Interaction of Inhibitor-2 with the Catalytic Subunit of Type 1 Protein Phosphatase

IDENTIFICATION OF A SEQUENCE ANALOGOUS TO THE CONSENSUS TYPE 1 PROTEIN PHOSPHATASE-BINDING MOTIF*

Inhibitor-2 (I-2) is the regulatory subunit of a cytosolic type 1 Ser/Thr protein phosphatase (PP1) and potently inhibits the activity of the free catalytic subunit (CS1). Previous work from the laboratory had proposed that the interaction of I-2 with CS1 involved multiple sites (Park, I. K., and DePaoli-Roach, A. A. (1994) J. Biol. Chem. 269, 28919–28928). The present study refines the earlier analysis and arrives at a more detailed model for the interaction between I-2 and CS1. Although the NH₂-terminal I-2 regions containing residues 1–35 and 1–64 have no inhibitory activity on their own, they increase the IC₅₀ for I-2 by ~30-fold, indicating the presence of a CS1-interacting site. Based on several experimental approaches, we have also identified the sequence Lys₁₄⁴-Leu-His-Tyr¹⁴⁷ as a second site of interaction that corresponds to the RXVF motif present in many CS1-binding proteins. The peptide I-2(135–151) significantly increases the IC₅₀ for I-2 and attenuates CS1 inhibition. Replacement of Leu and Tyr with Ala abolishes the ability to counteract inhibition by I-2. The I-2(135–151) peptide, but not I-2(1–35), also antagonizes inhibition of CS1 by DARPP-32 in a pattern similar to that of I-2. Furthermore, a peptide derived from the glycogen-binding subunit, R₅/₆₄₅₆₁(61–80), which contains a consensus CS1-binding motif, completely counteracts CS1 inhibition by I-2 and DARPP-32. The NH₂-terminal 35 residues of I-2 bind to CS1 at a site that is specific for I-2, whereas the KLHY sequence interacts with CS1 at a site shared with other interacting proteins. Other results suggest the presence of yet more sites of interaction. A model is presented in which multiple “anchoring interactions” serve to position a segment of I-2 such that it sterically occludes the catalytic pocket but need not make high affinity contacts itself.

Type 1 protein phosphatases (PP1) constitute a major proportion of the cellular Ser/Thr protein phosphatases and play important roles in the regulation of many cellular functions including metabolism, hormone receptor activation, muscle contraction, cell growth and division, and gene expression (1–4). The enzymes are oligomers that consist of one of four highly homologous catalytic subunits (CS1) and different regulatory/targeting subunits (5). These latter subunits target the holoenzyme to distinct subcellular compartments in proximity to physiological substrates, confer substrate specificity, and may be involved in the regulation of enzyme activity (6–8). About two dozen CS1-binding proteins have been identified. They include the glycogen-binding subunits, R₅/₆₄₅₆₁(9, 10) G₅, (11), PTG/R₅/₆₅(12–14), and R₆ (15), which target the phosphatase to glycogen, the myosin-associating subunits, M₁₁₀ (16), NIPP-1 (17), p99/PNUTS (18, 19), and Sds22 (20), which may direct the phosphatase to the nucleus. In addition, there are cytosolic protein inhibitors, inhibitor-1 (I-1) and its brain homologue DARPP-32, and inhibitor-2 (I-2) (21, 22). Other inhibitor proteins recently identified include CPI-17 (23), HCGV/I-3 (24), and PHI-1 (25). Only one CS1-binding protein is found in any given holoenzyme, suggesting that the interaction with regulatory subunits is mutually exclusive.

I-2 associates with CS1 to form the ATP-Mg²⁺−dependent protein phosphatase whose activity is regulated by the phosphorylation of I-2 (3, 4, 26, 27, 28). I-2 has two key properties, it inhibits free CS1 and it controls the cyclic inactivation/activation of CS1 in the ATP-Mg²⁺−dependent phosphatase complex. Inhibition occurs rapidly and leaves the CS1 in its “active” conformation even though its activity is blocked. Inactivation is slower and involves conversion of CS1 to an inactive conformation. The inactive CS1-I-2 complex can be reactivated through phosphorylation of I-2 at Thr³⁷ by glycogen synthase kinase-3 (27, 28) or the extracellular-regulated kinase 2 (29). I-2 can also be phosphorylated on Ser⁸⁶ by casein kinase II. This phosphorylation alone does not activate the complex but enhances the effect of glycogen synthase kinase-3 (27, 30, 31). Studies in our laboratory have indicated that different domains of I-2 are involved in inhibition, inactivation, and reactivation of the phosphatase (30). Deletion of the NH₂-terminal 35 residues of I-2 increased the IC₅₀ by two orders of magnitude, suggesting a role in inhibition. Within this domain, residues 10–13 (IKGI) were recently implicated in inhibition (32). The region surrounding Thr³⁷ was shown to be important for the inactivation of CS1, and the COOH-terminal region of I-2 may be required for reactivation of the inactive CS1-I-2 complex (30). Mutation of these domains individually was not sufficient to disrupt complex formation.

In contrast to I-2, I-1 and DARPP-32 require phosphorylation at a threonine residue by the cAMP-dependent protein kinase for inhibitory activity (21, 22, 33). CS1 is also inhibited by a number of naturally occurring toxins, including okadaic acid, microcystin, calyceulin A, and tautomycin (34). The crystal
structure of CS1α complexed with microcystin has provided a basis to understand the mechanism by which the toxin interacts at the active site to block access of the substrates (35). Genetic and mutagenesis studies have implicated the β12–β13 loop of CS1 in inhibition by both toxins and protein inhibitors (36, 37). Thus, although structurally different, the natural toxins bind to CS1 at a site that is shared or overlaps with that of the protein inhibitors, explaining their mutual competition (38, 39).

