Biochemical and Biophysical Research Communications **280,** 1–3 (2001)
doi:10.1006/bbrc.2000.4067, available online at http://www.idealibrary.com on IDEAL®

Binding of Phosphatase-1 δ to the Retinoblastoma Protein pRb Involves Domains That Include Substrate Recognition Residues and a pRB Binding Motif

Mariarita Bianchi and Emma Villa-Moruzzi[1]
Department of Experimental Pathology, University of Pisa, 56126 Pisa, Italy

Received November 27, 2000

Protein Ser/Thr phosphatase-1 (PP1) controls the retinoblastoma protein (pRb) function, including its dephosphorylation at mitotic exit. Since PP1δ was found to coimmunoprecipitate with pRb from mitotic and early G1 cells, we further investigated the PP1δ-pRb association using GST-full length and GST-deletion mutants of δ. GST-δ pulled-down pRb from G2, mitotic and G1 HeLa cells, thus confirming the coimmunoprecipitation results. Among the δ deletion mutants tested, pRb was pulled down by mutant 159–295, which reproduces the C-terminal domain of δ without the C-terminus, whereas the C-terminus alone did not pull-down pRb. Further fragmentation of the 159–295 mutant indicated that pRb was pulled down by fragment 195–260, which includes several residues involved in substrate binding, and by fragment 159–212, which contains the putative pRb-binding motif LxSxE. Altogether the results supported the hypothesis that PP1δ may contribute to the dephosphorylation of pRb at mitotic exit and that the PP1δ-pRb interaction may be at multiple sites. © 2001 Academic Press

Key Words: **protein phosphatase; retinoblastoma gene product; cell cycle.**

Protein Ser/Thr phosphatase-1 (PP1) is found in membranes and in specific cell structures (see e.g., 1). PP1 is a holoenzyme consisting of a catalytic subunit and of regulatory subunits, which target the enzyme to specific functions (2–3). In mammalian cells three major isoforms of the catalytic subunit are found (α, γ1 and δ) which are over 90% identical and display significant sequence differences only at the C-termini. Studies with isoform-specific antibodies (5) indicated differential tissue and subcellular localization of the isoforms (1, 5–7), suggesting association with specific regulatory subunits and differential functions. The C-terminal regions contain also regulatory sites that are phosphorylated by cdc2 (8–9) and by src and abl (10).

PP1 is cell cycle regulated. PP1 activity is low in mitosis (due to phosphorylation by cdc2-cyclin B, 11), maximal in G1 (11), and may decrease again in late G1/S (12), with no changes in the protein level (12). One of the relevant roles of PP1 in the cell cycle may be the dephosphorylation of pRb, the product of the retinoblatsoma gene, which acts as growth suppressor in G1 (see e.g., 13). The interaction between the PP1α2 isoform and pRb was first detected in a two-hybrid system and α2 was found to associate with pRb in mitosis and early G1 (14). PP1 was subsequently indicated as the enzyme that dephosphorylates pRb at the exit from mitosis (15, 16). We found that in mitotic cells the δ isoform associated with pRb and underwent fast activation at G1 entry, followed by decrease in the activity as the hypophosphorylated pRb appeared (17). Starting form late G1 the hyperphosphorylated pRb is produced, due to multiple Ser/Thr phosphorylations by cyclin-dependent kinases (see e.g., 13). PP1α seems to be the isoform that controls pRb function in G1 and at G1/S transition (12).

The present study shows the *in vitro* association of full-length and deletion mutants of PP1δ with pRb, indicating that the association involves residues relevant to substrate binding.

MATERIALS AND METHODS

Materials. PMSF, benzamidine, TPCK, leupeptin, protein A-Sepharose, protein A-peroxidase, nocodazole, media, and additives for cell culture were purchased from Sigma Chem. Co. Glutathione-Sepharose 4B, the pGEX vectors, anti-GST antibodies, Hyperfilms-

Abbreviations used: PP1, Serine/Threonine phosphatase of type-1; pRb, retinoblastoma gene product ($p110^{RB}$); GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonylfluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline.

[1] To whom correspondence should be addressed at Dipartimento di Patologia Sperimentale, Sezione di Patologia Generale, via Roma 55, 56126 Pisa, Italy. Fax: +39-050-554929. E-mail: villa@biomed.unipi.it.

