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In Vivo and *in Vitro* Binding of Microcystin to Protein Phosphatases 1 and 2A

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The hepatotoxic microcystins (Mcyst) are potent inhibitors of the ser/thr protein phosphatases (PP1 and PP2A) with IC_{50}'s of 0.1-1.0 nM. Mcyst and other PP inhibitors like okadaic acid or calyculin A interact with the C-terminal region of PP1 and PP2A. Using [^{125}I]-Mcyst and antibodies specific for PP1 and PP2A, we show by immunoprecipitation and autoradiography, that in hepatocytes Mcyst forms secondary covalent bonds with both PP1 and PP2A catalytic subunits. We demonstrate that the bond resulted from the reaction between the electrophilic α,β unsaturated carbonyl of the methyldehydroalanine residue of Mcyst and the thiol of Cys 273 located in the C-terminal of PP1 (Cys 266 in PP2A), since site-directed mutagenesis of Cys 273 to Ala in PP1α led to complete loss of ability for the formation of a covalent Mcyst-PP1 adduct.

The microcystins (Mcyst) are cyclic heptapeptide hepatotoxins produced by cyanobacteria that are potent and specific inhibitors of the the ser/thr protein phosphatases (PP) 1 and 2A (1,2). Mcyst are normally cell impermeant, but they accumulate in the liver by specific carrier-mediated transport in hepatocytes which results in PP inhibition and toxicity (3-6).

A number of other toxins, chemically unrelated to Mcyst, are potent inhibitors of PP activity. These include okadaic acid (OKA, a C_{38} fatty acid polyether) and calyculin A (an octamethyl polyhydroxylated C_{28} fatty acid). Binding of these inhibitors to PP1 and 2A catalytic subunits is mutually exclusive, indicating that these diverse toxins

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bind at a common site on PP1 and PP2A. Nevertheless, the affinity of these toxins for PP1 and 2A differ: Mcyst and calyculin A have IC_{50}'s in the range of 0.1-1 nM for both enzymes while OKA has greater affinity for PP2A than for PP1 (IC_{50} of 1 and 120 nM respectively) (7). Relative resistance to OKA in CHO cells is associated with a point mutation in PP2A (8). In these cells there is a Cys to Gly mutation at residue 269 in the C-terminal region of the protein. The possibility that this region of PP is important in determining affinity of the interaction of these inhibitors with PP was established by making a chimeric mutant in which residues 274-277 (GEFD) of PP1 were replaced by the corresponding PP2A residues (YRCG) 267-270. This chimeric mutant showed a ten-fold increase in sensitivity towards OKA (becoming more "PP2A-like"), indicating that this region of PP determines the specificity of protein-toxin interaction (7).

Following dosing of rats or mice with radiolabeled Mcyst, the toxin binds to hepatic protein (9-11). Several cycles of heat denaturation and pronase digestion were necessary for its release, suggesting covalent binding. SDS electrophoresis of liver cytosolic extracts from treated rats showed that [^{3}H]-Mcyst was exclusively associated with (a) protein(s) of molecular weight of 40 ± 1 kDa consistent in size with the catalytic subunits of PP (37 and 36 kDa for PP1 and PP2A, respectively) (10).

Toivola et al. in 1994 (12) showed that, although Mcyst is an equipotent inhibitor of PP1 and PP2A (1,2), *in vitro* incubation of Mcyst with hepatic cytosol resulted in secondary covalent binding of [^{3}H]-Mcyst to the PP2A but not PP1 catalytic subunit. The nature of the secondary covalent bond(s) was not established.

Mcyst, cyclo(D-Ala-L-X-erythro-β-methyl-Asp-L-Y-ADDA-D-Glu-*N*-methyldehydro-Ala) where X and Y denote variable L-amino acid residues and ADDA is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is chemically relatively stable. Mcyst forms adducts with GSH and Cys through nucleophilic reaction of the α,β-unsaturated carbonyl of *N*-methyldehydroalanine (Mdha) of Mcyst with the thiol group. These adducts were shown to retain full *in vivo* toxicity and inhibit PP activity with an affinity similar to that of the parent Mcyst (13). In addition, an aminoethanethiol adduct of Mcyst bound to Sepharose retained the capacity to bind and inhibit PP1 and PP2A (14). These findings indicate that the Mdha in Mcyst is not necessary for inhibition of PP activity and is not directly involved in the recognition and initial interaction of the toxin with the protein. It is therefore likely that, following the initial inhibitory interaction of Mcyst with PP, the Mdha group is free to bind covalently to suitably positioned thiols in the PP1 and PP2A catalytic subunits. By site-directed mutagenesis it has been previously shown that none of the six Cys residues common to PP1 and PP2A are essential for PP activity (15), so theoretically any of the six would be available for secondary covalent binding, if binding occurs with both PP1 and PP2A. If, as reported by Toivola et al (12) in *in vitro* incubations, binding is limited to PP2A and not PP1 then the Cys residue could be one only present in PP2A. As discussed above, it has already been shown by mutation that the binding region for the PP inhibitors OKA, calyculin A and Mcyst

