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Multiple Interactions within the AKAP220 Signaling Complex Contribute to Protein Phosphatase 1 Regulation*

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The phosphorylation status of cellular proteins is controlled by the opposing actions of protein kinases and phosphatases. Compartmentalization of these enzymes is critical for spatial and temporal control of these phosphorylation/dephosphorylation events. We previously reported that a 220-kDa A-kinase anchoring protein (AKAP220) coordinates the location of the cAMP-dependent protein kinase (PKA) and the type 1 protein phosphatase catalytic subunit (PP1c) (Schillace, R. V., and Scott, J. D. (1999) *Curr. Biol.* 9, 321–324). We now demonstrate that an AKAP220 fragment is a competitive inhibitor of PP1c activity (K_i = 2.9 ± 0.7 μM). Mapping studies and activity measurements indicate that several protein-protein interactions act synergistically to inhibit PP1. A consensus targeting motif, between residues 1195 and 1198 (Lys-Val-Gln-Phe), binds but does not affect enzyme activity, whereas determinants between residues 1711 and 1901 inhibit the phosphatase. Analysis of truncated PP1c and chimeric PP1/2A catalytic subunits suggests that AKAP220 inhibits the phosphatase in a manner distinct from all known PP1 inhibitors and toxins. Intermolecular interactions within the AKAP220 signaling complex further contribute to PP1 inhibition as addition of the PKA regulatory subunit (RII) enhances phosphatase inhibition. These experiments indicate that regulation of PP1 activity by AKAP220 involves a complex network of intra- and intermolecular interactions.

Extracellular signals conveyed by hormones and neurotransmitters are often relayed to precise intracellular sites through the generation of soluble second messengers such as calcium, phospholipid, or cAMP (1–3). Frequently, the targets for these molecules are second messenger-regulated protein kinases and phosphatases, which, in turn, catalyze changes in the phosphorylation status of key cellular proteins (4). Prototypic examples are the cAMP-dependent protein kinase (PKA)[1] and the type 1 protein phosphatase (PP1), both of which are broad specificity enzymes with ubiquitous patterns of expression. Accumulating evidence now suggests that subcellular location is a key factor in determining the substrate specificity of both enzyme classes (5, 6). In fact, families of anchoring and targeting proteins have been identified that tether PKA or PP1 to precise intracellular sites (7, 8).

The tetrameric PKA holoenzyme consists of a regulatory subunit dimer (R) and two inactive catalytic subunits (C) (9). Upon elevation of intracellular cAMP, the C subunit is released and becomes free to phosphorylate cellular proteins in that vicinity. The location of the PKA holoenzyme within the cell is controlled by high affinity protein-protein interactions between the R subunit dimer and A-kinase anchoring proteins (AKAPs) (10, 11). Over 40 AKAPs have been identified to date, which localize PKA and other enzymes to a variety of cellular membranes and distinct intracellular compartments (6, 12). Compartmentalized pools of kinase are maintained within the vicinity of activating elements such as G proteins and transmembrane receptors and in close proximity to selected substrates such as ion channels, mitochondrial proteins, cytoskeletal components, and cytoplasmic enzymes (13–19). This sophisticated level of molecular organization ensures selectivity in cAMP-responsive events (20).

PP1 location is controlled in a similar manner. The catalytic subunit of the phosphatase, PP1c, associates with numerous targeting subunits (21–23). Early examples are the G_M and liver glycogen-targeting subunit proteins, which target PP1 to skeletal muscle and glycogen particles, respectively. However, additional targeting subunits have been identified such as NIPP-1, p53-binding protein-2, PP1 nuclear-targeting subunit, which direct PP1 to the nucleus, whereas spinophillin attaches the phosphatase to the actin cytoskeleton at postsynaptic sites in neurons (22, 24–34). A common characteristic of this diverse protein family is their modular design that includes the presence of a consensus phosphatase-targeting motif that recognizes PP1c (35). The PP1c-targeting motif contains the sequence (Lys/Arg)-(Ile/Val)-Xaa-Phe (where Xaa is any amino acid) and has been shown to interact directly with a binding pocket on the surface of PP1c (35). This "KVXF" motif is often

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchoring protein; PP1, type 1 protein phosphatase; R, protein kinase A regulatory subunit; C, protein kinase A catalytic subunit; G_M, muscle glycogen-targeting subunit; NIPP-1, nuclear inhibitor of PP1; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; CRHM2, a chimera of PP1-(1–273) and PP2A-(267–309); DARPP-32, dopamine- and cAMP-regulated phosphoprotein of apparent M_r 32,000; NMDA, *N*-methyl-D-aspartic acid; oligos, oligonucleotides; FPLC, fast protein liquid chromatography; PCR, polymerase chain reaction.

considered a hallmark of PP1-targeting subunits, although several recent studies have suggested that there can be considerable degeneracy in the sequence (35–37). This has led to the proposal that other binding surfaces on targeting subunits and phosphatase inhibitor proteins contact PP1c (38–40).

