Phosphorylation of specific residues in the activation loops of AGC kinase group (protein kinase A, G, and C families) is required for activity of most of these kinases, including the catalytic subunit of PKA (PKAc). Although many phosphorylated AGC kinases are sensitive to phosphatase-mediated dephosphorylation, the PKAc activation loop uniquely resists dephosphorylation, rendering it “constitutively” phosphorylated in cells. Previous biophysical experiments and structural modeling have suggested that the N-terminal myristoylation signal and the C-terminal FXXF motif in PKAc regulate its thermal stability and catalysis. Here, using site-directed mutagenesis, molecular modeling, and in cell-free and cell-based systems, we demonstrate that substitutions of either the PKAc myristoylation signal or the FXXF motif only modestly reduce phosphorylation and fail to affect PKAc function in cells. However, we observed that these two sites cooperate with an N-terminal FXXW motif to cooperatively establish phosphatase resistance of PKAc while not affecting kinase-dependent phosphorylation of the activation loop. We noted that this tripartite cooperative mechanism of phosphatase resistance is functionally relevant, as demonstrated by changes in morphology, adhesion, and migration of human airway smooth muscle cells transfected with PKAc variants containing amino acid substitutions in these three sites. These findings establish that three allosteric sites located at the PKAc N and C termini coordinately regulate the phosphatase sensitivity of this enzyme. This cooperative mechanism of phosphatase resistance of AGC kinase opens new perspectives toward therapeutic manipulation of kinase signaling in disease.

cAMP-dependent protein kinase (PKA)² is an essential part of signaling networks across multiple cell types (1, 2). PKA is a member of the AGC kinase family, which also includes Akt and protein kinase C. Phosphorylation of a threonine residue in the activation loop (Thr¹⁹⁷) of its catalytic subunit (PKAc) is an essential prerequisite for PKA enzymatic activity and protein stability (1, 3). Maintaining phosphorylation of the centrally located activation loop is critical for catalytic activities in most AGC kinases (4, 5). However, PKA is unique in that it is relatively resistant to activation loop dephosphorylation compared with other AGC family members (6–8). Little is known about the structural motifs of PKA that underpin its activation loop dephosphorylation resistance.

Molecular mechanisms that gate access of phosphatases to protein kinases are increasingly being recognized as key elements of kinase steady-state activity. For example, in the presence of protective elements (membrane binding and C-terminal pocket and ATP pocket occupation), we and others have identified conserved residues within the Akt kinase core domain that interact directly with the activation loop to protect it from dephosphorylation by cellular phosphatases (9–12). Together, these elements establish a “phosphatase-resistant cage conformation” of the catalytic cleft that is shared among AGC kinases. In addition, we demonstrated previously that a molecular motif outside of the core domain of Akt allosterically contributes to phosphatase resistance of its activation loop residue (13). Based on these earlier findings, we hypothesized that sequences outside of the catalytic cleft and unique to PKA may also contribute to allosteric control of the phosphatase-resistant cage conformation of this AGC kinase.

Here we describe, for the first time, three molecular motifs located in the PKAc N and C termini that cooperatively stabilize the activation loop conformation and restrict phosphatase access to Thr¹⁹⁷. This conformation is critical for PKAc stability, expression, and function and contributes to the unique phosphorylation-resistant nature of PKA.