is in the C-terminal region of PP. These observations indicate that the most likely thiol residue that binds covalently to Mcyst must therefore be a Cys residue that is located in the C-terminal region.

The aims of this study were (1) to identify conclusively the proteins to which Mcyst binds covalently following dosing in hepatocytes, and (2) to characterize the interaction of Mcyst and its target proteins. By using antibodies capable of distinguishing PP1 from PP2A, we show in this report that, in hepatocytes treated with Mcyst, the toxin is covalently bound to both PP1 and PP2A. Further, by mutation we establish that the Cys residue involved in the covalent binding is a C-terminal Cys residue common to both PP1 and PP2A, Cys 273 for PP1 (Cys 266 in PP2A).

MATERIALS AND METHODS

Microcystin (Mcyst) and [^{125}I]-Mcyst. Mcyst-YM was isolated and purified from a bloom of the cyanobacterium *Microcystis aeruginosa* as described previously (16,17). Iodination of Mcyst-YM was by the lactoperoxidase (Sigma, St Louis, MO)/H2O2 method (17) using Na[^{125}I] (NEN-Dupont, Boston, MA).

PP1 and PP2A. PP1 and PP2A catalytic subunits were purified from rabbit skeletal muscle by standard procedures (18). In addition, recombinant PP1α was expressed in and purified from*E. coli* using either the pTTZ or pDR540 vector (19, 20). The PP1αC273A mutant of this second isolate was generated essentially as described by Zhang et al. (7) using a first step PCR amplification of the PP1α cDNA to generate a 3' fragment containing the mutated sequence, followed by a second PCR step in which this fragment was used as a primer to generate the full length coding sequence.

To confirm *in vitro* binding of Mcyst to PP2A, partially purified PP2A from human red cells (10 units of activity) was obtained from Upstate Biotechnology (Lake Placid, NY). PP1 and PP2A samples were incubated *in vitro* with [^{125}I]-Mcyst for 60-90 min at 37°C, and stored at -80°C for subsequent SDS-PAGE and Western blotting. The experimental details for the individual incubations are shown in the respective figure legends.

All other reagents were of analytical grade and were obtained from commercial sources.

Generation of Monospecific Antibodies to the Catalytic Subunits of PP1 and PP2A. Based on the known sequences of the PP1 and PP2A catalytic subunits (21-23) we selected the C-terminal peptides GRPITPPRNSAKAKK (for PP1) and GEPHVTRRTPDYFL (for PP2A) as immunogens. The peptides were synthesized on a 431A peptide synthesizer (Applied Biosystems). An additional cysteine was included at the N-terminus in both cases to ensure one-site coupling to the carrier protein. The peptides were coupled to keyhole limpet hemocyanin with m-maleimidobenzoyl-N-hydroxysuccinimide ester (24). Immunizations and bleeds were performed at Cocalico Biologicals, Reamstown, PA, according to the following protocol: Approximately 0.2 mg of peptide/carrier conjugate in Freund's complete adjuvant was used to immunize New Zealand white rabbits subcutaneously. The animals received up to three booster injections of 0.1 mg peptide/carrier conjugate in Freund's incomplete adjuvant at 4-week intervals. Test bleeds were obtained 1 week after the injections starting with the first boost. Antisera were analyzed by

ELISA (24). The IgG fraction was purified from each antiserum using protein A immobilized on agarose.