Although there is no extensive sequence homology among the different CS1 regulatory subunits, biochemical and crystallographic studies and peptide library screening have identified a consensus (K/R)(V/I/I/K/F/W) motif that is present in a number of CS1-binding proteins (40–42), including the glycogen-, myosin-, and nuclear-targeting subunits, I-1 and DARPP-32. The crystal structure of CS1 complexed with a 13-amino acid peptide containing this motif showed that the residues RRVSF occupies a hydrophobic groove, opposite to the catalytic site, flanked by a negatively charged region that accommodates the NH2-terminal basic residues in the peptide (41). DARPP-32 and I-1 contain the sequence KIQF, similar to RVX and I-1 contain the sequence KIQF, similar to RVX and I-1 contain the sequence, peptide complex were kindly provided by Dr. David Barford (Institute of Cancer Research, London, United Kingdom).

Preparation of Recombinant CS1α and γ CS1α and γ cDNAs were obtained by reverse transcription-polymerase chain reaction. CS1α cDNA was synthesized from rabbit skeletal muscle total RNA, using SUPERScriptII Rnase H reverse transcriptase (Life Technologies, Inc.). Oligonucleotides 5′-GGCATTGTCGACATATCTGGCTCATGAACTGG-3′ were used as the 5′-primer and 3′-primer, respectively. CS1γ cDNA was amplified from rat liver total cDNA (CLONTECH), using oligonucleotides 5′-ACGGATATGCCGATATCGAAT-3′ as the 5′-primer and 5′-AGTGTGACATACTGGTCTGGC-3′ as the 3′-primer. An NdeI site was engineered at the ATG start codon in the 5′-primers, and a SsiI site was inserted after the TAG stop codon in the 3′-primers (sites are underlined). The Ala, and I-2(1–114)X147AA, in which both Leu145 and Tyr147 were replaced by Ala, were generated by overlap extension polymerase chain reaction (Invitrogen) and sequenced. The CS1α and γ cDNA fragments were then excised and inserted into the pTactac vector (48) at the NdeI and SsiI sites. Protein expression and purification were performed by modification of published procedures (49). The proteins were expressed in Escherichia coli, with apparent homogeneity, greater than 95% as judged by SDS-PAGE, with a specific activity for both CS1α and γ of 13,000–18,000 units/mg.

Preparation of I-2 Site and I-1 Site Mutants—The wild type I-2(36–204) and I-2(1–114) were purified to apparent homogeneity, greater than 95% as judged by SDS-PAGE, with a specific activity for both CS1α and γ of 13,000–18,000 units/mg. Multiple sites of interaction have recently been shown to be critical for the interaction of CS1 with I-1 as assessed by the previously reported procedure (30) as modified by Dr. Tania Barsheskey at New England Biolabs. Briefly, the heat-treated cell lysate was centrifuged at 11,000 g for 20 min at 4 °C, and the cleared extract was loaded onto a Q-Sepharose fast flow column. The fast flow Q-Sepharose and reagents for enhanced chemiluminescence were purchased from Amersham Pharmacia Biotech. Toyopearl AF-Heparin-650 M was from Tosohaas. Microcystin-LR was from Life Technologies, Inc., and okadaic acid was from Roche Molecular Biochemicals. The following peptides, RRVSFADNPFGNFLVSVEK, 1-2(1–35), AASTASHRPIKGILKNKTSST-SSRVASEQPRGSV; 1-2(135–151), KKKRQFEMKRYNKQL and CS1α was originally proposed for I-2 (30), the notion has evolved that different regulatory proteins control enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Other Materials and Methods—**Glycogen phosphorylase, phosphorylase kinase, and CS1 were purified from rabbit skeletal muscle as described previously (27, 29). The C6 c catalytic subunit of the cyclic AMP-dependent protein kinase was a generous gift from Dr. Michael Uhler (University of Michigan, Ann Arbor, MI). The polyclonal anti-CS1α antibody raised in chicken against the CS1α peptide was provided by Dr. John Lawrence (University of Virginia, Charlottesville, VA). Horseradish peroxidase-conjugated rabbit anti-chicken antibodies were purchased from Sigma. Recombinant human DARPP-32 was from Chemicon. Restriction enzymes were obtained from New England Biolabs. The fast flow Q-Sepharose and reagents for enhanced chemiluminescence were purchased from Amersham Pharmac Biotech. Toyopearl AF-Heparin-650 M was from Tosohaas. Microcystin-LR was from Life Technologies, Inc., and okadaic acid was from Roche Molecular Biochemicals. The following peptides, RRVSFADNPFGNFLVSVEK, 1-2(1–35), AASTASHRPIKGILKNKTSST-SSRVASEQPRGSV; 1-2(135–151), KKKRQFEMKRYNKQL and CS1α was originally proposed for I-2 (30), the notion has evolved that different regulatory proteins control enzyme activity.

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was then diluted with 0.1 ml of 10 mM Tris-HCl, pH 7.5, 10% glycerol and extensively dialyzed against the same buffer to remove the unreacted ATP. The phosphorylation level was determined in a parallel reaction containing [γ-32P]ATP. The stoichiometry of phosphorylation was ~1 mol/mol.

**Phosphatase Activity Assay**—Phosphatase activity was measured as described previously (30) in the presence or absence of either I-2 or DARPP-32 and in combination with various peptides. CS1 purified from rabbit skeletal muscle (29) was used in most of the studies, because, unlike recombinant CS1, it does not require Mn2+ for activity, which could interfere with the inhibition assays. Furthermore, the native but not the recombinant CS1 is inhibited effectively by I-1 (51). To determine the effect of peptides on the I-2 IC50, fixed concentrations of the peptides and fixed amounts of I-2 were preincubated with CS1 at 30 °C for 5 min. Phosphatase activity was then measured as described above. The effect of the peptides was expressed either as their ability to alter the IC50 for I-2 or by their ability to release CS1 inhibition by I-2 or DARPP-32. One unit of phosphatase phosphatase activity is defined as the amount of enzyme that releases 1 nmol of phosphate/min at 30 °C.

