1} (sc-397), anti-cyclins A (sc-751) and B1 (sc-245) polyclonal antibodies and anti-E2F1 (sc-251) monoclonal antibody were from Santa Cruz Biotechnology. Anti-retinoblastoma (Rb) monoclonal antibody was from Pharmingen. H1 histone was from Boehringer. DEM and *N*-acetylcysteine (NAC) were from Sigma. DEM was added directly to the culture medium into the dishes from the concentrated solution to reach a final concentration of 1 mM. NAC was dissolved in phosphate buffered saline (PBS) solution at a final concentration of 30 mM.

*Corresponding author. Fax: (39)-81-7463650.
E-mail: cimino@dbbm.unina.it

Abbreviations: pRb, retinoblastoma protein; DEM, diethylmaleate; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; CDK, cyclin-dependent kinase; GSH, glutathione

PII: S0014-5793(00)01318-1

2.2. Western blot and immunoprecipitation analyses

Protein extracts were obtained by lysing the cells on ice in a buffer containing 1.6 mM KH_2PO_4, 12.5 mM K_2HPO_4, 1% Tryton, 3.4 mM SDS, 0.1 M NaCl, 11.5 mM Na-deoxycholate, 0.1% sodium azide, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each of aprotinin, leupeptin and pepstatin. Extracts used for Western blotting were obtained by centrifuging the lysate at 4°C at 1000×*g* for 10 min. For the immunoprecipitation experiments, confluent dishes were washed twice with PBS and the cells were lysed by the addition of 800 μl buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl; 0.1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride and 0.5% NP40, 1 mM PMSF and 1 μg/ml aprotinin, leupeptin and pepstatin. Cells were rocked at 4°C for 30 min and collected by centrifugation (4000 rpm for 10 min) at 4°C. The protein concentration was determined by using the Bradford method (Bio-Rad); for the immunoprecipitates 0.5–1 mg protein extracts were incubated with 1 μg anti-Rb antibody overnight at 4°C, centrifuged and the pellets washed in the lysis buffer without protease inhibitors. Fifty μg aliquots of proteins for Western blots and immunoprecipitates were loaded and electrophoresed through a SDS–polyacrylamide gel (polyacrylamide gel concentrations were 13 and 8% for p21 and pRb, respectively) and then transferred to Immobilon-P transfer membranes (Millipore); the incubation with anti-$p21^{waf1}$ antibody and with anti-Rb were according to the manufacturers. After incubation with rabbit horseradish peroxidase-conjugated secondary antibody, the blots were developed using enhanced chemiluminescence (Amersham). The H1 kinase assay was performed as described [12].

2.3. Cell free dephosphorylation assays

HL60 cells were pretreated with DEM for the indicated times. Whole cell extracts and immunoprecipitates were prepared immediately after treatment, as described in Section 2. For the cell free pRb dephosphorylation assays aliquots (50 μg) of protein per reaction or an immunoprecipitate (from 0.5–1 mg of protein) were suspended in PBS and incubated at 0 and 30°C for the indicated times. For $p21^{waf1}$ in vitro dephosphorylation by pRb-associated phosphatase, pRb immunoprecipitates were prepared from untreated and DEM pretreated HL60 cells and incubated at 0 and 30°C with aliquots (50 μg) of COS7 protein extract as a source of $p21^{waf1}$. After this incubation, the reactions were terminated by the addition of Laemmli buffer and the Western blot was performed with the antibodies indicated in the figure legends.

