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Phosphatase assay for multi-phosphorylated substrates using phosphatase specific-motif antibody

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Protein phosphorylation plays central roles in a wide variety of signal transduction pathways and most phosphorylated proteins contain multi-phosphorylated sites. PPM1 type Ser/Thr protein phosphatase family is known to show rigid substrate specificity unlike other Ser/Thr phosphatase PPP family including PP1, PP2A and PP2B. PPM1 type phosphatases are reported to play important roles in growth regulation and in cellular stress signalling. In this study, we developed a phosphatase assay of PPM1D using phosphatase motif-specific antibody. PPM1D is a member of PPM1 type Ser/Thr phosphatase and known to dephosphorylate Ser(P)-Gln sequence. The gene amplification and overexpression of PPM1D were reported in many human cancers. We generated the monoclonal antibody specific for the Ser(P)-Gln sequence, named 3G9-H11. The specificity of this method using ELISA enables the convenient measurement of the dephosphorylation level of only PPM1D target residues of substrate peptides with multiple phosphorylated sites in the presence of multiple phosphatases. In addition, the antibody was applicable to immunoblotting assay for PPM1D function analysis. These results suggested that this method should be very useful for the PPM1D phosphatase assay, including high-throughput analysis and screening of specific inhibitors as anti-cancer drugs. The method using phosphatase motif-specific antibody can be applied to other PPM1 phosphatase family.

Keywords: dephosphorylation motif/ELISA/PPM1D/p53/phosphatase assay/phosphatase-specific motif antibody.

Abbreviations: ATM, ataxia-telangiectasia mutated; DMEM, Dulbecco's minimum essential medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-Fluorenylmethoxycarbonyl; HAT medium, hypoxanthine/aminopterin/thymidine medium; HRP, horseradish peroxidase; IR, ionizing radiation; MDM2, murine double minute 2 protein; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween-20; PPM, Mg^{2+}- or Mn^{2+}-dependent protein phosphatase; PP2C, Protein Phosphatase type 2C; PPP, Phosphoprotein Phosphatase; PPM1D, Protein Phosphatase Magnesium-dependent 1; delta, S(P), phosphorylated Serine; T(P), phosphorylated Threonine; UNG2, uracil-DNA glycosylase 2; UV, ultraviolet; XPA, xeroderma pigmentosum group A; XPC, xeroderma pigmentosum group C; Y(P), phosphorylated Tyrosine.

Serine/threonine phosphatases (EC 3.1.3.16) are classified into two different families: the phosphoprotein phosphatases (PPP) and the phosphoprotein phosphatase M family (PPM) (*1*) The PPP family including PP1, PP2A and PP2B works as an oligomeric holoenzyme which is composed of a highly conserved catalytic subunit and one or two regulatory subunits. The regulatory subunits in PPP family attribute to diverse substrate specificity of each phosphatase. On the other hand, PPM type protein phosphatases, only contain a single subfamily PPM1 (formerly PP2C) work by themselves and determine substrates by recognizing primary sequences of dephosphorylation sites (*1*, *2*). The PPM1 phosphatases contain unique regions inside and/or outside of the catalytic domain and the regions seem to play important roles in the rigid substrate recognition (*1*, *2*). It is suggested that PPM1 phosphatases are broadly involved in regulating cell growth and cellular stress signalling. The functions and regulation mechanisms of most PPM1 phosphatases remain obscure. To further evaluate the phosphatase activity of PPM1 phosphatases, it is important to develop a sensitive detection method for this activity.

Malachite green is a cationic triphenylmethane dye that has been extensively used for assaying a number of phosphatases (*3*–*5*). However, its use requires large amounts of substrate since its sensitivity is limited. Also, it cannot be used to distinguish the activity of a particular phosphatase in the presence of multiple phosphatases as malachite green detects free orthophosphate released from phosphorylated substrate peptides. In addition, most substrate proteins are phosphorylated on multiple sites but malachite green methods will not identify the specific residues that are dephosphorylated by phosphatases (*6*–*8*). A method with an enhanced rapidity, sensitivity and selectivity

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for measuring PPM1 type phosphatase activity is therefore critical to perform functional assays.