**CS1-I-2 Complex Formation**—CS1-I-2 complexes were formed by incubating recombinant CS1 and I-2 proteins at a 1:1.3 ratio for 40 min at room temperature. Incubation was in the presence or absence of a 100-fold molar excess of I-2 (1–35), or a 200-fold molar excess of I-2 (135–151), or a combination of the two peptides. To detect the effect of microcystin-LR on complex formation, CS1 was preincubated with a 2-fold excess of I-2 for 40 min before the addition of microcystin at a concentration equimolar to I-2. All samples were analyzed by native PAGE performed similarly to Laemmli SDS-PAGE (52) except that SDS was not included in the stacking and separating gel buffer, which was at pH 8.8. Gels were run at low voltage (100 V) with cold buffer or at 4 °C to prevent heat denaturation of the proteins.

**CS1 Overlay**—Different forms of I-2 were separated on 13% Tricine-SDS-PAGE (53) and transferred onto nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk in TBST (Tris-buffered saline with 0.05% Tween-20) for 2 h before overnight incubation with 4 μg/ml of purified recombinant CS1a at 4 °C. Membranes were then washed and incubated with a 1:2000 peptide antibody for 2 h at room temperature. After treatment with horseradish peroxidase-conjugated rabbit-anti-chicken secondary antibodies, signals were detected by enhanced chemiluminescence.

**Molecular Modeling of the KLHY Sequence onto the CS1 Structure**—The structure of CS1 complexed with the Rαβ peptide (41) was utilized as the starting point for modeling the I-2 KLHY tetrapeptide bound to CS1. The program O (54) was used to change the amino acid side chains on the Rαβ peptide to those of the I-2 peptide. A library of standard amino acid side chain rotomers was used to find the best conformation of each mutated residue within the complex. Two amino acid side chains (Leu245 and Leu289) in the catalytic subunit were also rotated using the standard library to more commonly observed side chain rotomers to prevent unfavorable contacts. No atomic contacts with distances less than 2.6 Å were found in the modeled complex.

**Determination of Protein and Peptide Concentrations**—Concentrations of CS1a and γ, DARPP-32, and I-2 polypeptides were determined by the method of Bradford (55), using bovine serum albumin as standard. Peptide concentrations based on weight were confirmed by quantitative amino acid analysis performed in the Department of Biochemistry at Purdue University, West Lafayette, Indiana on a Beckman System Gold high pressure liquid chromatography system. For this determination, the peptide samples were hydrolyzed under vacuum in 6 N HCl vapor phase at 110 °C for 22 h. The hydrolysates were then derivatized with a Waters AceQ-Fluor Reagent Kit, and the amino acids were separated on a Waters AceQ-Tag column.

**RESULTS**

**Characterization of the Inhibitory Properties of I-2 Deletion Mutants**—Previous work from our laboratory had indicated that I-2 interacts with CS1 at multiple sites, and that the NH2-terminal 35 residues were required for high potency inhibition (30). An NH2-terminally truncated I-2, I-2(36–204), exhibited an IC50 for inhibition of CS1 that was ~200-fold higher than full-length I-2 (30). In the course of the present study, a second NH2-terminally truncated protein, I-2(12–204), was fortuitously generated by proteolysis during purification of the recombinant full-length I-2 (Fig. 1B). Loss of the NH2-terminal 11 residues increased the IC50 to 7 nm, 5-fold higher than that of the intact protein (Fig. 1A), a finding consistent with a critical role for this region in inhibition. Our previous data had indicated that I-2 lacking the COOH-terminal 59 residues, I-2(1–145), had an inhibitory potency similar to that of the wild type I-2 (30) (Fig. 1A). Therefore, we generated four NH2-terminal polypeptides, I-2(1–35), I-2(1–64), I-2(1–75), and I-2(1–114). All the polypeptides were purified to >95% homogeneity as estimated by Coomassie Blue staining of SDS-PAGE (Fig. 1C). The synthetic peptide comprising residues 1–35 had no inhibitory activity at concentrations up to 200 μM, and I-2(1–64) was not inhibitory at concentrations over 100 μM (56) (Fig. 1A). I-2(1–75) exhibited a weak inhibition, with an IC50 of ~50 μm, whereas I-2(1–114) displayed high inhibitory activity with an IC50 of 10 nm, only 6-fold greater than that of the wild type I-2, which under the same conditions was ~1.5 nm (Fig. 1A). Interestingly, the phosphatase activity was not completely inhibited by I-2(1–114) even at 100 μM concentration. This result is similar to that observed with I-2(1–145) (30) (Fig. 1A), supporting the proposal of additional CS1 interacting site(s) in the COOH-terminal 59 residues. The increased inhibitory potency of I-2(1–114) as compared with I-2(1–64) also implies that the region between 64–114 contributes significantly to the inhibitory properties of I-2.

**Antagonism of CS1 Inhibition by NH2-terminal Fragments of I-2**—The results described above indicated that the NH2-terminal region of I-2 has no inhibitory activity by itself, even though analysis of the NH2-terminally deleted I-2 mutants showed that it was important for inhibition (30). To further evaluate the effect of the NH2-terminal residues on CS1 activity, we tested the ability of the I-2(1–35) and I-2(1–64) to affect CS1 inhibition by full-length I-2, I-2(1–35) at 25 μM or I-2(1–64) at 1 μM caused 35- or 16-fold increases in the I-2 IC50, respectively (56) (Fig. 2A). Consistent with this result, I-2(1–35) also antagonized inhibition of enzyme activity, measured as release of CS1 inhibition by I-2 (Fig. 2B). At a concentration of 10 μM the I-2(1–35) peptide fully released I-2 inhibition. Half-maximal release was achieved at ~1 μM peptide. These results confirm that the NH2-terminal 35 residues of I-2 contain a binding site for CS1 even though alone they are not sufficient for inhibition.

