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Protein Phosphatase-1 Activation and Association with the Retinoblastoma Protein in Colcemid-Induced Apoptosis

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Protein phosphatase-1 (PP1) is cell cycle regulated and potentially related to apoptosis. We studied PP1 in HeLa cells exposed to colcemid, which leads first to mitotic block, then to cell death within 72 h. The soluble PP1 activity, which was low at 14 h (mitosis), was then reversibly activated (maximally around 48 h), with parallel changes in the protein levels of the α, γ1 and δ PP1 isoforms. PP1 activation suggested its involvement in dephosphorylating proteins relevant to apoptosis. Among these, we examined the retinoblastoma protein (pRb). This was found hyperphosphorylated at 14 h. Hypophosphorylated pRb appeared at 24 h, increased at 48 h, and was the only form left at 72 h. PP1 was found to associate with immunoprecipitated pRb, as indicated by PP1 activity assays on the pRb-immunocomplexes. The pRb-associated PP1 activity was low at 14 h, maximal at 24 h, low again by 72 h and was due to PP1δ. The presence of active PP1 suggests its involvement in pRb dephosphorylation.

Several model systems indicated that protein phosphorylation regulates apoptotic pathways. In fact proteins relevant to apoptosis, such as Bcl-2 (1), p53 (2), pRb (retinoblastoma gene product, 3) and caspases (4), are phosphorylated on Ser/Thr and phosphatase inhibitors can induce apoptosis (2, 5, 6). pRb is a key cell cycle regulator which also protects from cell death (7, 8). In G1 cells, pRb is hypophosphorylated and active as growth suppressor (9). In late G1/S pRb gets phosphorylated by cyclin-dependent kinases (10, 11). The hyperphosphorylated form is maintained till mitotic exit (10), when it is dephosphorylated by protein phosphatase-1 (PP1, 12, 24, 25). Also in apoptosis pRb is converted to the hypophosphorylated form, which is subsequently cleaved by proteases (e.g. 3, 13).

PP1 consists of a catalytic subunit bound to one among several possible regulatory subunits, which target the enzyme to cell structures (14, 15). In mammalian cells the catalytic subunit displays three main isoforms, α, γ1 and δ (14, 16). Studies with isoform-specific antibodies (17) indicated that the isoforms have differential sub-cellular localizations (18, 19), suggesting also different functions. PP1 activity oscillates during the cell cycle. PP1 is inactivated at M-phase, reactivated in G1, inactivated again in late G1/S and reactivated in G2 (20–22). Also the PP1 isoforms are differentially regulated (21) but their protein levels remain constant throughout the cell cycle (F. P. and E. V.-M, unpublished observation). This suggests that the regulation of PP1 is exerted through changes in its activity, rather than at protein level.

PP1 interacts with pRb during normal cell cycle (as first shown in a two-hybrid system, 23) and dephosphorylates pRb at the exit from mitosis (12, 24). We found that PP1δ was the isoform that co-immunoprecipitated with pRb in mitotic and early G1 cells (25). The pRb-associated PP1 was inactive in mitosis (when pRb is hyperphosphorylated) and activated at mitotic exit (when pRb gets dephosphorylated). Recent studies indicated that PP1α associates with pRb in late G1 (22) and PP1α inactivation is required for cell cycle progression (26).

The involvement of PP1 in apoptosis, though in opposite ways, was also indicated by the use of phosphatase inhibitors. In fact okadaic acid induced morphological changes typical of apoptosis in a number of cell types (2, 5, 6), suggesting an anti-apoptotic role for PP1. On the other hand, apoptosis induced by etoposide and other drugs was prevented by short-term exposure to okadaic acid, calyculin A or cantharidin (13).

Abbreviations used: PP1, Serine/Threonine phosphatase of type-1; pRb, retinoblastoma gene product ($p110^{RB}$); cdc2-cyclin B, cyclin-dependent protein kinase active at mitosis; PMSF, phenylmethylsulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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Although PP1 activation was not detected, these latter studies pointed to a role for PP1 upstream of major apoptotic events, such as intracellular acidification, DNA fragmentation, protease activation and pRb dephosphorylation (3, 13, 27).