One of the PPM1 the phosphatase, PPM1D (Wip1, PP2Cδ) was originally identified as a p53-inducible protein phosphatase that functions in the response to DNA damage by both UV and IR stimulation (*9*, *10*). The human *PPM1D* gene is located on chromosome 17q22/23 and its amplification and overexpression have been observed in several human cancers including breast cancer (*11–13*), ovarian clear cell adenocarcinomas (*14*), neuroblastomas (*15*) and pancreatic adenocarcinomas (*16*). It has also been reported that the phosphatase activity of PPM1D is essential for its oncogenic effects (*17*). This enzyme has thus received considerable attention as a potential anticancer drug target (*2*, *18*, *19*). It has been reported previously that PPM1D dephosphorylates p53, p38, ATM, Chk1, Chk2, H2AX, XPA, XPC and UNG2, all of which participate in cell cycle regulation and the repair of damaged DNA (*10*, *20–25*), and HDM2 and MDMX which have non-redundant roles in the regulation of p53 (*26*). Most of the sites targeted by PPM1D contain a 'Ser(P)-Gln' sequence [where Ser(P) represents a phosphorylated serine] and it was reported that other PPM1 type phosphatases, such as PPM1A can not dephosphorylate the 'Ser(P)-Gln' sequence (*27*). The exceptions are p38 and UNG2 which are dephosphorylated by PPM1D at 'Thr(P)-X-Tyr(P)' motifs (*20*, *25*). In addition to its oncogenic function, PPM1D-deficient mice have revealed that PPM1D also plays important roles in spermatogenesis, lymphoid cell function, longevity and cell cycle regulation (*28*). An alternative splicing variant, PPM1D430, has also been identified and differs from the common form of the protein (PPM1D605) in both its subcellular localization and tissue-distribution pattern (*3*). The catalytic domain is completely conserved in PPM1D splicing variants and it will be essential to clarify the differences in the targeted proteins and phosphatase activities between PPM1D605 and PPM1D430 to more fully understand the multi-biological functions of this phosphatase.

In this study, we put focus on oncogenic PPM1D as a PPM1 type protein phosphatase. Here, we report the development of an effective, sensitive and specific method for detecting PPM1D phosphatase activity using a PPM1D phosphatase-specific motif antibody, 3G9-H11. Analysis of PPM1D activity could be performed using this antibody that recognizes the Ser(P)-Gln motif with a high degree of sensitivity and selectivity, suggesting that it has great potential to properly clarify the biological functions of PPM1D.

Materials and Methods

Peptides

The antigenic peptide S(P)Q-Pep and other peptides were chemically synthesized via the Fmoc solid-phase method using the ABI433A peptide synthesizer (PE Applied Biosystems, Foster City, CA) (Table I). Peptides were deprotected at their side-chains and liberated from the resin using Reagent K [9 ml of trifluoroacetic acid (TFA), 0.5 ml of water, 0.5 ml of phenol, 0.5 ml of thioanisole, and 0.25 ml of ethanedithiol] for 3 h at room temperature. The crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a C8 column (VYDAC, Hsperia, CA, USA) and eluted with a linear gradient of 0.05% (v/v) TFA and acetonitrile. The identity of the products was confirmed by MALDI-TOF mass spectrometry using a Voyager DE-PRO system (PerSeptive Biosystems, Framingham, MA, USA). Peptide concentrations were measured by absorption at 280 nm ($\varepsilon = 1{,}280\ M^{-1}cm^{-1}$) in a denaturing solution containing 50 mM sodium phosphate (pH 6.5), 100 mM NaCl, and 6 M guanidium hydrochloride.

Preparation of monoclonal antibody

A monoclonal antibody against the PPM1D phosphatase-specific motif Ser(P)-Gln was produced using previously described methods (*29*). Briefly, the Ser(P)-Gln-containing peptide was first conjugated with keyhole limpet hemocyanin (KLH). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and then injected subcutaneously in the footpad of female BALB/c mice. After 9 days, the mice were sacrificed, and their inguinal lymph node cells were fused with P3x63Ag8U.1 myeloma cells using polyethylene glycol. Hybridomas, selected in HAT medium, were cultured in DMEM containing 10% fetal bovine serum. Culture supernatants were screened for the presence of antibodies that reacted with the peptide containing Ser(P)-Gln using an ELISA assay. Positive hybridomas were cloned via limiting dilution. Peptides used for screening and characterization of the motif-specific antibody were synthesized as described above. After cloning of the positive hybridomas, we selected a single clone as the Ser(P)-Gln specific monoclonal antibody producing clone (denoted 3G9-H11). To evaluate the subtype of this antibody, it was applied to an ELISA plate coated with goat IgGs specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA raised in goat and this was followed by addition of anti IgG-conjugated with HRP and substrate. The ELISA data indicated that the antibody was an IgM type (data not shown).