Several multivalent anchoring proteins have been identified that can simultaneously associate with kinases and phosphatases (41). The first example was the neuronal anchoring protein AKAP79 which maintains a signaling scaffold of PKA, protein kinase C, and the calcium-calmodulin-dependent phosphatase PP-2B (41–43). However, certain PP1-targeting subunits serve this function also. For example, the protein targeted to glycogen maintains a signaling scaffold of PP1 and several enzymes involved in glycogen metabolism (44). AKAP149 recruits the PKA holoenzyme and PP1c to the lamina of nuclear membranes (45). Likewise, the NMDA receptor-associated protein Yotiao maintains an anchored PKA holoenzyme and constitutively active PP1c to regulate tightly the phosphorylation status and activity of the NMDA receptor ion channel (36). In both cases, these more sophisticated mechanisms of phosphatase tethering sequester the PP1c with physiologically relevant substrates (12). Another example is the vesicular anchoring protein AKAP220, which we recently showed was capable of anchoring PKA and tethering PP1c (37, 46).

In this report we present evidence to suggest that PP1 targeting by AKAP220 involves a consensus KV*X*F motif and additional binding determinants located in the C-terminal region of the anchoring protein. We demonstrate that AKAP220 is a competitive inhibitor of PP1 activity. Analysis of a truncated and a chimeric PP1 enzyme suggests that phosphatase inhibition occurs by a mechanism distinct from many known PP1 inhibitors. Most remarkably, the ability of AKAP220 to function as an inhibitor of PP1 was further enhanced by anchoring of the R subunit of PKA. These experiments provide evidence for an additional level of control whereby the RII-AKAP220 interaction augments the down-regulation of PP1 activity.

MATERIALS AND METHODS

Generation of AKAP220 Fragments—Fragments of AKAP220 were constructed using restriction enzyme digest and PCR. Numbering of the AKAP220 fragments is based upon the amino acid sequence of the human ortholog (47). Fragment 910–1901 was subcloned using *Eco*RI restriction sites into Pet 30c (Novagen) for bacterial expression and purification. *Bam*HI digest of the 910–1901 fragment was used to construct fragment 910–1228; *Sac*I digest of the 910–1901 fragment was used to generate 1182–1901; and *Sac*I/*Hin*dIII double digest of the 910–1901 fragment generated fragment 1182–1591. PCR using oligos 5′ CGAGCTCGAACCCAAGGTTAAAAACCCTTGC and 5′ CCGCTCGAGGAGCCATCTTGCCCCAAACCTTCTA facilitated generation of fragment 1228–1901 by adding *Sac*I and *Xho*I sites. This fragment was then digested with *Hin*dIII to make fragment 1228–1591. PCR was again used to generate fragments 1591–1901 oligos 5′ CGAGCTCTACTGTGACCTTAAAGAACTCC and Pet 30 T7 terminator, 1591–1714 oligos 5′ CGAGCTCTACTGTGACCTTAAAGAACTCC and 5′ CCCAAGCTTGACAGACTCAGTTGACTGAAAGT, and 1711–1901 oligos 5′ CGAGCTCGAAGACTTTCAGTCAACTGAGTC and Pet30 T7 terminator. *Sac*I/*Hin*dIII double digest was then used to clone the PCR fragments into Pet 30. Point mutations were generated by quick change PCR mutagenesis (Stratagene) using different oligos for the Phe → Ala, Val → Ala, and FV → AA mutations. The Phe → Ala oligos used were 5′ GCACTCAGGGAAGAAGGTTCAGGCTGCAGAAGC and 5′ GCTTCTGCAGCCTGAACCTTCTTCCCTGAGTGC. The Val → Ala oligos used were 5′ GCACTCAGGGAAGAAGGCTCAGTTTGCAGAAGC and 5′ GCTTCTGCAAACTGAGCCTTCTTCCCTGAGTGC. The FV → AA oligos used were 5′ GCACTCAGGGAAGAAGGCTCAGGCTGCAGAAGC and 5′ GCTTCTGCAGCCTGAGCCTTCTTCCCTGAGTGC. Each construct was sequenced in both directions to confirm the presence of the PCR-induced mutation. All fragments were expressed as N-terminal His_6-tagged proteins in bacteria (BL21DE3) and purified via His tag purification using hi-trap chelating resin and FPLC (Amersham Pharmacia Biotech). Briefly, cells were pelleted by centrifugation at 5000 × *g* for 10 min and then sonicated in buffer A at 4 °C (20 mM HEPES, 500 mM NaCl, pH 7.9), and centrifuged at 35,000 × *g* at 4 °C for 30 min. Fragment 910–1901 was predominantly an insoluble protein; therefore, 6 M urea was added to Buffer A and a second round of sonication and centrifugation was conducted. Supernatants were filtered (0.2 μm) and then applied to hi-trap chelating resin (Amersham Pharmacia Biotech) using FPLC. Bound proteins were eluted with a stepwise gradient of imidazole (0–0.5 M) in buffer A. Fractions containing purified protein were identified by Coomassie staining of SDS-PAGE gels. Stepwise dialysis removed imidazole and urea from the protein preparations.

