Tyrosine-272 Is Involved in the Inhibition of Protein Phosphatase-1 by Multiple Toxins†

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ABSTRACT: Protein phosphatase-1 (PP1) is regulated by interaction with different subunits, which include several inhibitory proteins. It is also potently inhibited by several toxins of diverse origins. Recent work has identified a region near the C-terminus of PP1 (residues 274−277) whose modification was shown to moderate okadaic acid binding [Zhang et al. (1994) *J. Biol. Chem. 269*, 16997−17000]. In this study, the role of this region in toxin binding was explored by site-directed mutagenesis. A residue (Tyr-272) was identified whose mutation had dramatic effects on the spectrum of inhibitor sensitivity of PP1. The IC_{50}'s of a number of mutants of Tyr-272 toward okadaic acid, tautomycin, calyculin A, microcystin-LR, nodularin, inhibitor-2, and cantharidic acid were determined and compared to that of the wild-type enzyme. The sensitivity of PP1 toward tautomycin and calyculin A was markedly decreased, by as much as 3 orders of magnitude, with lesser effects on okadaic acid and nodularin, and with microcystin-LR and inhibitor-2 being the least affected. These studies show that Tyr-272 is of specific importance for the binding of these inhibitors and provide strong evidence for the postulate that these toxins all bind to a common inhibitor site on PP1. In addition, our studies show that Tyr-272 is not required for catalytic activity.

Protein phosphatase-1 is one of the four major classes of the Ser/Thr protein phosphatases, and has a key role in the regulation of metabolism, as well as in a number of important cellular functions [for reviews, see Shenolikar and Nairn (1991), Bollen and Stalmans (1992), and Cohen (1994)]. The catalytic subunit (PP1)[1] is a 37 kDa protein that is highly conserved in evolution, exhibits about 40% identity with the catalytic subunit of protein phosphatase-2A (PP2A) (da Cruz e Silva et al., 1987), and is also related more distantly to calcineurin (PP2B) (Shenolikar & Nairn, 1991). PP1 exists in multiple holoenzyme forms which arise from a combination of PP1 with different regulatory or targeting proteins (Hubbard & Cohen, 1993). These regulatory subunits include two heat-stable inhibitors, inhibitor-2 and a nuclear inhibitor (NIPP-1) (Beullens et al., 1992, 1993). In addition, a third heat-stable protein, inhibitor-1, and a related neural protein, DARPP-32, inhibit PP1 when in the phosphorylated state (Shenolikar & Nairn, 1991).

A number of toxins which are potent inhibitors of PP1 and PP2A have been discovered. Okadaic acid, a C38 polyether fatty acid, is the toxic agent responsible for diarrhetic shellfish poisoning when contaminated shellfish are ingested, and in addition is a powerful tumor promoter on skin (Suganuma et al., 1988; Fujiki et al., 1988). It inhibits PP1 with an IC_{50} that is 200−300-fold less than that for PP2A (Biojalan & Takai, 1988), and has become widely used as a research tool for the study of phosphorylation mechanisms (Cohen et al., 1990). Calyculin A, an octamethyl polyhydroxylated C28 fatty acid linked to two γ amino acids and esterified with phosphate, was isolated from marine sponges, and exhibits cytotoxic and tumor-promoting activities (Ishihara et al., 1989; Suganuma et al., 1990). The microcystins, a family of cyclic heptapeptide hepatotoxins that have been isolated from freshwater cyanobacteria (Carmichael, 1992, 1994), are potent inhibitors of PP1 and PP2A (Honkanen et al., 1990; MacKintosh et al., 1990). Microcystin also exhibits a liver tumor-promoting activity (Nishiwaki-Matsushima et al., 1992). The structurally related pentapeptide toxin, nodularin, is also a potent inhibitor of PP1 and PP2A. Tautomycin, an antibiotic produced by a soil bacterium, *Streptomyces spiroverticullatus*, has also been shown to be a potent inhibitor of PP1 and PP2A (MacKintosh & Klumpp, 1990). These inhibitors are all characterized by IC_{50}'s in the nanomolar range.

