

# Crystal Structure and Mutagenesis of a Protein Phosphatase-1:Calcineurin Hybrid Elucidate the Role of the $\beta$ 12– $\beta$ 13 Loop in Inhibitor Binding\*

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**Protein phosphatase-1 and protein phosphatase-2B (calcineurin) are eukaryotic serine/threonine phosphatases that share 40% sequence identity in their catalytic subunits. Despite the similarities in sequence, these phosphatases are widely divergent when it comes to inhibition by natural product toxins, such as microcystin-LR and okadaic acid. The most prominent region of non-conserved sequence between these phosphatases corresponds to the  $\beta$ 12– $\beta$ 13 loop of protein phosphatase-1, and the L7 loop of toxin-resistant calcineurin. In the present study, mutagenesis of residues 273–277 of the  $\beta$ 12– $\beta$ 13 loop of the protein phosphatase-1 catalytic subunit (PP-1c) to the corresponding residues in calcineurin (312–316), resulted in a chimeric mutant that showed a decrease in sensitivity to microcystin-LR, okadaic acid, and the endogenous PP-1c inhibitor protein inhibitor-2. A crystal structure of the chimeric mutant in complex with okadaic acid was determined to 2.0-Å resolution. The  $\beta$ 12– $\beta$ 13 loop region of the mutant superimposes closely with that of wild-type PP-1c bound to okadaic acid. Systematic mutation of each residue in the  $\beta$ 12– $\beta$ 13 loop of PP-1c showed that a single amino acid change (C273L) was the most influential in mediating sensitivity of PP-1c to toxins. Taken together, these data indicate that it is an individual amino acid residue substitution and not a change in the overall  $\beta$ 12– $\beta$ 13 loop conformation of protein phosphatase-1 that contributes to disrupting important interactions with inhibitors such as microcystin-LR and okadaic acid.**

Protein phosphatase-1 (PP-1)<sup>1</sup> is a member of the phospho-protein phosphatase gene family, whose members share 40% sequence identity in the catalytic subunit (PP-1c) and includes protein phosphatase-2A (PP-2A) and protein phosphatase-2B (PP-2B or calcineurin) (1). A striking structural similarity is seen upon superimposition of the crystal structures of PP-1c and the calcineurin catalytic subunit (calcineurin A), especially in the active site regions (the structure of PP-2A has not been determined). Despite the similarities in sequence and structure, PP-1c and calcineurin A have distinct inhibitor and substrate specificities. PP-1c is sensitive to the endogenous inhibitor proteins inhibitor-1 and inhibitor-2, as well as to the exogenous marine natural product inhibitors microcystin-LR (MCLR) and okadaic acid (OA). Calcineurin is markedly less sensitive to these inhibitors (250-fold for OA and over 1000-fold for the microcystins) (2–5).

One region of sequence dissimilarity between PP-1c, PP-2Ac, and calcineurin A is the  $\beta$ 12– $\beta$ 13 loop region of PP-1c/PP-2Ac (residues 273 to 277 in PP-1c and 266 to 270 in PP-2Ac), which corresponds to the L7 loop of calcineurin A (residues 312–316). Residues of the  $\beta$ 12– $\beta$ 13 loop region have previously been implicated in inhibitor selectivity (6–8). The importance of this region was first discovered when an okadaic acid-resistant isoform of PP-2A in Chinese hamster ovary cells was found to have a mutation in Cys<sup>269</sup> (9). The corresponding residues in PP-1c and calcineurin A are Phe<sup>276</sup> and Tyr<sup>315</sup>. Because PP-2Ac is much more sensitive to okadaic acid (IC<sub>50</sub> = 0.2 nM) than PP-1c (IC<sub>50</sub> = 20–50 nM) or calcineurin (IC<sub>50</sub> = 5  $\mu$ M), the presence of a cysteine at this position may be responsible for its higher sensitivity to okadaic acid. Further mutagenesis studies were carried out in PP-1c that confirmed the importance of the  $\beta$ 12– $\beta$ 13 loop region in inhibitor sensitivity, as well as in enzyme activity (6, 10–12).

Structural studies have shed light on the nature and importance of the  $\beta$ 12– $\beta$ 13 loop in PP-1c in the context of inhibitor binding. The hydroxyl of Tyr<sup>272</sup> in the  $\beta$ 12– $\beta$ 13 loop was analyzed in four different crystals of the same PP-1c-tungstate complex and found to have a metal to hydroxyl contact distance ranging from 3.0 to 4.3 Å, suggesting flexibility in the loop region (13). Flexibility is also noted in differences between the structures of PP-1c bound to MCLR, okadaic acid, tungstate, and calyculin A (13–16). The loop in the MCLR-bound structure folds back on itself, shifting the  $\beta$ 12– $\beta$ 13 loop relative to the tungstate-, OA-, and calyculin-bound PP-1c structures (15). These differences may be indicative of the covalent bond

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The atomic coordinates and structure factors (code 1U32) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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<sup>1</sup> The abbreviations used are: PP-1, protein phosphatase-1; MCLR, microcystin-LR; OA, okadaic acid.