Phosphatase Assay—Phosphorylase *b* was phosphorylated by phosphorylase kinase (in 100 mM Tris, 100 mM glycerophosphate, pH 8.2, 10 mM $MgCl_2$, 1 mM DTT, 2 mM CaCl) using [γ-^{32}P]ATP during a 3-h incubation at 30 °C (phosphorylase *b* and phosphorylase kinase, Sigma). The reaction was stopped by adding 50 mM NaF, 20 mM EDTA (final concentrations) for an additional 15 min at 30 °C. Phosphorylase *a* was then precipitated by incubation with an equal volume of saturated ammonium sulfate solution on ice for 30 min followed by a 10-min 14,000 × *g* spin. The pellet was resuspended in phosphorylase *a* solubilization buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 15 nM caffeine) and passed over a Pierce desalting column to remove remaining free ATP. Phosphorylase *a* eluted from the column with solubilization buffer in the first few fractions, whereas free nucleotide was retained on the column. Phosphatase assay was performed in a total volume of 30 μl at 30 °C. Recombinant PP1 (generously provided by Dr. Ernest Lee) or the native rabbit enzyme were diluted in phosphatase dilution buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1% bovine serum albumin, 1 mM DTT, 1 mM $MnCl_2$). AKAP220 fragments were diluted in phosphatase assay buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 3 mM EGTA, 1 mM DTT, 0.1% bovine serum albumin). PP1 and inhibitors were incubated for 5 min at 30 °C and then substrate was added. After 10 min at 30 °C, the reactions were stopped with 30% trichloroacetic acid. Following a 10-min incubation on ice and a 5-min, 14,000 × *g* spin, 100 μl of supernatant was removed, and the amount of ^{32}P radioactivity released was measured by liquid scintillation counting. Competitive inhibition assays were conducted over a 0.02–0.8 μM range of phosphorylase *a* and 0.01–10 μM AKAP220.

PP1 Overlay Assay—The PP1 overlay assay was performed essentially as described (37). Briefly, fragments were subjected to SDS-PAGE and transfer to Immobilon. The membranes were incubated with 0.5 μg of recombinant PP1cα in TTBS (0.03% Tween, Tris-buffered saline) for 2 h at room temperature. PP1 bound to the membrane was detected by Western blot using a polyclonal anti-PP1α antibody and chemiluminescence detection (Pierce).

RESULTS AND DISCUSSION

AKAP220 Is a Competitive Inhibitor of PP1—We have previously shown that AKAP220 interacts with the PKA holoenzyme and PP1 *in vitro* and inside cells (37, 46). To investigate further these events it was important to define the mechanism of AKAP220 interaction with the phosphatase. A recombinant fragment encompassing residues 910–1901 of human AKAP220 inhibited PP1 activity with an IC_{50} of 3.6 ± 0.7 μM ($n = 4$) when phosphorylase *a* was used as a substrate (Fig. 1*A*). Control experiments confirmed that this AKAP220 fragment inhibited recombinant PP1α or the purified rabbit enzyme to similar extent (Fig. 1*B*). Double-reciprocal plots were used to calculate an inhibition constant (K_i) of 2.9 ± 0.7 μM ($n = 3$) demonstrating that the AKAP220-(910–1901) fragment was a competitive inhibitor of PP1α activity (Fig. 1*C*).

One implication of these findings is that binding determinants on the anchoring protein must influence the active site of the phosphatase. Within the AKAP220 peptide is a core sequence of Lys-Val-Gln-Phe which is a recognizable characteristic of many PP1-targeting subunits (35). We have previously demonstrated that an AKAP220 peptide encompassing this sequence (residues 1185–1207) binds PP1α with nanomolar affinity (37). This sequence is unlikely to bind at the active site of the enzyme as other investigators have demonstrated that related "KV*X*F" peptides do not inhibit phosphatase activity (22, 26). More conclusive support for this view has been pro-

