

Identification of protein phosphatase 2A as the primary target for microcystin-LR in rat liver homogenates

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Abstract

The liver-specific toxin microcystin-LR (MC-LR) is a potent inhibitor of type 1 (PP1) and type 2A (PP2A) protein phosphatases. A tritiated form of the toxin, [³H]dihydromicrocystin-LR ([³H]DMC-LR), was used to identify target proteins in cellular fractions prepared from rat liver homogenates. About 80% of the [³H]DMC-LR bound to proteins was in the cytosolic fraction, which contained essentially all of the PP2A. In contrast, much of the PP1 was found in particulate fractions, each with only a few percent of the total protein-bound [³H]DMC-LR. Protein-bound [³H]DMC-LR in the cytosol co-eluted with PP2A, but not with PP-1 from a DEAE-Sephacel column. Native forms of liver cytoplasmic PP2A and PP1 separated by aminohexyl-Sepharose adsorption showed similar sensitivity to inhibition by MC-LR, and bound [³H]DMC-LR proportional to the amount of phosphatase activity. The results indicate that [³H]DMC-LR can bind both PP2A and PP1 in the liver which must be important for microcystin-induced toxicity, but is recovered mainly bound to PP2A in the cytosol.

Key words: Protein phosphatase 1; Protein phosphatase 2A; Microcystin-LR; Protein phosphorylation; Hepatotoxin; Rat liver homogenate

1. Introduction

Microcystins are potent liver toxins produced by different species of cyanobacteria (blue-green algae), which can form blooms in lakes and water reservoirs [1]. These toxins are cyclic heptapeptides and microcystin-LR (MC-LR), which is one of the more frequently studied forms of these peptides, has the basic structure cyclo(-D-Ala-L-X-erythro- α -methyl-Asp-L-Y-ADDA-D-Glu-N-methyldehydro-Ala) where X and Y denote variable L-amino acid residues [2–4] and ADDA is an abbreviation for the α -amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-phenyldeca-4,6-dienoic acid [2].

The primary target organ for microcystins is the liver [5,6], where the toxins cause complete disruption of the liver architecture, leading to rapid death of the animal

(the LD₅₀ in mouse (i.p.) is 50 μ g/kg) [7,8]. At the cellular level microcystins induce severe morphological changes and a total reorganization of the microfilaments [9]. The cellular specificity and organotropism of microcystins [5,10] is due to a selective transport system present only in hepatocytes, namely the multi-specific bile acid transport system [11].

In isolated hepatocytes microcystins induce an overall increase in phosphorylation of cytosolic and cytoskeletal proteins [12] and it has been shown in vitro that microcystins are potent inhibitors of the protein phosphatases 1 and 2A (PP1 and PP2A) [12–16]. PP1 and PP2A are the most abundant phosphatases in mammalian tissues with catalytic subunits of 38 and 36 kDa, respectively [17]. These phosphatases dephosphorylate serine/threonine residues on a great variety of regulatory and structural proteins [18]. MC-LR inhibits the purified catalytic subunits of PP1 and PP2A with approximately equal potency (range of reported IC₅₀ values: PP1 0.06–6 nM; PP2A 0.01–2 nM, [12–16], which gives this compound inhibitory properties similar to those of calyculin A [19]. Whereas MC-LR and calyculin A inhibit PP2A with similar potency as okadaic acid, they are 50- to 100-fold more effective than okadaic acid as PP1 inhibitors [19,20]. Immobilized MC-LR has been used to affinity purify PP2A [21]. However, the binding capacity (3 μ g PP2A/1 mg MC-LR) was only 0.03% of that ex-

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(α -amino-ethyl ether) *N,N,N',N'*-tetraacetic acid; [³H]DMC-LR, [³H]dihydromicrocystin-LR; kDa, kilodalton; LD₅₀, lethal dose, the concentration of chemical where 50% of test animals are killed; MC-LR, microcystin-LR; PPase, protein phosphatases; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; TCA, trichloroacetic acid.

pected, probably because coupling to the matrix involved one of the MC-LR carboxyl groups that is important for binding to PP2A [22].

