Molecular Pharmacology Fast Forward. Published on December 13, 2010 as doi: 10.1124/mol.110.065425
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MOL #65425

The large isoforms of AKAP18 mediate the Phosphorylation of Inhibitor-1 by PKA and the inhibition of PP1 Activity

Arpita Singh

John M. Redden

Michael S. Kapiloff,

Kimberly L. Dodge-Kafka

AS, JMR and KLD-K: Pat and Jim Calhoun Center for Cardiology,

University of Connecticut Health Center, Farmington, CT 06030

MSK: Depts of Pediatrics and Medicine, Interdisciplinary Stem Cell Institute,

University of Miami Miller School of Medicine, Miami, FL 33101

Molecular Pharmacology Fast Forward. Published on December 13, 2010 as DOI: 10.1124/mol.110.065425
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A: Running title: Inhibitor-1 – AKAP18 Complexes

B: Address correspondence to: Kimberly L. Dodge-Kafka, PhD, University of Connecticut Health Center, MC3946, EGO28, 263 Farmington Ave, Farmington, CT 06032 Phone: 860-679-2452; Fax 860-679-1426; E-mail: dodge@uchc.edu

C: Text pages: 28 pages

Figure pages: 6 pages

Abstract: 154 words

Introduction: 528 words

Discussion: 412 words

D. Abbreviations:

PP1: Protein phosphatase 1

I-1: Protein phosphatase 1 Inhibitor-1

PKA: cAMP-dependent protein kinase

AKAP: A-kinase anchoring protein

PKC: Protein Kinase C

CDK5: cyclin-dependent kinase 5

TCA: trichloroacetic acid

PP2A: Protein phosphatase 2A

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Abstract:

Inhibitor-1 (I-1) is phosphorylated on threonine residue 35 (Thr-35) by the cAMP-dependent protein kinase (PKA), inducing the potent inhibition of the serine-threonine-specific protein phosphatase 1 (PP1). We now report that the formation of a signaling complex containing PKA and I-1 by the A-kinase anchoring protein, AKAP18, facilitates this regulation in cells. AKAP18 directly bound I-1, and AKAP18/I-1 complexes were isolated from both rat heart extract and transfected heterologous cells. Importantly, prevention of PKA binding to the AKAP18 scaffold decreased I-1 phosphorylation by 48% in cells. Moreover, the I-1 target PP1 was also associated with AKAP18 complexes. The cAMP-mediated inhibition of phosphatase activity was contingent upon PKA binding to the scaffold. These observations reveal an additional level of complexity in PP1 regulation due to its association with AKAP18 multi-molecular signaling complexes, and suggest that targeting of AKAP18 complexes may be an alternative method to alter phosphatase activity and modulate specific substrate dephosphorylation.

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Introduction:

Protein phosphorylation is a key regulator of cellular physiology that affects essentially all biological processes. Despite our vast knowledge of the consequences of protein phosphorylation, the molecular mechanisms that confer specificity to the enzymes controlling this post-translational modification are not well understood, especially for the handful of phosphatases that catalyze the dephosphorylation of protein substrates (Cohen, 2002; Virshup and Shenolikar, 2009). Seminal investigations have demonstrated that the cell has evolved multiple strategies that control the location, activity and substrate specificity of each phosphatase (Cohen, 2002; Virshup and Shenolikar, 2009). Current research is focused on the contribution of individual multi-molecular signaling complexes to the specificity of intracellular signal transduction.

The first protein discovered to regulate phosphatase activity was the Protein Phosphatase Inhibitor-1 (I-1) (Huang and Glinsmann, 1976). I-1 is a 19 kDa, heat stable protein that was identified over 30 years ago as a regulator of glycogen metabolism (Huang and Glinsmann, 1976). While I-1 has no known intrinsic catalytic activity, its binding reduces the activity of protein phosphatase 1 (PP1). I-1 is highly expressed in the brain, where it plays a role in synaptic plasticity (Genous et al., 2002; Mansuy and Shenolikar, 2006). Although I-1 is expressed at low levels in the adult myocardium, recent work suggests that regulation of PP1 by I-1 at the sarcoplasmic reticulum is required for normal cardiac function, as well as for the response of the heart to disease (Nguyen et al., 2007; Nicolaou et al., 2009a). I-1 itself is phosphorylated by

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PKA, PKC and CDK5 (Huang and Glinsmann, 1976; Nguyen et al., 2007; Rodriquez et al., 2006; Sahin et al., 2006) and phosphorylation of I-1 at Thr-35 by PKA induces selective inhibition of PP1 (Huang and Glinsmann, 1976; Nicolaou et al., 2009a). This event is induced by β-adrenergic stimulation in cardiac myocytes, and potentiates the phosphorylation of key PKA substrates involved in excitation-contraction coupling by preventing their PP1-mediated dephosphorylation (Nicolaou et al., 2009a). As excessive PP1 activity resulting from a lack of I-1 function has been suggested to contribute to heart failure, a more complete understanding of the molecular regulation of this phosphatase may aid in the design of novel therapeutics to prevent or treat heart disease (Braz et al., 2004; Carr et al., 2002; El-Armouche et al., 2008; Nicolaou et al., 2009b).

The mechanisms conferring specificity on PKA phosphorylation have been of considerable interest in recent years, and research has shown that many PKA targets are co-localized with the kinase via the association with A-kinase anchoring proteins (AKAP) (Carnegie et al., 2009; Scott and Pawson, 2009). These scaffold proteins function to enhance the kinetics and specificity of PKA phosphorylation by sequestering the kinase with its target substrates, allowing for spatiotemporal control of cAMP signaling. Importantly, disruption of PKA binding to AKAPs in the heart significantly decreases the ability of the kinase to phosphorylate many key proteins such as troponin I, the ryanodine receptor and phospholamban (Mauban et al., 2009).

As I-1 is a target for PKA, we investigated whether an AKAP mediates this event. We discovered that I-1 binds the large isoforms of AKAP18 in the heart. AKAP18 potentiated the phosphorylation of I-1 by PKA, and disruption of PKA binding to the

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scaffold significantly attenuated Thr-35 phosphorylation in HEK293 cells. Moreover, PP1 was also associated with AKAP18 complexes, and the PKA-dependent inhibition of phosphatase activity required PKA-AKAP18 binding. These data imply that AKAP18 orchestrates the PKA-catalyzed phosphorylation of I-1 in the myocyte, providing an additional level of regulation of PP1 activity at the sarcoplasmic reticulum, where the large isoforms of the AKAP are enriched.

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Materials and Methods:

Antibodies:

The following antibodies were used for immunoblotting: rabbit polyclonal to inhibitor-1 (Abcam, 1:10,000), goat polyclonal to AKAP18 (received from Dr. John Scott, University of Washington, 1:1000), rabbit polyclonal to GFP (Invitrogen, 1:500), rabbit polyclonal phospho-specific antibody against Threonine-35 of I-1 (Cell Signaling, 1:1000), mouse monoclonal to PP1 catalytic subunit (Santa Cruz Biotechnology, 1:1000), mouse monoclonal to myc tag (Santa Cruz Biotechnology, 1:1000), mouse monoclonal to PKA RI subunit (BD Bioscience, 1:5000), mouse monoclonal to PKA-RII subunit (BD Bioscience, 1:5000), and mouse monoclonal to PKA catalytic subunit (BD Bioscience 1:5000). The following antibodies were used for immunoprecipitations: rabbit polyclonal to inhibitor-1 (Abcam, 2 μg), mouse monoclonal to PP1 catalytic subunit (Santa Cruz Biotechnology, 5 μg), mouse monoclonal to PP2A catalytic subunit (Santa Cruz Biotechnology, 5 μg), goat polyclonal to AKAP18 (received from Dr. John Scott, University of Washington, 5 μl), mouse monoclonal to myc tag (Santa Cruz Biotechnology, 5 μg), and mouse monoclonal to PKA-RII subunit (BD Biosciences, 2 μg).