3. Results and discussion

We previously showed that the exposure of cultured cells to DEM, a GSH-depleting agent, leads to two phenomena: an early event, represented by the dephosphorylation of $p21^{waf1}$ already evident 5 min after the exposure to DEM, and a late event, consisting of the progressive accumulation of $p21^{waf1}$ starting to be detectable 2-3 h after the exposure to DEM [6]. To explore the phosphorylation state of pRb following DEM treatment, HL60 cells were exposed for different times to various concentrations of DEM and protein extracts from these cells were analyzed by Western blotting with anti-pRb antibody. As shown in Fig. 1A, the Western blot of the extracts from untreated cells shows several bands recognized by the anti-pRb antibody. Slower and faster migrating bands are known to correspond to the hyper- and hypophosphorylated forms of pRb, respectively, considering that the alkaline phosphatase treatment abolishes the slower migrating bands and increases the amount of the fastest bands [13]. In the extracts from cells exposed to DEM, this shift of band migration toward the faster bands is already present after 5 min of treatment and progressively became more evident up to 1 h. To ascertain whether the observed phenomenon is caused by the modification of the intracellular redox conditions induced by DEM, HL60 cells were treated, before the exposure to DEM,

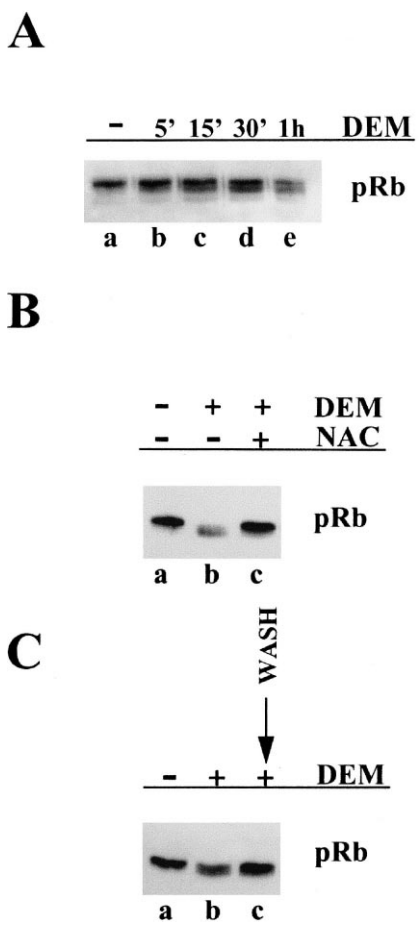

Fig. 1. Oxidative stress induces an early and reversible retinoblastoma protein (pRb) dephosphorylation. Panel A: HL60 cells were exposed to 1 mM DEM and harvested for protein extract preparation. The protein extracts were prepared as described in Section 2 and analyzed by Western blotting with anti-pRb antibody. Lane a, untreated cells; lanes b to e, cells treated with 1 mM DEM for the indicated times. Panel B: HL60 cells were pretreated with 30 mM NAC for 1 h and then with 1 mM DEM for 30 min. The cellular extracts were analyzed by Western blotting with anti-pRb antibody. Lane a, untreated cells; lanes b and c, cells treated with DEM; lane c, cells pretreated with NAC. Panel C: HL60 cells were treated with 1 mM DEM for 30 min. The cellular extracts were analyzed by Western blotting with anti-pRb antibody. Lane a, untreated cells; lane b, cells treated with DEM for 30 min; lane c, lane treated with DEM for 30 min, extensively washed with culture medium to remove DEM, incubated in fresh medium and harvested 30 min later for the preparation of cellular extracts.

with a GSH-precursor, *N*-acetylcysteine (NAC), which is well demonstrated to counteract the effects of DEM [6]. As shown in Fig. 1B, NAC pretreatment completely prevents the modification of pRb electrophoretic migration pattern. This experiment strongly supports the hypothesis that the observed phenomenon is a consequence of the modification of intracellular redox conditions. However, it must be noted that, at very early times, no significant modification of intracellular ROS concentration was observed (data not shown), thus suggesting either that very small changes of ROS concentration are already able to induce the phenomenon or that DEM acts by increasing ROS concentration only in specific cellular compartments. In any case, it is evident that the observed shift of bands towards the faster migrating ones is completely and