Results
The PKA catalytic subunit resists dephosphorylation by phosphatase enzymes
PKA and Akt kinases are members of the AGC kinase family and share threonine residues in the activation loop (Thr³⁰⁸ in Akt1 and Thr¹⁹⁷ in PKAc) that need to be phosphorylated for activity. In the absence of protective elements
membrane binding and C-terminal pocket and ATP pocket occupation), the prephosphorylated Akt activation loop is exquisitely sensitive to dephosphorylation. In contrast, the prephosphorylated PKAc activation loop constitutively resists dephosphorylation under cell-free native and quiescent cell culture conditions (6–8, 13). We validated different rates of Akt and PKAc dephosphorylation under consistent cell-free assay conditions by incubating 50 ng of recombinant PKAc phosphorylated at Thr197 and 50 ng of recombinant Akt phosphorylated at Thr308 with recombinant phosphatases. We used Escherichia coli-derived λ-phosphatase (Fig. 1A), which could release phosphate groups from phosphorylated serine, threonine, and tyrosine residues, and also the protein phosphatase 2A (PP2A) catalytic subunit (Fig. S2). PP2A is ubiquitously expressed in eukaryotes and releases phosphorylated serine/threonine residues with a broad substrate specificity. Consistent with our earlier observations (13), pThr308 within the Akt activation loop was readily dephosphorylated within 60 min of adding phosphatases to cell extracts. In contrast, less than 2% of PKAc Thr197 was dephosphorylated within this time frame. Thus, consistent with earlier reports, the purified PKA catalytic subunit is highly phosphatase-resistant compared with Akt.

Figure 1. Structural basis for differential phosphatase resistance between PKAc and Akt. A, recombinant (Recomb) PKAc is more resistant to dephosphorylation than recombinant phospho-Akt1 in a cell-free assay. Recombinant PKAc (50 ng) and recombinant Akt1 (50 ng) were incubated with recombinant bacteriophage λ phosphatase (40 units) for 60 min at 30 °C. Images show Akt1 Thr308 and PKAc Thr197 phosphorylation and total Akt and PKAc. Graphs show the percentage of pThr197 dephosphorylation. n = 4/group, *p < 0.01 for phosphatase versus phosphatase + ATP pocket inhibitor (1 μM A443654). B, amino acid homology comparison between human Akt2 and the human PKA catalytic subunit. Shown is the myristoylated (Myr) PKA–Mn-ATP–cAMP-dependent protein kinase inhibitor peptide structure (PDB code 4DG0). The PKAc myristoylated lipid (light blue), the FXXW motif in the N terminus (N-term, blue), and the C terminus (C-term) FXXF motif (red) form stable and high-affinity contacts.
Protein Kinase A phosphatase sensitivity

Structural determinants of the PKA catalytic subunit that affect activation loop phosphatase resistance

The catalytic domains of PKA and Akt share a high degree of structural and amino acid homology. Sequences outside of their protein kinase cores diverge significantly (PKA, PDB code 4DG0; Akt2, PDB code 1O6K; Fig. 1B). Based on molecular and thermal stability calculations, we hypothesized that N-terminal and C-terminal sequences unique to PKA underpin the resistance of pThr197 to phosphatase attack by restraining the flexibility of the catalytic cleft conformation. As illustrated in the structure of the PKA catalytic subunit containing the native myristoyl lipid (14) (PDB code 4DG0, Fig. 1B), the PKAc N terminus (residues 1–40) contains an α-helix and an N-terminal myristoylation site that pack stably against the protein kinase core (15). The corresponding amino acid sequences in Akt kinase are unstructured (16). Similarly, a PKAc C-terminal FXXF motif (347FXXF350) has been found previously to enhance thermal stability and enzymatic catalysis (17).

Our earlier Chemistry at HARvard Macromolecular Mechanics molecular simulation program-based analysis of the PKAc structure showed that the FXXF motif contained in the C terminus is well-ordered and is predicted to form “high-contact-order” interactions in the kinase domain to stabilize the tertiary structures (18). In contrast, the AKT kinase structure shows that the Akt C-terminal sequences around the F motif are disordered and that the AKT F motif interacts with the kinase domain in a more labile and dynamic fashion. The AKT kinase C terminus FXXF motif binds the kinase domain with weak affinity (3600 μM) (19). However, replacing the AKT C terminus FXXF motif with a high-affinity peptide (P1Ftide, 6 μM) markedly increased protection of the AKT activation loop from dephosphorylation (13), suggesting that the strength of the intramolecular interactions of the PKAc FXXF motif with the kinase domain may also regulate phosphatase protection of the activation loop. Based on these considerations, we decided to assess the relative contribution of N- and C-terminal sequences of the catalytic subunit of PKA to phosphatase sensitivity and resistance.