Expression Constructs:

For I-1 constructs, human I-1 was PCR amplified using a cDNA, supplied by Dr. Shirish Shenolikar, Duke University, and sub-cloned into the EcoRI-Xho sites of pGEX-4T1 and pCDNA3. Full-length human AKAP18γ (supplied by Dr. John Scott, University of

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Washington) as well as the AKAP18γ deletion constructs were cloned into either the EcoRI-BamHI sites of pEGFP-N1, or the EcoRI-HindIII sites of pET32a.

Experimental Procedures:

cAMP Agarose Purification:

Rat heart extract was prepared by homogenization of a single rat heart (PelFreeze) in 10 ml HSE lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA,1% Triton X-100, and protease inhibitors). Following centrifugation at 12,000 x g for 20 minutes at 4°C, 1 ml of supernatant (500 μg) was incubated overnight with 60 μl of Rp-cAMPs-agarose (Biolog, Bremen, Germany) in the presence and absence of 100 mM cAMP (Sigma). The beads were washed twice with HSE lysis buffer, followed by a high salt wash (20 mM HEPES [pH 7.4], 600 mM NaCl, 5 mM EDTA). Bound proteins were analyzed by immunoblotting.

For cAMP agarose pulldowns from HEK293 cells, cells were transfected at 70% confluency in 60 mm dishes using calcium phosphate. Cells were harvested and lysed 24 hours after transfection in 0.5 ml of HSE buffer containing protease inhibitors. Cell lysate was added to 60 μl of Rp-cAMPs-agarose and bound proteins were isolated as detailed above.

Pulldown Experiments:

Heart extract (1 ml) was prepared in HSE buffer as described above and incubated overnight with glutathione-resin pre-absorbed with bacterially expressed fusion proteins. The beads were then washed as described and bound proteins were identified by

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immunoblotting. Alternatively, for pulldown experiments using purified proteins, GST fusion proteins were incubated with S-tagged purified AKAP18 fragments bound to S-protein agarose in HSE buffer lacking EDTA. After an overnight incubation, beads were washed 3X in the HSE buffer and associated proteins were identified by immunoblot.

Immunoprecipitation:

For immunoprecipitation of AKAP18 constructs from heterologous cells, HEK293 cells were transfected at 70% confluency using the calcium phosphate method. 24 hours post transfection, cells were treated with 1 μM Isoproterenol for 5 minutes. Cells were rinsed in PBS, and then harvested and lysed in 0.5 ml of HSE buffer containing protease inhibitors plus phosphatase inhibitors (10 μM sodium pyrophosphate, 50 μM sodium fluoride). Supernatants were incubated with 4 μg of antibody and 20 μl of protein G-agarose beads. Bound proteins were analyzed by immunoblotting.

For immunoprecipitations from rat heart, extract was prepared as described above. Proteins were immunoprecitated from 1 ml of extract using specific antibodies bound to protein G-agarose. After extensive washing with HSE buffer, the associated proteins were determined by immunoblot.

Phosphatase Assay:

Phosphatase activity was measured as previously described using ^{32}P-labeled histone as a substrate (Dodge-Kafka et al., 2010). Histone was radiolabeled in a reaction containing 250 mM MOPS (pH 7.4), 2.5 mM MgAc, 100 mM beta-mercaptoethanol, purified PKA catalytic subunit, 1 mM ATP, 20 mM histone and 1 mCi [gamma-^{32}P-ATP

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(6000 Ci/mmol)]. The reaction was terminated by the addition of 50% TCA and [^{32}P]-histone was purified from free radionucleotide by centrifugation. After repeated washing, the [^{32}P]-histone was suspended in 200 µl of PP2A buffer (25 mM Tris, [ph 7.4], 1 mM DTT, and 10 mM MgCl2).

To measure phosphatase activity, immunoprecipitated complexes were washed twice in HSE buffer and again in PP2A buffer. The immunoprecipitates were incubated for 30 minutes at 30°C in 20 µl of PP2A buffer containing 100,000 cpm of [^{32}P]-histone in the presence and absence of inhibitors. Reactions were terminated by the addition of 100 µl of 20% TCA followed by 10 min centrifugation. TCA supernatants containing released $^{32}P0_4$ were measured using a scintillation counter.

PKA activity assay:

PKA activity was measured according to the method of Corbin and Reimann(Corbin and Reimann, 1974). Immunoprecipitated complexes were washed twice in HSE buffer and once in PKA assay buffer (50 mM Tris, pH 7.5 and 5 mM $MgCl_2$). The beads were then incubated in assay buffer containing 30 µM Kemptide 100 µM ATP, 5 µM γ^{32}-ATP and 3 µM cAMP. After incubating the samples at 30°C for 30 minutes, the reaction mixture was spotted onto phosphocellulose strips and washed five times in 75 mM phosphoric acid and once in 95% ethanol. Filters were air dried and counted.

Detection of I-1 phosphorylation:

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For I-1 phosphorylation experiments, 24 hours post transfection, cells were stimulated with 1 μM Isoproterenol for 5 minutes. Cells were rinsed in PBS and scraped into 0.5 ml of HSE buffer containing protease and phosphatase inhibitors (10 mM sodium pyrophosphate, 50 mM sodium fluoride). After centrifugation for 10 minutes at 13,000 x g, the clarified supernatant was added to 1 ml of isopropanol, and total cellular protein was precipitated by incubation at -20°C for 2 hours followed by centrifugation at 13,000 x g for 30 minutes. Precipitated proteins were resuspended in 200 μl of 2X SDS-PAGE sample buffer (0.5 M Tris-HCl, [pH 6.8], 1% glycerol, 10% SDS, and 0.5% beta-mercaptoethanol) that was added to the cellular pellet. For immunoblot analysis using a phospho-specific antibody, 20 μl of each isolated sample was subjected to SDS-PAGE.

For in *vitro* I-1 phosphorylation GST-fusion proteins were incubated with cellular extracts isolated from transfected HEK293 cells as described above. After an overnight incubation and washing of the beads with HSE buffer lacking EDTA, the complex was incubated in 25 μl of PKA assay buffer (25 mM Tris [pH 7.4], 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, and 3 mM cAMP) for 30 minutes at 37°C. The bound proteins were then subjected to immunoblot analysis.