Table I. Synthetic phosphopeptides.

Name	Sequence[a]
S(P)Q-Pep	Ac-GGG**S(P)**QGGG**S(P)**QGGGC-$_{NH2}$
p53(1-39)	Bio-ε-MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQA
p53(1-39)-9 P	Bio-ε-MEEPQSDP**S(P)**VEPPLSQETFSDLWKLLPENNVLSPLPSQA
p53(1-39)-15 P	Bio-ε-MEEPQSDPSVEPPL**S(P)**QETFSDLWKLLPENNVLSPLPSQA
p53(1-39)-20 P	Bio-ε-MEEPQSDPSVEPPLSQETF**S(P)**DLWKLLPENNVLSPLPSQA
p53(1-39)-33 P	Bio-ε-MEEPQSDPSVEPPLSQETFSDLWKLLPENNVL**S(P)**PLPSQA
p53(15 P)	Bio-ε-VEPPL**S(P)**QETFSDLW-$_{NH2}$
H2AX(139 P)	Bio-ε-KKATQA**S(P)**QEY-$_{NH2}$
ATM(1981 P)	Bio-ε-LAFEEG**S(P)**QSTTISS-$_{NH2}$
BRCA1(1387 P)	Bio-ε-DSSGLS**S(P)**QSDILTT-$_{NH2}$
p53(15 P,20 P)	Bio-ε-VEPPL**S(P)**QETF**S(P)**DLW-$_{NH2}$

[a]Phosphorylated amino acids are indicated in bold type. The Ser(P)-Gln motif is underlined. Bio, biotin; ε, aminocaproic acid; Ac, acetylation, S(P), phosphoserine (bolded).

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ELISA analysis using PPM1D phosphatase specific-motif antibody

Ninety-six-well modified flat-bottomed ELISA plates were coated with 50 µl of 1 mg/ml avidin for 2 h at room temperature. After two washes with 200 µl of PBST [phosphate-buffered saline (PBS) with 0.05% (v/v) Tween 20], the plate was incubated with 200 µl of 4% (w/v) BSA/phosphate-buffered saline solution for 2 h at room temperature. After five washes with 200 µl of PBST, 50 µl of biotinylated peptides were applied to avidin-coated ELISA plates for 2 h at room temperature. After five washes with 200 µl of PBST, 50 µl of 3G9-H11 antibody-containing medium diluted 1 : 50 in PBST was added to the wells for 2 h at room temperature. Another five washes with 200 µl of PBST were followed by a 30 min exposure with 50 µl of a 1 : 2000 solution of HRP-conjugated goat anti-mouse IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). After a final five washes with 200 µl of PBST, the wells were developed using 50 µl of o-phenylenediamine (Sigma Chemical Co., St. Louis, MO, USA). The reaction was stopped by addition of 50 µl of 40% (v/v) H_2SO_4. The absorbance at 490 nm reflected the amount of the antibody bound to the peptide analogs. The concentration curves were fitted to the following sigmoidal equation (1) using KaleidaGraph 4.0 software (Hulinks Inc., Tokyo Japan).

$$Y = B_{\min} + B_{\max}/(1 + \exp(-(x - EX_{50})/C)) \quad (1)$$

Where x is the amounts of coated peptide on ELISA plate, y is the relative binding of 3G9-H11 antibody to each peptide, $B_{\min}$ is the asymptotic minimum (background signal), $B_{\max}$ is calculated by the difference between the asymptotic maximum and $B_{\min}$, EX_{50} is the x value at the inflexion point (corresponding to the concentration producing 50% of the maximal absorbance), and C is the slope of the curve. The data were presented as mean ±SD (standard deviation).

PPM1D phosphatase assay using the PPM1D phosphatase-specific motif antibody

Recombinant His-tagged PPM1D(1–420) was purified from bacterial lysate as described in ref. (*2*). Biotinylated phosphopeptide (250 pmol) was incubated with recombinant PPM1D (0.2 pmol) in 50 µl of phosphatase buffer [50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.02% (v/v) 2-mercaptoethanol, 30 mM $MgCl_2$] at 30°C for 50 min. The phosphatase reaction was stopped by the addition of 187.5 µl of Gly–HCl (pH 2.0) followed by neutralization with 12.5 µl of Tris–HCl (pH 8.0). A reaction mixture containing PPM1D-treated peptides was diluted to solution including 5 or 10 pmol peptide. The diluted reaction mixture was applied to an avidin-coated ELISA plate and the phosphorylation level was detected with 3G9-H11 antibody as described above. Remaining amount of Ser(P)-Gln residues after phosphatase treatment were calculated from OD_{490} values using a standard curve in each case. Orthophosphates released from Ser(P)-Gln motif were calculated by subtracting remaining amount of Ser(P)-Gln residues after phosphatase treatment from amount of substrate without phosphatase treatment. The data were presented as mean ± SD.