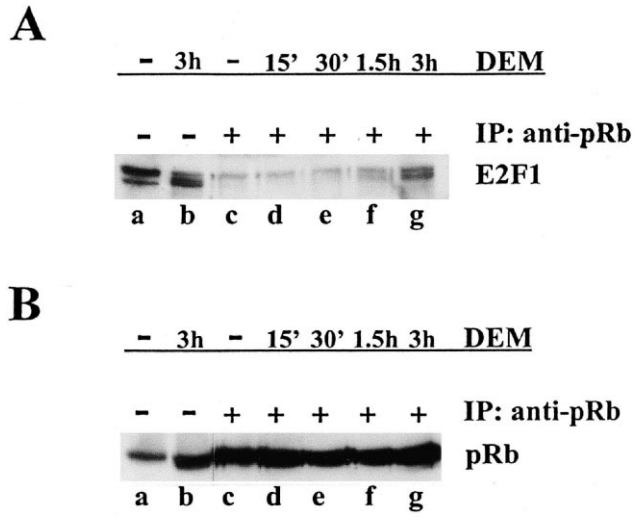

Fig. 2. Treatment of HL60 cells with DEM induces an increased association of E2F transcription factor to pRB. Panel A: HL60 cells were treated with 1 mM DEM and then harvested for the preparation of cellular extracts. The cellular extracts were analyzed by Western blotting with anti-E2F1 antibody (lanes a and b) or immunoprecipitated with anti-pRb antibody and analyzed by Western blotting with anti-E2F1 antibody (lanes c–g). Lane a, untreated cells; lane b, cells treated with 1 mM DEM for 3 h; lane c, untreated cells; lanes d to g, cells treated with 1 mM DEM for the indicated times. Panel B: HL60 cells were treated with 1 mM DEM and then harvested for the preparation of cellular extracts. The cellular extracts were analyzed by Western blotting with anti-pRb antibody (lanes a and b) or immunoprecipitated with anti-pRb antibody and analyzed by Western blotting with anti-pRb antibody (lanes c–g). Lane a, untreated cells; lane b, cells treated with 1 mM DEM for 3 h; lane c, untreated cells; lanes d to g, cells treated with 1 mM DEM for the indicated times.

rapidly reversible upon the removal of DEM. In fact, as shown in Fig. 1C, in HL60 cells exposed to DEM for 30 min, then washed with fresh culture medium for an additional 30 min, a complete recovery of the pRb electrophoretic pattern was observed. This excludes the possibility that the modification of the pRb pattern is a consequence of chemical modification of the protein by ROS, which are known to be generally irreversible [14].

The hypophosphorylated form of pRb is associated to E2F, while pRb phosphorylation causes the release of E2F from the complex and in turn the G1→S transition is allowed [9,10]. Therefore, we asked whether following the dephosphorylation of pRb upon DEM treatment, an increased amount of E2F complexed to pRb is found. Fig. 2A shows that at early times of DEM exposure pRb dephosphorylation does not lead to an increased E2F association, whereas increased amounts of E2F/pRb complexes are observed, as expected, after 1.5 and 3 h of DEM exposure, probably as a consequence of CDK inhibition by $p21^{waf1}$. This observation deserves further analysis aimed to evaluate the amino acid residues of pRb dephosphorylated as a consequence of the exposure to DEM, that could not be involved in the regulation of Rb/E2F interactions. Interestingly, treatment of HL60 cells with DEM affects also E2F protein: in fact, an inverted ratio between the upper and the lower bands in extracts from untreated and DEM-treated cells is observed (Fig. 2A, lanes a and b). However, both E2F forms are involved in pRB binding. These results suggest that another dephosphorylation process, analogous to