0006-291X/01 $35.00

ECL, and the ECL reagents were from Amersham Pharmacia Biotech. The restriction enzymes were from New England Biolabs. Taq DNA polymerase and the rapid DNA ligation kit were from Boehringer Mannheim. The chemicals for electrophoresis were from Bio-Rad. Immobilon-P was from Millipore. The PP1δ isoform-specific antibodies were raised in rabbits injected with the C-terminal peptide of δ (5). The affinity-purified anti-pRb polyclonal antibodies (C-15) were from Santa Cruz and recognized both hyper- and hypophosphorylated pRb forms.

Plasmids preparation, bacterial growth, and extracts. The PP1δ deletion mutants were obtained as GST-fusion proteins. The pcr amplimers were produced using full-length rat δ cDNA in pBluescript SK (supplied by Dr. M. Nagao, Japan), and cloned into the *Eco*RI and *Bam*HI sites of the pGEX4T-1 vector. Oligonucleotides were as follows. For mutant 6 (fragment 159–212): 5′ CTA GGA TCC CCT ATA GCT GCT AT 3′ and 5′ ATC GAA TTC TAC ATC CTT ATC TG 3′ (inverse). For mutant 7 (fragment 194–260): 5′ CTA GGA TCC GTA CCT GAT ACA GG and 5′ ATC GAA TTC TCG TTT AGC AAA AA (inverse). The oligonucleotides for mutant 2 (fragment 159–295) and 3 (fragment 295–327, reproducing the C-terminus of δ) were described elsewhere (M. Fresu, J. T. Parsons, and E. Villa-Moruzzi, submitted). The vector to express GST-full length δ was supplied by Dr. M. Ito (Mie, Japan). BL-21 protease-minus bacteria were used for GST-fusion protein production. The bacteria were grown at 37°C in LB-Ampicyllin, induced with 0.1 mM IPTG for 2 h, collected by centrifugation and resuspended in 25 mM Tris–Hcl, pH 7.5, 150 mM NaCl, 2 mM 2-mercaptoethanol (bacterial lysis buffer) added with protease inhibitors (0.02% benzamidine, 0.02% PMSF, 0.02% TPCK, and 4 μg/ml leupeptine). Following quick freezing and thawing, 0.25% Triton X-100, 25 U/ml DNase, 10 mM $MnCl_2$, and 10 mM $MgCl_2$ were added (final concentrations), followed by 30 min rotation at 4°C and 10,000*g* centrifugation for 20 min, to obtain the extract for pull-down assays.

Cell culture and extracts. Cells were grown at 37°C in water-saturated CO_2, in DMEM added with 8% fetal calf serum (11, 17). Following two washes in cold PBS, cells were lysed in 50 mM Tris–Hcl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 (cell lysis buffer) added with protease inhibitors.

Mitotic block, G1 and G2 cells. Hela cells were blocked in pseudomitosis as described previously (11, 17). In brief, HeLa cells were exposed to 50 ng/ml nocodazole for 16–18 h, the mitotic cells were collected by gentle pipetting, washed in PBS and lysed as above. The cells left on the plates, which were mainly in G2 (11), were lysed on the plate. G1 cells were obtained following growth for 48 h in DMEM added with 0.5% newborn calf serum. The cells were lysed on the plate after removing few occasional round cells.

Pull-down assays, immunoprecipitation, and immunoblotting. The GST-fusion proteins in the bacterial extracts were bound to 25–50 μl of glutathione-Sepharose beads, pretreated with lipid-free BSA. After 1.5 h rotation at 4°C and washing three times with bacterial lysis buffer (added with Triton X-100, benzamidine and PMSF, as above) and once in cell lysis buffer, the beads were mixed with the cell extracts, rotated for 1.5 h at 4°C, washed three times with cell lysis buffer (added with benzamidine and PMSF, as above) and boiled in Laemmli buffer. Anti-pRb (purified polyclonal rabbit antibodies, 20 μl/ml of cell extract) were bound to Protein A-Sepharose beads (5), followed by incubation and processing as in the pull-down assays. Electrophoresis was on 7.5% polyacrylamide-SDS gel and Immobilon-P was used for transblotting. The membrane was probed with the indicated antibodies (diluted either 1:1000 in PBS or as specifically indicated by the supplier), followed by protein A-peroxidase or peroxidase-conjugated antibody and the ECL (enhanced chemiluminescence) reagent (5). For reprobing with a second antibody, the membranes were previously incubated in 5 mM phosphate buffer, 2% SDS, and 2 mM 2-mercaptoethanol at 60°C for 30 min.