Animals and Cells. Male Swiss Webster ND4 mice (Harlan Sprague Dawley Inc., Indianapolis) 20-30 g in weight were injected intraperitoneally with a solution of [^{125}I]-Mcyst in water with or without unlabeled Mcyst. Mice were killed 60 min after injection, the liver removed for the preparation of extracts. Male C3H/HE mice (8 week old) were used for the preparation of hepatocytes by collagenase perfusion (25). Incubations (3-8 x 10^6 cells/ml) were carried out in Krebs-Henseleit buffer containing 12.5 mM Hepes, pH 7.4 at 37°C. 30-60 min min after addition of [^{125}I]-Mcyst cells were washed in phosphate buffered saline and lysed.

Tissue/Cell Extract Preparation for SDS-PAGE or Immunoprecipitation (IP). Weighed portions of the liver were homogenized with 5-10 volumes of lysis buffer, hepatocytes (3-8x10^6) were lysed in 0.3-0.6 ml of the same buffer (50 mM Tris/HCl pH 7.5 containing 100 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 2% Nonidet-P40, 0.2 % sodium dodecyl sulfate (SDS), 0.5% deoxycholate and the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), benzamidine (1 mM), pepstatin (1 µg/ml) and leupeptin (0.5 µg/ml)) at 4°C, and then centrifuged at 10,000 x g for 10 min. The supernatants were used for SDS-PAGE, Western Blotting and IP.

Gel Electrophoresis and Western Blotting. Aliquots of the lysates were taken for protein assay. 20-40 µl of samples (40-100 µg protein) were diluted in one volume sample buffer (2x, 5% v/v mercaptoethanol) and boiled for 5 min. These were loaded onto 12.5% SDS-PAGE gels, electrophoresed (Bio-Rad Mini-gel apparatus, Hercules, CA), transferred to PVDF membranes (Immobilon membranes, Millipore, Bedford, MA) and immunoprobed with antibody for PP1 or PP2A (24). Bound primary antibodies were detected using biotinylated secondary antibody (anti-rabbit IgG, Vector Laboratories, Burlingame, CA) followed by addition of avidin and biotinylated alkaline phosphatase (VECTASTAIN ABC-AP kit, Vector Laboratories) and development of color with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad). The migration of the molecular weight standard proteins carbonic anhydrase (32.5 kDa) and of ovalbumin (49.5 kDa) are shown in the figures. Protein bound [^{125}I]-Mcyst was localized by autoradiography of the membranes.

Immunoprecipitation. Extracts from lysed hepatocytes were cleared by centrifugation. Supernatants containing 400-800 µg protein in 200 µl of lysis buffer (see above) were precleared by incubation with 4 µl of preimmune rabbit serum for 60 min at 4°C and added to 100 µl of protein A agarose beads (Immunopure Immobilized Protein A (Pierce, Rockford IL) prewashed with lysis buffer) and incubated for a further 30 min at 4°C. The supernatant fraction was then incubated with anti-PP1 or anti-PP2A antibody for 2-4 hr, then 100 µl of Protein A Sepharose beads were added to the extracts and incubated overnight with gentle rotation at 4°C. The immunoprecipitates were washed four times with wash buffer (lysis buffer containing no SDS and 0.2% Nonidet-P40). Finally SDS sample buffer (containing 10 mM N-ethylmaleimide and no mercaptoethanol) was added to the beads. After 5 min boiling, the proteins were separated by SDS-PAGE followed by Western blotting and autoradiography.

RESULTS

Characterization of Antibodies against PP Catalytic Subunits. Antibodies to the C-terminus of PP1 reacted with purified PP1 (Fig. 1C, lane 1) and also with a protein of the same apparent molecular mass (37 kDa) in cell lysates (Fig. 1C, lane 3), whereas antibodies to the C-terminus of PP2A reacted with purified PP2A (Fig. 1D, lane 2)