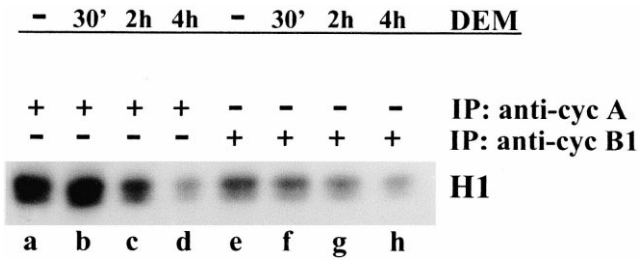

Fig. 3. Effects of DEM treatment on the inhibition of cyclin A and cyclin B1 activity by $p21^{waf1}$. HL60 cells were exposed to 1 mM DEM for the indicated times and harvested for the preparation of cellular extracts and immunoprecipitates as described in Section 2. Lanes a–d, extracts were immunoprecipitated with anti-cyclin A antibody; lanes e–h, extracts were immunoprecipitated with anti-cyclin B1 antibody. Lanes a and e, untreated cells; lanes b and f, cells treated with DEM for 30 min; lanes c and g, cells treated with DEM for 2 h; lanes d and h, cells treated with DEM for 4 h.

pRb dephosphorylation, takes place upon DEM treatment for E2F transcription factor.

An important point to be addressed concerns the mechanism through which DEM induces the dephosphorylation of pRb. pRb dephosphorylation could be due to the induction of $p21^{waf1}$ and the consequent inhibition of kinase activities which phosphorylate pRb or to an induction of protein Ser–Thr phosphatase activities. Since we observed that $p21^{waf1}$ accumulation, which follows DEM treatment, is a late phenomenon, compared to the observed pRb dephosphorylation, it seems unlikely that this dephosphorylation can be a consequence of CDK inhibition. However, we measured the changes of cyclin A- and cyclin B1-associated kinase activities, as an indirect measurement of $p21^{waf1}$-induced inhibition. During the first 30 min of incubation of HL60 cells with DEM we did not observe any change of cyclin/CDK activity, as demonstrated by the phosphorylation levels of H1 histone used as a substrate of the cyclin A (Fig. 3, lane b versus lane a) and cyclin B1 immunocomplexes (Fig. 3, lane f versus lane e). On the contrary, appreciable inhibition of the kinase activity was observed 2 and 4 h after DEM exposures (Fig. 3, lanes c and d versus lane a for cyclin A and lanes g and h versus lane e for cyclin B1) and this is in agreement with the timing of $p21^{waf1}$ accumulation that follows DEM treatment [6].

The lack of a measurable inhibition of CDK activities, even 30 min after the exposure of cells to DEM, suggests the possibility that pRb dephosphorylation could be due to the acti-

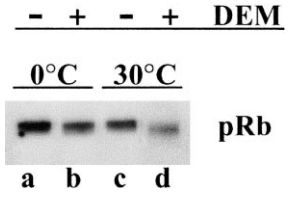

Fig. 4. pRb dephosphorylation under cell-free conditions. HL60 cells were treated with 1 mM DEM for 5 min and then harvested for the preparation of cellular extracts. The cellular extracts were analyzed by Western blotting with anti-pRb antibody. Lanes a and b: cellular extracts from untreated (lane a) and DEM-treated (lane b) HL60 cells were incubated at 0°C for 90 min; lanes c and d: cellular extracts from untreated cells (lane c) and DEM-treated cells (lane d), were incubated at 30°C for 90 min.