Results:

AKAP18 associates with Inhibitor-1 in vivo

The formation of signaling complexes by AKAPs that include PKA and individual PKA substrates contributes to the efficiency and fidelity of cAMP signal transduction(Carnegie et al., 2009). As I-1 is an important PKA target, we considered I-1 might associate with anchored PKA in the heart. To test this idea, we isolated PKA-AKAP complexes from rat heart extract by affinity chromatography using Rp-cAMPs-agarose that binds the PKA regulatory subunits. This compound is a competitive inhibitor of PKA, so it does not stimulate activation of the kinase, thus leaving the holoenzyme intact. I-1 was consistently detected in the Rp-cAMPs pulldowns, but not under conditions which excess cAMP was added to the extracts to block specific binding to the beads (Figure 1A). As a control, both the PKA regulatory subunit type I, type II, as well as the PKA catalytic subunit were found specifically in cAMP-agarose pulldowns. To confirm that the PKA holoenzyme containing the catalytic subunit associated with I-1, we used GST-tagged, purified I-1 to pulldown PKA from rat heart extract. As shown in Figure 1B, kinase activity was found specifically in GST-I-1 complexes, and not with GST alone. Kinase activity was specific for PKA, as the addition of the PKA specific inhibitor PKI inhibited PKA activity in the pulldowns. Furthermore, PKA catalytic subunit as well as PKA regulatory type I and type II subunits were found in GST-I-1 pulldowns from rat heart extract. Importantly, the percentage of PKA-RII was enriched 20 fold over RI, suggesting this AKAP is a primarily RII binding AKAP. In summary, these data show that I-1 associates with a PKA complex in the heart.

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Next we sought to identify which AKAP might mediate this interaction between PKA and Inhibitor-1 in the heart. To do this, we isolated proteins in rat heart extracts that associated with I-1 using GST-tagged Inhibitor-1 protein, and then blotted for some highly characterized AKAPs found in the heart. As shown in Figure 2A, a high molecular weight AKAP18 isoform was readily detected in GST-I-1 complexes, in addition to what are potentially lower molecular weight AKAP18 isoforms that were less prevalent. As a control, I-1 did not associate with mAKAP, another muscle enriched AKAP involved in the induction of cardiac hypertrophy (Supplementary Figure 1)(Dodge-Kafka et al., 2010). The molecular weight of the intense, 45 kDa band implied that the major isoform pulled down by GST-I-1 was either AKAP18δ or AKAP18γ. Importantly, this interaction was not observed using GST alone, demonstrating the specificity of the interaction. The association of AKAP18 with I-1 was confirmed using an I-1 antibody to immunoprecipitate the scaffold from HEK293 cells expressing GFP-tagged AKAP18γ and I-1 (Figure 2B). Additionally, complexes containing PKA were purified from the HEK293 cell extracts using Rp-cAMPs agarose. I-1 co-purified with the agarose only when co-expressed with the anchoring protein, mimicking the results found with the endogenously expressed I-1 and AKAP18 in the heart (Figure 2C). Together, these data suggest that AKAP18, I-1, and PKA form a multi-molecular signaling complex in the heart as well as when expressed in heterologous HEK293 cells.

I-1 Directly Binds a Long Isoform of AKAP18

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AKAP18γ is one of the larger protein isoforms encoded by the alternatively spliced *AKAP18* gene (schematic shown in Figure 3A). The smaller AKAP18α is N-terminally myristoylated and palmitoylated, allowing for attachment to the plasma membrane where it facilitates PKA phosphorylation of the L-type calcium channel(Fraser et al., 1998b). In contrast, the large isoforms of AKAP18 are targeted to the sarcoplasmic reticulum where they from a signaling complex with phospholamban(Lygren et al., 2007). In order to define which isoform of AKAP18 is the primary target for I-1 binding, we performed pulldown assays using bacterially expressed GST-tagged I-1 with these two isoforms of AKAP18. GST-I-1 incubated with either bacterially expressed, S-tagged AKAP18α or AKAP18γ was subjected to S-tag pulldown assay. GST-I-1 was found in the S-protein agarose only in the presence of the larger isoform AKAP18γ. Importantly, these data also demonstrate that I-1 and the scaffolding protein can bind directly in a purified system.

Expression of AKAP18 enhances I-1 phosphorylation on Threonine 35

The role of an AKAP is to anchor the kinase in close proximity to its target in order to enhance PKA phosphorylation(Carnegie et al., 2009). Therefore, AKAP18 should enhance PKA-mediated phosphorylation of I-1 by assembling a signaling complex containing both I-1 and PKA. To test whether PKA binding to AKAP18 is important for I-1 phosphorylation, we first confirmed that a ternary complex consisting of AKAP18, PKA and I-1 could be isolated from transfected HEK293 cells. Cell extracts were prepared from HEK293 cells expressing AKAP18γ or a C-terminal truncated AKAP18γ mutant lacking the PKA binding site (AKAP18γ 1-268). GST-I-1 was

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incubated with the isolated cell extracts, and PKA activity assays were performed on the pulldowns (Figure 4A). While I-1 bound both full length AKAP18γ and AKAP18γ 1-268 (Figure 4B, top panel), kinase activity was only detected with full length AKAP18γ (Figure 4A). Kinase activity was blocked by the addition of PKI, demonstrating the specificity of the reaction to measure PKA activity assay. In support of this finding, PKA-RII was not detected in GST-I-1 pulldowns from cells expressing AKAP18γ 1-268 (Figure 4B, middle panel, GST-protein stain shown in Supplementary Figure 2A). These data show that AKAP18γ can serve to couple PKA to I-1 in a heterologous system.

Next, we determined if the associated kinase could phosphorylate I-1 at Thr-35. Consistent with the results of the PKA activity assays, cAMP-dependent phosphorylation of I-1 was greatly enhanced in GST-I-1 pulldowns containing full-length AKAP18γ compared to those containing the AKAP18γ PKA-binding mutant (Figure 4C). In order to show that AKAP18 enhances the PKA phosphorylation of I-1 in response to G-protein coupled receptor stimulation, the AKAP18γ proteins were co-expressed with I-1 in HEK293 cells. In the absence of stimulation, no I-1 phosphorylation was detected using the phospho-specific antibody. However, β-adrenergic stimulation with 1 μM isoproterenol for 5 minutes rapidly enhanced I-1 phosphorylation when AKAP18γ was co-expressed (Figure 4D) in a PKA dependent manner (Supplementary Figure 2B). Importantly, this stimulated increase was significantly attenuated in cells transfected with I-1 and AKAP18γ lacking the PKA binding domain (42±5.7% of wildtype, Figure 4E). Together, these data show that targeting of PKA to I-1 via the association with AKAP18 facilitates I-1 phosphorylation by the kinase.

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AKAP18 bind PP1 in vivo

Because AKAPs often serve as scaffolds for multi-molecular signaling complexes that include both kinase and phosphatases, we considered that the I-1 target PP1 might also be present in AKAP18γ complexes. To test this hypothesis, protein complexes were immunoprecipitated from rat heart extract using an antibody that recognizes the catalytic subunit of PP1 (Figure 5A). A high molecular weight isoform of AKAP18 co-precipitated with the PP1 antibody, but not with IgG control, implying that PP1 and AKAP18γ and/or AKAP18δ associate *in vivo*. In reciprocal experiments, STREP-tagged AKAP18γ was used to pulldown PP1 from rat heart extract (Figure 5B). PP1 did not associate with STREP-beads alone, demonstrating the specificity of this interaction. Furthermore, an okadaic acid-sensitive phosphatase activity was found in AKAP18 immunoprecipitates isolated from rat heart extract (Figure 5C). Taken together, these data show that PP1 and AKAP18 form a complex in the rat heart.