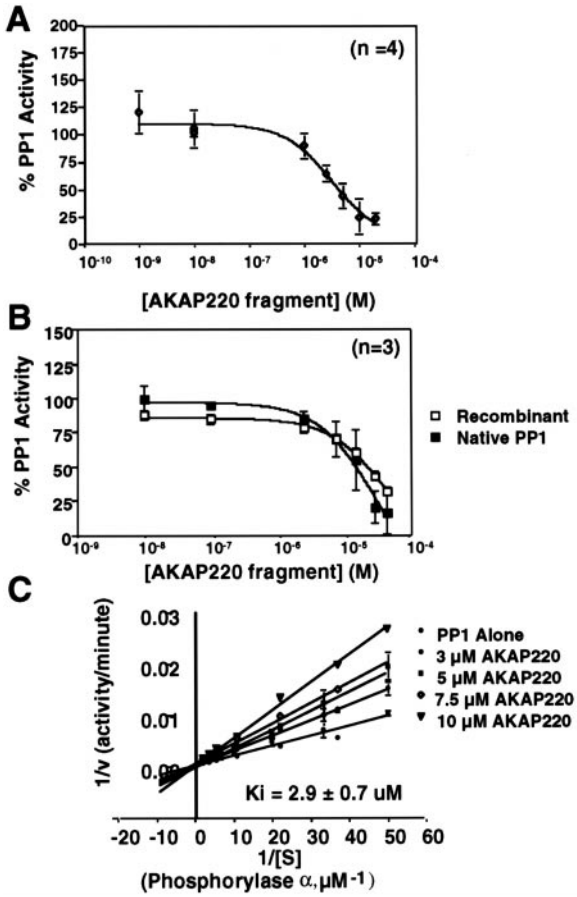

FIG. 1. **AKAP220 is a PP1 inhibitor.** Purified recombinant PP1α and native PP1c isolated from rabbit skeletal muscle were incubated with the AKAP220-(910–1091) fragment over a concentration range from 1 nM to 100 μM. Phosphatase activity was measured as described under "Materials and Methods" using phosphorylase *a* as a substrate. PP1 activity is presented as a percentage of phosphorylase *a* dephosphorylation in the absence of AKAP fragment. *A* depicts data collected from an average of four independent experiments. *B* depicts a comparison of the inhibition profiles for recombinant (*open squares*) and native (*closed squares*) PP1 catalytic subunits over a similar concentration range of AKAP220-(910–1901) fragments, with the data collected from three independent experiments. *C,* detailed kinetic analysis of AKAP220-(910–1901) fragment as a PP1α inhibitor was performed in the presence of AKAP220-(910–1901) 3–10 μM. Michaelis-Menten analysis was used to calculate the inhibition constant.

vided by crystallographic analysis of PP1c complexed with a peptide derived from the glycogen-targeting subunit G_M. These elegant studies show that the KV*X*F sequence binds to a hydrophobic surface that is distal to the catalytic center of the phosphatase (35). These protein-protein interactions must represent a principle targeting interaction as delivery of KV*X*F peptides into tissue culture cells and dissociated striatal neurons disrupts PP1 location (21, 22, 48–50). One relevant example is peptide-mediated disruption of PP1 from a phosphatase-kinase signaling complex maintained by Yotiao, a scaffolding protein that binds to the cytoplasmic tail of the NMDA-type glutamate receptor ion channel (36, 51). Interruption of the Yotiao/PP1 interaction uncouples phosphatase regulation of the ion channel and enhances cAMP-responsive currents (19, 36). In some respects Yotiao and AKAP220 have related roles. Both anchoring proteins maintain a PP1-PKA signaling complex and target the enzymes to precise locations within cells. However, one important distinction is that the 910–1901 fragment of AKAP220 is a reasonably potent competitive inhibitor of PP1 activity. Thus, there must be additional binding sites on AKAP220 that inhibit the phosphatase.