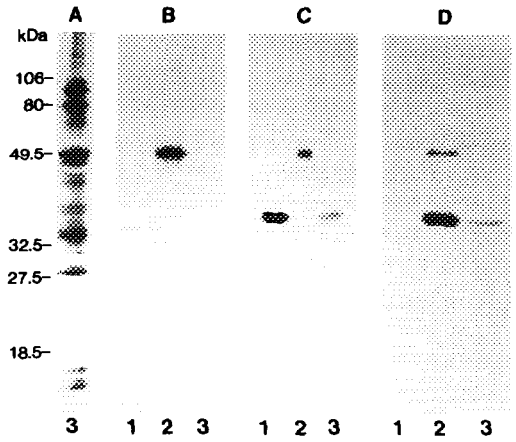

Figure 1. Characterization of antibodies against the catalytic subunits of protein phosphatases 1 and 2A. Approximately 50 ng of purified PP1 (lane 1), 50 ng of purified PP2A (lane 2) or 30 µg of cellular protein (lane 3) were separated by 12.5 % SDS-PAGE and then transferred to Immobilon-P membrane as described under METHODS. The membrane was then cut and stained or immunostained with the following reagents: **(A)** 0.1 % (v/v) India ink in tris-buffered saline (25 mM tris/Cl pH 8.0, 2.7 mM KCl, 137 mM NaCl) containing 0.2 % Tween-20, **(B)** anti-rabbit IgG (1:200 dilution), **(C)** purified anti-PP1 antibodies, **(D)** purified anti-PP2A antibodies.

and a protein of corresponding molecular mass (36 kDa) in cell lysates (Fig. 1D, lane 3). Neither antibody cross-reacted with the other respective phosphatase (Fig. 1C, lane 2 and Fig, 1D, lane 1). A ~50 kDa protein present in the PP2A preparation which apparently reacted with both antibodies (Figs. 1C and 1D, lane 2) was determined to be recognized by secondary antibody only (Fig. 1B). The PP1 antibody was specific for the α-isoform and did not cross-react with any of the other known recombinant isoforms (not shown). Both antibodies reacted with the respective native protein as well, as they were found to be capable of immunoprecipitation.

Mcyst Binds Both PP1 and PP2A in Murine Hepatocytes. The identity of the protein(s) bound to Mcyst was established by demonstrating that for extracts of treated mouse liver and hepatocytes, after SDS-PAGE and Western blotting, [^{125}I[-Mcyst radioactivity was bound to the catalytic subunits of PP (Fig. 2A, lanes 2,3 & 5,6). *In vitro* preincubation of the purified catalytic subunit of PP1 or of partially purified PP2A (included as positive controls) with [^{125}I]-Mcyst resulted in the same specific association of Mcyst radioactivity with the proteins that resisted dissociation by boiling in SDS under reducing conditions (Fig. 2A, lanes 1 & 4 and 2B, lanes 3 & 6). Because of the similar MWs of PP1 and PP2A catalytic subunits (37 and 36 kDa, respectively), it was not possible from Western blots and autoradiograms of hepatocyte extracts to differentiate between binding to PP1, or PP2A, or both. IP of mouse hepatocyte extracts showed that [^{125}I]-Mcyst binding occurred with both PP1 and PP2A (Fig. 2B, lanes 1,2 and 4,5).

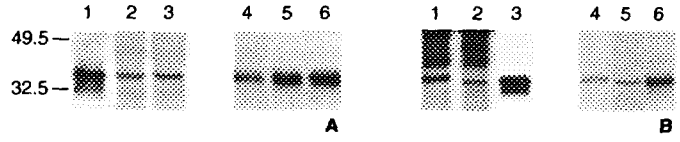

Figure 2. Protein binding of [^{125}I]-Mcyst in murine hepatocytes and liver by Western blot and autoradiography. (A) SDS-PAGE electrophoresis followed by Western blotting with anti-PP1 (lanes 1-3) and autoradiography (lanes 4-6) of lysed murine hepatocytes after incubation for 60 min, 37°C of 5 x 10^6 cell/ml with 0.05 µg of Mcyst containing 0.4 µCi of [^{125}I]-Mcyst (lanes 2 and 5) and of lysed extract from murine liver prepared 60 min after injection with 0.3 µg of Mcyst containing 0.5-1 µCi of [^{125}I]-Mcyst (lanes 3 and 6). Lanes 1 and 4 are the Western blot and autoradiogram showing binding of ^{125}I-Mcyst to purified rPP1α after *in vitro* incubation (60 min at 37°C). **(B)** SDS-PAGE electrophoresis of PP1 and PP2A immunoprecipitates (IP) of murine hepatocytes preincubated with [^{125}I]-Mcyst: lane 1 IP with anti-PP1, lane 2 IP with anti-PP2A, lanes 4 and 5 corresponding autoradiograms. The higher bands for the IP samples represent unspecific Igs recognized by the secondary antibody. Lanes 3 and 6 are the Western blot and autoradiogram showing binding of [^{125}I]-Mcyst to PP2A after *in vitro* incubation (60 min at 37°C).