I-1 and I-2 have been reported to compete for CS1 inhibition (46), and a DARPP-32 peptide containing the KIQF sequence antagonized inhibition by I-2 (44). Interaction of I-1 and its homolog DARPP-32 with CS1 involves two subdomains, one surrounding the phosphorylated Thr47/Thr55 and the other containing the KIQF motif (43, 44). Both subdomains are located within the NH2-terminal 38 residues. Therefore, we questioned whether the I-2(1–35) peptide could also antagonize inhibition by DARPP-32. Under the conditions used, phosphorylated DARPP-32 had an IC50 of ~1 nm, a value similar to that reported by other laboratories (33, 44). As shown in Fig. 2B, I-2(1–35) was more than 100-fold less effective in releasing inhibition by DARPP-32 than by I-2 (56). As a control, an unrelated peptide, RRAAEELDSRAGPSPQL, based on the se-
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Fig. 1. Analysis of wild type and mutant I-2. A, inhibition of CS1 by wild type and mutant I-2. The indicated concentrations of various I-2 polypeptides were preincubated with 4–7 mU of rabbit skeletal muscle CS1 at 30 °C for 5 min prior to the determination of the phosphatase activity as described under "Experimental Procedures." Phosphatase activity is expressed as percent of the activity in the absence of I-2. The IC₅₀ was extrapolated from the inhibition curve. Data are presented as mean ± S.D. from three to five experiments each carried out in duplicate. B, SDS-PAGE of purified wild type and mutant I-2 polypeptides. Lane 1, wild type I-2; lane 2, I-2(12–204); lane 3, I-2(36–204), lane 4, I-2, Tricine-SDS-PAGE of purified I-2(1–114); lane 5, I-2(1–145); lane 6, I-2(1–204). Each lane contains 0.5 μg of purified protein. C, Tricine-SDS-PAGE of purified full-length and COOH-terminally truncated I-2. Lane 1, full-length I-2; lane 2, I-2(1–114); lane 3, I-2(1–75); lane 4, I-2(1–64). Each lane contains 1 μg of purified I-2.

Fig. 2. Effect of I-2(1–35) and I-2(1–64) on CS1 inhibition by I-2 or DARPP-32. A, shift of I-2 IC₅₀ by I-2(1–35) or I-2(1–64). Different amounts of I-2 were incubated with 4–6 mU of rabbit skeletal muscle CS1 in the absence (closed squares) or presence of 25 μM of I-2(21–35) peptide (open circles) or 1 μM of I-2(21–64) (open triangles) for 5 min at 30 °C prior to phosphatase activity determination. The data shown represent an average of two experiments, each carried out in duplicate. B, effect of I-2(1–35) on the release of CS1 inhibition by I-2 or DARPP-32. The indicated concentrations of I-2(21–35) peptide were incubated with CS1 in the absence (closed squares) or presence of 4 μM of I-2 (open squares) or 2 μM of phosphorylated DARPP-32 (open triangles) for 5 min at 30 °C prior to determination of the phosphatase activity. Phosphatase activity is expressed as the percent of the activity in the absence of inhibitors and peptides. Data are presented as mean ± S.D. from three independent experiments with duplicate measurements.

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observed with all the I-2 mutants analyzed (Fig. 3). The binding of CS1 was specific because no signal was detected with bovine serum albumin (Fig. 3, lane 5). Deletion of the NH₂-terminal 11 residues of I-2, which interrupted the IKGI motif after the Lys, had little effect on interaction with CS1 and caused only ~20% decrease in binding, as estimated by densitometry. Deletion of 35 residues reduced binding by 60%. Interaction of the NH₂-terminal polypeptides, I-2(21–145), I-2(1–114), I-2(1–75), and I-2(1–64) with CS1 was reduced but still significant (Fig. 3). Interestingly, in the overlay assay, I-2(1–145) showed weaker interaction, ~25%, with CS1 as compared with the wild type, even though its inhibitory potency was similar to that of the full-length I-2. An independent analysis of interactions in solution, monitoring the formation of the complexes either by native gel electrophoresis or isoelectric focusing, showed that all truncated I-2 forms associated with CS1 (data not shown). These data are consistent with those of the overlay assays. The results directly demonstrate that sites of interaction with CS1 are present in both the NH₂ and the COOH termini of I-2.

Effect of RGL(61–80) and I-2(135–151) on the Inhibition of CS1 by I-2 or DARPP-32—To determine whether I-2 interacts with CS1 at the same site as other PP1-binding proteins, we analyzed the ability of a RGL peptide (61–80), which contains the prototypical RVSF sequence, to alter I-2 or DARPP-32 inhibition of CS1. As shown in Fig. 4A, the peptide antagonized inhibition by I-2 and DARPP-32 with the same potency. Full release of inhibition was achieved at 1 μM peptide, and 50% release was attained at 0.1 μM. At 1 μM, the peptide also caused a ~25-fold increase in I-2 IC₅₀ (data not shown). As observed by Johnson et al. (40) with the RGL peptide containing residues...
63–75, the 60–81 peptide alone caused a small but consistent increase in phosphatase activity (Fig. 4A). These results support the hypothesis that I-2 shares a similar or an overlapping binding site on CS1 with RGL and DARPP-32.