PPM1D phosphatase assay using malachite green

Biotinylated phosphopeptide was incubated with recombinant PPM1D (4 nM) under the same conditions used for the PPM1D phosphatase assay using 3G9-H11 antibody. The phosphatase reaction was stopped by addition of 100 µl of malachite green solution and orthophosphates released from phosphopeptides were detected at OD_{620} following the protocols provided by the manufacturer (Biomol Int., Philadelphia, PA, USA). The data were presented as mean ± SD.

PPM1D phosphatase assay in phosphatase mixture solution

Recombinant His-tagged PPM1A was purified from bacterial lysate. About 0.25 nmol of p53 (15 P,20 P) peptide was incubated with PPM1A (20 nM) and/or PPM1D (4 nM) for 10 min at 30°C in a 50 µl reaction mixture. Orthophosphate release from the peptides was calculated as described above in 'Materials and Methods' section. The data were presented as mean ± SD.

Western blotting and immunoprecipitation

MCF7 cells were seeded in plastic dishes 24 h prior to transfection with a siRNA specific for all PPM1D variants (5′-GAAGUGGACA AUCAGGGAAACUUUA-3′). Cells were transfected with siRNA duplexes (final 20 nM) using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 48 h the cells were harvested with ice-cold lysis buffer [50 mM Tris–HCl at pH 7.5, 5 mM, EDTA, 150 mM NaCl, 1% (v/v) Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium molybdate] containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). In adriamycin-treated cells the cells were treated with 345 nM adriamycin for 16 h incubation then harvested with ice-cold lysis buffer. Aliquots of cell lysates (20 µg protein) were resolved by SDS–PAGE and transferred onto polyvinylidene difloride membranes. After blocking with 4% (w/v) skimmed milk in PBS, the membranes were probed with appropriate antibodies and bands were visualized using enhanced chemiluminescence detection (Pierce, Rockford, IL). For immunoprecipitation of endogenous p53 from MCF7 cells, cell extracts (1.5 mg protein) were incubated with 3 µg of anti-p53 polyclonal antibody (FL393, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 4°C, mixed with 50 µl of ExactaCruz^{TM} B beads suspension (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated for an additional 1 h. Immunoprecipitates were washed five times with lysis buffer and subjected to SDS–PAGE. Immunoblotting analysis was then performed with indicated antibodies. The following antibodies were used: anti-actin (Ab-1) mouse mAb (JLA20) (Calbiochem, LaJolla, CA, USA); anti-p53 mAb DO-1 (Santa Cruz Biotechnology); anti-phospho-p53 (Ser15) (16G8) monoclonal antibody (Cell Signalling technology, Danvers, MA, USA); and an anti-PPM1D polyclonal antibody generated against KLH-CVKSLEEDPWPRVNSKDHI-$_{NH2}$ (*3*).

Results and Discussion

Characterization of a PPM1D phosphatase specific-motif monoclonal antibody

To produce PPM1D substrate motif-specific antibody, we designed and synthesized a phosphopeptide, termed S(P)Q-Pep, which contained two Ser(P)-Gln sequences (Table I). ELISA analysis revealed that the PPM1D phosphatase-specific motif antibody, 3G9-H11 showed high specificity also to the p53(1-39)-15 P product containing a Ser(P)-Gln motif but did not react with unphosphorylated peptide or other p53 phosphopeptides (Fig. 1A). ELISA analysis also demonstrated that the antibody clearly recognized only 5 pmol of p53(15 P), ATM and H2AX peptides, all of which correspond to PPM1D targeted substrates, although at different affinities (Fig. 1B). The values of a half maximal binding of the antibody for p53(15 P), ATM and H2AX peptides were 1.5, 7.9, 4.5 pmol/well, respectively. We further analysed whether this antibody would recognize the putative PPM1D-target BRCA1 and whether phosphorylated residues surrounding the Ser(P)-Gln motif would affect this binding using p53(15 P,20 P). The 3G9-H11 antibody recognized the BRCA1 peptide with a higher affinity than either ATM or H2AX and the value of half of maximal binding for BRCA1(1387 P) was 3.7 pmol/well (Fig. 1C). Furthermore the antibody recognized 0.2 pmol of p53(15 P,20 P) which was the highest affinity found among the peptides we tested. These results suggested that 3G9-H11 antibody is applicable to highly sensitive and selective methods for detecting PPM1D phosphatase activity.