Thus, PP1 is the target of a number of structurally dissimilar inhibitors, and the identification of their binding site(s) is of both biological and biochemical interest. Given the acute sensitivities to the toxins, PP1 and PP2A can be considered to be the cellular receptors for these toxins, providing a basis for their cytotoxicities. Recent mutational studies have provided evidence for the potential localization of the okadaic acid binding region. A mutant CHO cell line that showed increased resistance to okadaic acid possessed a PP2A gene that had undergone a point mutation such that Cys-269 was converted to glycine (Shima et al., 1994). On this basis, a chimeric PP1 mutant was constructed in which residues 274−277 (GEFD) of PP1 were substituted by the cognate region of PP2A (YRCG, residues 267−270) (Zhang et al., 1994a). This mutant displayed a 10-fold increase in

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[1] Abbreviations: PP1, catalytic subunit of protein phosphatase-1; PP2A, catalytic subunit of protein phosphatase-2A; PCR, polymerase chain reaction.

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                        270       280       290       300
PP1α  261 RQLVTLFSAPNYCGEFDNAGAMMSVDETLMCSFQILKPAD
PP2A  254 •NV••I•••••••YRCG•QA•I•EL•D••KY••LQFD••P
                             269
                        310       320       330
PP1α  301 KNKGKYGQFSGLNPGGRPITPPRNSAKAKK*
PP2A  294  RRGEPHTRRTPDYFL*
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FIGURE 1: Alignment of the C-termini of PP1 and PP2A. The diagram shows the alignment of the C-terminal residues 261−330 of PP1α (Bai et al., 1988) and 254−308 of PP2A (Da Cruz e Silva et al., 1987). Sequence identities are shown as solid circles.

sensitivity to okadaic acid. In the present study, the region between residues 268 and 277 was surveyed by site-directed mutagenesis, and has revealed the presence of a residue, Tyr-272, that is critical for toxin sensitivity and whose mutation dramatically alters the inhibitory response of PP1 in a broad manner. Moreover, these studies show that Tyr-272 is not required for catalysis.

MATERIALS AND METHODS

Materials. Okadaic acid was purchased from Kamiya Corp.; microcystin-LR, tautomycin, and calyculin A were purchased from Calbiochem. Okadaic acid was also obtained as a gift from Dr. D. Baden, University Miami. Inhibitor-2 was the purified recombinant rabbit muscle protein expressed in *Escherichia coli* (Zhang et al., 1994b). Cantharidic acid was a generous gift of Dr. D. Brautigan, Brown University.

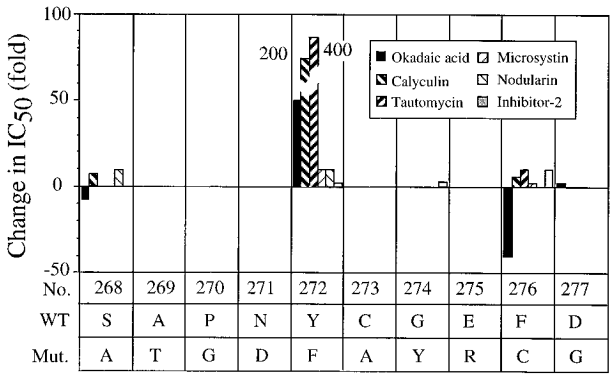
Generation of Mutants. Mutants of PP1α were generated essentially as described by Zhang et al. (1994a) using a first-step PCR amplification of the PP1α cDNA (Bai et al., 1988) to generate a 3′ fragment containing the mutated sequence, followed a second PCR step in which this fragment was used as a primer to generate the full-length coding sequence. The sequence of each mutant was determined by DNA sequencing to confirm the construction. The cDNAs were all inserted into the pTACTAC vector as previously described (Zhang et al., 1992).

Purification of PP1 Mutants. The PP1 mutant constructs were grown in *E. coli* host cells in 4 × 1 L batches as described previously (Zhang et al., 1992, 1994a). The cells were harvested, and the mutants were each purified to near-homogeneity by the column chromatography procedures described by Zhang et al. (1993a). The procedure involved successive chromatography on DEAE-Sepharose, heparin−Sepharose, polylysine−agarose, and Q-Sepharose. The final yields of enzyme protein were in the range of 10−20 mg.