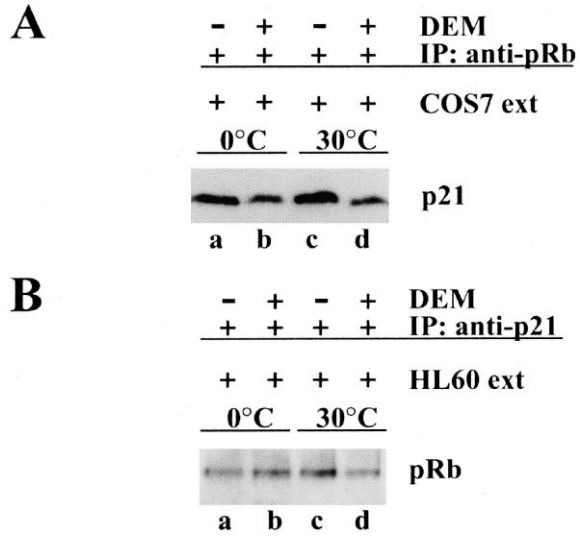

Fig. 5. Protein phosphatase(s) associated to $p21^{waf1}$ and to pRb. Panel A: $p21^{waf1}$ dephosphorylation by the pRb-associated phosphatase(s). HL60 cells were treated with 1 mM DEM for 30 min and then harvested for the preparation of cellular extracts. The cellular extracts were immunoprecipitated with anti-pRb antibody, incubated with 50 μg COS7 protein extracts and analyzed by Western blotting with anti-$p21^{waf1}$ antibody. Lanes a and c, pRb immunoprecipitates from untreated cells; lanes b and d, pRb immunoprecipitates from cells treated with DEM for 30 min; lanes a and b, pRb immunoprecipitates were incubated on ice for 90 min; lanes c and d, pRb immunoprecipitates were incubated at 30°C for 90 min. Panel B: pRb dephosphorylation by the $p21^{waf1}$-associated phosphatase(s). COS7 cells were treated with 1 mM DEM for 30 min and then harvested for the preparation of cellular extracts. The cellular extracts were immunoprecipitated with anti-$p21^{waf1}$ antibody, incubated with 50 μg HL60 protein extracts and analyzed by Western blotting with anti-pRb antibody. Lanes a and c, $p21^{waf1}$ immunoprecipitates from untreated cells; lanes b and d, $p21^{waf1}$ immunoprecipitates from cells treated with DEM for 30 min; lanes a and b, $p21^{waf1}$ immunoprecipitates were incubated on ice for 90 min; lanes c and d, $p21^{waf1}$ immunoprecipitates were incubated at 30°C for 90 min.

vation of protein Ser–Thr phosphatases. If this is the case we should observe that the dephosphorylation of pRb continues in vitro on incubation under the proper conditions. To test this point, protein extracts from untreated HL60 cells or from cells treated with DEM for 5 min were incubated at 0 or 30°C for 90 min. As shown in Fig. 4, DEM-dependent pRb dephosphorylation is more evident in cell extracts kept at 30°C for 90 min (lane d) than in the extracts kept at 0°C for 90 min (lane b). This observation is clearly DEM-dependent, because no dephosphorylation was observed in the extracts from cells not exposed to DEM (lanes a and c).

The dephosphorylation of pRb by protein phosphatases was also demonstrated under different experimental conditions [15,16]. In the case of treatment with anticancer drugs, these phosphatase activities were found to be associated with pRb itself, as demonstrated by co-immunoprecipitation experiments with anti-pRb antibodies [15]. Considering that we already demonstrated a DEM-induced dephosphorylation of $p21^{waf1}$ upon the increase of ROS levels [11], it could be hypothesized that the $p21^{waf1}$ dephosphorylation is due to the phosphatase activities associated with pRb protein. To address this point, pRb immunoprecipitates were prepared from untreated and DEM-treated HL60 cells and incubated with protein extracts from COS 7 cells, that contain considerable amounts of $p21^{waf1}$, at 30°C for 90 min. Fig. 5A shows the Western blot of these incubation mixtures with anti-$p21^{waf1}$ antibody; the $p21^{waf1}$ dephosphorylation was observed only in samples from DEM-treated cells (lane d), while no $p21^{waf1}$ dephosphorylation was observed when the samples from DEM-treated cells were kept on ice (lane b) or when immunoprecipitates from untreated control cells were incubated at 30°C (lane c). To evaluate whether a similar phosphatase activity is also associated to $p21^{waf1}$ we immunoprecipitated protein extracts from DEM-treated COS7 cells with anti-$p21^{waf1}$ antibodies and incubated these immunoprecipitates with extracts from untreated HL60 cells. As reported in Fig. 5B, the Western blot of these extracts with anti-pRb antibodies shows that neither at 0°C nor at 30°C is pRb migration affected by the exposure to anti-$p21^{waf1}$ immunoprecipitates. This demonstrates that $p21^{waf1}$ immunoprecipitated from COS7 cells is not associated with phosphatases.