The 3G9-H11 antibody was also found to recognize universal PPM1D target peptides containing

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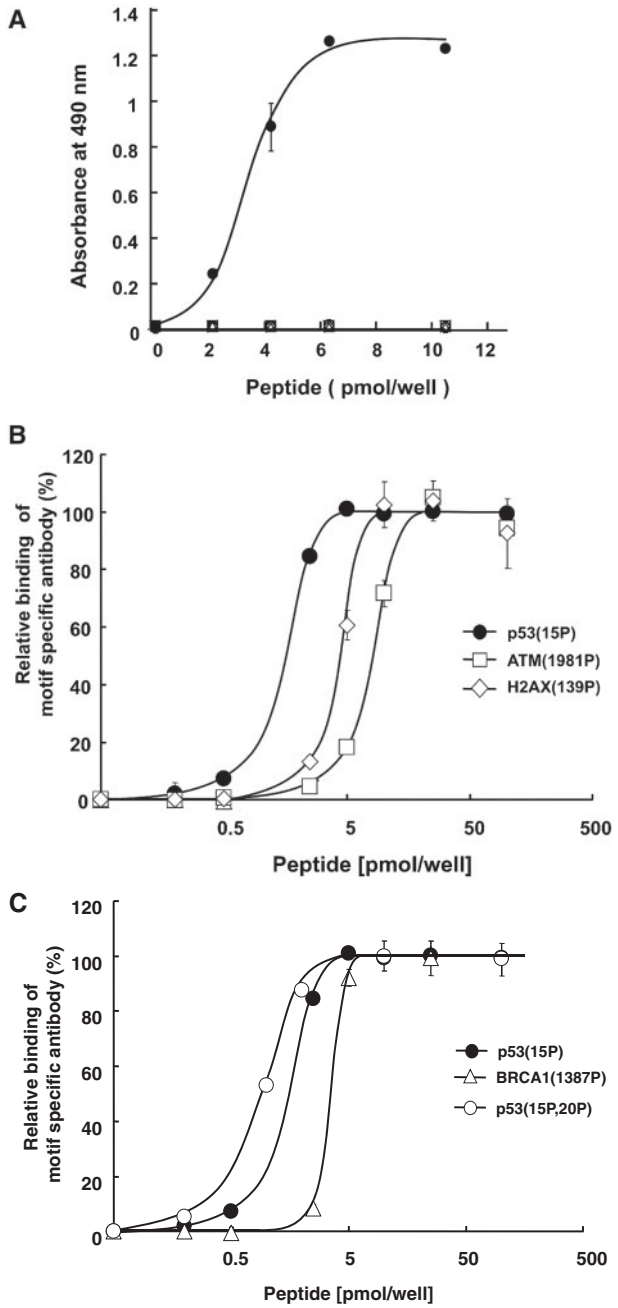

Fig. 1 Detection of p53 phospho-peptide analogs using the 3G9-H11 antibody. (A) Specificity of 3G9-H11 antibody for various phosphorylated peptides derived from p53. ELISA wells were coated with each peptide: p53(1–39) (open diamond), p53(1–39)-15 P (filled circle), p53(1–39)-18 P (inverted open triangle), p53(1–39)-20 P (open square), p53(1–39)-37 P (filled diamond), p53(303–325)-315 P (open circle) and p53(361–393)-376 P (open triangle). Binding of the antibody was measured using the absorbance at 490 nm. (B) The levels of the antibody binding to PPM1D substrate peptides containing Ser(P)-Gln sequence were plotted versus concentration of each peptide. Binding was measured by the absorbance at 490 nm and the maximum values were deemed to indicate 100% binding of the antibody. Curve fitting was carried out using KaleidaGraph 4.0 (Hulinks). (C) Plot showing the amount of antibody bound to the putative PPM1D substrate BRCA1(1387 P) and double-phosphorylated p53 peptide p53(15 P,20 P). The detection of antibody and the curve fitting was carried out as described in (B). The data were presented as mean ± SD ($n = 9$).