We studied PP1 and pRb in HeLa cells made apoptotic by the continuous exposure to colcemid, a drug that induces mitotic arrest. If the drug is not removed, the cells are unable to exit from mitosis (i.e., the cells keep condensed chromatin and elevated cdc2-cyclin B activity, 28) and go directly into apoptosis. In this system we detected activation of PP1 and association of active PP1δ with pRb at the time of its dephosphorylation.

MATERIALS AND METHODS

Materials. Colcemid was from Fluka. Okadaic acid was from Moana Bio products. Triton X-100 and leupeptin were from Boehringer. The chemicals for agarose electrophoresis were from Gibco BRL. [^{32}P]ATP and the ECL chemiluminescence system were from Amersham. The anti-pRb C-15 rabbit antibody (from Santa Cruz Biotechnology Inc.) was raised against a C-terminal peptide of p110 pRb and recognized both phosphorylated and non-phosphorylated pRb. All the other products and cell culture media were from Sigma.

Induction of apoptosis and cell harvesting. HeLa cells were grown on 150-mm plates (21). Exponentially growing cells were exposed to 70 ng/ml colcemid (28) for up to 72 h. At each time-point, the apoptotic cells were collected by gentle pipetting, washed in cold PBS and either analyzed for apoptosis or broken with a glass homogenizer for cell fractionation or lyzed for total cell extract. The asynchronous (time 0) cells were washed and scraped in cold PBS and either trypsinized for apoptotic counting or used for cell fractionation or extraction.

Apoptotic cells counting. The frequency of apoptotic cells was determinated by the Typan blue exclusion assay. Cells were resuspended in Hanks' balanced salt solution, mixed with 0.4% Trypan blue, incubated for 10 min and counted in a hemocytometer chamber. Non-viable cells stained blue.

Analysis of DNA fragmentation. Cells were lysed in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA and 0.5% SDS. The lysate was digested with 0.1 mg/ml proteinase K at 55°C for 17 h, extracted with 25:24:1 phenol/chloroform/isoamyl alcohol and precipitated with 100% ethanol. The nucleic acids were resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and separated on a 1% agarose-ethidiun bromide gel, using 0.8 mM Tris-acetate, 0.004 mM EDTA as electroforesis buffer.

Cell fractionation. PBS-washed cells were resuspended in 10 mM HEPES, pH 7.4, 0.1 mM EDTA, 10 mM NaCl, 15 mM 2-mercaptoethanol and protease inhibitors (0.004% benzamidine, 0.004% PMSF, 0.002% TPCK, 4 μg/ml leupeptin) at 4°C, and broken using a glass (Dounce) homogenizer. Separation of the soluble and nuclear/chromosomal fractions was by low-speed centrifugation (2,000 $\times$ g for 11 min at 4°C, 20).

Total cell extract. PBS-washed cells were lysed at 4°C in 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 (lysis buffer), 7.5 mM 2-mercaptoethanol and protease inhibitors as above. 1 μM okadaic acid was added to preserve pRb phosphorylation when the extracts were used to visualize pRb. After 20 min rotation the extracts were centrifuged at 12,000 $\times$ g for 5 min.

Immunoprecipitation and Western blotting. 10 μl/ml of anti-PP1α, anti-PP1γ1 or anti-PP1δ hyperimmune rabbit sera (17, 21) or 2.5 μg/ml of affinity-purified C-15 anti-pRb antibody were used for immunoprecipitation with Protein A-Sepharose. (a) For Western blotting, the immunocomplexes were washed three times with lysis buffer containing 0.004% benzamidine and 0.004% PMSF, resuspended in Laemmli buffer and boiled. This was followed by 9% SDS-PAGE electrophoresis and blotting onto Immobilon-P (according to the manufacturer's instructions). Immunodetection used protein A-peroxidase and the chemiluminescence ECL system. For reprobing, the membrane was stripped by incubating for 30 min at 60°C in 5 mM phosphate buffer pH 7.5, 2% SDS and 2 mM 2-mercaptoethanol. In some cases proteins were concentrated before gel loading by precipitation with 7% TCA. (b) For PP1 assay, the immunoprecipitates were washed three times with lysis buffer as above, once in phosphatase assay buffer and resuspended in 15 μl of assay buffer (29). Control immunocomplexes were prepared using non-related rabbit IgGs.