Some attempts have been made to identify the subcellular binding target for MC-LR. These studies have revealed that when whole liver is perfused, or hepatocytes in suspension are incubated, with [3 H]DMC-LR, the radioactivity is mainly found in the cytosolic fractions [23]. It has been shown that radiolabelled MC-LR in liver cytosol binds to a monomeric protein with a molecular weight of 40 kDa [24]. Whereas PP2A is known to be predominantly cytosolic, much of PP1 is located in particulate fractions. The present study was performed in order to determine whether PP1 and/or PP2A are the proteins that bind [3 H]DMC-LR in liver homogenates.

2. Materials and methods

2.1. Toxin and chemicals

MC-LR was isolated and purified from toxic blooms of the cyanobacterium *Microcystis aeruginosa*, and the pure toxin was tritiated to yield 3 H-labelled dihydromicrocystin-LR ([3 H]DMC-LR; specific activity 170 mCi/mmol; 142 mCi/mmol by gel filtration binding assay) as described elsewhere [5]. The identity of the labelled compound was confirmed by fast atomic bombardment mass spectrometry (Toivola, D., Poon, G. and Eriksson, J., unpublished results). All salts and organic compounds were of reagent grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Liver homogenization, [3 H]DMC-LR treatment and subcellular fractionation of homogenates

Sprague-Dawley rats were anaesthetized (Nembutal, i.p.), the liver was rapidly removed, rinsed in wash buffer (50 mM imidazole, pH 7.4, 150 mM NaCl) and minced in homogenization buffer (50 mM imidazole, pH 7.4, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose, 50 mM 2-mercaptoethanol), supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (5 mM) and benzamide (5 mM). The liver was homogenized and 5 μ l of [3 H]DMC-LR in methanol was added to the crude homogenate (5 μ g/30 ml homogenate equivalent to 0.17 μ M, 100 times the IC_{50} for PP1 and PP2A). The homogenate was centrifuged to pellet the nuclear and cytoskeletal fraction (1,000 \times g, 10 min), the mitochondria, lysosomes and peroxisomes (10,000 \times g, 30 min) and the microsomal and plasma membrane fraction (100,000 \times g, 60 min). The final supernatant (100,000 \times g) contains the cytosol. Pellets were dissolved in homogenization buffer and aliquots of every fraction were then diluted in 2 \times sample buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS, 20% glycine, 0.01% Bromophenol blue, 0.3% 2-mercaptoethanol and 6 M urea). Samples were separated by polyacrylamide gel electrophoresis (PAGE) and analyzed for PP2A and PP1 by Western blotting as described below. The experiments were repeated with livers from 3 different animals with similar results.

2.3. Determination of bound [3 H]DMC-LR in subcellular fractions

Samples of each subcellular fraction were treated with 1% Triton X-100 (to solubilize membranes) and the proteins were then precipitated by addition of TCA to a final concentration of 10%. This procedure separated protein-bound [3 H]DMC-LR from excess unbound [3 H]DMC-LR in each subcellular fraction. The radioactivity in the protein pellet after centrifugation was regarded as [3 H]DMC-LR bound to proteins, whereas the radioactivity in the TCA supernatant was considered to be unbound [3 H]DMC-LR, which does not precipitate in 10% TCA.

2.4. Identification of the [3 H]DMC-LR binding proteins in liver cytosol

Because approximately 80% of [3 H]DMC-LR bound to proteins was found in the cytosol after subcellular fractionation, the cytosol was fractionated by DEAE-Sepharose CL-6B (Pharmacia) and Bio-Gel

A-0.5m (Bio-Rad) column chromatography. These methods did not involve denaturation of the proteins, as did acid precipitation, and afforded observations of MC-LR effects 100 times over the IC_{50} for PP1 or PP2A. The chromatography buffer used was 50 mM imidazole, pH 7.4, 5 mM EDTA, 5 mM EGTA, 10% glycerol and 50 mM 2-mercaptoethanol. A sample was applied to the 18ml DEAE and eluted with a salt gradient from 0 to 0.5 M NaCl (total volume 300 ml). The flow rate was 32 ml/h and the fractions eluted were collected every 10 min. The cytosolic proteins were also separated according to size on a Bio-Gel A-0.5m column in the same buffer. Individual fractions from Bio-Gel chromatography were tested for protein phosphatase activity [25].