Ser(P)-Gln, however, the affinity depended on the residues flanking this motif. The high affinity peptides contained flanking acidic residues, including phosphorylated residues, in their C-termini, which are consistent with the substrate preferences shown by PPM1D. These data indicated that acidic residues surrounding the Ser(P)-Gln motif would increase the affinity of the antibody to PPM1D substrate peptides. Such affinity enhancement by acidic residues surrounding Ser(P)-Gln motif in peptide was seen in PPM1D substrate recognition, suggesting that the binding mode is similar between 3G9-H11 and PPM1D itself (*2*).

PPM1D phosphatase activity analysis method using the PPM1D phosphatase-specific motif antibody 3G9-H11

The 3G9-H11 antibody was able to recognize PPM1D substrate peptides including p53(15 P) at sub-pmol order in ELISA assay. We next used the antibody for PPM1D activity assay to develop a highly sensitive and selective method to detect PPM1D phosphatase activity. p53(15 P), H2AX(139 P) and ATM(1981 P) have been established previously as PPM1D targets *in vitro* and *in vivo*. These peptides were dephosphorylated by recombinant PPM1D and the reaction mixture was then directly applied to an avidin coated ELISA plate. This analysis exhibited that 4.83 ± 0.06 pmol phosphate was released from 5 pmol of p53(15 P) after PPM1D treatment (Table II). On the other hand, using the malachite green method we could not detect dephosphorylation of p53(15 P) peptide when used at 50 pmol (Table II). In addition, the noise versus signal ratio (S/N ratio) obtained from the method using 3G9-H11 antibody was 40.8 for 5 pmol of p53(15 P) whereas no clear difference was detected in the malachite green method at 50 pmol of p53(15 P), resulting in an S/N ratio of 1.0 at OD_{620}. These data suggested that the motif-specific antibody method could detect PPM1D activity with at least 10-fold higher sensitivity than malachite green method.

In terms of the substrate preference by PPM1D, we found by ELISA that only 40% of H2AX(139 P) was dephosphorylated by PPM1D (Table II), indicating that PPM1D dephosphorylation preference to p53(15 P) and ATM(1981 P) over H2AX(139 P). This is consistent with previously reported data showing that the affinity of PPM1D to the phosphorylated Ser139 of H2AX is less than to the phosphorylated p53 peptide at Ser15 and phosphorylated ATM peptide at Ser1981 (*21*, *27*). In addition, the PPM1D *k*cat/*K*m value for phosphorylated Ser139 in the H2AX peptide is lower than that of the phosphorylated p53 peptide at Ser15 and ATM at Ser1981 (*21*, *27*, *30*, *31*). These data suggest that the motif-specific antibody method can provide quantitative analysis and evaluate the substrate preferences of PPM1D.

We applied the motif-specific antibody method to the detection of phosphatase activity of PPM1D against putative PPM1D targets on BRCA1(1387 P) and p53 (15 P,20 P). The analyses showed that BRCA1(1387 P) and p53 (15 P,20 P) are good

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Table II. PPM1D activity analysis using the s phosphatase-specific motif antibody, 3G9-H11.

Method	Peptide	Substrate (pmol)	Released Pi (pmol)	S/N ratio	Dephosphorylated Peptide (%)
PPM1D substrate motif-specific antibody	p53(15 P)	5	4.83 ± 0.06	40.8	96.6
	ATM(1981 P)	10[a]	10.00 ± 0.25	46.3	100
	H2AX(139 P)	5	1.98 ± 0.91	2.8	39.6
	BRCA1(1387 P)	5	3.75 ± 0.40	74.6	75.0
	p53(15 P,20 P)	5	4.82 ± 0.03	12.4	96.4
Malachite Green	p53(15 P)	50	Not detectable	1.0	Not detectable
	p53(15 P)	100	90.1 ± 19.11	1.6	90.1
	p53(15 P)	200	168.5 ± 25.48	2.7	84.3
	p53(15 P)	1000	824.9 ± 0.031	12.7	82.4

[a]For the ATM(1981 P) peptide, 10 pmol of PPM1D-treated peptide was applied because the sensitivity of the 3G9-H11 antibody against the ATM peptide is weaker compared with other peptides (Fig. 1B). The data were presented as mean ± SD ($n = 6$).

substrates for PPM1D like p53(15 P) and ATM(1981 P) (Table II). The phosphorylation of Ser1387 in BRCA1 has been reported previously to be required for the S-phase checkpoint following exposure to ionizing irradiation (*32*). A reduction of PPM1D has also been correlated with a robust intra-S phase checkpoint following DNA damage (*10*). These findings suggest that the phosphorylated Ser1387 in BRCA1 is a likely target of PPM1D and that PPM1D may regulate the DNA damage response *via* BRCA1.