PP1 assay. PP1 was assayed by the release of [^{32}P]H_3PO_4 from [^{32}P]-rabbit muscle phosphorylase *a* 1–2 $\times$ 10^5 cpm/nmol (29), in the presence of 5 nM okadaic acid, to inhibit PP2A. The trypsin-treatment (20 μg/ml) was for 3.5 min at 30°C, followed by soybean trypsin inhibitor (120 μg/ml). The phosphatase activity associated with the anti-pRb immunocomplexes was calculated after subtracting the activity assayed in control complexes (29).

RESULTS

(1) Induction of Apoptosis in HeLa Cells with Colcemid

Asynchronous HeLa cells were exposed to 70 ng/ml colcemid for up to 72 h. This drug concentration induces mitotic arrest in about 12–14 h, prevents exit form mitosis and leads to apoptosis, which begins at about 24 h (28). Only the round mitotic or apoptotic cells were collected, which represented 50–60% of the cells at 14 h, 70–80% at 24 h, and 95% at 48 h and 72 h. For time 0, asynchronous cells were harvested by plate scraping. Progression into apoptosis was monitored by the number of cells unable to exclude Trypan blue and by the appearance of the DNA ladder. 40% of the cells by 24 h and the vast majority of them by 72 h were unable to exclude Trypan blue (Fig. 1). The DNA ladder became relevant at 48 h and 72 h (Fig. 1, inset).

(2) Changes in PP1 Activity in Apoptotic Cells

We previously reported that the soluble PP1 activity decreased in mitosis and increased again in early G1. This was due to changes in enzyme activity that did not affect the protein levels of the PP1 isoforms (21). In the nuclear/chromosomal fractions of the same cells PP1 displayed little changes (20, 21). In the present study we found that inactivation of the soluble PP1 occurred also in colcemid-induced mitotic arrest (Fig. 2S, compare time 0 with 14 h). Subsequently, as cells progressed into apoptosis, the PP1 activity increased, reaching a peak at 48 h, followed by a final decrease by 72 h. The changes were more pronounced when the PP1 assays were preceded by a short trypsin-treatment (Fig. 2), which removes PP1 inhibitors and regulatory proteins. This suggested that the PP1 catalytic subunit, rather than its regulatory proteins, was affected. This, in turn, might be due to changes in either PP1