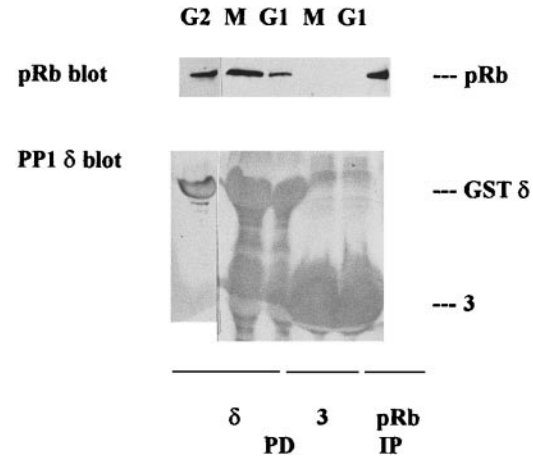

FIG. 1. Association of pRb with PP1δ in HeLa cells synchronized in G2, M and G1 phase. GST-PP1δ (δ) or the GST-deletion mutant 295–327 of δ (3) were used to pull-down (PD) pRb from 2 mg of HeLa cell extracts, as indicated. pRb was also immunoprecipitated from 0.4 mg of mitotic cell extract, as positive control (pRB IP). Following electrophoresis and Western blotting, the same membrane was first probed with the anti-pRb and then with the anti-PP1δ C-terminal peptide antibodies (following removal of the antibody-protein A-peroxidase complex, as described under Materials and Methods). The data are representative of three independent experiments.

RESULTS AND DISCUSSION

Association of pRb with Full-Length PP1δ, but Not with the C-Terminal Domain of δ

Since δ is the PP1 isoform that coimmunoprecipitated with pRb from mitotic cell extracts (17), full-length PP1δ, fused to the GST protein, was used to study its association with pRb in pull-down assays. GST-δ was able to pull-down pRb from mitotic HeLa cell extracts, as well as from G2 and G1 cells (Fig. 1). The PP1 isoforms are highly homologous and only the C-termini are isoform-specific. For this reason we prepared the C-terminal of δ, also as GST-fusion protein (defined as fragment 3) and used it in pull-down assays. Fragment 3 was not able to pull-down pRb, both from mitotic and G1 cells (Fig. 1). The same result was also obtained using GST alone (not shown).

The results confirmed the interaction between PP1δ and pRb, as previously detected in immunoprecipitates. The amount of pRb pulled-down from G1 cells was lower than what pulled down from the same amount of G2 and mitotic cells extracts. This might indicate preferential interaction of δ with hyperphosphorylated pRb, or might simply reflect the difficulty to extract pRb, which is tightly associated with chromatine in G1 cells.

Association of pRb with Specific PP1δ Domains

In order to investigate the domain interacting with pRb, more deletion mutants of PP1δ were prepared, all

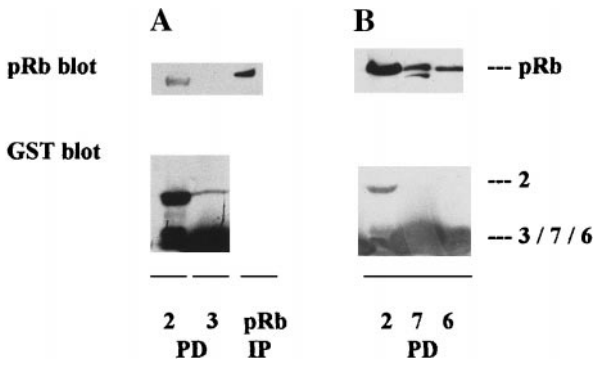

FIG. 2. Association of pRb with the PP1δ deletion mutants 2, 3, 7, and 6. The GST-159-295 mutant (2) and GST-295-327 (3) (A) or mutants GST-194-260 (7) and GST-159-212 (6) (B) were used to pull-down pRb from 2 mg of mitotic HeLa cell extract, as described in Fig. 1. Immunoprecipitated pRb is also shown in A. The first probe was with anti-pRb antibodies and the second with anti-GST antibodies (see Fig. 1). The data are representative of two independent experiments.

produced as GST-fusion proteins, and used in pull-down assays. The source of pRb was mitotic cell extract, since it contains hyperphosphorylated pRb, which is the putative PP1δ substrate. The deletion mutant of δ contains residues 159–295 (defined as fragment 2) did pull-down pRb (Fig. 2A). This indicated that the interaction mapped to the C-subdomain of PP1δ (18), and further confirmed that it excluded the C-terminus.