Assays for PP1 Activity. Assays for PP1 activity were performed using ^{32}P-labeled phosphorylase *a* as previously described (Zhang et al., 1992) in the presence of Mn^{2+}. IC_{50}'s for each toxin were determined, and in each case, changes in sensitivity were based on determination of the IC_{50} for PP1 in the same experiment as described by Zhang et al. (1994a).

RESULTS

A Cysteine at Residue 276 Enhances Okadaic Acid Sensitivity. The comparison of the C-terminal sequences of PP1 and PP2A is shown in Figure 1. PP1 and PP2A have about 40% sequence identity, but diverge quite strongly in sequence similarity after residue 300 of PP1. The location of the region of PP1 that we previously identified as being involved in okadaic acid binding (residues 274−277), based



No.	268	269	270	271	272	273	274	275	276	277
WT	S	A	P	N	Y	C	G	E	F	D
Mut.	A	T	G	D	F	A	Y	R	C	G

FIGURE 2: Effects of mutations at residues 268−277 on the toxin sensitivities of PP1. The single-point mutants S268A, A269T, P270G, N271D, Y272F, C273A, G274Y, E275R, F276C, and D277G were constructed, expressed, and purified to homogeneity as described under Materials and Methods. The IC_{50}'s for okadaic acid, microcystin-LR, nodularin, tautomycin, calyculin A, and inhibitor-2 were determined for each of these mutants. The IC_{50} for the wild-type enzyme was determined at the same time as for the mutant for each inhibitor in the same experiment. Data are expressed as the fold changes in IC_{50}. Positive values represent an increase in IC_{50}, and negative values are the fold decreases in IC_{50}.

on the behavior of a chimeric construct, is close to the C-terminus of the conserved region of PP1 and PP2A (Zhang et al., 1994a). Immediately N-terminal to the region from 261 to 273 of PP1 is highly conserved with PP2A (Figure 1), and appeared to be a logical region for further investigation by site-directed mutagenesis since both PP1 and PP2A are potently inhibited by the toxins okadaic acid, calyculin A, tautomycin, microcystin-LR, and nodularin. Single mutants of residues 268−277 were constructed and expressed in *E. coli*. The mutants were purified to homogeneity as determined by SDS−PAGE (Materials and Methods). The mutants were all expressed at between 2 and 4% of soluble protein, similar to the wild-type enzyme, and had specific activities that ranged between 5000 and 12 000 units/mg of protein. The latter range is similar to that encountered with routine preparations of wild-type recombinant PP1 (Zhang et al., 1992). The effects of okadaic acid, calyculin A, tautomycin, microcystin-LR, nodularin, and inhibitor-2 on these mutants were examined. [Cantharidic acid (Honkanen, 1993), a less potent inhibitor of PP1 and PP2A, with IC_{50}'s in the micromolar range, was also tested for all the mutants described here, but no significant changes were observed.] Inhibition curves were generated as described under Materials and Methods, and for each determination, a control curve for the wild-type PP1 was done at the same time. The effects of these mutations on inhibitor sensitivities are summarized in Figure 2 as the fold changes of IC_{50} values. Representative inhibition data are shown in Figure 3 for the Y272F mutant. Large changes (>10-fold) in inhibitor response are seen in only two cases, *i.e.*, modification of residues Y272 and F276 (Figure 2). In the case of residues 274−277, the changes (Y, R, C, G) were individual substitutions for the amino acids present in PP2A (Figure 1). Of the four residues, only the mutation of residue F276 to cysteine had any positive effect on okadaic acid binding, and resulted in a *ca.* 40-fold decrease in IC_{50}. Examination of the sensitivities of the F276C mutation toward other toxins showed that these were only slightly affected, and in most cases resulted in an increase in IC_{50}. The "YRCG" mutant (Zhang et al., 1994a) was further mutated so that Cys-276 was mutated to glycine, mimicking the original mutation observed in PP2A by Shima