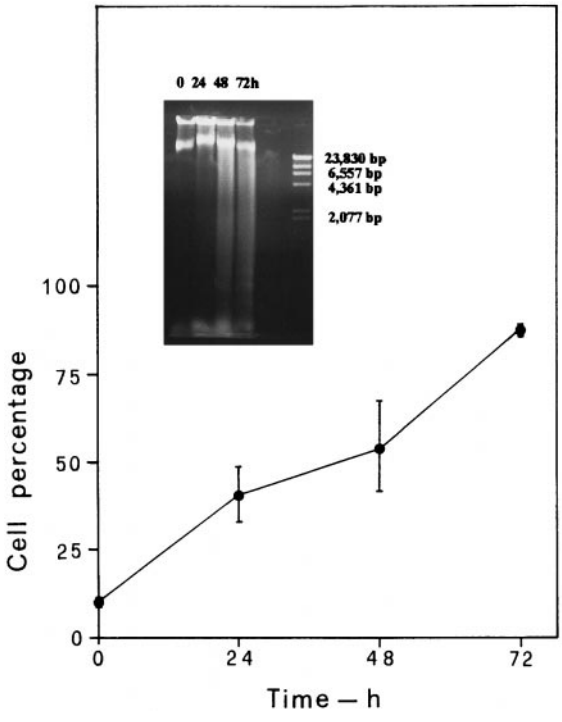

FIG. 1. Induction of apoptosis in asynchronous HeLa cells exposed to colcemid. Cells were treated with 70 ng/ml colcemid and harvested as indicated. The percentage of apoptotic cells was determined by Trypan blue exclusion assay, scoring a minimum of 100 cells per time-point. Data are mean values ± SEM of three determinations. (Inset) DNA fragmentation visualized as oligonucleosome-sized fragments in ethidium bromide-stained agarose gel. The sizes of marker DNA fragments are indicated to the right.

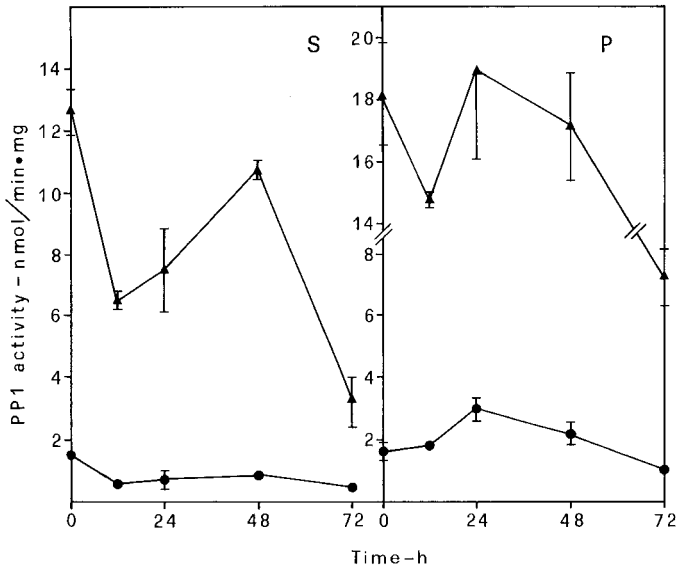

FIG. 2. PP1 activity in the soluble fraction (S) and in the low-speed pellet (nuclear/chromosomal fraction, P) of HeLa cells exposed to colcemid. Cells were harvested from two 150-mm plates per time point and fractionated and PP1 was assayed as basal activity (closed circles) or after trypsin-treatment (which removes PP1 inhibitors, closed triangles) in the presence of 5 nM okadaic acid (to inhibit PP2A). Data are mean values ± SEM of three determinations.

conformation or protein level. Western blotting and probing with the isoform-specific antibodies indicated that the protein of all the three isoforms increased at 24 and 48 h, and decreased at 72 h (Fig. 3). This may account, at least in part, for the observed activity oscillations. We also analyzed the activity of each isoform following immunoprecipitation and assay of the associated PP1 activity. The results confirmed a peak of activity at 48 h for all the isoforms and subsequent decrease (not shown).

The PP1 activity was assayed also on the pellet fraction, which contains chromosomes and membrane aggregates. The changes were less relevant than in the soluble fraction, with an activation peak at 24 h and subsequent decrease (Fig. 2, P). Also the immunoblot indicated minor changes in the amount of the three isoforms (Fig. 3).

(3) Phosphorylation Levels of pRb in Apoptotic Cells

The activation of PP1 suggested its role in dephosphorylating substrates involved in apoptotic progression, such as pRb. In order to investigate its phosphorylation state, pRb was immunoprecipitated with the C-15 antibody from cells exposed to colcemid for up to 72 h. Following Western blotting, the immunocomplexes were probed with the same antibody, which detects both phospho- and dephospho-pRb. As expected from previous results (25), pRb was fully phosphorylated at 14 h (mitosis), since only the hyperphosphorylated upper band was detected (Fig. 4, inset; for comparison, both hyper- and hypo-phosphorylated bands can be seen in pRb from time 0 asynchronous cells). Dephosphorylation started at 24 h, when the lower pRb band appeared, and became more relevant at 48 h.