One of the biggest advantages in the PPM1D activity assay using the motif-specific antibody is that the method can detect the dephosphorylation level of only Ser(P)-Gln sites of substrate peptides with multiple phosphorylated sites in the presence of multiple phosphatases with different specificity, whereas malachite green method can not. The PPM1 type phosphatase family, including PPM1D, is known to display rigid substrate specificity, e.g. PPM1D dephosphorylates Ser15 but not Ser20 on p53 (*27*, *33*) and PPM1A dephosphorylates Ser20 but little Ser15 (Supplementary Fig. S1). To demonstrate the utility of our PPM1D phosphatase assay, the p53(15 P,20 P) peptide was treated with PPM1D and/or PPM1A and the reaction mixture was subjected to ELISA using the 3G9-H11 antibody and malachite green assays. First following dephosphorylation by PPM1D alone, the orthophosphate release from p53(15 P,20 P) was calculated to be 0.17 ± 0.01 nmol using the motif-specific antibody method and 0.16 ± 0.02 using malachite green, indicating that the 3G9-H11 antibody is useful for quantitatively analyse PPM1D (Fig. 2). PPM1A treatment showed that 0.06 ± 0.01 nmol of released orthophosphate was released from p53(15 P,20 P) peptide by malachite green method, whereas no signal was detected by 3G9-H11 methods. The result suggested that the detected orthophosphate using malachite green method was derived from phosphorylated Ser20. Next when

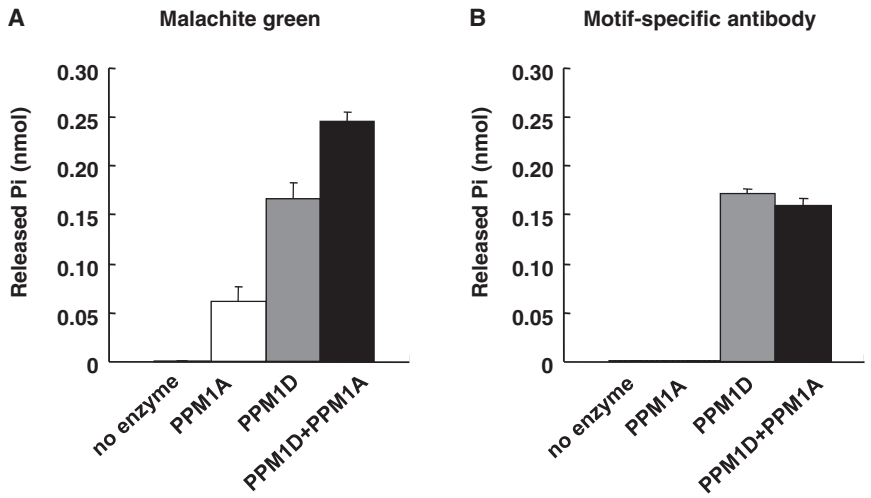

Fig. 2 Specificity of the 3G9-H11 antibody in reactions containing multi-phosphorylated peptides and a mixture of phosphatases. (A) Phosphatase activities of PPM1A and PPM1D towards the double-phosphorylated peptide p53(15 P,20 P) detected using malachite green. A 5 μM concentration of p53(15 P,20 P) peptide was incubated with PPM1D (4 nM) alone or with a PPM1A (20 nM)/PPM1D (4 nM) mixture for 10 min at 30°C in 50 μl reaction. The released phosphate was measured using a standard curve (phosphate versus OD_{620}).(B) Phosphatase activities of PPM1A and PPM1D towards double-phosphorylated peptide p53(15 P,20 P) detected by ELISA using the 3G9-H11 antibody. p53(15 P,20 P) peptide was incubated with PPM1A, PPM1D or mixture of PPM1A and PPM1D, respectively. In no enzyme, p53(15 P,20 P) peptide was treated by same operation without enzyme. Phosphatase reactions was carried out as in (A) and stopped by the addition of Gly–HCl (pH 2.0) following neutralization with Tris–HCl (pH 8.0). Reaction mixtures containing 5 pmol of p53(15 P,20 P) peptides treated with or without phosphatase were applied to avidin-coated ELISA plate and phosphorylation levels were detected with the antibody. Released phosphate were calculated from the OD_{490} for p53(15 P,20 P) peptide shown in Figure 1C. The data were presented as mean ± SD ($n = 6$).