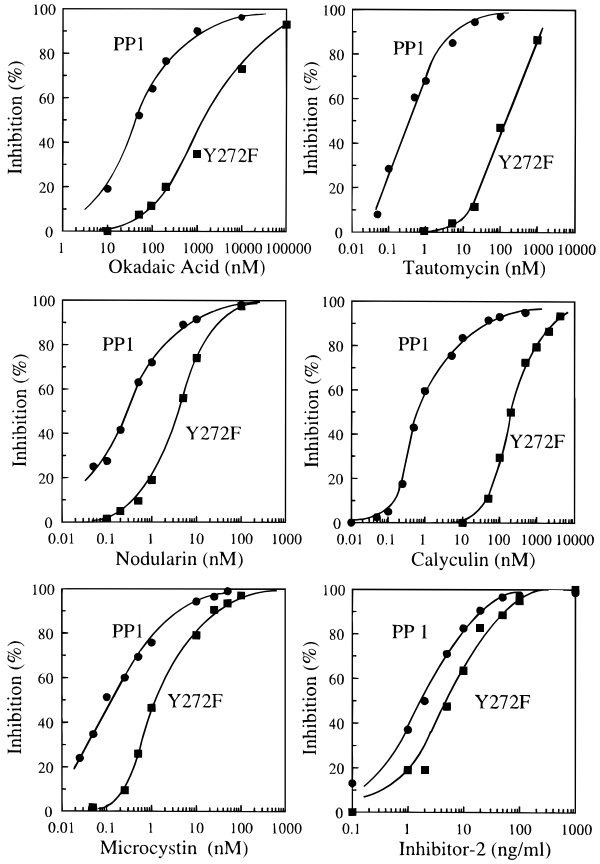


FIGURE 3: Inhibition curves for the Y272F mutant of PP1. The inhibition of the Y272F mutant is shown as a function of inhibitor concentration. Inhibitors tested were okadaic acid, tautomycin, calyculin A, microcystin-LR, nodularin, and inhibitor-2. Enzyme activity was assayed using phosphorylase *a* as the substrate (Materials and Methods). The inhibition of the wild-type enzyme was determined at the same time. These inhibition curves were used to estimate the fold changes in the IC_{50}'s.

et al. (1994). This would be predicted to result in a decrease in okadaic acid sensitivity, and is exactly what was observed, as the IC_{50} was now increased over 10-fold over the original "YRCG" mutant (not shown). The effect of a cysteine at position 276 in increasing sensitivity is specific for okadaic acid. Additional mutants which extended the similarity of PP1 to PP2A (see Figure 1) toward the C-terminus were constructed by further mutagenesis of the "YRCG" mutant by mutation of A279 to Q, and then a further mutation of G280 to A. These two mutants did not result in any further increase in sensitivity to okadaic acid (not shown), and behaved like the wild-type enzyme. Thus, it can be concluded that the greater sensitivity of PP2A toward okadaic acid as compared to PP1 is in part dictated by the presence of a cysteine residue at position 269 of PP2A and its absence at the comparable position (residue 276) in PP1. The mutation of one other residue, S268, also resulted in a small increase in okadaic sensitivity, suggesting that this residue also is involved in some way in okadaic acid sensitivity.

Tyr-272 Is Critical for Toxin Binding. Modification of the residues S268, A269, P270, N271, Y272, and C273 which are conserved between PP1 and PP2A shows that only modification of Y272 led to loss of okadaic acid sensitivity (Figure 2). In this case, the modification was the highly conservative substitution of phenylalanine for tyrosine, which was sufficient to cause a 50-fold increase in IC_{50} for okadaic acid. Relatively modest changes (<10-fold) in IC_{50} were

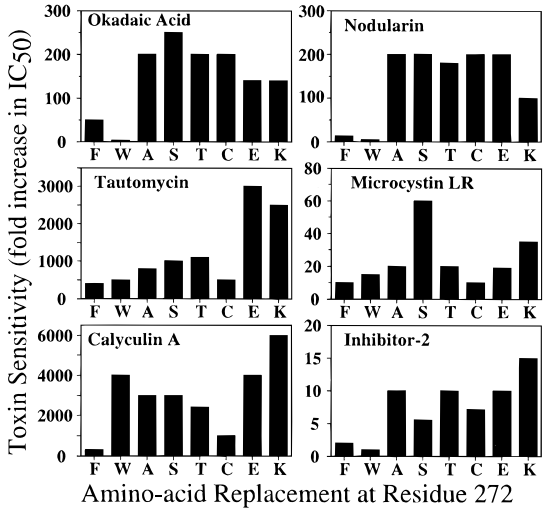


FIGURE 4: Effects of mutagenesis of Y272 of PP1 on toxin sensitivity. Mutations of Y272 to F, W, K, E, S, A, C, and T were constructed, expressed, and purified to homogeneity. The IC_{50}'s of okadaic acid, microcystin-LR, nodularin, tautomycin, calyculin A, and inhibitor-2 were determined and compared to those of the wild type by analysis as described in Figure 3. Data are shown as fold changes in IC_{50}.

observed for microcystin-LR, nodularin, and inhibitor-2. Larger changes in sensitivity involving 300−400-fold increases in IC_{50}, respectively, were observed for calyculin A and tautomycin.