Disabling myristoylation and the FXXF motif cooperatively reduces phosphatase resistance of the PKA catalytic subunit

Our previous work with myristoylated Akt revealed that mutating the FXXF motif effectively reduced Akt phosphorylation by over 90% (Fig. S3) (10, 13). To test whether phosphorylation of myristoylated PKA was similarly affected by its FXXF motif, we abolished, by site-directed mutagenesis, the myristoylation and C-terminal sequences unique to PKA underpin the resistance of pThr197 to phosphatase attack by restraining the flexibility of the catalytic cleft conformation. As illustrated in the structure of the PKA catalytic subunit containing the native myristoyl lipid (14) (PDB code 4DG0, Fig. 1B), the PKAc N terminus (residues 1–40) contains an α-helix and an N-terminal myristoylation site that pack stably against the protein kinase core (15). The corresponding amino acid sequences in Akt kinase are unstructured (16). Similarly, a PKAc C-terminal FXXF motif (347FXXF350) has been found previously to enhance thermal stability and enzymatic catalysis (17).

The N-terminal structure of the PKA catalytic subunit shows that the myristoylation sequence (residues 1–10) and the α-helix (residues 11–34) stably pack against the kinase domain (Fig. 3A, reviewed in Ref. 15). By mutational analysis, we determined that, even when the myristoylation signal was preserved, partial deletion of the α-helix (Δ24–34) resulted in reduced PKAc activation loop phosphorylation (Fig. 3A). Further alanine scanning and mutational analysis determined that only Phe26 and Trp30 (subsequently termed the FXXW motif) are required for protection of the activation loop against dephosphorylation (Fig. 3B). These data are consistent with previous work by Taylor and co-workers (20, 21) demonstrating that Phe26 and Trp30 occupy a deep hydrophobic pocket within the kinase core and that mutating Trp30 increases PKA instability.

To ascertain the relative contribution of the FXXW motif to activation loop phosphorylation, we disabled the amino acid terminal FXXW motif (F26A/W30A) and the C-terminal FXXF motif (F347A/F350A) alone or in combination. Upon transfection into HASM cells, we observed that, even in the presence of myristoylation sequences, the combined mutations FXXW and FXXF reduced Thr197 phosphorylation in a cooperative manner. Combined mutations reduced phosphorylation to 68% ± 7% (p < 0.05) compared with 21% ± 18% reduction in the FXXW mutation and 25% ± 12% reduction in the FXXF mutation (Fig. 3C and Figs. S4, A and B, and S6).

Quantification of the data, summarized in Fig. S4B, showed the effects of mutating all combinations of the three molecular motifs under investigation. Mutating both G1A/FXXF more than additively decreased pThr197 phosphorylation compared with mutating each motif alone (Fig. 2D). Mutating FXXW/FXXF motifs also more than additively decreased phosphorylation compared with mutating each motif alone (Fig. 3C). Interestingly, mutating both sites at the N terminus (G1A and FXXW) did not further decrease pThr197 phosphorylation (G1A/FXXW, 29% ± 26%; G1A, 23% ± 18%; FXXW, 21% ± 18%; Fig. S4, A and B). Collectively, these results suggest that intramolecular interactions of two N-terminal nodes (myristoylation signal and the FXXF motif) and a C-terminal FXXF motif cooperated to protect the PKAc activation loop against dephosphorylation in a superadditive manner.