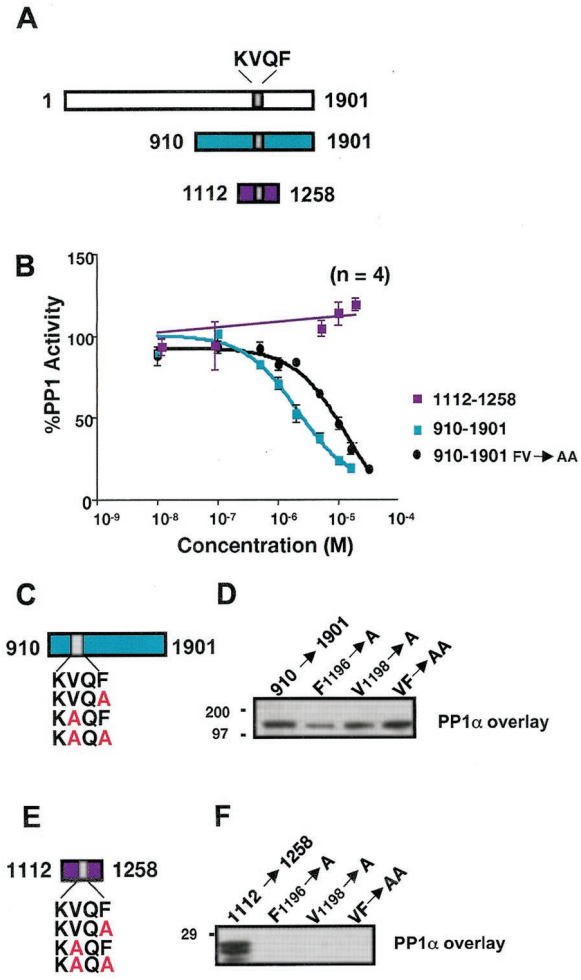

FIG. 2. **The consensus targeting domain in AKAP220 is not required for inhibition of PP1.** AKAP220 fragments were analyzed for inhibition of recombinant PP1α activity using phosphorylase *a* as a substrate. *A,* schematic diagram depicts the size of each fragment in relation to the full-length anchoring protein. The first and last residue of each fragment is indicated, and the consensus PP1-targeting motif is *highlighted*. *B,* the PP1α inhibitory properties of three AKAP220 fragments were assayed as described under "Materials and Methods." Dose-response curves for each fragment are presented: residues 1112–1258 (*black squares*), residues 910–1901 (*filled squares*), and a mutant form of the AKAP220-(910–1901) fragment with a disrupted targeting motif (*closed circles*). Each curve represents averaged data from four independent experiments. *C,* a schematic diagram showing a series of mutations in the consensus targeting (KV*X*F) motif of the AKAP220-(910–1901) fragment. Amino acids are shown in the *one-letter code. D,* samples of each mutant AKAP220-(910–1901) fragment (indicated *above* each lane) were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose filter. Solid-phase binding to recombinant PP1α was assessed by an overlay assay. Molecular weight markers are indicated. *E,* a schematic diagram showing a series of mutations in the consensus targeting (KV*X*F) motif of the AKAP220-(1112–1258) fragment. Amino acids are in the *one-letter code. F,* samples of each mutant AKAP220-(1112–1285) fragment (indicated above each lane) were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose filter. Solid-phase binding to recombinant PP1cα was assessed by an overlay assay. Molecular weight markers are indicated.

Multiple Sites of Interaction between AKAP220 and PP1—Two recombinant fragments of human AKAP220 were further characterized to locate sites on the anchoring protein that inhibit the phosphatase (Fig. 2*A*). A large C-terminal fragment, AKAP220-(910–1901), inhibits the phosphatase with an IC_{50} of 3.6 ± 0.7 μM (n = 4, Fig. 2*B*). Solid-phase binding experiments using an overlay procedure confirmed that this AKAP220 fragment retained the ability to bind PP-1 (Fig. 2*D*). Furthermore,

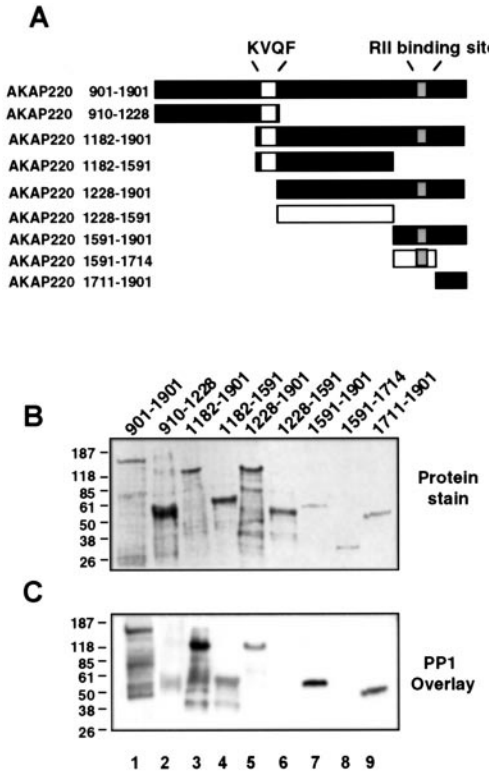

FIG. 3. **Mapping additional PP1 binding determinants in AKAP220.** AKAP220 fragments were screened for interaction with PP1α using the overlay assay. *A,* schematic diagram depicts the size of each fragment. AKAP220 fragments retaining the ability to interact with PP1α are indicated in *black.* The first and last residues of fragment are indicated; the PP1-targeting motif and RII binding sites are *boxed.* Recombinant AKAP220 fragments were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose filter. *B,* protein staining shows the relative purity of each AKAP220 fragment. *C,* detection of solid-phase phosphatase binding used the PP1α overlay. The first and last residues of each fragment are indicated *above* and the lane numbers are indicated *below*. The mobility of molecular weight markers is indicated.

