

Identification of Alternative Splicing Variants of PPM1 Protein Phosphatase PPM1D

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Introduction

PPM1D, a member of the PPM1 type phosphatases which contains 605 amino acids, was originally identified as a p53-inducible protein phosphatase in response to DNA damage by ultraviolet (UV) and infrared (IR) stimulation. The phosphatase was described as a negative regulator of p53 pathway through its ability to attenuate p38 MAPK signaling [1, 2]. Recently, many other tumor suppressor proteins, such as Chk1, Chk2, ATM, and p53 itself have been identified as targets of PPM1D that are inactivated by the resulting dephosphorylation [3, 4]. The gene amplification of PPM1D has also been observed in several human cancers including breast cancer, neuroblastoma, medulloblastoma, ovarian clear cell adenocarcinoma, and pancreatic adenocarcinoma. PPM1D-deficient mice and cells derived from them show a tumor-resistant phenotype, further suggesting that PPM1D plays a role as an oncogenic protein. On the other hand, Fiscella and coworker reported that PPM1D overexpression leads to growth suppression in a human glioblastoma cell line, suggesting that PPM1D might contribute to growth inhibitory pathways [5]. The mechanism on the different effects by PPM1D overexpression has remained obscure. In this study, we identified an alternative splicing variant of PPM1D (PPM1Das) in addition to the originally reported PPM1D protein in breast cancer MCF7 cells and characterized these two enzymes at the mRNA and protein levels.

Results and Discussion

In order to analyze the possibility that the point mutation of PPM1D affects the cell cycle regulation, cDNA clones of PPM1D were isolated from breast cancer MCF7 cells. mRNA from the cells was used for cDNA synthesis reaction with oligo(dT)₁₂₋₁₈ primer and full-length cDNA clones of PPM1D were amplified with PPM1D-specific primers. Sequence analysis of PPM1D cDNA from MCF7 cells showed that there are no mutations in cDNA of PPM1D coding 605 amino acid residues, designated as PPM1Df. However, a slight longer cDNA was also isolated from MCF7 cells. Sequence analysis revealed that the cDNA encoded an alternative splicing variant of PPM1D, designated as PPM1Das. In the mRNA of the variant, a new exon is inserted between exon 5 and exon 6, resulting in coding 430 residues proteins constituted of 1-420 residues of PPM1Df and additional PPM1Das-specific 10 residues (Fig. 1). Generally many phosphatases contain a regulatory domain in the C-terminal region and control their phosphatase activities and protein-protein interactions. One of the PPM1 family proteins, PPM1B was also reported to generate alternative

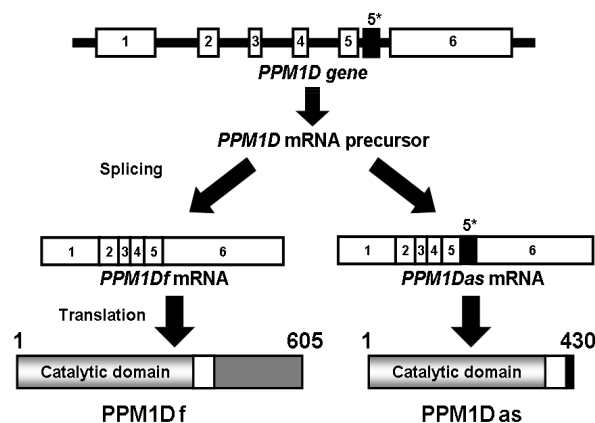


Fig. 1. A schematic representation of the PPM1D alternative splicing variants.

splicing variants at their C-terminal regions. Kusuda *et al.* reported that these PPM1B isoforms showed different affinity and activity against phosphorylated proteins [6]. These facts suggested that PPM1Das can possess distinct functions from PPM1Df through different regulations of localization, modification and stabilization.

RT-PCR analysis exhibited that the PPM1Das also existed in other cells, including T47D (breast cancer cells), MDB-MB-231 (breast cancer cells), and H1299 (lung cancer cells). The ratio of mRNA level of PPM1Df and PPM1Das were different among each cells, suggesting that the transcriptional regulations of these splicing variants are different in each cell (Fig. 2).

To evaluate whether PPM1Das retained phosphatase activity, we expressed a PPM1Das protein in *E. coli* and analyzed its phosphatase activity. The recombinant PPM1Das dephosphorylated the p53 phosphorylated peptide at position 15, which site was reported to be dephosphorylated by PPM1D *in vitro* and *in vivo* [3, 7]. Substrate recognition analysis using p53 phosphorylated peptide analogs revealed that the substrate preference of PPM1Das is almost the same with that of PPM1D(1-420), which property is known to be comparable to that of PPM1Df. Furthermore PPM1Df are known to interact with other proteins through the catalytic domain, and the phosphatase activity was essential to exert the oncogenic effect by PPM1D [3]. These suggested that PPM1Das might also function in cell cycle regulation.