2.5. Gel electrophoresis and Western blotting

Every two DEAE fractions and every four Bio-Gel fractions were pooled. Proteins in the pooled fractions were precipitated with 12% TCA plus 0.0125% deoxycholic acid (final concentrations, w/v). The proteins were pelleted and washed 3 times with -20°C acetone. Pellets were allowed to dry before they were dissolved in 2 \times sample buffer. After separation on 12% polyacrylamide gels, proteins were electrotransferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). Sheep anti-PP1 affinity-purified antibodies [26] and rabbit anti-PP2A peptide antibodies [27], were used to identify the proteins. Binding of primary antibodies to the membranes was detected by using alkaline phosphatase-conjugated secondary antibodies (Promega Corp., Madison, WI, USA, and Boehringer, Mannheim, Germany), or the ECL Western blotting detection system (Amersham, UK). Purified PP1 and PP2A were used as positive controls. Low molecular weight standards from Pharmacia (Uppsala, Sweden) were used to calibrate protein size. Standards were stained with Ponceau S (0.5% Ponceau S, 10% acetic acid). The relative intensity of Western Blot bands was measured by a densitometer.

2.6. Further purification of PP1 and PP2A

Untreated rat liver homogenate was fractionated by centrifugation as described above, and the cytosol was applied to a 5 ml DEAE-column. The column was washed with the chromatography buffer and a fraction containing PP1 and PP2A was eluted with 0.5 M NaCl (the elution and collection of the protein fraction was monitored with the Bradford protein assay). The fraction (3 ml) was further applied to a 1 ml aminohexyl-Sepharose CL-6B column (AH-Sepharose) and PP1, which does not absorb well to AH-Sepharose, was eluted with 0.5 M NaCl (fraction 1). PP2A was eluted with 1.5 M NaCl in the same buffer (fraction 2). The two fractions were assayed for protein phosphatase activity with or without 2 nM okadaic acid to distinguish between PP1 and PP2A. The PPase inhibition of a concentration series of MC-LR (0.1–5 nM) was also measured [25].

Cytosol from untreated homogenate was also run on a Bio-Gel A-0.5m column. Protein phosphatase activity was measured in every second fraction, as well as the inhibition of the PPase-activity, with 2 nM of okadaic acid. A unit of activity dephosphorylates 1 nmol/min of 10 mM phosphorylase α at 30°C . The inhibition by MC-LR (0.1–5 nM) was measured on the fractions with ABC and AC forms of PP2A that were resolved by this chromatography.

2.7. Gel-filtration binding assay

The binding of [3 H]DMC-LR to partially purified fractions of PP1 and PP2A from AH-Sepharose (see above) was determined by a gel-filtration assay. 50 μ l of each sample was incubated for 30 min with 1,000 nM [3 H]DMC-LR at 22°C , and then applied to 300 μ l of Sephadex G-25 Fine. The samples were centrifuged briefly (1–2 s) at low speed through the Sephadex, and the flow through and another 50 μ l of buffer that was applied to the column were collected. The radioactivity of each sample was measured. The DEAE flow through from the purification steps of fractions 1 and 2 was used as a negative control (data not shown).