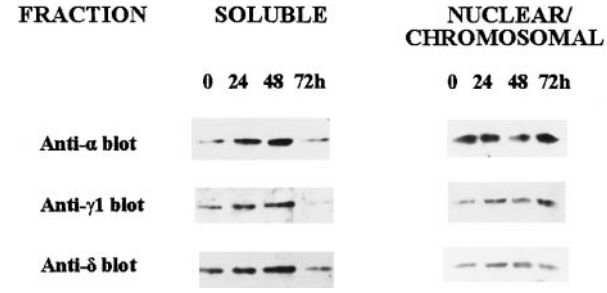

FIG. 3. PP1 α, $\gamma 1$ and δ isoforms in soluble and low-speed pellet (nuclear/chromosomal) fractions of HeLa cells exposed to colcemid. Cells were collected and fractionated as in Fig. 2. 30 μg of protein was applied to each lane. Following electrophoresis and Western blotting, the same membrane was probed with the anti-PP1δ and anti-PP1γ1 antibodies in sequence (removing the antibody-protein A-peroxidase complex after the first probing, as described in Materials and Methods). A separate electrophoresis was used for the anti-PP1α blot. The data are representative of three independent experiments.

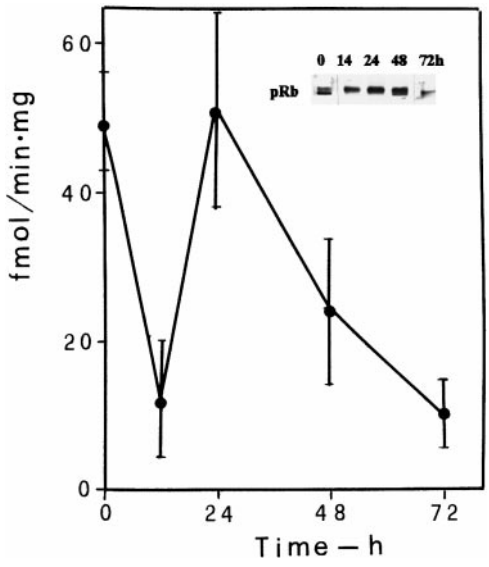

FIG. 4. Association of PP1 activity with pRb immunoprecipitated from colcemid-treated HeLa cells. pRb immunocomplexes, obtained from 3 mg of total extract for each time point, were used to assay the associated PP1. The activity was calculated after subtracting the activity non-specifically bound to control immunocomplexes. Data are mean values ±SEM of three determinations. (Inset) Phosphorylation status of pRb in cells exposed to colcemid. pRb was immunoprecipitated from cell extracts prepared in the presence of 1 μM okadaic acid, to prevent dephosphorylation. After electrophoresis and blotting, immunodetection used the C-15 anti-pRb antibody.

Hypophosphorylated pRb was the only form present at 72 h, when also much less pRb was detected, probably due to proteolysis.

(4) Association of PP1 with pRb in Apoptotic Cells

The results indicating that also in our system pRb underwent apoptotic dephosphorylation prompted us to investigate whether PP1 was associated with the pRb immunocomplexes. These latter were prepared as above, but using extracts without okadaic acid, to allow PP1 assay. Little activity was assayed at 14 h, thus confirming previous results with mitotic cells (25). However, the associated PP1 activity increased at 24 h, and then decreased again, reaching a minimum at 72 h. Thus, more PP1 activity was present when pRb dephosphorylation started (24 h) and less activity when a relevant amount of pRb was already in the dephosphorylated form (48 h). When comparing the activity and immunoblot results of Fig. 4, the possibility should be considered that in the absence of okadaic acid some pRb dephosphorylation has occurred. This may cause detachment of PP1 from pRb, with consequent underestimating of the PP1 bound to pRb. Nevertheless, the results clearly indicated that active PP1 was associated with pRb and suggested its involvement in pRb dephosphorylation.