It has been speculated that residues Lys\textsuperscript{135}, Lys–Arg–Gln–Phe\textsuperscript{139} in I-2 resemble the RKKIQF motif in DARPP-32 (44). However, close scrutiny of the I-2 sequence revealed another region, Lys\textsuperscript{142}–Arg–Lys–Leu–His–Tyr–Asn\textsuperscript{148}, that we believe shows closer similarity to the consensus motif. A synthetic peptide, I-2(135–151), containing both candidate sequences, released CS1 inhibition by either I-2 or DARPP-32 in a very similar pattern (56) (Fig. 4B). Maximal effect was observed at 25 \( \mu \)M and 50% release was attained at ~7 \( \mu \)M. Similarly to the RGL peptide, I-2(135–151) caused a ~20% increase in phosphatase activity at concentrations up to 10 \( \mu \)M and became inhibitory at higher concentrations. This inhibitory effect may account for the apparent inability to completely relieve the inhibition by I-2 or DARPP-32. The I-2(135–151) peptide at 25 \( \mu \)M also increased the IC\textsubscript{50} for I-2 by ~5-fold (data not shown). These results suggest that the region between 135–151 in I-2 may interact with CS1 at a site that is shared with other CS1-binding proteins.

We have previously shown that mutations of Thr\textsuperscript{72} and Ser\textsuperscript{86} had no effect on the inhibitory or inactivating properties of I-2, even though activation of the CS1-I-2 complex was altered. However, deletion of residues 76–85 or substitution of amino acids 77–81 to Pro resulted in incomplete inactivation of the complex, without significantly affecting inhibitory potency (30). To further address the role of this region of I-2, a synthetic peptide comprising residues 70–93 was tested for its ability to antagonize inhibition by I-2. The peptide either by itself or in combination with I-2 had no effect on CS1 activity (data not shown).

Identification of KLHY as a CS1 Binding Motif in I-2— Either of the two sequences KRQF or KLHY in the I-2(135–151) peptide could account for the observed antagonism of I-2 inhibition. Three approaches were taken to identify the residues involved. Taking advantage of the presence of an intervening methionine (Met\textsuperscript{142}) in I-2(135–151), the peptide was cleaved with CNBr to generate two short peptide fragments. One, I-2(135–141), contained the KRQF sequence and the other, I-2(142–151), the KLHY sequence. The I-2(142–151) peptide but not I-2(135–141) retained the ability to release I-2 inhibition, albeit to a lesser extent as compared with the full-length peptide (data not shown).

Further support for the thesis that KLHY and not KRQF is a CS1-binding site was gained by introducing point mutations in full-length I-2. Consistent with the analysis of the I-2(135–151) peptide, mutation of Phe\textsuperscript{139} to Ala in F139A mutant peptide had no effect on the inhibitory or inactivating properties of I-2, even though activation of the CS1-I-2 complex was altered. However, deletion of residues 76–85 or substitution of amino acids 77–81 to Pro resulted in incomplete inactivation of the complex, without significantly affecting inhibitory potency (30). To further address the role of this region of I-2, a synthetic peptide comprising residues 70–93 was tested for its ability to antagonize inhibition by I-2. The peptide either by itself or in combination with I-2 had no effect on CS1 activity (data not shown).

The second approach utilized two synthetic mutant peptides: I-2(135–151)L145A/Y147A, where both Leu\textsuperscript{145} and Tyr\textsuperscript{147} were substituted by Ala, and I-2(135–151)F139A, in which Phe\textsuperscript{139} was replaced by Ala. The F139A mutant peptide behaved similarly to the wild type peptide (Fig. 4C) and caused maximal release of CS1 inhibition by both I-2 and DARPP-32 at ~25 \( \mu \)M. The concentration required for half-maximal effect by the F139A or the wild type peptide was very similar, ~8–10 \( \mu \)M. The F139A mutant peptide alone also caused a small but significant activation at concentrations below 10 \( \mu \)M, similar to what was observed with the wild type peptide (Fig. 4, B and C). In contrast, the L145A/Y147A mutant peptide did not antagonize CS1 inhibition by either I-2 or DARPP-32, and by itself it did not activate (Fig. 4D). These data support the proposal that Leu\textsuperscript{145} and Tyr\textsuperscript{147} are involved in the interaction with CS1.

Further support for the thesis that KLHY and not KRQF is a CS1-binding site was gained by introducing point mutations in full-length I-2. Consistent with the analysis of the I-2(135–151) peptide, mutation of Phe\textsuperscript{139} to Ala in F139A mutant peptide had no effect on the inhibitory or inactivating properties of I-2, even though activation of the CS1-I-2 complex was altered.
Fig. 5. Inhibitory activities of I-2(F139A) and I-2(L145A/Y147A). Different amounts of wild type I-2 (squares), I-2(F139A) (circles), or I-2(L145A/Y147A) (triangles) were incubated with -4 mU of rabbit skeletal muscle CS1 at 30 °C for 5 min, prior to the phosphatase activity determination. Data shown are the average from two experiments with duplicate measurements.

Fig. 6. Molecular modeling of the KLHY interaction with CS1. KLHY was modeled onto the RVSF position in the crystal structure of CS1 and RGL RVSF peptide complex. Top, ribbon diagram of the CS1 and stick representation of the RGL peptide. The active site is indicated by the arrow. Bottom, space filling model showing the interaction of RVSF (right, dark) or KLHY (left, dark) with a portion of CS1 (gray). Leu would not be favorable at position 2 due to its bulky side chain. However, modeling of a bound KLHY tetrapeptide molecule using the coordinates from the CS1 and RGL peptide complex showed that the KLHY sequence made interactions with CS1 similar to the RGL peptide (Fig. 6). The side chain of Lys forms an ion pair comparable to Arg. The side chain of His can form hydrogen bonds with the carbonyl oxygen of Thr288 on CS1 through a water molecule, as is seen for Ser67 in the RGL peptide. In contrast to the predictions (41), our model, Leu makes favorable Van der Waal’s contacts with the side chains of Leu243, Ile289, and Leu289 in the CS1 molecule, which create the hydrophobic pocket. In fact, it appears that Leu fits more completely in this hydrophobic pocket and contacts more hydrophobic surface area than Val in the RGL peptide. The aromatic ring of Tyr maintains the same hydrophobic contact with Phe257 in CS1, as is seen for Phe in the RGL peptide. Furthermore, the hydroxyl group of Tyr is hydrogen-bonded (3.3 Å) to the carbonyl oxygen of Arg63 in the CS1 molecule. Preceding the KLHY sequence are two basic residues, another feature of the consensus PP1-binding motif. These residues may further interact with the adjacent acidic channel and thus strengthen the interaction at this site, as proposed by Egloff et al. (41). On the other hand, the Arg in the KRQF is too big to fit into the Val position without disturbing the structure of the peptide or CS1. Also, replacing an aliphatic residue with Arg would obviously weaken the hydrophobic interaction.