3. Results

To identify binding proteins for MC-LR, [3 H]DMC-LR was added to liver homogenates. The distribution of [3 H]DMC-LR in subcellular fractions prepared by differ-

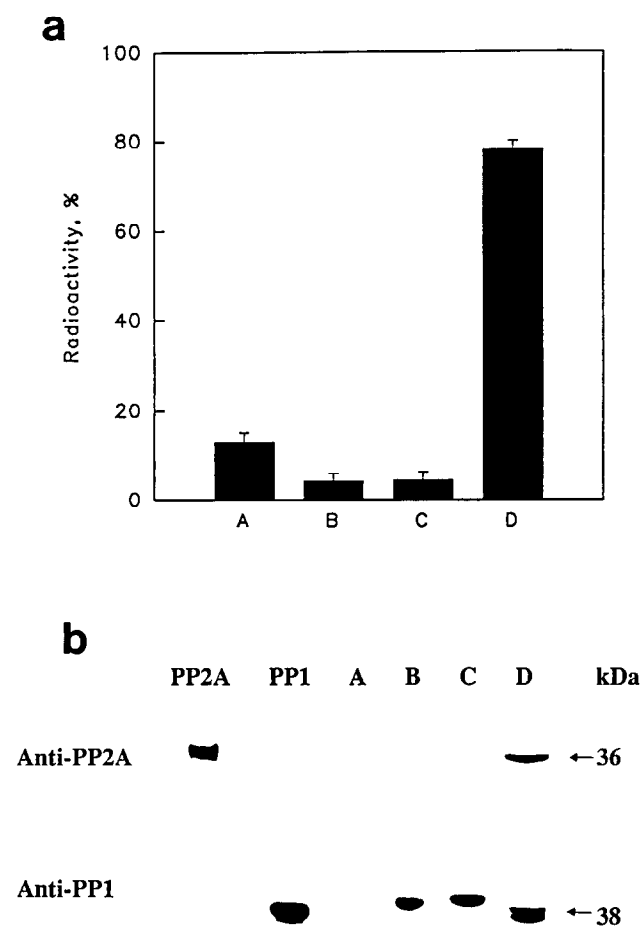


Fig. 1. Distribution of [^3H]DMC-LR in subcellular fractions of liver homogenates. Rat liver homogenates were treated with [^3H]DMC-LR and subjected to subcellular fractionation as described in section 2. (a) The amount of protein-bound ^3H -MCLR in each fraction is expressed as the percentage of total protein-bound radioactivity. The total (100%) was determined as the sum of the individual subcellular fractions. Each bar represents the mean of 4 replicate experiments \pm S.D. (b) Aliquots of the subcellular fractions were run on SDS-PAGE and Western blotted against PP2A and PP1 as described in section 2. Lanes: A, $1,000 \times g$ pellet; B, $10,000 \times g$ pellet; C, $100,000 \times g$ pellet; D, $100,000 \times g$ (60 min) supernatant. Samples of purified PP1 and PP2A were run as standards. Molecular weights are indicated at the right.

ential centrifugation was determined using acid precipitation of proteins to separate toxin–protein complexes from toxin not bound to protein, which was soluble in 10% TCA. The sum of the protein-bound [^3H]DMC-LR of the individual fractions was taken as 100%. As shown in Fig. 1a, proteins that bound [^3H]DMC-LR were predominantly ($78 \pm 2.0\%$) in the cytosolic fraction of the liver. Besides the cytosol, $13 \pm 2.3\%$ of [^3H]DMC-LR was found in the $1,000 \times g$ pellet, and $4.3 \pm 1.7\%$, and $4.6 \pm 1.9\%$ was in the $10,000 \times g$ and $100,000 \times g$ pellets, respectively (Fig. 1a). Therefore, only a minor fraction of toxin bound to membrane or cytoskeletal proteins. Particulate samples treated with Triton X-100 prior to the precipitation, in order to better solubilize proteins,

did not show any significant differences from samples not treated with Triton X-100 (data not shown). Distribution of PP1 and PP2A in subcellular fractions was determined by Western immunoblotting. Nearly all of the PP2A was detected in the $100,000 \times g$ supernatant as a 36 kDa protein (Fig. 1b, lane D). In contrast, the catalytic subunit of PP1 (38 kDa) was detected in the $10,000 \times g$ and $100,000 \times g$ pellets (Fig. 1b, lanes B and C) as well as in the $100,000 \times g$ supernatant (Fig. 1b, lane D).