Next, we confirmed the PP1/AKAP18γ complexes could be reconstituted in transfected HEK293 cells. In support of our findings that PP1 and AKAP18 formed a complex in rat heart cell extract, PP1 also associated with AKAP18γ in transfected HEK293 cells (Figure 5D). Importantly, this association was lost when cells not expressing the anchoring protein were immunoprecipitated with the AKAP18 antibody, demonstrating the specificity of the interaction. Importantly, we were unable to immunoprecipitate the scaffold with a PP2A catalytic subunit antibody (Figure 5E), even though phosphatase activity was found in both PP2A and PP1 immunoprecipitations, (Supplementary Figure 2), suggesting that under these conditions, AKAP18γ selectively

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binds PP1. Taken together, our data demonstrate a complex consisting of AKAP18 and PP1 that can be isolated from both rat heart extract as well as from transfected HEK293 cells.

AKAP18 is the scaffold for a PKA, PP1 and I-1 signaling complex

The data presented here suggest that I-1, PKA and PP1 all associate with AKAP18 in both transfected cells and heart extract. One important implication of these data is that the anchoring protein may sequester these enzymes into the same signaling complex, and that compartmentation is dependent on AKAP18. To test this hypothesis, we investigated the importance of AKAP18γ expression for formation of the complex. HEK293 cells were transfected with I-1 in the presence and absence of AKAP18γ, while endogenous PP1 catalytic and PKA regulatory subunit were used for these experiments. Each protein was immunoprecipitated, and association of the other three proteins was determined by western blot. As shown in Figure 6, the expression of AKAP18γ was required for the association of the PKA-RII with I-1 and PP1, as well as association of PP1 to both AKAP18γ and the PKA-RII. Furthermore, I-1 was only found in PKA -RII immunocomplexes upon expression of AKAP18γ. While, I-1 and PP1 can associate in the absence of AKAP18γ, co-expression of the anchoring protein significantly increased the association of the two proteins. Controls demonstrating the specificity of each immunoprecipitation are shown in Supplementary Figure 4. The data presented in Figure 6 effectively argues the importance of AKAP18 for linking PKA to I-1 and PP1.

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AKAP18 orchestrates the PKA-mediated inhibition of PP1 activity

Phosphorylation of I-1 by PKA induces potent inhibition of PP1 activity(Huang and Glinsmann, 1976). Our data suggest AKAP18-bound PKA facilitates this phosphorylation, and hence, should enhance the ability of I-1 to inhibit the phosphatase. To study this potential functional consequence of AKAP18 complex formation, we first isolated AKAP18 complexes from rat heart extract, and determined the effect of cAMP on the associated endogenous PP1 activity. Activation of PKA in the complex by the addition of 3 μM cAMP inhibited PP1 activity (95±4.67%) present in the AKAP18 immunoprecipitates (Figure 7A). This inhibition was attributed to the activation of PKA, as inhibition was reversed by inclusion of the PKA inhibitor PKI (50 nM). These data demonstrate that the PKA-stimulated inhibition of AKAP18-bound PP1 is functional in native AKAP18 complexes isolated from rat heart.

Showing the necessity of PKA anchoring to AKAP18 for PP1 inhibition extended these results. HEK293 cells were transfected with I-1 and either full-length AKAP18γ or the AKAP18γ PKA binding mutant. After stimulation with 1 μM isoproterenol for 5 minutes, complexes were isolated using an antibody that recognizes the anchoring protein, and the associated phosphatase activity was measured. As shown in Figure 7B, PKA anchoring to the complex was required for effective inhibition of the associated phosphatase activity, as the cAMP-induced inhibition of PP1 was lost when AKAP18 could not bind PKA. This result is not due to a loss of complex formation, as similar amounts of AKAP18 and I-1 were found in PP1 immunocomplexes (Figure 7C). Taken together, these data show that the binding of I-1, PP1 and PKA by AKAP18 permits the efficient regulation of PP1 activity by cAMP signaling in cells.

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Discussion

In this report, we identify the first interaction between Inhibitor-1 and an A-kinase anchoring protein, AKAP18. Functional AKAP18 complexes containing I-1, the target PP1, and PKA were isolated from both rat heart extract and transfected HEK293 cells. The functional significance of these interactions was demonstrated in two ways. First, complex formation facilitated the cellular phosphorylation of I-1, as disruption of PKA association with AKAP18 significantly decreased phosphorylation of I-1 at Thr-35. Second, PKA binding to AKAP18 was required for isoproterenol-induced inhibition of the associated PP1 activity. This information establishes AKAP18 as a regulator of both I-1 and PP1 activity and advances our understanding of phosphatase regulation.

PKA phosphorylation of I-1 at Thr-35 and the resulting inhibition of PP1 activity acts as a feed-forward mechanism to enhance PKA phosphorylation of downstream effectors that would otherwise be dephosphorylated by PP1 (Nicolaou et al., 2009a; Pathak et al., 2005). PKA anchoring to AKAP18 is now shown to play an integral role in this process, since disruption of PKA anchoring to AKAP18 prevented cAMP-mediated inhibition of the PP1 activity associated with the complex. As a result, in cells such as cardiac myocytes that express AKAP18γ and AKAP18δ, these complexes should be highly sensitive to cAMP signals.

Previous work has demonstrated the existence of multi-protein complexes containing I-1 and PP1. An I-1/PP1 complex mediated by interactions with the Growth arrest and DNA damage protein 34 (GADD34) was found in active squirrel brains (Connor et al., 2001). GADD34 was shown to enhance the I-1-mediated inhibition of

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PP1 in order to regulate the phosphorylation of the transcription factor eIF-2α. While GADD34 is found in the heart, the role of this complex in the regulation of PP1 in this tissue is unknown (Morton et al., 2006). An important difference between GADD34 and AKAP18 in the regulation of PP1 activity is that the AKAP also anchors PKA to the complex, enhancing I-1 phosphorylation. Thus, the ability of AKAP18 to sequester I-1 and PKA ensures spatial-temporal control of I-1 phosphorylation.

Several AKAPs have been previously implicated to bind to and regulate phosphatase activity. For example, the direct association between AKAP220 and PP1 inhibits phosphatase activity (Schillace et al., 2001). Additionally, mAKAP binds to PP2A and mediates a cAMP-induced increase of phosphatase activity through PKA phosphorylation of PP2A-B56δ subunit(Dodge-Kafka et al., 2010). In contrast to these other complexes, PP1 bound to AKAP18 is active, and PKA phosphorylation of the phosphatase's regulator results in its inhibition. To date, this is the first identification of an AKAP-mediated regulation of I-1 phosphorylation.