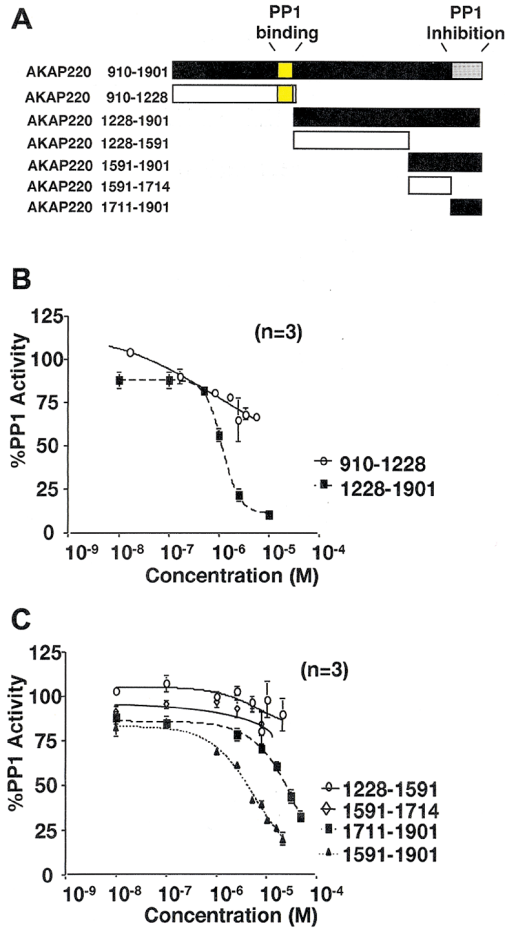

FIG. 4. **Mapping the PP1 inhibitory site on AKAP220.** The inhibitory properties of selected AKAP220 fragments toward recombinant PP1α were measured using phosphorylase *a* as a substrate. *A,* a schematic diagram depicts the size of each fragment. *Filled boxes* represent AKAP220 fragments that inhibit PP1α activity. The first and last residues of fragment are indicated; the PP1-targeting motif and PP1 inhibitory region are *highlighted*. *B,* dose-response curves comparing the inhibitory potency of AKAP220-(910–1228) (*open circles*) and the AKAP220-(1228–1901) (*closed boxes*) fragments. *C,* dose-response curves for the AKAP220-(1228–1591) fragment (*open circles*), AKAP220-(1591–1714) fragment (*open diamond*), AKAP220-(1711–1901) fragment (*closed squares*), and AKAP220-(1591–1901) fragment (*closed triangles*). All data represent an average of three independent experiments.

mutation of the KVQF motif does not significantly impair the inhibitory potency of the AKAP220-(910–1901) fragment (Fig. 2*B*). Substitution of Val-1196 → Ala, Phe-1198 → Ala, or replacement of both residues with alanine (Fig. 2*C*) within the context of the AKAP220-(901–1901) fragment had no qualitative effect on PP1 interaction (Fig. 2*D*). However, phosphatase binding was abolished when the same panel of mutants was screened for PP1 interaction within the context of a smaller fragment, AKAP220-(1112–1258) (Fig. 2, *E* and *F*). Control experiments confirmed that the wild-type AKAP220-(1112–1258) fragment bound PP1 in the overlay assay (Fig. 2*E*) and activity measurements confirmed that this 146-residue fragment which spans the KVQF sequence does not inhibit the phosphatase (Fig. 2*B*). Additional control experiments confirmed that equal amounts of each AKAP220 fragment were used in the overlay blots (data not shown). Collectively, these results allow us to conclude that inhibition of PP1 activity involves the C-terminal half of AKAP220 and does not require binding through the KVQF sequence.

Mapping a Second PP1-binding Site on AKAP220—A family of AKAP220 fragments spanning selected regions of the anchoring protein were generated to identify further phosphatase-binding sites and PP1 inhibitory determinants (Fig. 3*A*). Each AKAP220 fragment was expressed in *E. coli* as a His_6 fusion protein and affinity-purified using FPLC His tag technology. Approximately equal amounts of each purified protein fragment were used (Fig. 3*B*), and interaction with PP1 was assessed by the overlay assay (Fig. 3*C*). All binding studies were performed at least three times. As expected all AKAP220 fragments including the KVQF sequence bound PP1 (Fig. 3*C, lanes 1–4*), although weaker binding was observed with AKAP220-(910–1228), a fragment that contained the KVQF sequence at the extreme C terminus (Fig. 3*C*, *lane 2*). Most importantly, certain fragments such as AKAP220-(1228–1901) that lack the KVQF sequence also retained the ability to interact with PP1 as assessed by the overlay assay (Fig. 3*C, lane 5*). This led to the analysis of additional fragments spanning this region (Fig. 3*A*) which permitted the mapping of supplementary PP1 binding determinants between residues 1711 and 1901 of AKAP220 (Fig. 3*C, lanes 6–9*). Thus, at least two regions of AKAP220 interact with PP1 as follows: a conserved targeting motif between residues 1195 and 1198, and site(s) located between residues 1711 and 1901 of the anchoring protein (Fig. 4*A*).

A logical next step was to ascertain if the C-terminal binding region of AKAP220 influenced PP1 activity. Inhibition profiles for a representative selection of AKAP220 fragments are presented in Fig. 4, *B* and *C*. Initial experiments showed that

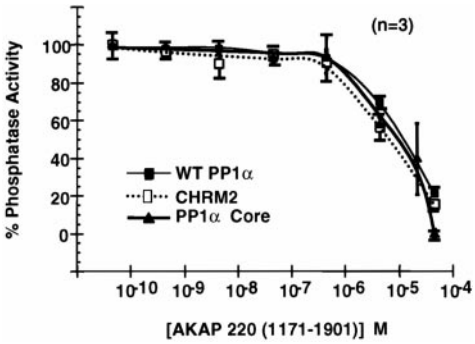

FIG. 5. **The β12/13 loop is not required for PP1 inhibition by AKAP220.** The sensitivity of PP1α (*solid squares*), CRHM2, the PP1/2A chimera (*open squares*), and the PP1α core, residues 41–269 (*filled triangles*), to inhibition by the AKAP220-(1711–1901) fragment was measured using phosphorylase *a* as a substrate. Activity is presented as the percentage of phosphatase activity measured in the absence of the AKAP220 inhibitory fragment. Each experiment was performed three times, and the data are shown with standard errors.