The above results strongly suggested that Y272 is important for the binding of okadaic acid, and more so for calyculin A and tautomycin. In order to further investigate this possibility, a number of mutations of Y272 were made, in which Y272 was mutated to Trp, Lys, Glu, Ser, Ala, Cys, and Thr. Each mutant protein was purified to homogeneity, and the fold changes in IC_{50}'s were compared to the wild-type enzyme for the various toxins (Figure 4). The first general conclusion that can be made is that modifications of Tyr-272 produce strong effects on inhibitor sensitivity in all cases. The increases in IC_{50} for tautomycin ranged from 500- to 1500-fold, while those for calyculin A increased by 1000−6000-fold. The loss of sensitivity was such that at a calyculin A concentration of 100 μM, which completely inhibits the wild-type enzyme, these mutants retained significant levels of activity (70, 90, 87, 86, 95, 83, 100, and 83%, for the Y272F, Y272W, Y272A, Y272S, Y272T, Y272C, Y272K, and Y272E mutants respectively). For okadaic acid, the increases in IC_{50} were generally about 200-fold. In the case of the peptide-based toxins, the fold increases in IC_{50} for nodularin were similar to those for okadaic acid, while those for microcystin-LR and inhibitor-2 were lower (Figure 4). Surprisingly, nodularin was more strongly affected than the structurally related microcystin-LR, with ca. 300-fold decreases observed for five of the mutants. The actual IC_{50} values for the inhibition of the mutants by the toxins are tabulated in Table 1. It is seen that mutations of Y272 can increase the IC_{50}'s to the micromolar range for okadaic acid, calyculin A, and tautomycin. The results clearly suggest that Y272 is involved in intimate contact with the inhibitors, in particular with okadaic acid, calyculin A, and tautomycin.

DISCUSSION

The studies reported here provide strong evidence for the hypothesis that the toxins and inhibitors all bind to the same

Table 1: Inhibition of PP1 Mutations at Residue Y272[a]

enzyme	oka	mic	nod	caly	taut	I-2
WT	200	0.3	0.5	0.5	1.1	13
F	2000	1	4	200	120	20
W	600	2	2	1600	1000	12
A	33000	10	120	1200	560	100
S	50000	24	150	1200	550	70
T	42000	10	90	1200	800	144
C	22000	2	110	500	300	98
E	30000	8	130	2000	1800	140
K	30000	14	70	3000	2600	180

[a] The table shows the IC_{50}'s for okadaic acid (oka), microcystin-LR (mic), nodularin (nod), calyculin A (caly), and tautomycin (taut) in nanomolar; data for inhibitor-2 (I-2) are in nanograms per milliliter. WT refers to the wild-type enzyme. Values for the wild-type are the average values for 8 determinatons.

proximal region in PP1. Previously, it had been shown that prior binding of microcystin, nodularin, and calyculin A would prevent binding of [^{3}H]okadaic acid to cell extract protein (Suganuma et al., 1990; Matsushima et al., 1990; Yoshizawa et al., 1990). Other studies using an indirect kinetic assay also indicated that prior binding of okadaic acid and the heat-stable protein inhibitors, inhibitor-1 and inhibitor-2, prevented PP1 from interacting with microcystin (MacKintosh et al., 1990). Fluorescence anisotropy experiments have also shown that okadaic acid and inhibitor-2 compete for binding to PP1 (Picking et al., 1991). The idea that these inhibitors may share a common site is surprising in view of their diverse chemical structures, although there are some general similarities. Okadaic acid and tautomycin share strong similarities in the carbon backbone for the first 16 carbons, while microcystin, nodularin, and inhibitor-2 are peptide compounds. The case of inhibitor-2 presents an anomaly, since it is generally regarded as having no effect on PP2A, unlike the toxin inhibitors which affect both PP1 and PP2A (Bollen & Stalmans, 1992). However, recent studies indicate that inhibitor-2 binding to PP1 may involve multiple sites (Park & DePaoli-Roach, 1994), so that this might explain the different response of PP2A. It may be that the structures of the toxins share some topographical similarities, such that they present common surface features that are accommodated in the inhibitor binding site. However, the only solution structure for the toxins that has been determined is for the microcystins by 2D nuclear magnetic resonance spectroscopy (Rudolph-Böhner et al., 1994). An additional consideration is that because of the different structures of these inhibitors, the contact sites may not all be identical. This would explain the disparity in the effects observed on mutation of Tyr-272 on inhibitor sensitivity, if one considers that changes of greater than a thousandfold are observed with calyculin A and tautomycin, while those for microcystin-LR are less than a hundredfold.