In the heart, I-1 activity is spatially restricted and selectively regulates PP1 activity at the sarcoplasmic reticulum, where PP1 opposes phospholamban phosphorylation (Nicolaou et al., 2009a; Pathak et al., 2005). In contrast to the smaller isoforms of AKAP18 (α and β) that are targeted to the plasma membrane, the long isoforms (γ and δ) bind directly to phospholamban, facilitating PKA phosphorylation of phospholamban (Lygren et al., 2007). Our results imply that AKAP18 may facilitate the localization of I-1 to the sarcoplasmic reticulum, further promoting phospholamban phosphorylation. Future investigations in our laboratory will address how the

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intracellular targeting of I-1 by the long isoforms of AKAP18 affects the kinetics and sensitivity of phospholamban phosphorylation.

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Authorship Contribution:

Participated in Research Design: Singh, Redden, Kapiloff, and Dodge-Kafka

Conducted experiments: Singh, Redden and Dodge-Kafka

Contributed new reagents or analytic tools: none

Performed data analysis: Singh, Redden, Kapiloff, and Dodge-Kafka

Wrote or contributed to the writing of the manuscript: Singh, Redden, Kapiloff, and Dodge-Kafka

Other: Dodge-Kafka and Kapiloff acquired funding for the reseach.

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Footnotes:

This work was supported by the National Institute of Heart, Blood Lung [Grants 1R01-HL82705 and R01-HL075398].

Molecular Pharmacology Fast Forward. Published on December 13, 2010 as DOI: 10.1124/mol.110.065425
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Figure legends:

Figure 1: Inhibitor-1 associates with PKA in heart cells

A. Rat heart extracts were incubated with Rp-cAMPs-agarose in the presence or absence of excess cAMP (50 mM). I-1 in whole extract (Ex, 2% of total) and the cAMP-associated fraction of I-1, PKA-RI, PKA-RII, and PKA-catalytic subunit were determined by immunoblotting. n=3. B. Rat heart extract was incubated with either GST-I-1 (3 μg) or control GST protein, and the bound proteins were assayed for PKA activity using Kemptide as a substrate. n=3, *p<0.008, **P<0.01. C. PKA-RI, PKA-RII, and PKA-catalytic subunit present in GST control and GST-I-1 pulldowns were detected by immunoblot. Extract is 5% of the total protein used for each pulldown.

Figure 2: AKAP18 associates with I-1

A. Isolated rat heart extract was incubated with either GST-I-1 or control GST-protein. AKAP18 was detected by western blot (left) and total protein by Ponceau stain (right) n=3. B. HEK293 cells expressing GFP-AKAP18γ in the presence and absence of I-1 were used for immunoprecipitations with an I-1 antibody, and AKAP18γ association was determined by immunoblot using a GFP antibody. C. HEK293 cells were transfected with Inhibitor-1 in the presence and absence of GFP-tagged AKAP18γ. Isolated extracts were incubated with cAMP agarose and immunoblotting demonstrated association of I-1. Immunoblots of whole cell extracts (2.5% of total) are shown as controls for both B and C. n=3 for all panels.

Figure 3. Direct binding of AKAP18γ and Inhibitor-1. A. Schematic diagram depicting the known AKAP18 splice variants. PLN, phospholamban; LTCC, L-type calcium channel. B. GST-I-1 fusion protein (3 μg) was incubated with S-tagged AKAP18α or S-tagged AKAP18γ fusion protein (3 μg), or S-protein agarose alone. S-tagged pulldown assays were performed and association of GST-I-1 was determined by western blot. Total protein was detected by Ponceau stain.

Figure 4: AKAP18 orchestrates I-1 phosphorylation by linking PKA with the Inhibitor. A. GST-I-1 fusion protein (3 μg) was incubated with HEK293 cell lysate extracted from prepared from cells expressing AKAP18γ, or AKAP18γ 1-268. PKA activity was determined in each GST pulldown in the presence and absence of 50 nM PKI. B. Pulldowns were performed as described in A and the association of the AKAP, as well as PKA-RII with GST-I-1 was demonstrated by immunoblot. C. GST-I-1 fusion protein (3 μg) was incubated with HEK293 cell lysate as prepared in A, and PKA associated with the GST pulldowns was activated by the addition of cAMP. I-1 phosphorylation was detected by western blot using an antibody that specifically recognized the inhibitor when phosphorylated at Thr-35 (upper panel). Total I-1 in each pulldown is shown in the middle panel while protein expression of the different AKAP18 constructs is shown in the bottom panel. D. HEK293 cells expressing I-1 and either full-length AKAP18γ or AKAP18γ 1-268 were stimulated with 1 μM Isoproterenol for 5 minutes. Whole cell lysate were analyzed by western blot using antibodies against phospho-I-1 Thr-35 (upper panel), I-1 (middle panel) and GFP for the AKAP18

constructs (lower panel). E. Densitometric quantification of I-1 phosphorylation shown in C normalized to isoproterenol, AKAP18γ sample. n=3 for each panel, *p<0.05.

Figure 5: AKAP18 is a PP1 anchoring protein

A. Protein complexes were immunoprecipitated from rat heart extract with either an antibody specific to PP1 catalytic subunit or control IgG and detected using an AKAP18 antibody. Extract represents 2.5% of total input. B. Rat heart extracts were incubated with either STREP-tagged (ST) AKAP18γ fusion protein or control STREP protein. PP1 in the pulldowns and total extract (2.5% of total input) were detected with an antibody to PP1 catalytic subunit. C. Protein complexes were immunoprecipitated from rat heart extract with either an antibody specific to AKAP18 or control IgG and assayed for phosphatase activity in the presence or absence of okadaic acid (100 nM). Amount of PP1 in each group is demonstrated by western blot analysis of the immunoprecipitate. D. Protein complexes were immunoprecipitated from lysates prepared from control HEK293 cells or cells expressing GFP-tagged AKAP18γ using an AKAP18 antibody. Precipitated PP1 was detected using a catalytic subunit antibody. E. Protein complexes were immunoprecipitated from lysates prepared from HEK293 cells expressing GFP-tagged AKAP18γ using either a PP1 or PP2A catalytic subunit antibody (designated as PP: Protein Phosphatase). AKAP18 in the immunoprecipitates and AKAP18, PP1 and PP2A in the total extract (2.5% of total) were detected by immunoblot. n=3 for all panels.

Figure 6: AKAP18 sequesters PKA, PP1 and I-1 to a specific signaling complex

Molecular Pharmacology Fast Forward. Published on December 13, 2010 as DOI: 10.1124/mol.110.065425
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Protein complexes were immunoprecipitated from lysates prepared from HEK293 cells transfected with I-1 plus and minus GFP-AKAP18γ using antibodies to AKAP18, I-1, PP1 and PKA-RII. Proteins in the extracts (bottom panels) and immunoprecipitates (top panels) were detected with the respective antibodies. Total protein (5% of input) is shown below. n=3 for each.

Figure 7: PKA binding to AKAP18 is necessary for PKA-induced inhibition of PP1. A. Protein complexes were immunoprecipitated from rat heart extract with either an antibody specific to AKAP18 or control IgG and assayed for phosphatase activity in the presence and absence of CPT-cAMP (3 μM) or PKI (50 nM) B. Phosphatase activity associated with protein complexes immunoprecipitated using an antibody specific for myc-tag from lysates prepared from HEK293 cells expressing I-1 and either myc-tagged full-length AKAP18γ or AKAP18γ 1-268 was assayed. Endogenous PP1 was used. Cells were stimulated with 1 μM isoproterenol for 5 minutes as indicated. AKAP18 proteins in the total lysates were detected with a myc antibody. n=3 for each panel, *$p<0.05$. C. Cell lysates were prepared as in B. PP1 immunocomplexes were isolated and association of AKAP18 and I-1 was determined by western blot. n=3 for each.