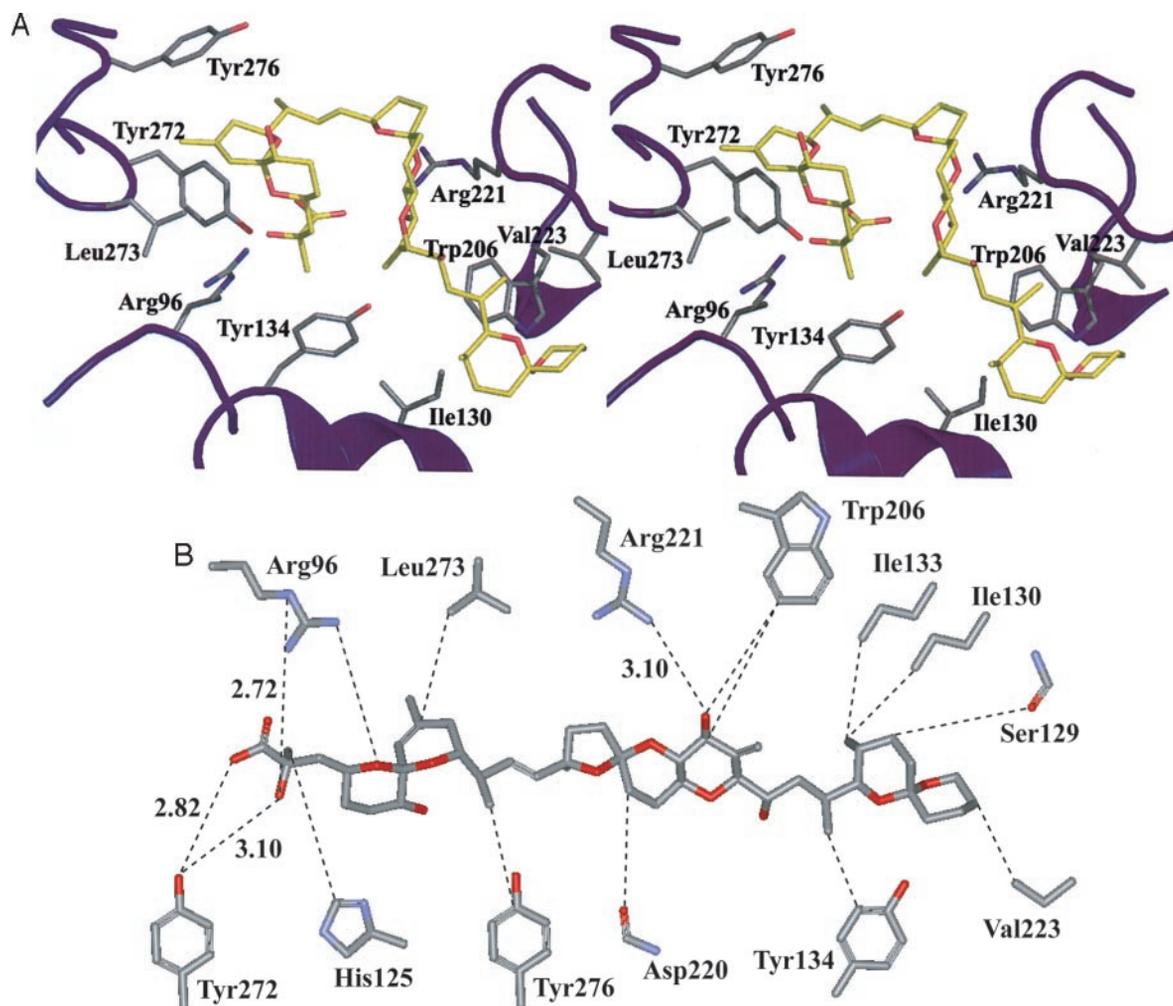


FIG. 2. *A*, stereo representation of the active site of the PP-1c-loop-OA complex showing pertinent active site residues. The inhibitor is shown with yellow carbons. The numbering of active site residues is the same as wild-type PP-1c. *B*, active site contacts seen the PP-1c-loop-OA complex. OA is shown as a linear representation. All contacts within 4 Å are shown and potential hydrogen bonding interactions have their distances shown.

the difference density using the structure of OA bound to wild type PP-1c as a starting model. The protein-inhibitor model was subjected to rigid body refinement in CNS prior to manually fitting the model using the program XtalView (26, 27). The model was then subjected to iterative rounds of macromolecular refinement using CNS with a maximum likelihood target. The crystallographic data are listed in Table I. The final model consisted of density for residues 6–298 and was checked for validity using WHATCHECK and PROCHECK (28, 29). PROCHECK showed that 97% of residues were in allowed Ramachandran plot ranges with an overall G factor of 0.2.

**Coordinates**—The atomic coordinates and structure factors have been deposited in the Protein Data Bank with code 1U32.

## RESULTS

**Inhibition and Substrate Specificity of Wild-type and Loop Mutant PP-1c**—To understand the role of the  $\beta$ 12– $\beta$ 13 loop region of PP-1c in inhibitor selectivity further, we examined the inhibitory effects of both natural product inhibitors and an endogenous inhibitor protein (inhibitor-2) on a mutant of PP-1c in the  $\beta$ 12– $\beta$ 13 loop region (Table II). This mutant (herein referred to as the “loop mutant”) has residues 273–277 of PP-1c substituted with the corresponding residues of calcineurin A (residues 312–316) (Fig. 1). This loop mutant has allowed us to investigate the role of this non-conserved portion of the type 1, 2A, and 2B (calcineurin) phosphatases in inhibitor selectivity.

Wild-type PP-1c was inhibited by okadaic acid with an  $IC_{50}$  of 30 nM and microcystin-LR with an  $IC_{50}$  of 0.1 nM. The loop mutant was ~4-fold less sensitive to okadaic acid ( $IC_{50}$  = 110–120 nM) and at least 6-fold less sensitive to microcystin-LR

( $IC_{50}$  = >0.6 nM). Inhibitor-2 inhibited PP-1c with an  $IC_{50}$  of 1–3 nM. The loop mutant was less sensitive to inhibitor-2 inhibition ( $IC_{50}$  = 5–6 nM). The resistance of the loop mutant of PP-1c to all inhibitors studied is consistent with the hypothesis that the residues of the  $\beta$ 12– $\beta$ 13 loop region of PP-1c play a role in inhibitor selectivity.

**Crystal Structure of PP-1c-loop-OA Complex**—The structure observed of the loop mutant PP-1c bound to okadaic acid is very similar to other PP-1c structures (0.22 Å  $C_{\alpha}$  r.m.s. deviation to wild-type PP-1c:OA, 0.55/0.54 Å  $C_{\alpha}$  r.m.s. deviation to PP-1c:MCLR (two in the asymmetric unit), 0.51/0.53 Å  $C_{\alpha}$  r.m.s. deviation to PP-1c:tungstate (two in the asymmetric unit), 0.45/0.48 Å  $C_{\alpha}$  r.m.s. deviation to PP-1c:calyculin (two in the asymmetric unit), all measurements taken over 284  $C_{\alpha}$  atoms). The orientation of OA in the active site is indistinguishable from that seen with the wild-type enzyme, with the OA adopting a cyclic conformation via an intramolecular hydrogen bond between the  $C_1$ -acid and the  $C_{24}$ -hydroxyl (Fig. 2). Similarly, all contacts between the enzyme and the inhibitor are seen in both the wild-type and loop mutant complexes. Namely, this involves the hydrophobic double ring spiroketal moiety of OA binding into the hydrophobic cleft of PP-1c created by residues Ile<sup>130</sup>, Ile<sup>133</sup>, Trp<sup>206</sup>, and Val<sup>223</sup> and hydrogen bonding interactions between Tyr<sup>272</sup> and the  $C_1$ -acid and  $C_2$ -hydroxyl, Arg<sup>221</sup> and the  $C_{24}$ -hydroxyl and Arg<sup>96</sup> ( $N^{\epsilon}$ ) and the  $C_2$ -hydroxyl. The conclusion that hydrophobic energy drives complex formation is supported by this structure because there are very few pro-