We analyzed the [^3H]DMC-LR protein complexes by chromatographic resolution and immunoblotting. Cytosol proteins eluted from a DEAE column showed one main peak with bound [^3H]DMC-LR (Fig. 2a, centered at fraction 35) that eluted at about 250 mM NaCl. A peak of [^3H]DMC-LR eluted in the flow through fractions (Fig. 2a, fractions 1–14), but was determined to be free, rather than protein-bound toxin, based on its solubility in 10% TCA. Gel electrophoresis and immunoblotting of the proteins from DEAE fractions show that PP2A (36 kDa) was detected in fractions 30–41 (Fig. 2b), with the maximum PP2A in fractions 36–37 (Fig. 2a,

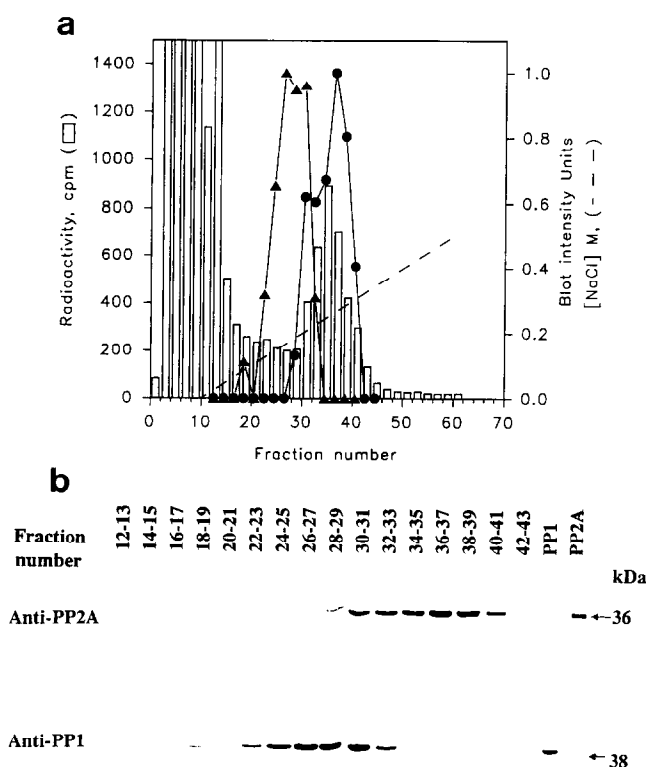


Fig. 2. Binding of [^3H]DMC-LR to cytosol proteins separated by ion-exchange chromatography. Cytosol ($100,000 \times g$ supernatant) from [^3H]DMC-LR-treated liver homogenates was applied to a DEAE-Sepharose column. (a) The counts per minute (cpm) in 500 μl of every second eluted fraction (bars) and the relative intensity of PP2A (●) and PP1 (▲) immunoblotting (see section 2) are presented in relation to the salt gradient (---) and fraction number. (b) The proteins in the DEAE fractions were concentrated by acid precipitation, subjected to SDS-PAGE, and Western blotted against PP2A and PP1 as described in section 2. Samples of purified PP2A and PP1 were run as standards in the right-hand lanes. The molecular weights are indicated at the right.

filled circles). The PP1 catalytic subunit was found as a 38 kDa protein in fractions 24–33 (Fig. 2b) that eluted (Fig. 2a, filled triangles) just before PP2A. Co-elution of PP2A with [3 H]DMC-LR suggests that a stable complex formed in rat liver cytosol. A complex between [3 H]DMC-LR and PP-1 was not recovered after DEAE chromatography.