Tripartite cooperative regulation of PKAc dephosphorylation sensitivity by the N-terminal FXXW and myristoylation sites and the C-terminal FXXF motif

The N-terminal structure of the PKA catalytic subunit shows that the myristoylation sequence (residues 1–10) and the α-helix (residues 11–34) stably pack against the kinase domain (Fig. 3A, reviewed in Ref. 15). By mutational analysis, we determined that, even when the myristoylation signal was preserved, partial deletion of the α-helix (Δ24–34) resulted in reduced PKAc activation loop phosphorylation (Fig. 3A). Further alanine scanning and mutational analysis determined that only Phe26 and Trp30 (subsequently termed the FXXW motif) are required for protection of the activation loop against dephosphorylation (Fig. 3B). These data are consistent with previous work by Taylor and co-workers (20, 21) demonstrating that Phe26 and Trp30 occupy a deep hydrophobic pocket within the kinase core and that mutating Trp30 increases PKA instability.

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Rephosphorylation of PKAc mutants in cells and phosphatase sensitivity

Decreased phosphorylation in cells could be due to reduced phosphorylation, increased dephosphorylation, or a combination of both. To distinguish effects of PKA mutations on PKAc phosphorylation versus PKAc dephosphorylation, we took advantage of the prior observation that PDK1 phosphorylates the activation loop of many AGC kinases, including PKAc (22).

Specifically we cotransfected PKAc WT or a phosphorylation-defective PKAc construct (F26A/W30A/F347A/F350A) with a construct encoding PDK1. In the presence of the phos-
Phosphatase inhibitor pervanadate, these constructs were efficiently phosphorylated in HEK293 cells (Fig. 4A).

Flash-frozen cell extracts prepared from maximally phosphorylated WT and F26A/W30A/F347A/F350A mutant allowed us to monitor time-dependent dephosphorylation of PKAc variants independent of concurrent changes in phosphorylation. Time-dependent dephosphorylation was assessed in EDTA detergent cell-free extracts as described recently (13). As expected, PKAc WT was only marginally dephosphorylated (6% ± 4%) after 30 min of incubation at 30 °C (Fig. 4B). In contrast, the PKAc-F26A/W30A/F347A/F350A mutant was dephosphorylated by 52% ± 6% (p < 0.01 compared with WT PKAc) under the same experimental conditions.

Because transfected PKAc mutant proteins often expressed at a lower level, we developed an immunoprecipitation-based phosphatase assay where equal amounts of PKAc WT and mutant proteins were used. The PKAc catalytic subunit fused with three copies of the HA epitope was deleted (aa 13–34 or 24–34). The constructs were transfected into HEK293 cells. Protein lysates were subjected to immunoblot analysis using antibodies for phospho-PKA Thr197 and the HA tag. Molecular mapping of amino acids affects PKA phosphorylation in the PKAc N terminus. Phe26 and Trp30 regulate Thr197 phosphorylation. HEK293 cells were transfected with PKAc-3xHA WT or the indicated mutants. Protein lysates were subjected to immunoblot analysis using antibodies for phospho-PKA Thr197 and the anti-HA tag. The graph shows the percentage of HA-PKAc dephosphorylation (Dephos %). n = 17, 10, 9, and 8 for the indicated groups. *, p < 0.01 FW/FF versus FW or FF.