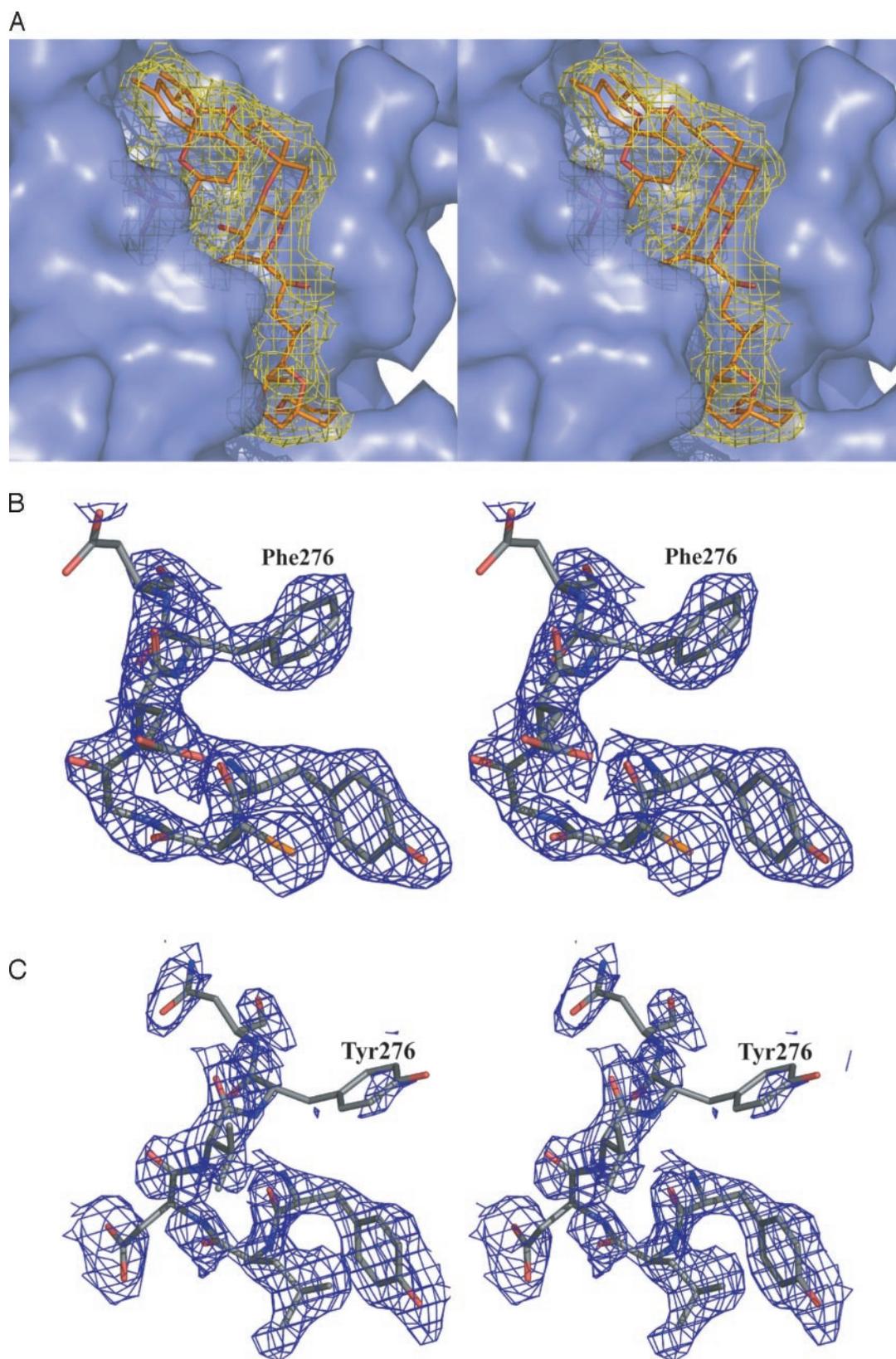


FIG. 3. *A*, stereo representation of the electron density of okadaic acid bound to the chimeric PP1. The protein is shown as a semi-transparent surface. *B*, stereo representation of the electron density of the  $\beta 12$ - $\beta 13$  loop seen in the wild-type PP-1c-OA complex. *C*, stereo representation of the electron density of the  $\beta 12$ - $\beta 13$  loop seen in the PP-1c-loop-OA complex. All density shown is a  $\sigma_A$ -weighted  $2F_o - F_c$  map contoured at  $1\sigma$ .

tein-inhibitor interactions (given the size of the inhibitor) and the strength of these interactions is on a par with the interactions seen in the wild-type PP-1c-okadaic acid complex (15).

The residue changes within the  $\beta 12$ - $\beta 13$  loop involve substitution of the sequence  $^{273}$ CGEFD $^{277}$  with LDVYN from cal-

calcineurin A. Because the orientation of the backbone atoms in both the wild-type and the loop mutant remains the same, this leaves side chain alterations as possible reasons for inhibitor specificity. Although these substitutions may be expected to affect overall negative charge in the  $\beta 12$ - $\beta 13$  loop of the chi-