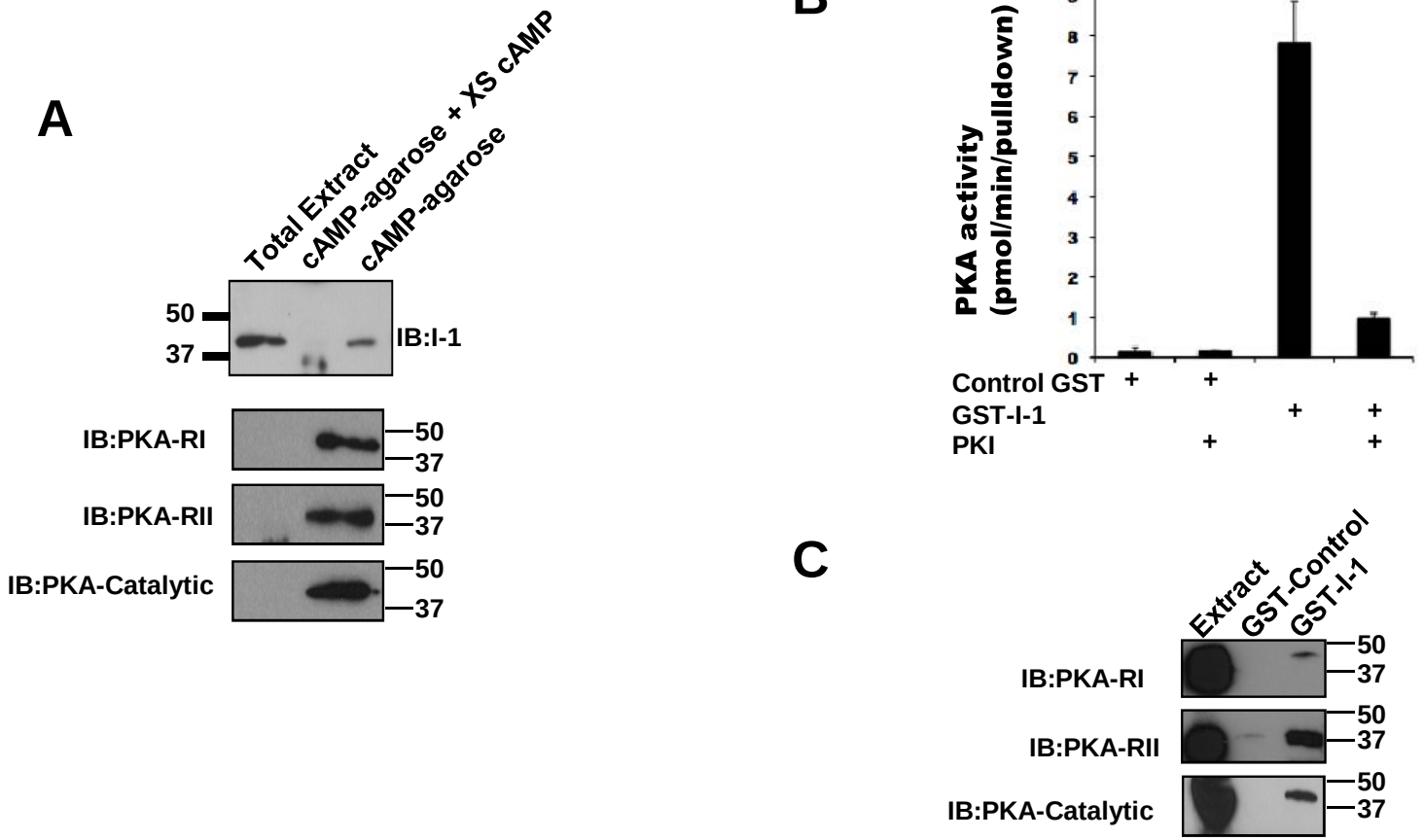


Figure 1

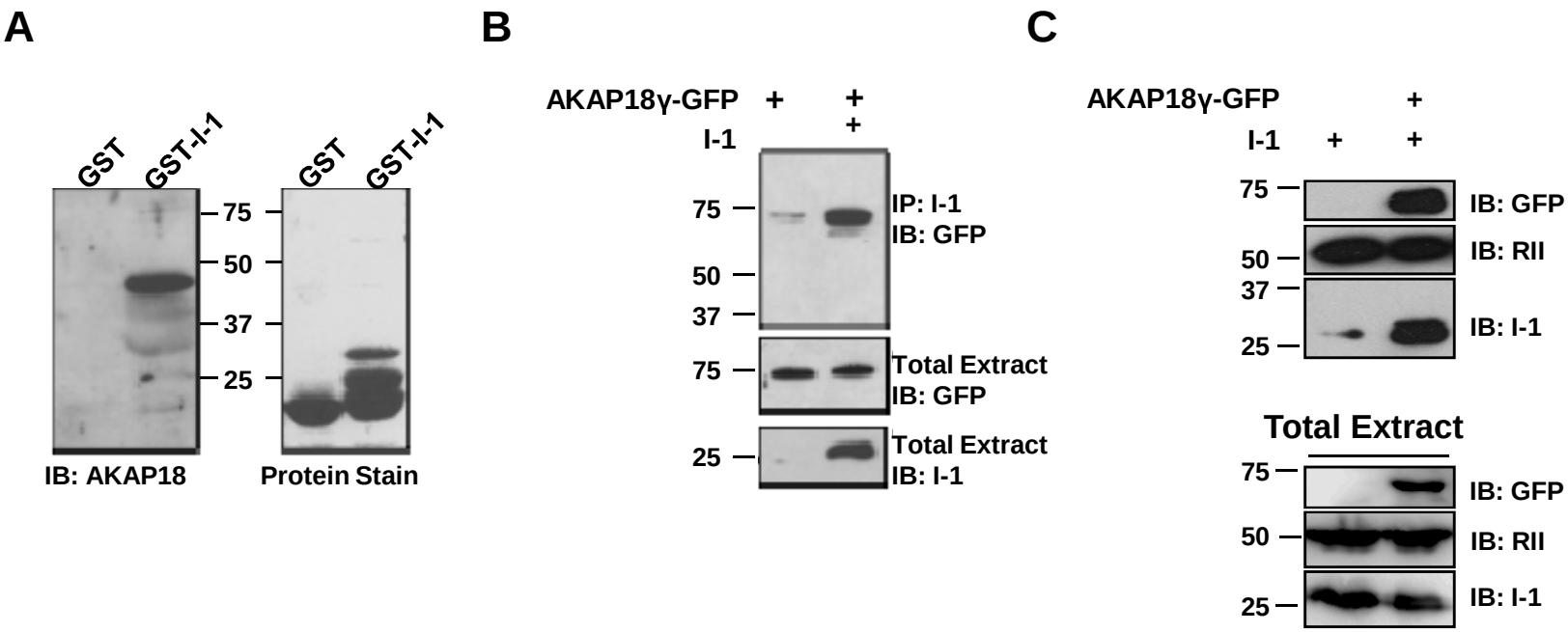
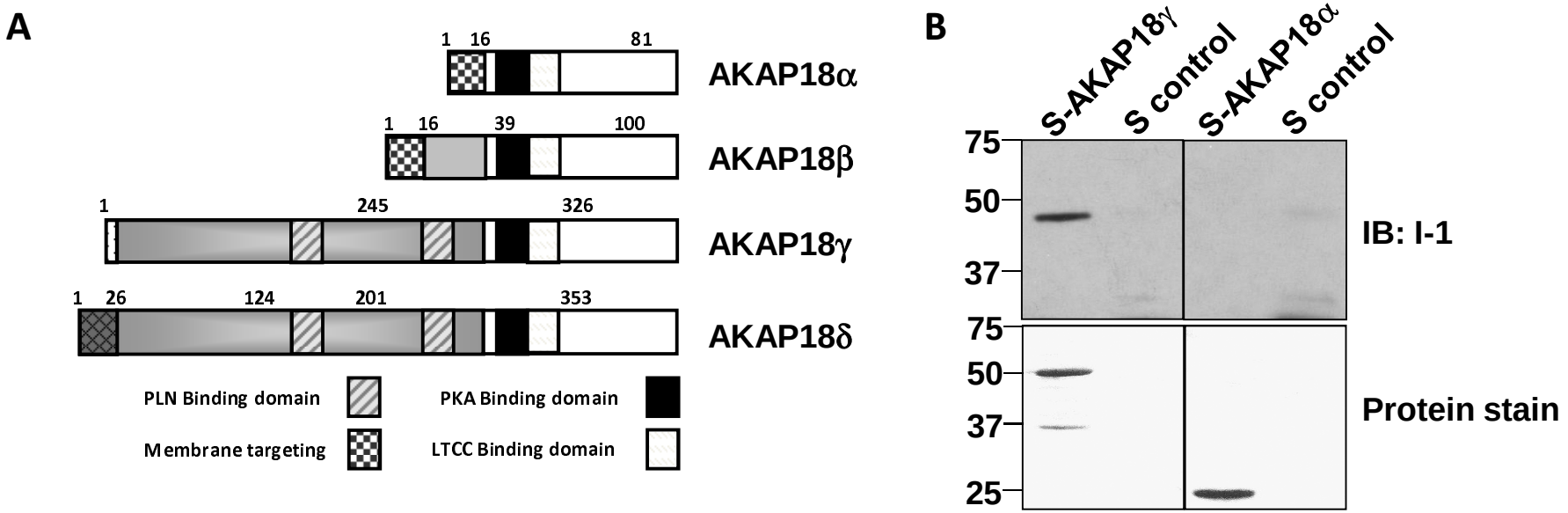