Determination of Cys Residue in PP That Binds Mcyst. The identity of the residue in PP1 and PP2A that is involved in the secondary covalent binding of Mcyst with PP was determined by comparing the interaction of Mcyst with rPP1 and a PP1 mutant (C273A) in which the Cys 273 residue in the carboxy terminal has been changed to Ala (Figure 3). SDS-PAGE followed by autoradiography of rPP1 showed that there is binding between Mcyst and rPP1 (Figure 3B, lanes 1-3). This is completely abolished in the C273A mutant (Figure 3B, lanes 4-6). Quantitative scanning of the autoradiographs showed that radioactivity associated with the PP1 bands of the C273A mutant was ≤ 5% of that of the wild type PP1 for equivalent loading.

DISCUSSION

We have shown here that Mcyst, a potent and specific inhibitor of PP1 and PP2A activity, is covalently bound to both PP1 and PP2A catalytic subunits in hepatocytes

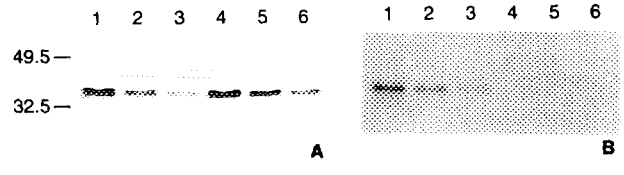

Figure 3. Effect of replacement of Cys273 on PP1 binding to [^{125}I]-Mcyst. Recombinant wild-type PP1α or single-point mutant PP1αC273A (constructed, expressed and purified to homogeneity) (40 pmol) were incubated with [^{125}I]-Mcyst (50 pmol) for 90 min at 37°C. Dilutions of the samples were subjected to SDS-PAGE. Load per well: lanes 1 and 4: 25 ng; lanes 2 and 5: 12.5 ng; lanes 3 and 6: 6.25 ng of PP1 (lanes 1-3) or PP1αC273A (lanes 4-6). **(A)** Western blots against PP1 (see Methods). **(B)** Corresponding autoradiograms.

incubated with the toxin. A previous report indicated a differential binding of Mcyst to PP2A only and not with PP1 (12) following *in vitro* incubation of liver cytosol with Mcyst. We cannot fully explain the discrepancy between these observations and our results. It is possible that it arose from the different incubation protocols: dosing of mice or incubation of intact hepatocytes with Mcyst in our experiments, *vs* addition of the toxin to liver homogenate by Toivola et al. (12).

The nature of the groups in Mcyst and PP involved in the binding was not previously known. It was speculated that an ester or an isopeptide bond between the free carboxyls of Mcyst and PP could be the cross link (12). While the free carboxyl groups are necessary for PP inhibition by Mcyst (26), there is no evidence of intrinsic chemical reactivity of these groups, while the MdhA residue, although not required for PP inhibition, is by itself more reactive, readily forming adducts with thiols *in vitro* (13), making it a a more likely candidate for bond formation with PP. Since we found that Mcyst forms covalent bonds with both PP1 and PP2A, the thiol group needed to be present in equivalent positions in both enzymes. Six Cys residues are shared by PP1 and PP2A, only one located in the carboxy terminal (C273 for PP1 and C266 for PP2A), the binding region for PP inhibitors (7). We found that the bond between rPP1 and Mcyst could not form when Cys 273 was mutated to Ala (Figure 3). The possibility that this loss of binding was simply a reflection of conformational changes in the mutant is unlikely since PP1αC273A has a similar specific activity towards phosphorylase *a* as the wild-type PP1. There are also no significant differences between the wild-type and C273A mutant in IC_{50} values for the inhibitors Mcyst, OKA, nodularin, tautomycin and inhibitor-2 (L. Zhang et al., manuscript submitted). These observations clearly show that the formation of Mcyst PP adducts is an event separate from the inhibition by this toxin of PP activity. Furthermore, the identification of C273 as being the residue involved in covalent adduct formation now places the identification of the binding of Mcyst to this region of the C-terminus on a firm basis. This binding explains the persistence of Mcyst in liver of mice dosed with Mcyst, a significant portion of the dose remaining even after six days (10). It also explains the potency of Mcyst as tumor promoter (27) since it would be expected that even small increases in the level of protein phosphorylation in cells resulting from partial PP inhibition with concomitant increases in kinase activity, will lead, over time, to profound changes in the control of cell growth and differentiation. Both PP1 and PP2A have been shown to to be key components of cell cycle control (28,29).

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