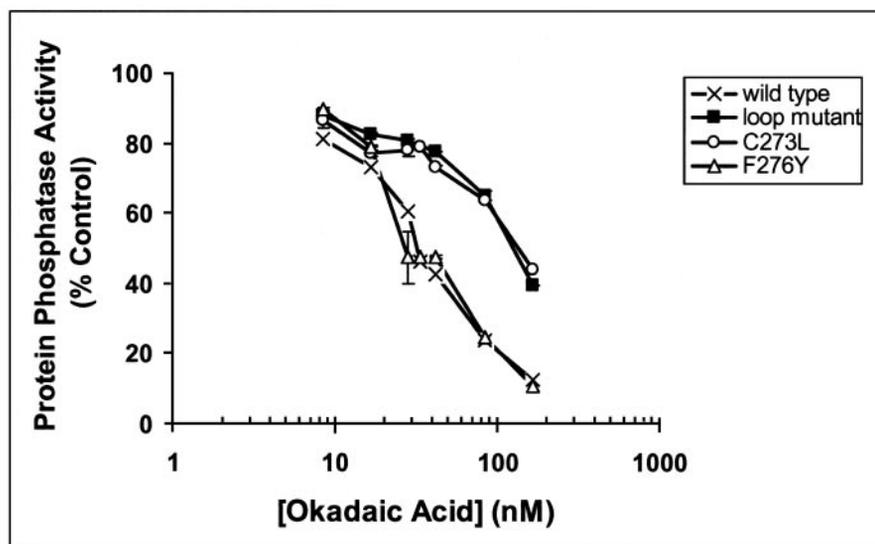
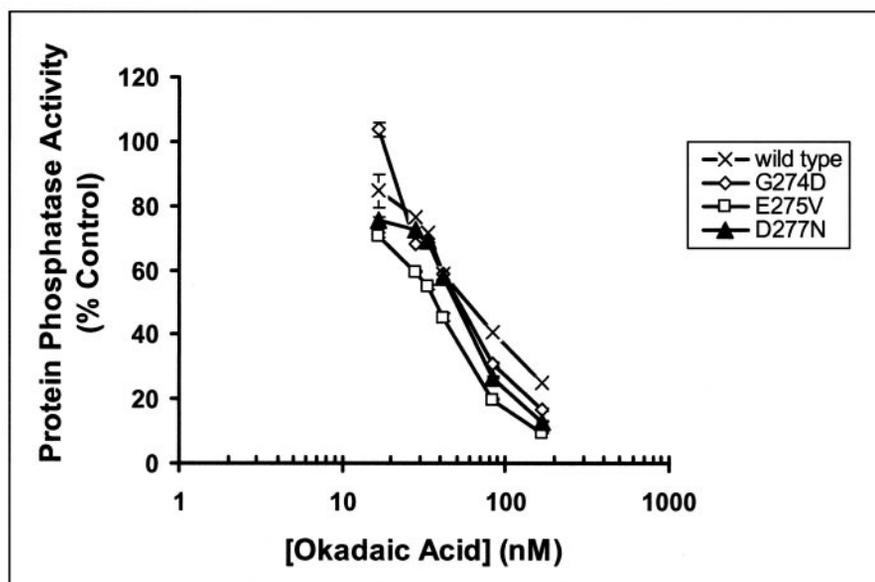


FIG. 4. Inhibition of wild-type, loop mutant, and point mutants of PP-1c by okadaic acid. The activity of recombinant wild-type, loop mutant, and point mutant PP-1c was assayed using [ $^{32}$ P]phosphorylase  $\alpha$  as a substrate, in the presence of increasing amounts of okadaic acid. The results shown are representative of at least four duplicate assays.



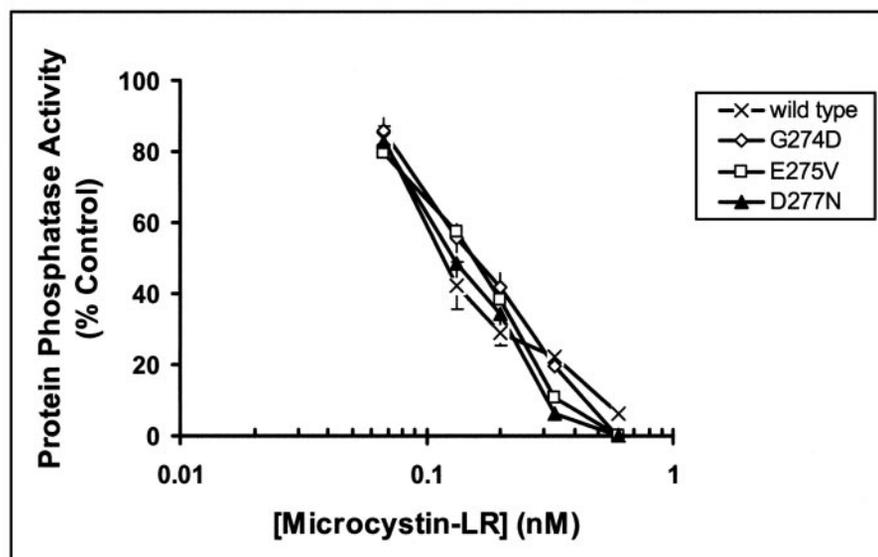
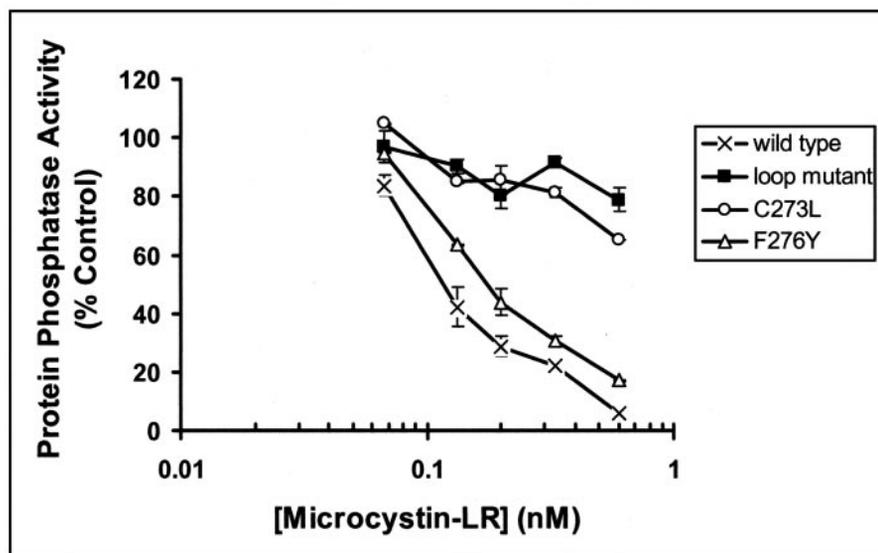
meric mutant, the two most prominent residues that have potential inhibitor interactions are Leu<sup>273</sup> and Tyr<sup>276</sup>. The leucine (Leu<sup>273</sup>) lies near a hydrophobic area of OA (C<sub>10</sub>-methyl, Fig. 2), potentially securing a hydrophobic section of the molecule. With respect to the position of Tyr<sup>276</sup>, the equivalent residue in wild-type PP-1c (Phe<sup>276</sup>) lies near the same hydrophobic section of OA, again potentially securing this area. The electron density for Phe<sup>276</sup> is very well defined in wild-type PP-1c; it has an average side chain thermal factor of 24 Å<sup>2</sup> above the average thermal factor for the molecule (Fig. 3). In the loop mutant the electron density of Tyr<sup>276</sup> is very poorly defined with an average side chain thermal factor of 31 Å<sup>2</sup> above the average thermal factor for the rest of the molecule (in comparison all four structures of calcineurin have values for the equivalent residue (Tyr<sup>315</sup>) that are maximally 19 Å<sup>2</sup> above the average thermal factor for the rest of the enzyme). The tyrosine side chain in this position would not have the potential for hydrophobic interactions that the phenylalanine in wild-type PP-1c possesses. The conclusion that hydrophobic energy drives inhibitor binding in the phosphoprotein phosphatase M family may involve binding of the hydrophobic tail (that many