Alternatively, [3 H]DMC-LR binding was also analyzed by gel filtration chromatography. The protein-bound peak of [3 H]DMC-LR was eluted at an apparent molecular weight of greater than 100 kDa from Bio-Gel A-0.5m (not shown). Immunoblotting detected PP2A in fractions exactly coinciding with the elution of the peak of protein-bound [3 H]DMC-LR. PP1 was eluted in fractions at the leading edge of the peak of [3 H]DMC-LR (not shown).

These results showed co-elution of PP2A and protein-bound [3 H]DMC-LR. However, both PP1 and PP2A purified catalytic subunits are known to be inhibited by MC-LR. To examine the binding and inhibition of the liver cytosolic forms of these phosphatases by MC-LR, they were further purified. The rat liver cytosol containing PP1 and PP2A was purified on DEAE-Sepharose CL6B, and further purified on AH-Sepharose to separate PP1 (fraction 1) and PP2A (fraction 2). As expected from the subcellular distribution (Fig. 1), the amount of PP2A was much higher than the amount of PP1 in rat liver cytosol, evidenced by the 5-fold higher recovery of protein phosphatase activity (Table 1). The identity of the phosphatases in fractions 1 and 2 as PP1 and PP2A, respectively, was confirmed by their different sensitivity to inhibition by 2 nM okadaic acid (Table 1). Both fractions 1 and 2 from AH-Sepharose bound radiolabelled toxin in a gel-filtration binding assay, and the amount of [3 H]DMC-LR bound was proportional to the phosphorylase phosphatase activity (Table 1). Based on the specific radioactivity of [3 H]DMC-LR and assuming a specific activity of 1,000 nmol/min/mg for PP2A (using phosphorylase a as substrate) the binding corresponds to 1 mol [3 H]DMC-LR per mol of PP2A.

Furthermore, the PP1 in fraction 1 and PP2A in fraction 2 both showed dose-dependent inhibition by MC-LR with IC_{50} 's of 0.3 and 0.5 nM, respectively (Fig. 3). [3 H]DMC-LR also inhibited both protein phosphatases at these concentrations, showing that the radiolabelled

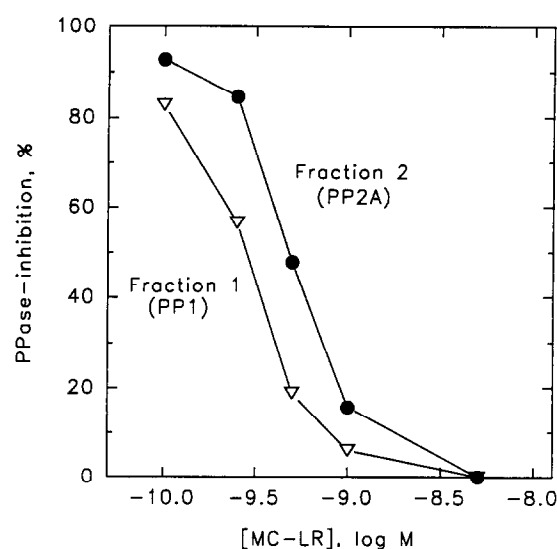


Fig. 3. Inhibition of phosphorylase phosphatase activity of PP1 (fraction 1) and PP2A (fraction 2) purified from rat liver cytosol by DEAE and AH-Sepharose column chromatography using various concentrations of MC-LR. Fraction 1 (▽); fraction 2 (●).

toxin itself was a potent inhibitor. The ABC hetrotrimer and AC heterodimer forms of rat liver PP2A were separated by Bio-Gel A-0.5m chromatography and resolved from residual PP1 by AH-Sepharose adsorption and elution. Both forms of PP2A were inhibited by nanomolar concentrations of MC-LR; the ABC form was inhibited like the fraction 1 sample and the AC form like the fraction 2 sample (see Fig. 3). These results showed that liver cytosolic forms of both PP1 and PP2A were potently inhibited by MC-LR.