AKAP220-(1228–1901) inhibited PP1 activity with an IC_{50} of 3 ± 0.3 μM (n = 3) (Fig. 4*B, closed squares*), whereas AKAP220-(910–1228), an upstream fragment that encompasses the targeting motif, had little effect on phosphatase activity (Fig. 4*B, open circles*). Further analysis indicated that PP1 inhibitory determinants were located in the last 300 residues of the anchoring protein (Fig. 4*C*). Interestingly, residues 1711–1901 inhibited PP1 α with an IC_{50} of 48.9 ± 4.6 μM (n = 3) (Fig. 4*C, closed squares*), whereas a larger fragment that included the RII binding domain (AKAP220-(1591–1901)) inhibited the phosphatase to a 10-fold greater extent (IC_{50} of 4.1 ± 1.1 μM (n = 3); Fig. 4*C, closed triangles*). Thus it would appear that multiple interactions including an inhibitory site located between residues 1711 and 1901 promote tight binding and inhibition of the phosphatase. This finding is consistent with structure-function analysis on several protein inhibitors of PP1 such as inhibitor-1 (I-1), inhibitor-2 (I-2), NIPP-1, and DARPP-32 (24, 25, 29, 38, 39, 49, 50, 52–54).

Analysis of Truncated and Chimeric Phosphatase Catalytic Subunits—Shenolikar and colleagues (40, 55) have established the importance of the β12–13 loop in PP1 as a region required for inhibition of PP1c by protein inhibitors such as I-1, I-2, and NIPP-1 and environmental toxins. To examine the mechanism of PP1 inhibition by AKAP220, we first analyzed a chimeric PP1α catalytic subunit containing C-terminal sequences from PP2A (55). This reengineered phosphatase, termed CRHM2, is sensitive to toxins, such as microcystin, tautomycin, and fostreicin, but is not inhibited by the phosphorylated inhibitor proteins including I-1, I-2, or NIPP-1 (39, 55). When incubated with AKAP220, the PP1/2A chimera (Fig. 5, *open squares*) is inhibited to a similar extent as the wild-type PP1 (Fig. 5, *closed squares*). In addition, we analyzed a truncated PP1α catalytic subunit lacking the variable N- and C-terminal sequences, termed PP1 core, that was previously shown to be insensitive to both protein and small molecule inhibitors. The PP1 core was also inhibited by AKAP220 in a manner similar to the wild-type phosphatase (Fig. 5, *filled triangles*). These results indicate that AKAP220 inhibits phosphatase activity by a mechanism distinct from known PP1 inhibitors. To emphasize this point, fostreicin, an anti-cancer drug that inhibits the PP1 catalytic core competed with AKAP220 for PP1 inhibition (data not shown). Thus, the data suggest that the β12–13 loop in the PP1 catalytic subunit is not essential for its inhibition by AKAP220. Furthermore, residues 1711–1901 of the anchoring protein bind the phosphatase in a manner distinct from many known PP1 inhibitors, proteins, and toxins.

RII Enhances Inhibition of PP1 by AKAP220—Although

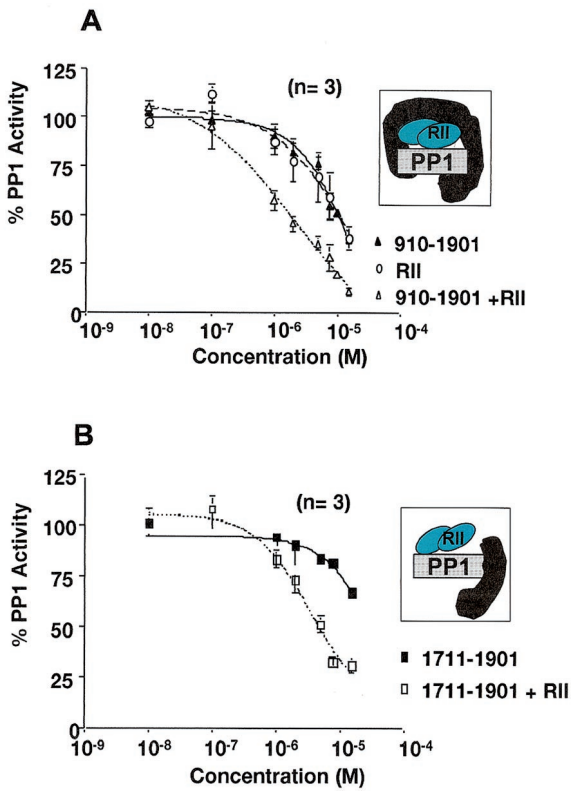

FIG. 6. **RII binding enhances PP1 inhibition by AKAP220.** The inhibitory properties of selected AKAP220 fragments toward PP1α were measured in the presence of RII. *A,* dose-response curves for AKAP220-(910–1901) fragment (*closed triangles*), RII (*open circles*), and AKAP220-(910–1901) fragment plus RII (*open triangles*) are presented. *Inset* demonstrates the potential role of AKAP220 to coordinate RII anchoring in a manner that optimizes inhibitory contact with the phosphatase. *B,* dose-response curves for the AKAP220-(1711–1901) fragment in the presence (*open squares*) and absence of RII (*closed squares*). *Inset* depicts how RII and the AKAP220 fragment act synergistically to inhibit the phosphatase. All experiments were performed at least three times.