of the inhibitors contain) and also a hydrophobic binding closer to the active site involving residue 276.

*Inhibitor Binding Is Partially Determined by Residues of the  $\beta$ 12– $\beta$ 13 Loop*—Our kinetic data show that the L7 loop of calcineurin A and  $\beta$ 12– $\beta$ 13 loop of PP-1c partially determine inhibitor specificity. Within this loop, there are few candidate residues that could confer this specificity. Tyr<sup>272</sup> has the greatest potential for impact because it forms the most intimate contacts with the inhibitors, hydrogen bonding with the C<sub>1</sub> acid moiety of OA, the phosphate group of calyculin A, and the acidic moiety in MCLR. Tyr<sup>272</sup> is also important in binding of calyculin A and tautomycin because the mutation Y272F causes a 200- and 400-fold decrease in sensitivity to these toxins (11). This residue is, however, conserved as a tyrosine in both PP-1c and calcineurin A. To determine the individual contributions of the remaining residues within the  $\beta$ 12– $\beta$ 13 loop toward inhibitor resistance, we undertook to mutate systematically and express the single point mutations of residues 273–277 from PP-1c to their corresponding residues in calcineurin A.

With respect to okadaic acid inhibition (Figs. 4, A and B),

FIG. 5. Inhibition of wild-type, loop mutant, and point mutants of PP-1c by microcystin-LR. The activity of recombinant wild-type, loop mutant, and point mutant PP-1c was assayed using [ $^{32}$ P]phosphorylase *a* as a substrate, in the presence of increasing amounts of microcystin-LR. The results shown are representative of at least four duplicate assays.



PP-1c G274D, E275V, F276T, and D277N were inhibited by this toxin as potently as the wild-type enzyme ( $IC_{50} = 30\text{--}50$  nM). In contrast, a single point mutant, PP-1c C273L ( $IC_{50} = 120$  nM), showed ~4-fold resistance to inhibition over wild type. This resistance was close to that exhibited by the full PP-1c loop mutant ( $IC_{50} = 110$  nM).

With respect to microcystin-LR inhibition (Fig. 5, A and B), PP-1c G274D, E275V, F276Y, and D277N mutant enzymes were again inhibited by this toxin as potently as the wild-type enzyme ( $IC_{50} = 0.1\text{--}0.2$  nM). In contrast, the single point mutant PP-1c C273L ( $IC_{50} = > 0.6$  nM) was as strongly resistant to toxin inhibition as the full PP-1c loop mutant ( $IC_{50} = > 0.6$  nM).

Unlike toxin inhibition of PP-1c mutants, the PP-1c C273L mutant was not resistant to inhibition by inhibitor-2 (Fig. 6) and this mutant, as well as PP-1c G274D, E275V, and D277N mutant enzymes, was inhibited as strongly as the wild-type enzyme ( $IC_{50} = 1\text{--}3$  nM). Only the PP-1c loop mutant showed resistance to inhibition by inhibitor-2 ( $IC_{50} = 5\text{--}6$  nM).

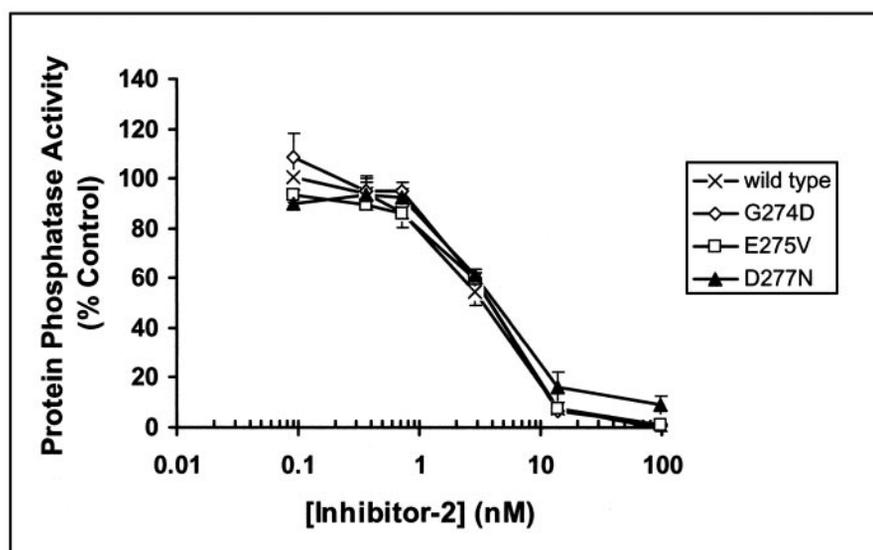
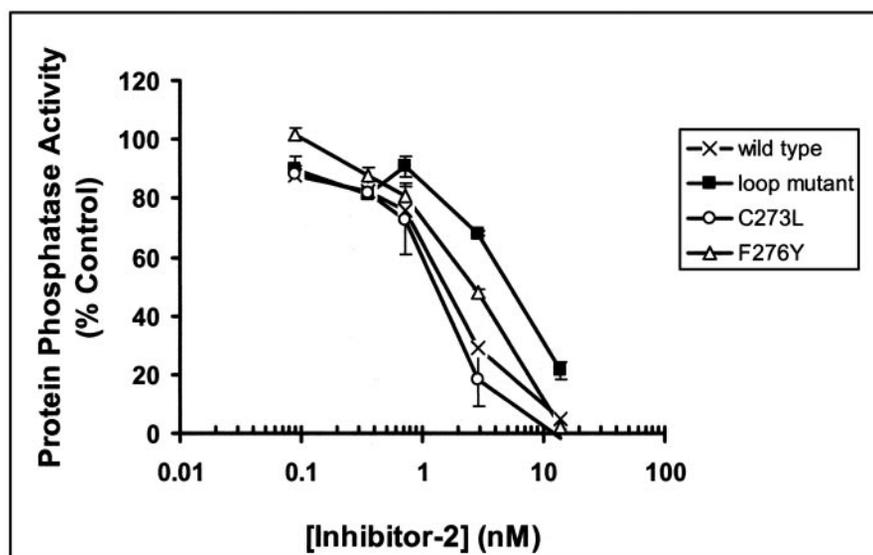
#### DISCUSSION