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p53(15 P,20 P) was dephosphorylated using the PPM1A/PPM1D mixture, the level of released phosphate was calculated to be 0.24 ± 0.01 nmol using the malachite green method. The value of released Pi by PPM1A/PPM1D mixture was well consistent with that of calculated sum of PPM1A and PPM1D alone (Fig. 2A). On the other hands, the motif-specific antibody method successfully calculated the amount of released phosphates (0.16 ± 0.01 nmol) from Ser15 residues following PPM1D/PPM1A treatment, yielding the almost same value as that found following sole PPM1D treatment (Fig. 2B). These data suggested that motif-specific antibody method is taken full advantage to quantitate the phosphorylation level of PPM1D target residues of multi-phosphorylated peptides or proteins in the presence of phosphatase mixture solution from cell lysate.

Detection of PPM1D phosphatase activity by immunoblotting using 3G9-H11

The PPM1D gene maps to 17q23 and its amplification and overexpression have been observed in several human cancers including breast cancer (*11–13*). MCF7 cells are a breast cancer cell line with an amplified *PPM1D* gene (*11*). It has been reported that the accumulation of p53 protein level and phosphorylation level at Ser15 was seen in MCF7 cells after adriamycin stimulation and PPM1D knock down (*3*, *17*). In order to analyse whether the 3G9-H11 antibody can recognize phosphorylation level of endogenous p53 at Ser15 in the cell, the antibody was used for western blotting analysis. The enhancement of the 3G9-H11 antibody binding to p53 was detected after adriamycin stimulation, as reported (Fig. 3). The binding of the 3G9-H11 antibody in PPM1D siRNA treated cells was also enhanced compared to control cells and recognition mode of p53 by the 3G9-H11 antibody was almost parallel to commercially available anti-p53(15 P) antibody (Fig. 3). This indicates that the 3G9-H11 antibody can detect PPM1D-specific activity in cells. These data further suggested that the motif-specific antibody may detect PPM1D activity against not only p53 but also other PPM1D target proteins containing a Ser(P)-Gln motif such as ATM, H2AX and BRCA1.

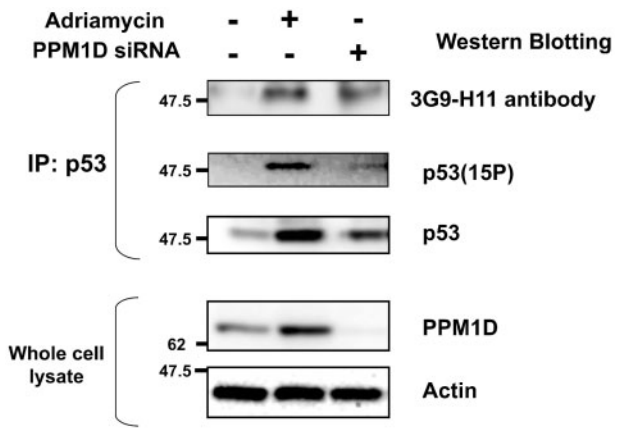

Fig. 3 Application of the 3G9-H11 antibody to the analysis of endogenous PPM1D activity in MCF7 cells.MCF7 cells were transfected with control siRNA or PPM1D specific siRNA and incubated for 2 days. Treated cells were stimulated with 345 nM ADR for 16 h before harvesting. Immunoprecipitations were performed with anti-p53 antibody (FL493) followed by western blotting with the indicated antibodies. Whole cell lysates were also probed with PPM1D and actin antibodies. The data were representative of two independent experiments.

In summary, we have developed the highly sensitive and selective method for detecting phosphatase activity using the phosphatase-specific motif antibody. This assay has distinct advantages over anti-phosphoserine or anti-phosphopeptide antibodies as it can distinguish between PPM1D targeting residues and can be used for multiple target proteins with high sensitivity and quantity. Hence, this phosphatase assay method should be useful for analyzing the biological functions and screening specific inhibitor of PPM1D. The method using phosphatase motif-specific antibody can be applied to other PPM1 phosphatase family.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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