Effect of Microcystin on CS1/I-2 Complex Formation—Studies from several laboratories (36, 37, 39), including our own (38), have indicated that the protein inhibitors of PP1 including I-1, DARPP-32, and I-2 share a site of interaction on CS1 with small molecule toxins, such as microcystin and okadaic acid, located on the β12-β13 loop. Utilizing native PAGE, we showed direct competition between microcystin and I-2 for binding to recombinant CS1 (Fig. 7A). Microcystin at a concentration equimolar with I-2 impaired the formation of the CS1-I-2 complex (Fig. 7A, lanes 2 and 3), and disrupted the pre-formed complex (Fig. 7A, lane 4). Note that, for reasons that are not understood, free CS1 does not enter the native gel. Neither decreasing the concentration of acrylamide or bisacrylamide nor inverting the polarity of the current allowed CS1 to enter the gel (data not shown). Incubation of CS1 with β-octylglucoside or a chaotropic salt, such as lithium chloride, was also ineffective.

Effect of the RGL and I-2 Peptides on the Inhibition of CS1 by Okadaic Acid—The notion that okadaic acid and I-2 bind to CS1 at a shared site is supported by fluorescence anisotropy (38) as well as biochemical (39) and mutagenesis studies (36). To examine whether either the NH2-terminal 35 residues or the 135–151 region of I-2 interacted with CS1 at the same site as okadaic acid, peptide competition assays were conducted. None of the peptides tested, I-2(1–35), I-2(135–151), I-2(70–93), or RGL(60–81), antagonized inhibition by okadaic acid, even at concentrations significantly higher than those effective for competition with I-2 (Fig. 7B). These results suggest that a distinct region of I-2 may be involved in the competition with okadaic acid implying that I-2 interacts with CS1 at an additional site.

Competition of I-2(135–151) and RGL(61–80) Peptides for CS1 Inhibition by I-2(1–114) and Complex Formation—The observation that I-2(1–114) was an effective inhibitor (IC50 of 10 nM) is intriguing, since the KLHY motif lies COOH-terminal to residue 114. Competition assays showed that I-2-(135–151) and RGL(61–80) did not impair the formation of the CS1-I-2 complex (Fig. 7A, lane 3). Microcystin at a concentration equimolar with I-2 impaired the formation of the CS1-I-2 complex (Fig. 7A, lane 4). I-2(135–151) by itself was less effective (Fig. 9A, lane 3), but in combination with I-2(1–35), the complex formation was reduced by 60% (Fig. 9A, lane 5). However, neither I-2(1–35), I-2(135–151), nor the combination of both peptides disrupted the complex formed between CS1 and the full-length I-2 (Fig. 9B), consistent with the existence of additional interacting sites in the COOH-terminal 114–204 region of I-2.

DISCUSSION

I-2 was initially identified as one of two heat stable protein inhibitors of PP1, although it subsequently became clear that I-2 is a regulatory subunit of the ATP-Mg2+-dependent phosphatase, whose activity state is modulated by phosphorylation of I-2. Previous work from our laboratory had suggested the
are consistent with a model in which the NH2-terminal 35 motif, common to many CS1-binding proteins (41). Our results suggest that it is not a radius (3.5 nm), stability to heat and acid treatment, and the low content of hydrophobic amino acids, which may point to the presence of three or more other contacts shared by other CS1-binding proteins. Several lines of evidence also point to the presence of three or more other contacts shared by other CS1-binding proteins. The NH2-terminal region, subdomain 1, contains the IKGI sequence (32) and binds to site A. The region comprised of residues 135–151, subdomain 3, contains the KLHY motif and binds to site C. Residues within the KLHY sequence interact with CS1 at a site that is specific for I-2, whereas the KLHY sequence interacts with CS1 at a site that is specific for I-2. One site may be located within residues 59–64 of I-2, another in the COOH-terminal 59 amino acids of I-2, and an additional site may also be present around Trp46, corresponding to Phe33 in the KLHY motif, common to many CS1-binding proteins (41). Our results are consistent with a model in which the NH2-terminal 35 residues of I-2 bind to CS1 at a site that is specific for I-2, whereas the KLHY sequence interacts with CS1 at a site shared by other CS1-binding proteins. Several lines of evidence also point to the presence of three or more other contacts between CS1 and I-2. One site may be located within residues 64–114, another in the COOH-terminal 59 amino acids of I-2, and an additional site may also be present around Trp46, corresponding to Phe33 in the Drosophila I-2 (57). Mutation of Trp46 to Ala in rabbit I-2 resulted in a 10-fold increase in the IC50 (data not shown).