(5) Identification of the Associated PP1 as PP1δ

In order to identify the PP1 isoform involved, pRb was immunoprecipitated from an extract obtained from cells exposed to colcemid for 24 h, when the associated activity was maximal. Subsequent Western blotting with the isoform-specific antibodies detected only PP1δ (Fig. 5). Neither α nor γ1 were found in parallel experiments (not shown). "Pull-down" experiments of pRb using recombinant PP1δ-GST fusion protein confirmed that PP1δ was able to associate with pRb from apoptotic cell extracts (not shown).

DISCUSSION

Previous reports indicated the involvement of Ser/Thr phosphatases in apoptosis. In fact several effects of apoptosis-inducing drugs, including pRb dephosphorylation, were blocked by phosphatase inhibitors (13, 27). In order to investigate the PP1 changes in apoptosis and its to association with pRb, we used HeLa cells made apoptotic by the continuous exposure to colcemid (28). The results showed reversible PP1 activation, which involved also increase in the levels of the PP1 isoforms. This latter result was different from what observed in normal cell cycle, when the PP1 protein levels did not change and the activity oscillations were attributed to regulation by proteins or inhibitory phosphorylation of the catalytic subunit (21, 22, 26, 30). The detection of increased PP1 protein levels in colcemid-induced apoptosis was surprising and further stressed the differences between these terminally committed cells and normal cell cycle.

PP1 activation suggested its involvement in dephosphorylations needed at an early and intermediate phase of apoptosis. One of the proteins that undergo dephosphorylation in apoptosis is pRb. However, previous studies did not clarify which phosphatase was

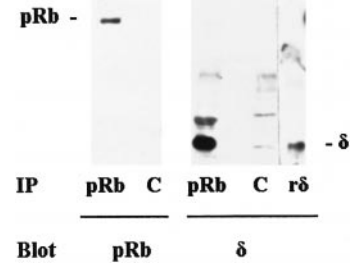

FIG. 5. Association of PP1δ with pRb immunoprecipitated from cells exposed to colcemid for 24 h. pRb was immunoprecipitated as in Fig. 4 from 10 mg of total extract (pRb IP). The control immunoprecipitate (C IP) was prepared with unrelated IgGs. After electrophoresis and Western blotting, the membranes were probed first with the anti-PP1δ antibody (δ Blot), followed by removal of the antibody-protein A-peroxidase complexes and second probing with the anti-pRb antibody (pRb Blot). 25 ng of recombinant PP1δ was added to the electrophoresis as positive control (rδ).

involved and total cellular PP1 was not found to be activated (3, 13). Also in our apoptotic system pRb was converted to the hypophosphorylated form. Analysis of the pRb-immunocomplexes demonstrated the presence of active PP1, identified as PP1δ, at the time when this dephosphorylation occurred. Consequently, the associated PP1 is most likely the enzyme that brings-about pRb dephosphorylation.

The discrepancy between the present data and the previous literature reports that did not detect PP1 changes in apoptosis, may be related to the different model systems or to differences in the conditions used to assay PP1. Specifically, the use of a high concentration of the substrate (phosphorylase *a*) in the assays of the pRb-immunocomplexes may be critical to compete with pRb, which is probably a preferred substrate for the pRb-phosphatase.

It is presently not known if the binding of PP1δ to pRb is direct or mediated by some other protein (e.g. a PP1 regulatory subunit) and only "in vitro" association studies will help to clarify this point. It is conceivable that the pRb-associated PP1δ represents only a portion of the total cellular PP1δ. In fact each PP1 isoform may associate with different regulatory subunits and thus regulate different pathways. Since the PP1 changes shown in Fig. 2 apply to all the PP1 isoforms, the results suggest that PP1 activation might influence also other regulatory pathways.

Finally, from the present and previous data we can conclude that PP1δ is the isoform that associates with pRb not only in mitosis (25), but also in colcemid-induced apoptosis. On the contrary, PP1α associates with pRb in normal G1 (22) but not in apoptosis. It would be interesting to see if this selectivity towards a PP1 isoform occurs also in other apoptotic models and which other substrates might associate with and be dephosphorylated by PP1.

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