The loss of sensitivity observed when Tyr-272 is mutated indicates that it is likely to be involved in close contact with the toxins. Evidence that we have correctly identified a region involved in toxin binding comes from other studies which have indicated that microcystin-LR forms covalent adducts with PP1 and/or PP2A (Robinson et al., 1991; Toviola et al., 1994; Moorhead et al., 1994). A potential candidate for the site of addition is a cysteine residue, since it has been shown that microcystin forms covalent adducts with free thiols via reaction of the α,β-unsaturated carbonyl of *N*-methyldehydroalanine (Kondo et al., 1991). If the effects of mutation of Tyr-272 are due to its direct involvement in binding to the toxins, a likely candidate is the immediately adjacent residue, Cys-273 (which is conserved in PP2A). To test this, the C273A mutant was examined for its ability to form an adduct with microcystin. The results were negative (Runnegar et al., 1995). Since we have shown that the inhibition of the C273A mutant and the inhibition of the C273S mutants by microcystin-LR are similar to that of the wild type (Runnegar et al., 1995; Zhang et al., 1993b), this failure cannot be due to a loss of binding of the toxin, leading to the conclusion that the adduct involves reaction at Cys-273. This supports the interpretation of our data that there is a direct physical interaction of Tyr-272 with the toxins and that the loss of toxin sensitivity as a result of mutations of Tyr-272 is not due to secondary conformational effects.

During the course of this work, the crystal structures of two serine/threonine phosphatases were solved, those of a calcineurin (PP2B)−FKBP12−FK506 complex (Griffith et al., 1995) and the PP1α-microcystin−LR complex (Goldberg et al., 1995). The latter now provides a structural basis for interpreting our findings, directly in the case of microcystin-LR and inferentially in the case of the other inhibitors. The crystal structures of the calcineurin A subunit and PP1 reveal that the core regions are structurally conserved, and both have two metals at the active site. This metal pair is Fe^{3+} and Zn^{2+} in the case of calcineurin (Griffith et al., 1995), while in the case of PP1 this is inferred to be two Mn^{2+} ions (Goldberg et al., 1995). The PP1 structure reveals the active site to be at the intersection of a striking topographical feature consisting of a Y-shaped groove. The arms of this groove are formed by a C-terminal groove, an acidic groove, and a hydrophobic groove that may be the substrate binding site. The active site containing the metal ions is situated at the head of the hydrophobic groove (Goldberg et al., 1995). Microcystin-LR binds into this groove, and is in intimate contact with the active site. Relevant to this discussion are two aspects of its binding. First, microcystin-LR is revealed to be covalently attached via the *N*-methyldehydroalanine group to Cys-273 (Goldberg et al., 1995), consistent with our observations of the failure of the C273A mutant to form a covalent adduct (Runnegar et al., 1995). Second, the leucine residue of microcystin-LR is shown to be closely packed against the side chain of Tyr-272. A water molecule (W1) is hydrogen-bonded to the phenolic group of Tyr-272 as well as to the carboxylate group of the γ-linked glutamate of microcystin-LR (Goldberg et al., 1995). The observation that the conservative mutation of Tyr-272 to Phe involving the loss of the phenolic residue affects the inhibition of PP1 by microcystin-LR is thus consistent with the structure. Mutation of Tyr-272 has more marked effects on the inhibition by other toxins (okadaic acid, calyculin A, and tautomycin) than are observed with microcystin-LR, strongly suggesting that the phenolic hydroxyl plays a role in their interaction with PP1. It should be noted that the other mutations, which involve loss of the aromatic function, produce even larger perturbations, suggesting that this function is also important in toxin binding.