Figure 2



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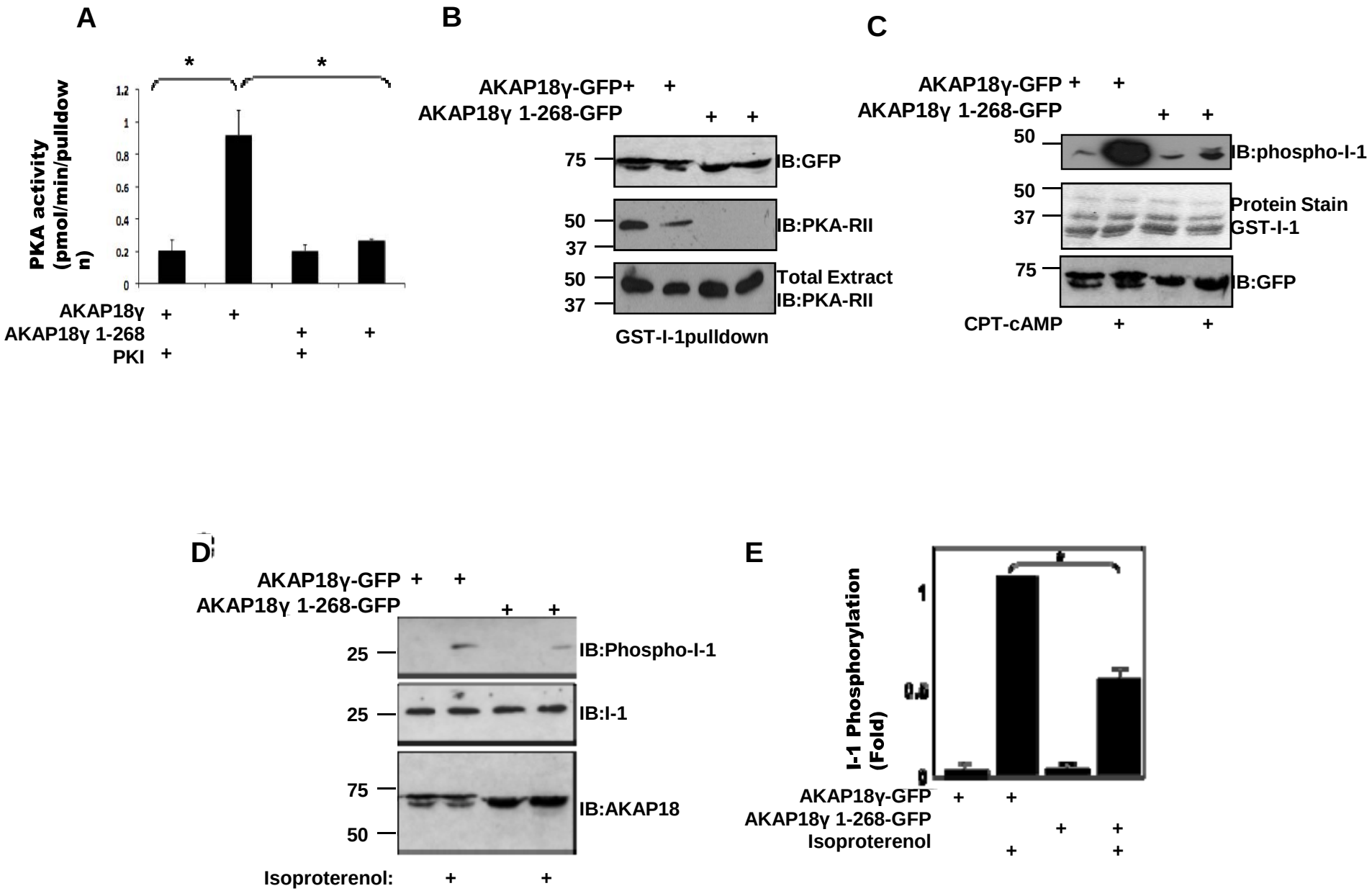