**Figure 3. N-terminal FXXW contributes to activation loop phosphorylation protection in concert with the C-Terminal FXXF motif.** A, the PKAc N-terminal α-helix (blue, aa 13–40) forms stable contacts with the kinase core amino acids. The PKA catalytic subunit fused with three copies of the HA epitope was deleted (aa 13–34 or 24–34). The constructs were transfected into HEK293 cells. Protein lysates were subjected to immunoblot analysis using antibodies for phospho-PKA Thr197 and the HA tag. B, molecular mapping of amino acids affects PKA phosphorylation in the PKAc N terminus. Phe26 and Trp30 regulate Thr197 phosphorylation. HEK293 cells were transfected with PKAc-3xHA WT or the indicated mutants. Protein lysates were subjected to immunoblot analysis using antibodies for phospho-PKA Thr197 and the anti-HA tag. The graph shows the percentage of HA-PKAc dephosphorylation (Dephos %). n = 17, 10, 9, and 8 for the indicated groups. *, p < 0.01 FW/FF versus FW or FF.
We immunoprecipitated equal amounts of PKAc WT and PKAc mutants from the cell extracts. Incubating the immunoprecipitates in a buffer containing recombinant phosphatase showed that PKAc mutants were dephosphorylated faster than WT-PKAc (G1A/F347A/F350A, 84% ± 4%; G1A/F26A/W30A/F347A/F350A, 74% ± 8%; PKAc WT, 41% ± 9%, p < 0.05; Fig. 4C). Taken together, these results indicate that the mutations affecting the FXXF and FXXW motifs do not interfere with the ability of PKAc to be phosphorylated and that the combined mutations affect PKAc dephosphorylation resistance independent of effects on phosphorylation per se.

**N-terminal FXXW and C-terminal FXXF motifs protect PKAc function**

We next tested the effects of modifying PKAc dephosphorylation resistance on PKA function in cells. PKA activation by...
cAMP is known to regulate HASM cell contraction/relaxation/motility (2). Transient overexpression of the WT PKAc construct in HASM cells resulted in a rounded cell morphology (normally HASM cells are elongated in morphology) within 24 h, whereas overexpressing the catalytically inactive PKAc-T197A mutant did not affect cell morphology in either HASM or HEK293 cells (Fig. 5A and Fig. S7A). Cells expressing either G1A or F347A/F350A PKAc still had the rounded HASM cell...
morphism. However, cells expressing mutant constructs (G1A/F347A/F350A) did not undergo morphological transition (Fig. 5B), consistent with PKAc loss of function. Similarly, G1A/F26A/W30A/F347A/F350A mutant- or F26A/W30A/F347A/F350A-transfected cells did not undergo morphological transition (data not shown).

Next we tested the effect of PKA construct expression in HASM cell adhesion and spreading by use of the xCELLigence (ACEA Biosciences) system, which allows noninvasive electric cell substrate impedance measurements. Impaired cell spreading manifests as reduced impedance of confluent cell layers in this assay. Six hours after transfection, equal numbers of cells were placed into electronic microtiter plates. Starting 2 h after plating, PKAc WT–expressing cells had reduced impedance values compared with the G1A/F347A/F350A mutant and vector control, consistent with faster adhesion and spreading in the absence of PKA activity (Fig. 5C).

Finally, PKA is known to inhibit migration of HASM cells (23). We therefore examined the effects of PKAc variants with differential phosphatase sensitivity on HASM cell migration by tracking cell migration into the 500-µm space created by a silicone insert (Ibidi). Transfected mutant–expressing cells were tracked by cotransfected GFP plasmid. PKAc WT–expressing cells failed to migrate into the gap space after 72 h, whereas cells expressing the PKAc G1A/F347A/F350F mutant migrated into the gap space, similar to vector control–transfected cells (Fig. 5D and Fig. S7B). These results suggest that the cooperative preservation of PKA Thr<sup>197</sup> phosphorylation between the PKAc myristoylation signal, the N-terminal FXFW motif, and the C-terminal FXXF motif is vital for PKA function.