While microcystin-LR makes other contacts within the hydrophobic groove of PP1 as revealed by the crystal structure (Goldberg et al., 1995), the interaction with Tyr-272 is clearly an important one as shown by the studies described here. The binding of microcystin-LR to the

hydrophobic groove, as well as its close interaction with Tyr-272 within the active site, provides a satisfactory explanation for the potency of its inhibition as well as a basis for the view that these structurally disparate inhibitors bind to the same site. In the context of the crystal structure, it may be proposed that the toxins bind to PP1 in a similar manner to microcystin; this would involve binding within the hydrophobic groove proposed to be the peptide substrate binding site as well as a close contact with the active site. In addition to phosphorylase *a*, a small substrate, *p*-nitrophenyl phosphate, is also hydrolyzed by PP1, albeit with a K_m roughly 1000-fold greater than for phosphorylase *a* (Li, 1979). This activity is also inhibited by the toxins (Takai & Mieskes, 1991; An & Carmichael, 1994). This inhibition is consistent with the crystal structure data that show that the microcystin binding involves occupancy both of the putative polypeptide binding region and also of the active site region. In the case of calcineurin, it is of interest that cyclophilin−cyclosporin A or FKBP−FK506 inhibits its activity toward phosphopeptide substrates but not its activity toward *p*-nitrophenyl phosphate (Liu et al., 1991). Our findings do not preclude the possibility that the various toxins can be accommodated in an overlapping manner within the other two arms of the Y-shaped groove, but do place a constraint that an interaction with Tyr-272 must be involved, i.e., that there is a common mechanism of inhibition that minimally involves binding of the toxin at the active site. Since the crystal structure of free PP1 is unknown, it is not certain whether inhibitor binding is also accompanied by significant structural perturbation of the protein, but this is clearly not a necessary requirement for inhibition, at least in the case of microcystin-LR.

The crystal structure confirms the correctness of the mutational data which indicate an interaction of Tyr-272 with microcystin-LR. The findings that perturbations of the inhibitor sensitivity of the Tyr-272 mutants toward the toxins (okadaic acid, calyculin A, and tautomycin) are much greater than those observed for microcystin-LR further strengthen the view that these are binding to PP1 in a similar manner that involves binding to the active site region. In this context, some comment of the spatial location of Tyr-272 is merited. The region that we have mutated from residues 268−277 lies in a surface-exposed loop region connecting β12 and β13. This loop lies at the intersection of the Y groove, proximal to the active site. Indeed, Tyr-272 is close enough to the two metal ions so that the phenolic group is proposed to be liganded to one of the water molecules ("W1") in the active site which is within 3 Å of the modeled position of the phosphorus atom of the substrate. The phenolic function of Tyr-272 is suggested as a candidate for the source of the nucleophile that is involved in the catalytic mechanism, either directly or indirectly through a hydroxide ion from the water molecule ("W1") to which the phenolic group is hydrogen-bonded (Goldberg et al., 1995). Our mutational studies provide a clarification of the identity of the nucleophile involved in catalysis, since they eliminate an obligate participation of the phenolic residue of Tyr-272.

A striking biological aspect of the existence of a number of toxins that affect PP1 and PP2A can now be brought into the context of their targeting of the active site and the substrate binding regions. Indeed, the use of PP1 inhibition is a useful method for the assay of microcystins and okadaic acid (Takai & Mieskes, 1991; An & Carmichael, 1994), and a potential method for screening for new toxins. Finally, the dramatic effects on toxin sensitivity of mutations of Tyr-272, especially in the case of tautomycin and calyculin A, are of sufficient magnitude that several of these mutants are active in the presence of concentrations of inhibitor that would completely suppress wild-type PP1 and PP2A activities. Thus, the Y272 mutants of PP1 are reagents that could be of utility in defining the involvement of PP1 in the dephosphorylation of any given substrate in the presence of wild-type PP1 and PP2A activities, when combined with the use of calyculin A or tautomycin.

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