Protein phosphatase-1 and protein phosphatase-2B (calcineurin) share 40% sequence identity in their catalytic sub-

units. Despite the similarities in sequence, these phosphatases are widely divergent when it comes to inhibition by both endogenous proteins, such as protein phosphatase inhibitor-2, and natural product toxins, such as microcystin-LR and okadaic acid. One of the more prominent regions of non-conserved sequence between these phosphatases corresponds to the  $\beta 12\text{--}\beta 13$  loop of protein phosphatase-1, and the L7 loop of calcineurin. In the present study, mutagenesis of residues 273–277 of the  $\beta 12\text{--}\beta 13$  loop of protein phosphatase-1 to the corresponding residues in calcineurin (312–316), resulted in a chimeric mutant that showed a decrease in sensitivity to microcystin-LR, okadaic acid, and inhibitor-2.

A crystal structure of the chimeric mutant in complex with okadaic acid was determined to 2.0-Å resolution. The purpose of determining this structure was to observe directly the differences in the  $\beta 12\text{--}\beta 13$  loop structure between wild-type PP-1c and the loop mutant PP-1c, both bound to OA. This was examined in the context of the large structural changes seen in the PP-1c-MCLR complex that may have implicated structural changes in the loop as being indicative of inhibitor specificity. However, the backbone conformation of the loop (and the entire active site) is virtually unchanged in both the wild-type com-

FIG. 6. Inhibition of wild-type, loop mutant, and point mutants of PP-1c by inhibitor-2. The activity of recombinant wild-type, loop mutant, and point mutant PP-1c was assayed using [ $^{32}$ P]phosphorylase *a* as a substrate, in the presence of increasing amounts of inhibitor-2. The results shown are representative of at least four duplicate assays.



plex and the loop mutant (Fig. 7). When compared in a larger context, the loop has an extremely similar orientation in the tungstate-, OA-, and calyculin-bound forms. More strikingly, the L7 loop of calcineurin also has a virtually identical conformation to all these structures. The only anomaly is the PP-1c: MCLR structure where the covalent linkage to Cys<sup>273</sup> may affect the loop position. This supports the proposition that the primary sequence, rather than the overall  $\beta$ 12– $\beta$ 13 loop structure, determines the inhibitor specificity.

A change in  $IC_{50}$  with chimeras of the  $\beta$ 12– $\beta$ 13 loop region was reported before by Connor *et al.* (7), when they substituted the C terminus of PP-1c (residues 274 onwards) with the corresponding C terminus of PP-2Ac (7). The chimeric protein produced was not inhibited by either NIPP-1 or thiophosphorylated inhibitor-1 (neither of which inhibit PP-2A). Interestingly, the chimeric protein produced had the dose-response characteristics of PP-1 with respect to fostriecin and tautomycin, suggesting that the  $\beta$ 12– $\beta$ 13 loop region is less important for inhibition by these natural products.

We undertook to systematically mutate all residues in the  $\beta$ 12– $\beta$ 13 loop region of PP-1c to their corresponding residues in calcineurin A. The point mutants created were PP-1c C273L, G274D, G275V, F276W, and D277N. We did not mutate Tyr<sup>272</sup>

as this residue is a Tyr in calcineurin A and our structural data showed that it adopted an identical conformation in the loop mutant PP-1c-OA complex (Fig. 7). The necessity to create single mutations in the  $\beta$ 12– $\beta$ 13 loop region of PP-1c was suggested following the finding of a poorly defined electron density for Tyr<sup>276</sup> in the loop mutant crystal structure. The corresponding residue in PP-2Ac, Cys<sup>269</sup>, has already been found to be important for okadaic acid sensitivity. Mutation of Phe<sup>276</sup> to Cys in PP-1c increases the sensitivity of PP-1c to okadaic acid 40-fold, indicating that an even smaller hydrophobic residue at this position may facilitate tighter binding of okadaic acid (10).

Systematic mutation of each residue in the  $\beta$ 12– $\beta$ 13 loop of PP-1c to their corresponding calcineurin A residue showed that a single amino acid change (C273L) was the most influential in mediating sensitivity of PP-1c to the toxin inhibitors microcystin-LR and okadaic acid. Taken together, these data indicate that an individual amino acid residue substitution (C273L, as occurs in calcineurin) and not a change in the overall  $\beta$ 12– $\beta$ 13 loop conformation of PP-1c contributes to disrupting important interactions with inhibitors such as microcystin-LR and okadaic acid. MacKintosh *et al.* (18) first showed that Cys<sup>273</sup> binds covalently to microcystin-LR and determined that abolition of