PKA and PP1 bind to distinct regions of AKAP220, it was of interest to establish if there was any cooperative effect of PKA anchoring on phosphatase activity. Earlier reports (56, 57) had suggested that RII was a noncompetitive inhibitor of PP1 with phosphorylase *a* as a substrate. Since the C terminus of AKAP220 binds both RII and PP1, it was important to explore the potential influence of these protein interactions on inhibition of the phosphatase. Recombinant RII inhibited PP1 with an IC_{50} = 2.2 ± 0.7 μM (n = 3) (Fig. 6*A, open circles*), a similar inhibitory potency to the AKAP220-(910–1901) fragment (Fig. 6*A, closed triangles*). However, PP1 inhibition was enhanced 4-fold (IC_{50} = 0.59 ± 0.2 μM (n = 3)) when RII was added to the enzyme reaction (Fig. 6*A, open triangles*). Since the AKAP220-(910–1901) fragment contains determinants of PKA anchoring, we reasoned that RII could enhance PP1 inhibition in either an additive or a cooperative manner (Fig. 5*A, inset*). Additive effects would require that both RII and AKAP220 inhibit PP1 by binding at different sites on the phosphatase. Cooperative effects could occur if the RII-AKAP220 complex constrained a preferred conformation or exposed additional determinants within the anchoring protein that enhanced PP1 inhibition. In an attempt to delineate between these two possible mechanisms, we used AKAP220-(1711–1901), a fragment which lacks the RII-binding site (Fig. 3*A*). This fragment inhibited PP1 with an IC_{50} = 34.5 ± 5.9 μM (n = 3) in the absence of RII (Fig. 6*B, closed squares*). However, PP1 inhibition was enhanced ~28-fold in the presence of equimolar concentrations of RII

(IC_{50} = 1.2 ± 0.2 μM (n = 3) Fig. 6*B, open squares*). Control binding experiments confirmed that the AKAP220-(1711–1901) fragment does not bind RII *in vitro* (data not shown). These results suggest that AKAP220 and RII are more likely to work additively to inhibit PP1 by binding to separate sites on the phosphatase (Fig. 6*B, inset*). The greater inhibitory potency of the larger AKAP220 fragment implies that RII association with the anchoring protein may orient the regulatory subunit to permit optimal inhibitory contact with the phosphatase (Fig. 6*A, inset*).

Conclusion—The substrate specificity of the type I protein phosphatase catalytic subunit PP1c is thought to be influenced in large part through association with targeting subunits (58). This growing family of proteins not only controls the subcellular location of PP1 leading to selective dephosphorylation of certain substrates but also influences the catalytic efficiency of the enzyme (21, 22, 36, 48, 59–61). To perform both functions it has been proposed that PP1-targeting subunits and inhibitor proteins interact with multiple sites on the phosphatase (38, 55, 62). Subcellular targeting is mediated in part through a loosely conserved tetrapeptide sequence KV*X*F found in many PP1-regulatory proteins (35). Additional binding sites participate in allosteric interactions that tailor the substrate specificity of PP1c (40, 63–65).

In this report we demonstrate that two or more binding surfaces on AKAP220 act synergistically to target and inhibit the phosphatase. On the basis of these observations, we now propose a more sophisticated model for AKAP220-mediated PP1 targeting. Our data suggest that a consensus-targeting motif including residues 1195–1198 of the anchoring protein is responsible for localizing the phosphatase, whereas inhibitory sites between residues 1711 and 1901 maintain the enzyme in an inactive state. Our kinetic evidence indicates that AKAP220 is a competitive inhibitor of PP1c activity (Fig. 1*C*). These latter findings are consistent with accumulating evidence that other multivalent anchoring proteins such as AKAP79/150 and gravin bind and inhibit their anchored enzymes (66–68). A previously unappreciated level of phosphatase regulation appears to involve interaction with other proteins in the AKAP220-signaling complex. Although free RII has been reported to inhibit PP1 in a noncompetitive manner (56, 57), its recruitment into the AKAP220 signaling scaffold enhances phosphatase inhibition (Fig. 6). This represents a new concept in AKAP signaling in which intermolecular interactions within the signaling complex influence the activity of other anchored enzymes. Another tier of regulation may be phosphorylation of the anchored signaling components by PKA. For example, thiophosphorylated RII is a more potent inhibitor of PP1 (57), and PKA phosphorylation of regulatory molecules such as inhibitor 1 or DARPP-32 enhances phosphatase inhibition (50). In this regard our preliminary studies suggest that AKAP220 is a PKA substrate.[2] Future studies will focus on whether the anchoring protein is phosphorylated *in vivo* and if there are effects on phosphatase inhibition. The complexity of these interactions will be more fully apparent when a three-dimensional structure of the PKA-AKAP220-PP1 complex is solved.

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