Taking into account these and previous studies, we propose a model for the complex interaction between I-2 and CS1 as depicted in Fig. 10. The physical properties of I-2, including the low sedimentation coefficient (s20,w = 1.75 S) and large Stokes radius (3.5 nm), stability to heat and acid treatment, and the low content of hydrophobic amino acids, suggest that it is not a globular protein (58). Circular dichroism studies indicate that I-2 has ∼50% random coil structure, which could correlate with an elongated shape. An extended conformation would provide a structural basis for a model in which I-2 wraps around CS1 to establish multiple contacts. The NH2-terminal region, subdomain 1, contains the IKGI sequence (32) and binds to site A. The region comprised of residues 135–151, subdomain 3, contains the KLHY motif and binds to site C. Residues within positions 64–114, subdomain 2, interact with site B, and a region near the COOH terminus, subdomain 4, may bind to site

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FIG. 10. Model of interaction between I-2 and CS1. A schematic diagram of the regions of I-2 that interact with CS1 is shown in the upper part. The IKGI and the KLHY motifs are indicated as subdomains 1 and 3, respectively. Subdomains 5, 2, and 4 denote, respectively, the region of I-2 comprising Trp46, residues 64–114, and a site in the COOH terminus of I-2. The lower part shows a cartoon of I-2 (thick line) wrapped around the CS1. The interacting sites on CS1 are indicated by capital letters A, B, C, D, and E. Interaction of I-2 with CS1 positions the masking region in front of the active site (*), thus blocking the access of substrates. Sites A and C are located at the back of the active site and indicated the binding sites for IKGI and the KLHY, respectively. Site B may correspond to the β12-β13 loop of CS1. Site D is a site that may interact with the COOH terminus of I-2, and site E indicates the Trp46 binding site. Weakening of any of the interactions renders the masking region more mobile thus allowing access of substrates to the active site.

D. Subdomain 5, which surrounds Trp46 may interact at a distinct site, site E. Sites A and C are both located on CS1 opposite to the active site (41, 59). Site C, the site shared with other CS1-binding proteins, is characterized by a hydrophobic channel and adjacent acidic residues. Site A is unique for I-2 binding, is characterized by a cluster of charged amino acids (59), and may be the site where the first I-2 contact is established (30). Although we have no direct evidence, we speculate that site B may be the β12-β13 loop. Based on our model, it is unlikely that the Trp46 subdomain 5 binds to site B or C, because the I-2 (1–64) is not inhibitory. The finding that the Drosophila I-2 F33A mutant (equivalent to the rabbit W46A mutant) is more sensitive to antagonism by peptides containing the canonical RXXF sequence, as compared with wild type I-2 (57), cannot be taken as proof that the PhexThr35 region interacts at the common CS1 site. It is equally possible that the mutation affects another contact whose disruption weakens the interaction with CS1, so that binding of the peptide to site C more readily releases inhibition. More importantly, it has not been shown that peptides encompassing PhexThr35-Trp46 can antagonize other CS1-binding proteins that have the consensus motif. Thus, we favor positioning the Trp46 subdomain 5 as interacting at a separate site, site E.

We propose that interactions at sites A, B, and C serve as anchors to bring a "masking region" close to the active site, thus blocking access to substrates. This mechanism is reminiscent of the inhibition of calcineurin by the FKBP12-FK506 complex, which does not inhibit the phosphatase by direct interaction with the active site. Instead, the FKBP12-FK506 complex interacts with calcineurin such that access of the substrate to the catalytic pocket is physically hindered (60). An important feature of our model is that the anchoring sites on I-2 are important to achieve inhibition but the primary sequence in the masking region is relatively less important, since it would not contact the catalytic site directly. This explains why mutations in this region, between Thr72 and Ser86, do not alter the inhibitory potency of I-2 (30, 31). The lack of inhibitory activity for the NH2-terminal peptides, I-2(1–35) and I-2(1–64), can be explained by the absence of an anchor in their COOH termini. The reduced potency of the NH2-terminally truncated I-2 polypeptides would result from the removal of the strong NH2-terminal anchor, IKGI. Competition with peptides at any of the anchoring sites will destabilize the CS1-I-2 complex making it easier to dislodge the masking region and allow partial access to the catalytic pocket. The anchoring mechanism can also account for the ability of several truncated I-2 mutants still to interact with CS1, albeit with much lower affinity than wild-type I-2 (Fig. 3).

In the ATP-Mg2+-dependent protein phosphatase, activation of CS1 by I-2 involves the conversion of the inactive CS1 to an active state following phosphorylation of I-2 at Thr72 (61). This event induces conformational changes in I-2 (38) that may loosen one or more of the anchors such that the masking region moves and no longer occludes the catalytic site. However, since there is no dissociation of the complex, some anchoring interactions must persist after phosphorylation. As dephosphorylation of Thr72 is required for activity toward exogenous substrates (61), our model positions Thr72 in front of the active site, but not necessarily in direct contact with CS1. In fact, an I-2 peptide containing residues 70–93 was not inhibitory and did not antagonize inhibition by I-2, suggesting that this region does not directly contribute greatly to CS1 binding. Previous work had shown that I-2(1–145) had the same inhibitory potency as wild type I-2 and formed a complex with CS1 that was not activated by glycosgen synthase kinase-3 (30). The retention of the basic and the Leu residues in subdomain 3 may be sufficient for some degree of interaction at site C, which is further strengthened by the anchors in subdomains 1 and 2. These contacts, by positioning the masking domain in front of the catalytic pocket, could account for the similar inhibitory potencies of I-2(1–145) and wild type I-2. The weaker binding to CS1, as detected in the overlay assay, may be due to faster on- and off rates, caused by the absence of the binding site in the COOH-terminal domain. As to the failure of glycosgen synthase kinase-3 to activate the CS1-I-2(1–145) complex, it is possible that the conformational changes required for activation do not occur without full interaction with the KLHY sequence and/or with subdomain 4. It should be noted, however, that the CS1 was in the inactive form, because treatment with trypsin alone did not result in activation (30).