Based on this result, further deletion mutants of δ were produced and used in pull-down assays. Among these, both fragment 7 (residues 194–260) and 6 (residues 159–212) pulled-down pRb. Fragment 7 is of particular interest, since it displays several residues involved in substrate recognition and catalysis (19). Specifically, Arg 220 (corresponding to Arg 221 in PP1α) was reported to interact with a phosphate oxygen of the substrate and to position it for catalysis. Asp 207 and Trp 205 are both contributing to the correct metal binding at the PP1 catalytic site. Moreover, also His 247 and His 172 bind to one of the two metals of PP1 (20). Altogether, the binding of pRb to a fragment containing so many sites involved in substrate recognition supports the hypothesis that pRb is a substrate for PP1δ (15–17, 12).

Also fragment 6 binds pRb and includes the metal-binding His 172. But, most relevantly, fragment 6 includes the putative pRb-binding motifs LxSxE. This is similar to the LxCxE motif found in several pRb-binding proteins and is also present in PP1α2, the PP1 isoform found to associate with pRb in a two-hybrid screen (14). This result may indicate that the interaction between PP1δ and pRb is at multiple sites.

Altogether the results indicated the direct association between PP1δ and pRb, but also suggested that the interaction may be complex, involving multiple sites in addition to the substrate binding site. Moreover, considering that PP1 is a holoenzyme, the presence of an additional PP1 regulatory subunit that modulates the PP1-pRb intraction cannot be discarded (see e.g., 21).

ACKNOWLEDGMENTS

The financial supports of Telethon-Italy, MURST (Rome) and the University of Pisa to the project is gratefully acknowledged. M.B. is recipient of a fellowship from Telethon-Italy.

REFERENCES

1. Andreassen, P. R., Lacroix, F. B., Villa-Moruzzi, E., and Margolis, R. L. (1998) *J. Cell Biol.* **141,** 1207–1215.
2. Cohen, P. (1989) *Annu. Rev. Biochem.* **58,** 453–508.
3. Hubbard, M., and Cohen, P. (1993) *Trend Biochem. Sci.* **18,** 172–177.
4. Sasaki, K., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T., and Nagao, M. (1990) *Jpn. J. Cancer Res.* **81,** 1272–1280.
5. Tognarini, M., and Villa-Moruzzi, E. Methods in Molecular Biology (Ludlow, J. W., Ed.), Vol. 93, pp. 169–183, Humana Press, Totowa, NJ.
6. Murata, K., Hirano, K., Villa-Moruzzi, E., Hartshorne, D. J., and Brautigan, D. L. (1997) *Mol. Biol. Cell* **8,** 663–673.
7. Villa-Moruzzi, E., Tognarini, M, Cecchini, G., and Marchisio, P. C. (1998) *Cell Adh. Commun.* **5,** 297–305.
8. Villa-Moruzzi, E. (1992) *FEBS Lett.* **304,** 211–215.
9. Dohadwala, M., Da Cruz e Silva, E. F., Hall, F. L., Williams, R. T., Carbonaro-Hall, D. A., Nairn, A. C., Greengard, P., and Berndt, N. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6408–6412.
10. Villa-Moruzzi, E., Dalla Zonca, P., and Crabb, J. W. (1991) *FEBS Lett.* **293,** 67–71.
11. Puntoni, F., and Villa-Moruzzi, E. (1997) *Arch. Biochem. Biophys.* **340,** 177–184.
12. Liu, C. W. Y., Wang, R.-H., Dohadwala, M., Schoenthal, A. H., Villa-Moruzzi, E., and Berndt, N. (1999) *J. Biol. Chem.* **274,** 29470–29475.
13. Wiman, K. G. (1993) *FASEB J.* **7,** 841–845.
14. Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H., and Elledge, S. J. (1993) *Genes Develop.* **7,** 555–569.
15. Ludlow, J. W., Glendening, C. L., Livingston, D. M., and DeCaprio, J. A. (1993) *Mol. Cell. Biol.* **13,** 357–372.
16. Nelson, D. A., Krucher, N. A., and Ludlow, J. W. (1997) *J. Biol. Chem.* **272,** 4528–4535.
17. Puntoni, F., and Villa-Moruzzi, E. (1997) *Biochem. Biophys. Res. Commun.* **235,** 704–708.
18. Goldberg, J., Huang, H, Kwon, Y., Greengard, P., Nairn, G., and Kuriyan, J. (1995) *Nature* **376,** 745–753.
19. Zhang, L., and Lee E. Y. C. (1997) *Biochemistry* **36,** 8209–8214.
20. Zhang, J., Zhang, Z., Brew, K., and Lee E. Y. C. (1996) *Biochemistry* **35,** 6276–6282.
21. Allen, P. B., Kwon, Y.-G., Nairn, A., and Greengard, P. (1998) *J. Biol. Chem.* **273,** 4089–4095.