**Discussion**

Maintaining phosphorylation of the centrally located activation loop is critical for the catalytic activities of most AGC kinases (4, 5), including PKA’s Thr<sup>197</sup> site. Compared with other AGC kinases, the PKA activation loop resists dephosphorylation in both cell-free and cell-based assay formats more effectively (6–8, 13). Here we define a cooperative mechanism by which three distinct regulatory sites embedded in the PKA kinase domain inhibit Thr<sup>197</sup> dephosphorylation. The three sites are the native N-terminal myristoylation site, an N-terminal FXFW motif adjacent to the myristoylation site; and an FXXF motif at the extreme C terminus (Fig. 6C). These three motifs allosterically contribute to establishment of a strong network of electrostatic interactions that stabilize directional interactions of His<sup>87</sup> and Arg<sup>165</sup> within the phosphorylated activation loop, restricting phosphatase access to Thr<sup>197</sup> (Fig. 6C). This PKA conformation is reminiscent of the Akt kinase activation loop (Thr<sup>308</sup>) structure (His<sup>194</sup> and Arg<sup>273</sup> in Akt1) reported by us previously, which similarly shields Akt from phosphatase attack (10). As determined previously (9), PKAc dephosphorylation is enhanced by mutating amino acids surrounding PKA Thr<sup>197</sup> (C199A and R194A). Thus, it is likely that the three distantly located regulatory sites allosterically contact these closer sites to restrain phosphatase access to pThr<sup>197</sup>.

Prior biophysical, enzymatic, and structural analyses of purified PKAc protein suggested that each of the three PKAc motifs (myristoylation signal, N-terminal FXFW motif, and C-terminal FXXF motif) regulate thermal stability and/or catalytic function (17, 24–26). The data suggest that these domains do not act in isolation regarding PKA dephosphorylation resistance. In support of this conclusion, mutational “inactivation” of individual motifs only modestly reduced activation loop phosphorylation (20%–30%) and only marginally reduced the biological effects of transiently overexpressed PKAc variants overexpressed in human airway smooth muscle cells. In contrast, activation loop phosphorylation was significantly reduced when we simultaneously mutated the myristoylation signal and the FXXF motif, and this effect was associated with compromised function of transduced HASMCs. Similarly, mutational inactivation of the FXFW motif also cooperatively reduced activation loop phosphorylation and compromised PKAc function in HASMCs. However, mutating the myristoylation signal and the FXXW motif (the two N-terminally located motifs) did not further reduce PKAc phosphorylation, suggesting that the constitutive protection of the PKAc activation loop required one of the two N-terminally located motifs (myristoylation signal or FXWW) to independently cooperate with the C-terminal FXXF motif.

Our data suggest that the myristic acid lipid provides a stabilizing effect on PKAc (Fig. 6). It is interesting that the myristic acid lipid could interact intracellularly with a hydrophobic surface within PKAc (Myr-in) and with the cell membrane (Myr-out) (26, 27). The Myr-in→Myr-out conformational change has been shown to be thermodynamically accessible within the NMR timescale, according to recent NMR studies (26, 27).

The identification of molecular motifs that render the PKAc activation loop constitutively phosphorylated invites comparison with other AGC kinases, such as protein kinase C and Akt, which are sensitive to dephosphorylation by phosphatases. We suggest that similar allosteric mechanisms regulate both PKAc constitutive phosphatase protection and the “dynamic” phos-
phatase protection in Akt kinase and protein kinase C, albeit with differential contributions of each regulating motif (ATP binding, N-terminal hydrophobic surface/membrane binding, and C-terminal FXXF motif).

PKA structures, with or without a bound ATP analog, crystallize in an active “closed” conformation (14). In contrast, in the absence of bound ATP, Akt adopts an inactive “open” conformation. Thus, ATP binding pocket occupation plays a more prominent role in regulating Akt kinase dephosphorylation because of the requirement of bound ATP to establish an active closed conformation (10, 16, 19). In addition, although PKAc is constitutively exposed to a hydrophobic environment because of N-terminal myristic fatty acid, Akt kinase may only dynamically engage a hydrophobic environment upon binding of the N-terminal pleckstrin homology domain to PtdIns(3,4)P₂/PtdIns(3,4,5)P₃ lipids, produced by PI3K. In addition, the PKAc N-terminal FXXW motif critically contributes to stability and phosphatase resistance. The corresponding amino acids in the Akt kinase domain (Akt linker region, aa 114–144) lack the FXXW motif and show no electron density (28). It is possible that the absence of this motif underpins the dynamic control of phosphatase resistance in Akt kinase.