The much greater inhibitory potency of I-2(1–114) as compared with I-2(1–64) implies that there are at least two anchors within residues 1–114 that effectively position the masking region. One is the NH2-terminal IKGI sequence and the other is most likely located within residues 64–114. The ability of I-2(135–151) and the BGLP(61–80) peptides to antagonize inhibition by I-2(1–114) (Fig. 8) suggested that one of the anchors in the I-114 region interacts with CS1 at a site that is close to or overlapping with site C. Indeed, recent work (59) has shown that mutation of CS1 residues near site C decreases the sensitivity to inhibition by I-2, but not by I-1, indicating that the I-2-specific site A is close to site C. The proximity of sites A and C may allow for binding at one site to affect interaction at the other. This could explain why the I-2(135–151) peptide attenuates inhibition by I-2(1–114) and the weak antagonizing effect of the I-2(1–35) peptide on DARPP-32 inhibition (Fig. 2B). However, the presence of subdomain 1 alone cannot account for the inhibitory potency of I-2(1–114) because I-2(1–64), which contains the same region, is not inhibitory. An additional site must be present between residues 64 and 114.

The molecular basis for CS1 inhibition by I-2 is different from that by DARPP-32 or I-1, which do not form stable complexes with the catalytic subunit. However, although the three inhibitors do not share obvious primary structure homology, they are mutually exclusive for inhibition of CS1 indicating that they share common binding site(s). Inhibition by DARPP-32/I-1 involves the interaction of phosphothreonine-34/35 di-
rectly with the active site (43, 44), whereas inhibition by I-2 does not involve a similar interaction of the phosphorylated Thr72. Nevertheless, all these inhibitors make multiple contacts with CS1, and they share at least two binding sites, one where the KLHY sequence in I-2 and the KIQF sequence in I-2 binds and the other on the KLHY sequence in I-2 and the KIQF sequence in I-1. The regions of the inhibitors that interact with the β12-β13 loop (residues 267–282), where the toxins also bind (35–37), the regions of the inhibitors that interact with CS1, and as determined by the overlay assay, is much weaker as compared with the full-length I-2 (Fig. 3), but the IC50 is similar to that of the wild type I-2 (Fig. 1A). Our model for interaction of I-2 with CS1 readily accommodates uncoupling of binding and inhibition. A single site is sufficient for binding but not for inhibition, which appears to require interaction at two or more sites straddling the masking region. Furthermore, as suggested by the peptide competition and the CS1 overlay studies, interaction at the individual sites may be of low affinity but together create high affinity for CS1.

Huang et al. (32) reported that a peptide containing the KLHY sequence of I-2 did not compete for inhibition of CS1 by DARPP32, but increased the IC50 for I-2 by 3-fold. Although these results are in apparent disagreement with ours, which show that a similar peptide antagonized inhibition by DARPP-32 and I-2 with the same potency, other data in the same report (32) implied the presence of a CS1-binding site in the region comprising Lys144-Leu-His-Tyr147. Notably, CS1 could bind to I-2(1–160) but not to I-2(1–145). Furthermore, the 5-fold increase in the IC50 for I-2 that we observed in the presence of I-2(135–151) is similar to effects reported by Huang et al. (32) using the DARPP-32(6–38) or the M110-(1–40) peptides, both of which contain the canonical RVXF motif. Additional evidence that the KLHY sequence is analogous to the consensus CS1-binding motif comes from our finding that the I-2(135–151) peptide also attenuated inhibition of CS1 by Glic8p (data not shown), the yeast homolog of I-2. Most importantly, a peptide in which the Leu145 and Tyr147 were replaced by Ala completely lost the ability to antagonize inhibition of CS1 by both I-2 and DARPP-32 (Fig. 4D), as well as by Glic8p (data not shown). Finally, modeling KLHY onto the structure of RVSF complexed with CS1 showed that the KLHY can be readily accommodated in the CS1 binding pocket, contrary to the predictions that a Leu at the position of the Val or Ile would not be favorable (41).

Not all CS1-binding consensus motifs need to bind with equal affinity to site C. A peptide containing KSVTW in p99 showed —100-fold lower potency in antagonizing p99 inhibition of CS1, compared with a p53BP2 peptide, which harbored the RVKF sequence (18). However, substitution of Trp with Phe in the p99 peptide did not increase its ability to release p9 inhibition, supporting the idea that the affinity of the binding protein for CS1 is not determined solely by the conserved residues. Similarly, the lower efficacy of the I-2(135–151) peptide to affect the IC50 for I-2 as compared with the RGL(61–80) peptide indicates that binding of the KLHY sequence by itself to CS1 is not very strong. When Leu145 and Tyr147 were mutated to Ala in full-length I-2 a more potent inhibitor was created, with 4-fold lower IC50. A similar, ∼2-fold higher potency was also observed by Huang et al. (32) for the I-2/Y147A mutant. The reason that the Ala substitution renders I-2 a more powerful inhibitor is not completely clear. However, RGL peptides containing the RVKF sequence as well as the I-2(135–151) peptide by themselves slightly activate CS1. One possibility is that interaction at the C site, which is behind the catalytic pocket, affects the conformation of the active site to cause a small activation. Thus, loss of this interaction paradoxically leads to a more potent inhibitor.

In conclusion, it is clear that the interaction of I-2 with CS1 involves multiple sites, some of which are specific for I-2, such as site A where the NH2-terminal subdomain 1 binds, whereas others are shared with other CS1-binding proteins. This implies that interaction of the various CS1-binding proteins with CS1 at shared as well as unique sites may allow for different modes of regulation of the large number of PP1 holoenzymes and therefore their involvement in diverse cellular functions. The experiments presented support the hypothesis that the KLHY is the I-2 homolog of the RVXF motif. These findings expand the degeneracy of the consensus sequence to (R/K)(V/I/L)(X/F/W/Y) to consist of a branched aliphatic residue at position 2 and an aromatic residue at position 4. This more degenerate motif will be important for the identification and analysis of new CS1-binding proteins. However, final definition of the detailed interaction between I-2 and CS1 will have to await the determination of the three-dimensional structure of the complex.

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