In order to identify endogenous PPM1D proteins in cells, polyclonal antibodies specific for PPM1Das and PPM1Df were generated by immunization against rabbit

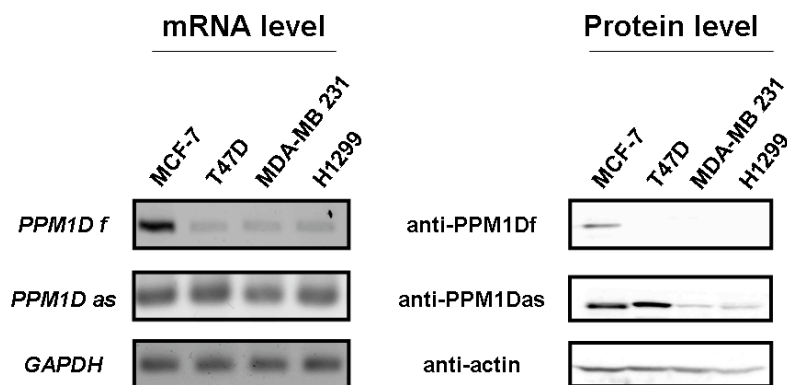


Fig. 2. Expression of PPM1D splicing variants both in mRNA and protein levels

Specific sequence in C-terminal domain of each protein was selected as antigen. These antigen peptides were synthesized by Fmoc chemistry. Western blotting analysis demonstrated that PPM1Df was strongly detected by the PPM1Df-specific antibody in MCF7 cells, however there were no or weak signals observed in other cells. In contrast, PPM1Das was expressed in all breast cancer cells tested, including MCF7, T47D, MDA-MB-231 cells and lung cancer H1299 cells (Fig. 2). Interestingly, the ratios of protein levels of PPM1Df and PPM1Das were also different among these cells. Expression level of PPM1Das protein in T47D cells was much higher than those of other cells, suggesting that PPM1Das may especially play more important roles in such cell.

In order to analyze the localization of exogenous PPM1Df and PPM1Das proteins, we performed the subcellular fractionation analysis using PPM1D variants specific antibodies. The result indicated that both PPM1Df and PPM1Das were localized in nucleus in MCF7 and T47D cells. We previously reported that the PPM1D catalytic domain contains characteristic basic region with a putative nuclear localization signal (NLS), KRPR sequence. To evaluate whether this sequence of PPM1D works as NLS, EGFP fusion proteins were expressed in lung cancer H1299 cells. As expected, not only EGFP-PPM1Das but also EGFP-PPM1D(1-378) was localized predominantly in the nucleolus. Substitution of the Asn-Gly-Ser sequence for the basic region induced distribution of the protein throughout the cells. These data suggested that the KRPR sequence in catalytic domain of PPM1Das can also function as NLS.

In summary, we identified the alternative splicing variant of PPM1D, PPM1Das, and detected the expression of the enzyme both at mRNA and protein levels. Both of PPM1Df and PPM1Das should play important roles in cell cycle regulation through dephosphorylation of tumor suppressor proteins, such as p53, Chk1, and p38. Ratios of expression level in these PPM1D variant proteins were different in various cells, suggesting that expression of PPM1D splicing variants may be controlled by cell-specific regulations. Previous PPM1D studies were performed without discrimination between PPM1Df and PPM1Das. Thus, our study using PPM1Das and PPM1Df specific antibody will help to understand the function of

PPM1D and further to clarify the cell cycle regulation by the PPM1D proteins.

References

1. Takekawa, M., Adachi, M., Nakahata, A., Nakayama, I., Itoh, F., Tsukuda, H., Taya, Y., and Imai, K. (2000) *EMBO J.* **19**, 6517-6526.
2. Bulavin, D.V., Phillips, C., Nannenga, B., Timofeev, O., Donehower, L.A., Anderson, C.W., Appella, E., and Fornace, A.J. Jr. (2004) *Nat. Genet.* **36**, 343-350.
3. Lu, X., Nannenga, B., and Donehower, L.A. (2005) *Genes Dev.* **19**, 1162-1174.
4. Oliva-Trastoy, M., Berthonaud, V., Chevalier, A., Ducrot, C., Marsolier-Kergoat, M.C., Mann, C., and Leteurtre, F. (2007) *Oncogene* **26**, 1449-1458.
5. Fiscella, M., Zhang, H., Fan, S., Sakaguchi, K., Shen, S., Mercer, W.E., Woude, G.V., O'Connor, P.M., Appella, E. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 6048-6053.
6. Kusuda, K., Kobayashi, T., Ikeda, S., Ohnishi, M., Chida, N., Yanagawa, Y., Shineha, R., Nishihira, T., Satomi, S., Hiraga, A., and Tamura, S. (1998) *Biochem. J.* **332**, 243-250.
7. Shreeram, S., Demidov, O.N., Hee, W.K., Yamaguchi, H., Onishi, N., Kek, C., Timofeev, O.N., Dudgeon, C., Fornace, A.J., Anderson, C.W., Minami, Y., Appella, E., and Bulavin, D.V. (2006) *Mol. Cell* **23**, 757-764.