4. Discussion

Previous studies have demonstrated that nanomolar concentrations of MC-LR inhibit the catalytic subunits of both PP1 and PP2A in vitro [12–16]. However, the properties of the purified catalytic subunits are known to be markedly different from those of the holoenzymes with respect to substrate reactivity and inhibitor sensitivity [18,28–30]. Therefore we wanted to study the binding of MC-LR to native forms of protein phosphatases found in the liver.

Approximately 80% of the radiolabelled MC-LR bound to proteins was detected in the cytosolic fraction. This agrees with previous studies [21,23] on the distribution of [3 H]DMC-LR in either perfused liver or isolated hepatocytes. Therefore it seems to make little difference if the MC-LR was added to liver homogenates or to intact cells. In agreement with previous reports [18], we detected PP2A primarily in the cytosolic fraction, whereas much of the PP1 was in the mitochondrial and post-mitochondrial particulate fractions. Furthermore,

Table 1
Properties of rat liver cytosolic phosphatases

Fraction	Phosphorylase phosphatase (pmol/min)	Inhibition of Pase-activity by 2 nM okadaic acid (%)	[3 H]DMC-LR bound (cpm)	Ratio [3 H]DMC-LR/PPase activity
1	60	< 15	570	9.5 (PP1)
2	318	83	2985	9.4 (PP2A)

the binding assay of [3 H]DMC-LR to PP1 and PP2A partially purified from the cytosol clearly showed that there was much more PP2A than PP1 in the cytosol of rat liver. The [3 H]DMC-LR binding to PP1 and PP2A was directly proportional to the abundance of the respective enzymes in liver cytosol.

Our results indicated that the toxin in the cytosolic fraction was associated mostly with PP2A. The DEAE chromatography showed recovery of a complex of [3 H]DMC-LR with PP2A, but not with PP1. It was estimated that MC-LR formed a stoichiometric complex with PP2A in an equilibrium binding assay. However, [3 H]DMC-LR inhibited both PP2A and PP1 recovered from liver cytosol with equal potency. Co-crystallization of MC-LR with PP1 has been reported [31], removing any questions about whether the toxin can complex with both PP1 and PP2A. Therefore binding of [3 H]DMC-LR to PP2A in a complex that was the only one recovered from the liver homogenate cytosol probably reflects a difference in the stability of the toxin–PP2A complex relative to the toxin–PP-1 complex. It is conceivable that a covalent link was formed between [3 H]DMC-LR and PP2A, not PP1, forming a non-dissociable and a dissociable complex, respectively. An ester or an isopeptide bond with one of the two carboxyl sidechains in MC-LR could be possible crosslinks.

Injection of MC-LR into mice or rats causes rapid dissociation of the liver cells with complete disruption of liver morphology [7,8,32]. These morphological effects correspond well with the marked cytoskeletal rearrangements observed in freshly isolated hepatocytes exposed to MC-LR [9,12]. The changes in cytoskeletal microfilament organization induced by MC-LR are correlated with increases in overall protein phosphorylation [12]. Furthermore, the phosphorylation state of the liver intermediate filaments, cytokeratin 8 and 18, is markedly altered upon treatment of liver cells with MC-LR [12,33–35]. Correspondingly, calyculin A has been shown to induce hyperphosphorylation of vimentin, the principal intermediate filament in BHK-21 fibroblasts [36,37]. Selective inhibition [36] or microinjection of PP1 [38] has implicated PP1 as the predominant phosphatase in the dephosphorylation of vimentin and myosin light chains. Thus, PP1 has a major role in the maintenance of cytoskeletal networks and apparently is inhibited during liver toxicity produced by MC-LR. Consistent with inhibition of PP1 in vivo by MC-LR there are observations that the toxin induces rapid liver glycogenolysis [8,33] and increases glucose release from hepatocytes [12]. The results in the present study show that inhibition of PP2A is also involved in the effects of MC-LR. This may result in kinase activation, since it is known that the activity of kinases, such as MAP-kinases, is regulated by the activity of PP2A [39]. In this regard, formation of a stable complex of MC-LR with PP2A could provide an explanation for promotion of liver tumors by MC-LR [40–42].

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