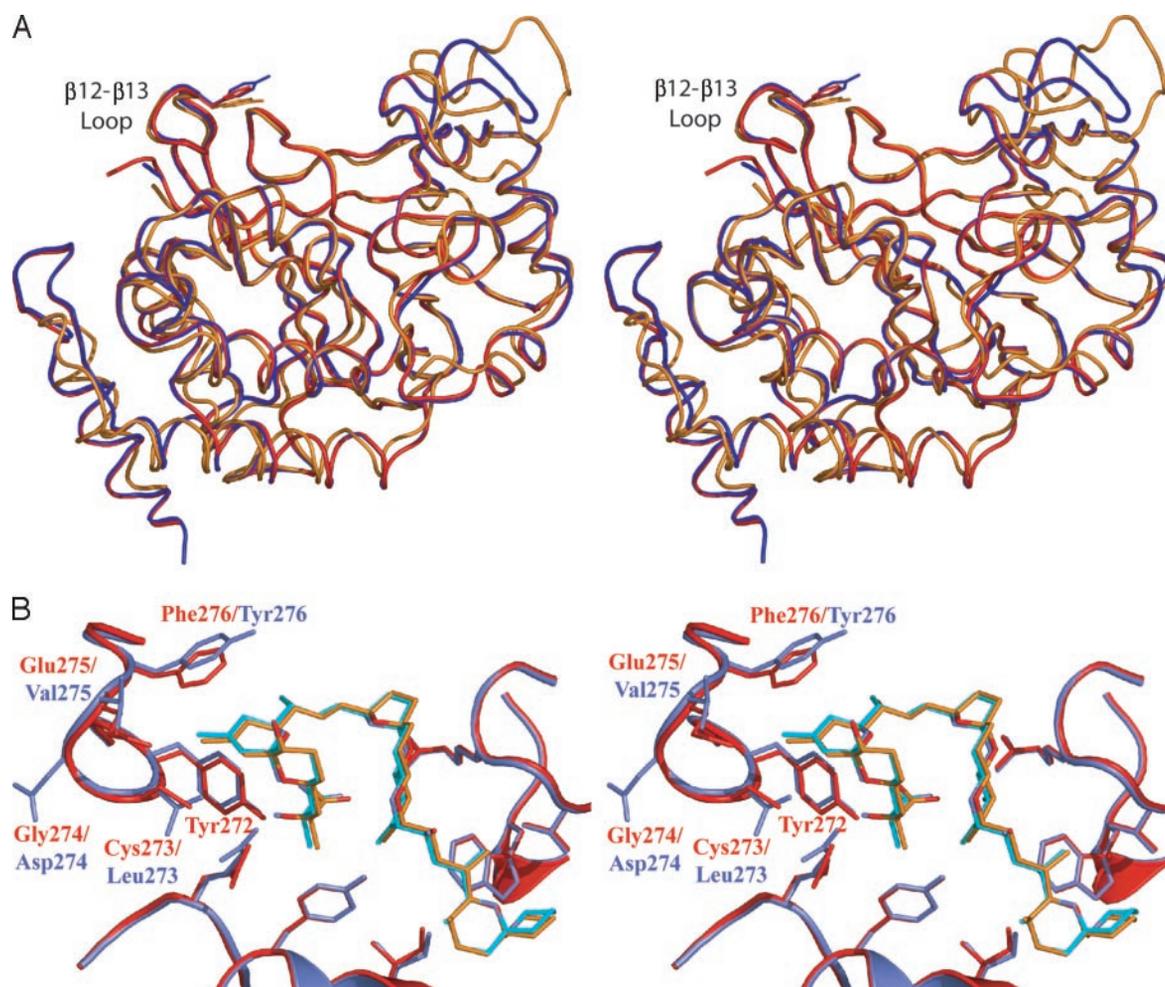


FIG. 7. A, stereo representation of the overlay between wild-type PP-1c:OA (red), PP-1c-loop:OA (blue), and calcineurin (orange). Phe<sup>276</sup> (PP-1c, or equivalent) is shown as sticks. B, alignment of PP-1c (red) bound to OA (carbon atoms colored orange) with PP-1c-loop mutant (blue) bound to OA (carbon atoms colored light blue).

covalent binding (via mutation to Ala, Ser, or Leu) increased the IC<sub>50</sub> for toxin inhibition of PP-1c by 5–20-fold. This is entirely consistent with our mutational analyses of the  $\beta$ 12– $\beta$ 13 loop presented here. However, we have also shown that covalent modification of Cys<sup>273</sup> by microcystin-LR occurs very slowly over several hours and only after the phosphatase is fully inhibited by the toxin (17, 19). Taken together, the findings of these studies suggest that covalent modification of Cys<sup>273</sup> is adventitious with respect to inhibition of the phosphatase and that this residue clearly plays a role in the initial inhibition of PP-1c by the microcystin toxins (19).

Consistent with its important role in toxin interactions, Cys<sup>273</sup> is conserved in virtually all known members of the PP-1c family. A notable exception being PP-1c from *Dictyostelium discoideum* (social amoeba) (30), where it is changed to Phe. The sensitivity to microcystin inhibition of this form of PP-1c was reported as ~2-fold less than a “mutant” containing Cys<sup>269</sup> (the equivalent residue in this organism). It is possible that Phe<sup>273</sup> is more favorable for toxin inhibition in this position than Leu<sup>273</sup> (present study and MacKintosh *et al.* (18)), however, these experiments were conducted on unpurified recombinant protein from crude *E. coli* extracts. PP-1c from several plants, including *Brassica napus*, *Arabidopsis thaliana* TOPP 8, and *Phaseolus vulgaris*, have Cys<sup>273</sup> changed to Gly (31–33). Crude extract preparations of *B. napus* PP-1c were reported to be as sensitive to inhibitors as mammalian PP-1c (34, 35), however, it will be interesting to see whether homo-

geneous preparations of highly purified PP-1c from these organisms have any resistance to microcystin inhibition.

In contrast to the role of Cys<sup>273</sup> in mediating toxin inhibition of PP-1c, replacement of Cys<sup>273</sup> with Leu did not affect inhibition of PP-1c by inhibitor-2. The only substitution that affected inhibitor-2 inhibition was F276Y, clearly indicating that the mode of inhibition of PP-1c by this endogenous inhibitor protein is distinct from that of the natural product/toxin inhibitors. The effect of substitutions in the  $\beta$ 12– $\beta$ 13 loop of PP-1c on inhibition by inhibitor-2 are difficult to interpret because the three-dimensional structure of this protein bound to PP-1c is unknown. The differences in sensitivity to the inhibitor protein 2 between wild-type PP-1c and calcineurin A are much more dramatic than the differences between wild-type PP-1c and loop mutant PP-1c, clearly indicating that the  $\beta$ 12– $\beta$ 13 loop of PP-1c is not the only region playing a role in binding of this inhibitor. Taken together, these data are consistent with the most recent models of inhibitor-2 binding (8).