Figure 4

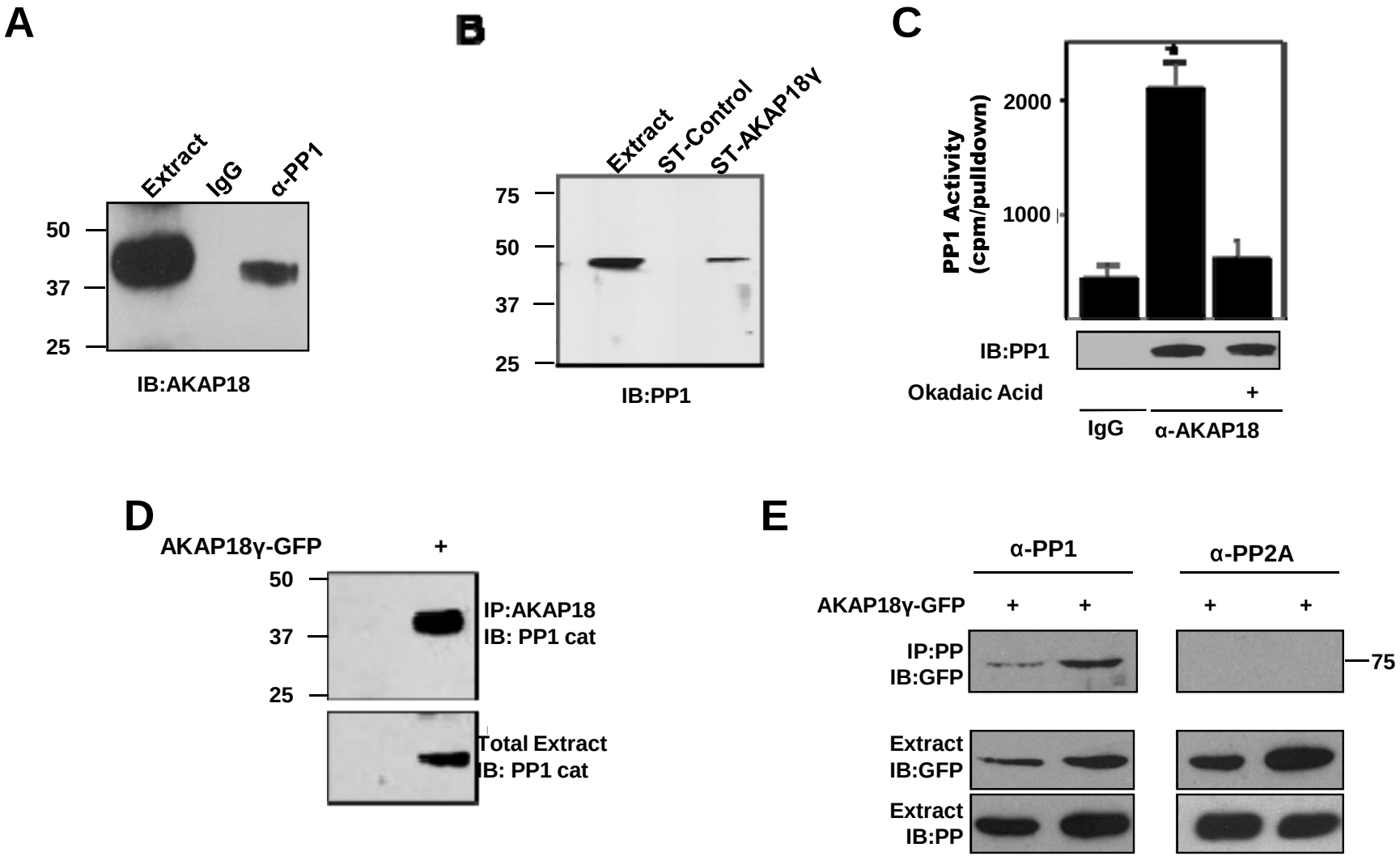

Figure 5

Figure 6

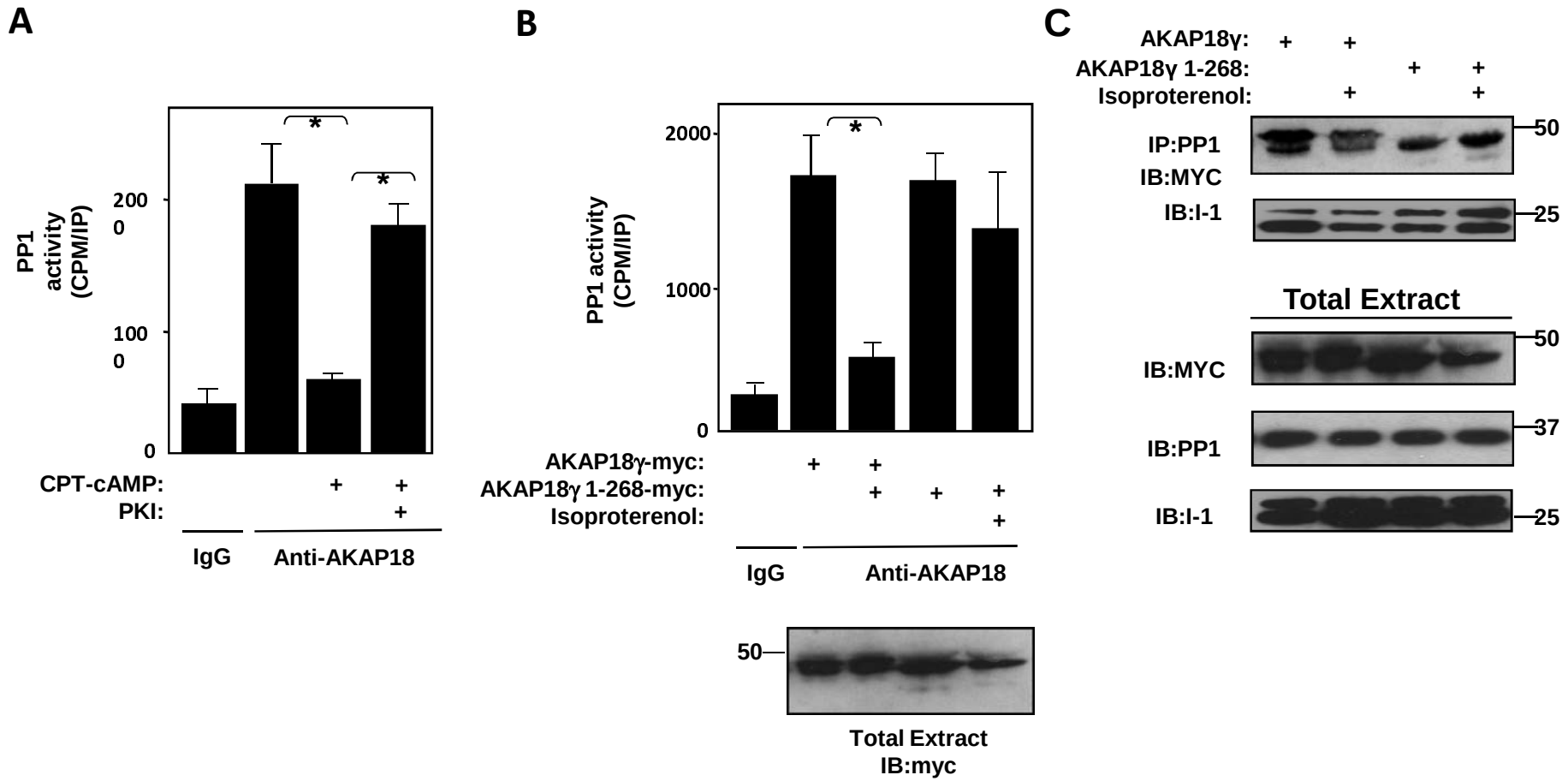

Figure 7