In summary, the results reported in this paper provide evidence for the ability of intracellular redox changes to cause an early dephosphorylation of pRb. This phenomenon precedes the accumulation of $p21^{waf1}$ that leads to the inhibition of CDK activity. The two effects act synergistically to obtain the dephosphorylation of pRb and to avoid its rephosphorylation by CDKs. However, in cells exposed to oxidants, the early dephosphorylation of pRb does not lead to the sequestration of E2F in the dephospho-pRb/E2F complexes. This does not exclude that the early pRb dephosphorylation caused by DEM could affect other pRb function.

While protein kinase activities regulating the response to mitogenic stimuli and cell cycle progression are well known, less information is available on protein phosphatases which are expected to contribute, to the same extent as the kinases, to the regulation of these phenomena. The experimental system that we used appears to be suitable to study the regulation of these protein phosphatases and their role in the molecular machinery responsible for the control of cell proliferation.

Acknowledgements: This work was supported by grants from the Italian National Research Council (CNR) PF 'Biotecnologie' and PSt/74 'Biologia dell'invecchiamento e sue conseguenze sul sistema assistenziale', from Associazione Italiana per la Ricerca sul Cancro (AIRC), and from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (COFIN 98).

References

[1] Yu, B.P. (1994) Physiol. Rev. 74, 139–162.
[2] Reid, T.M. and Loeb, L.A. (1993) Proc. Natl. Acad. Sci. USA 90, 3904–3907.
[3] Richter, C., Park, J.W. and Ames, B.N. (1988) Proc. Natl. Acad. Sci. USA 85, 6465–6467.
[4] Sundaresan, M., Yu, Z.-X., Ferrans, V.J., Irani, K. and Finkel, T. (1995) Science 270, 296–299.
[5] Cimino, F., Esposito, F., Ammendola, R. and Russo, T. (1997) Curr. Topic Cell. Regul. 35, 123–147.
[6] Russo, T., Zambrano, N., Esposito, F., Ammendola, R., Cimino, F., Fiscella, M., Jackman, J., O'Connor, P.M., Anderson, C.W. and Appella, E. (1995) J. Biol. Chem. 270, 29331–29386.
[7] El-Deiry, W.S., Tokino, T., Velculesku, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993) Cell 75, 817–825.
[8] Esposito, F., Cuccovillo, F., Vanoni, M., Cimino, F., Anderson, C.W., Appella, E. and Russo, T. (1997) Eur. J. Biochem. 245, 730–737.
[9] Mittnacht, S. (1998) Curr. Opin. Genet. Dev. 8, 21–27.

[10] Dyson, N. (1998) Genes Dev. 12, 2245–2262.
[11] Esposito, F., Cuccovillo, F., Russo, L., Casella, F. and Russo, T. (1998) Cell Death Differ. 5, 940–945.
[12] Dou, Q.P., Levin, A.H., Zhao, S. and Pardee, A.B. (1993) Cancer Res. 53, 1493–1497.
[13] Ludlow, J.W., De Caprio, J.A., Huang, C., Lee, W.H., Paucha, E. and Livingston, D.M. (1989) Cell 56, 57–65.
[14] Stadtman, E.R. (1993) Annu. Rev. Biochem. 62, 797–821.
[15] Dou, Q.P., An, B. and Will, P. (1995) Proc. Natl. Acad. Sci. USA 92, 9019–9023.
[16] Nelson, D.A., Krucher, N.A. and Ludlow, J.W. (1997) J. Biol. Chem. 272, 4528–4536.