The importance of the  $\beta$ 12– $\beta$ 13 loop was emphasized in a recent structure of the complex between PP-1c and a 34-kDa fragment from myosin phosphatase targeting subunit (MYPT-1) (36). This structure showed some active site remodeling with targeting subunit binding to the catalytic subunit but left the  $\beta$ 12– $\beta$ 13 loop in a prominent position to influence enzymatic activity and inhibition. Elucidation of the sequence-specific role of the  $\beta$ 12– $\beta$ 13 loop of PP-1c in mediating inhibition of this enzyme by toxins will now facilitate rational protein

engineering of calcineurin A that is sensitive to toxin inhibition. This would be an essential first step in designing active site-directed inhibitors that would be specific for calcineurin.

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## REFERENCES

- Cohen, P. (1994) *Bioessays* **16**, 583–588
- Shepeck, J. E., 2nd, Gauss, C. M., and Chamberlin, A. R. (1997) *Bioorg. Med. Chem.* **5**, 1739–1750
- Dawson, J. F., and Holmes, C. F. (1999) *Front. Biosci.* **4**, D646–D658
- Bialojan, C., and Takai, A. (1988) *Biochem. J.* **256**, 283–290
- MacKintosh, C., and MacKintosh, R. W. (1994) *Trends Biochem. Sci.* **19**, 444–448
- Watanabe, T., Huang, H. B., Horiuchi, A., da Cruze Silva, E. F., Hsieh-Wilson, L., Allen, P. B., Shenolikar, S., Greengard, P., and Nairn, A. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3080–3085
- Connor, J. H., Kleeman, T., Barik, S., Honkanen, R. E., and Shenolikar, S. (1999) *J. Biol. Chem.* **274**, 22366–22372
- Yang, J., Hurley, T. D., and DePaoli-Roach, A. A. (2000) *J. Biol. Chem.* **275**, 22635–22644
- Shima, H., Tohda, H., Aonuma, S., Nakayasu, M., DePaoli-Roach, A. A., Sugimura, T., and Nagao, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9267–9271
- Zhang, Z., Zhao, S., Long, F., Zhang, L., Bai, G., Shima, H., Nagao, M., and Lee, E. Y. (1994) *J. Biol. Chem.* **269**, 16997–17000
- Zhang, L., Zhang, Z., Long, F., and Lee, E. Y. (1996) *Biochemistry* **35**, 1606–1611
- Wei, Q., and Lee, E. Y. (1997) *Biochemistry* **36**, 7418–7424
- Egloff, M. P., Cohen, P. T., Reinemer, P., and Barford, D. (1995) *J. Mol. Biol.* **254**, 942–959
- Goldberg, J., Huang, H. B., Kwon, Y. G., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) *Nature* **376**, 745–753
- Maynes, J. T., Bateman, K. S., Cherney, M. M., Das, A. K., Luu, H. A., Holmes, C. F., and James, M. N. (2001) *J. Biol. Chem.* **276**, 44078–44082
- Kita, A., Matsunaga, S., Takai, A., Kataiwa, H., Wakimoto, T., Fusetani, N., Isobe, M., and Miki, K. (2002) *Structure (Lond.)* **10**, 715–724
- Craig, M., Luu, H. A., McCready, T. L., Williams, D., Andersen, R. J., and Holmes, C. F. (1996) *Biochem. Cell Biol.* **74**, 569–578
- MacKintosh, R. W., Dalby, K. N., Campbell, D. G., Cohen, P. T., Cohen, P., and MacKintosh, C. (1995) *FEBS Lett.* **371**, 236–240
- Holmes, C. F., Maynes, J. T., Perreault, K. R., Dawson, J. F., and James, M. N. (2002) *Curr. Med. Chem.* **9**, 1981–1989
- Dawson, J. F., Luu, H. A., Bagu, J. R., and Holmes, C. F. (2000) *Biochem. Biophys. Res. Commun.* **270**, 543–549
- McCready, T. L., Islam, B. F., Schmitz, F. J., Luu, H. A., Dawson, J. F., and Holmes, C. F. (2000) *J. Biol. Chem.* **275**, 4192–4198
- Alessi, D. R., Street, A. J., Cohen, P., and Cohen, P. T. W. (1993) *Eur. J. Biochem.* **213**, 1055–1066
- Zhang, Z. J., Bai, G., Deanszirattu, S., Browner, M. F., and Lee, E. Y. C. (1992) *J. Biol. Chem.* **267**, 1484–1490
- Otwinowski, M., and Minor, W. (1997) *Methods Enzymol.* **276**, 307–326
- Navaza, J., and Saludjian, P. (1997) *Methods Enzymol.* **276**, 581–594
- McRee, D. E. (1999) *Struct. Biol.* **125**, 156–165
- Brunger, A. T., Adams, P. D., Clore, G. M., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D* **54**, 905–921
- Hooft, R. W., Vriend, G., Sander, C., and Abola, E. E. (1996) *Nature* **381**, 272
- Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) *J. Mol. Biol.* **231**, 1049–1067
- Andrioli, L. P., Zaini, P. A., Viviani, W., and Da Silva, A. M. (2003) *Biochem. J.* **373**, 703–711
- MacKintosh, R. W., Haycox, G., Hardie, D. G., and Cohen, P. T. (1990) *FEBS Lett.* **276**, 156–160
- Lin, Q., Li, J., Smith, R. D., and Walker, J. C. (1998) *Plant Mol. Biol.* **37**, 471–481
- Zimmerlin, A., Jupe, S. C., and Bolwell, G. P. (1995) *Plant Mol. Biol.* **28**, 363–368
- MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P., and Codd, G. A. (1990) *FEBS Lett.* **264**, 187–192
- MacKintosh, C., and Cohen, P. (1989) *Biochem. J.* **262**, 335–339
- Terrak, M., Kerff, F., Langsetmo, K., Tao, T., and Dominguez, R. (2004) *Nature* **429**, 780–784