Finally, PKAc and Akt kinase require the C-terminal FXXF motif for phosphatase protection (Fig. 2 and Ref. 13). Although the PKA C-terminal FXXF motif forms high-affinity electrostatic interactions with the N-terminal lobe, the Akt C-terminal FXXF binds to the Akt kinase domain with comparatively low affinity (19). Importantly, we demonstrated previously that
engineering the Akt C-terminal FXXF motif to bind with higher affinity to the N lobe increases dephosphorylation resistance (13). Akt and PKA kinases share structural elements to enhance dephosphorylation resistance. However, differences in sequences surrounding these sites may underlie the resistance of PKA, but not that of Akt kinase, to cellular phosphatases in the cytosol.

The identification of molecular motifs that render the PKAc activation loop constitutively phosphorylated invites comparison with other AGC kinases, such as protein kinase C and Akt, which are sensitive to dephosphorylation by phosphatases. Phosphorylated activation loops are required for catalysis in all of these kinases (4, 29). However, in contrast to Akt kinase, phosphorylation of the PKA activation loop occurs during synthesis of the enzyme, not during kinase activation. The “prephosphorylated” active PKA catalytic subunit is folded into inactive larger structures via steric hindrance interactions with regulatory subdomains. Binding to the second messenger cAMP unleashes the catalytic activities of “primed” PKAc (30–32).

Although the E. coli–expressed PKAc undergoes autophosphorylation at the activation loop, PDK1 (3-phosphoinositide–dependent protein kinase 1) is most likely responsible for PKA activation loop phosphorylation in mammalian cells. PKAc mutant proteins that disrupted PDK1 phosphorylation in vitro also disrupted phosphorylation, whereas PKAc autophosphorylation–disrupting mutant protein was properly phosphorylated in mammalian cells (33). It is unclear whether PKD1 is the only kinase responsible for PKA activation loop phosphorylation. Embryonic stem cells with the PDK1 gene knocked out do not completely abolish phosphorylation of PKAc (34), leading to the suggestion that a heterologous kinase with in vitro characteristics of PDK1 could contribute to phosphorylation of PKAc in mammalian cells (22). Considering that the fully phosphorylated PKA catalytic subunit also becomes highly resistant to phosphatases in cells and in cell-free assays (6–8), the constitutive nature of PKAc phosphorylation is likely achieved via a combined effect of PDK1 phosphorylation, phosphatase resistance, as well as weak autophosphorylation. Supporting this idea, we showed here that our phosphatase-sensitive mutants could be rephosphorylated in cells with a combination of phosphatase inhibitor and enforced PDK1 expression (Fig. 4).

Evolution tracing showed that multiple PKAc genes are present in budding yeast (35), suggesting that constitutive activation loop phosphorylation is an early evolutionary event, whereas dynamic regulation of the activation loop in Akt kinases is adopted later. Protein kinase A is a vital molecular sensor and signaling intermediary that coordinates cellular responses to signals emanating from the intracellular milieu and the extracellular environment. A teleological explanation is that constitutive phosphorylation likely reflects nature’s preference to not have PKA activity dynamically regulated by phosphorylation, relying instead on compartmentalized regulation of PKA by cAMP and subcellular localization of the enzyme by A-kinase anchoring proteins. Perhaps the need to regulate A-kinase anchoring proteins and other regulatory elements of PKA activity by kinases requires PKA to be relatively immune to control by kinases. Regardless of the true reason for constitutive PKA activity, understanding the mechanisms of protection from phosphatases and the consequences of PKA dephosphorylation may provide unique opportunities for PKA therapeutic targeting.

Biophysical studies showed that PKAc with defective activation loop phosphorylation reduces protein expression, folding, and stability (36, 37). Also, alkylation and oxidative agents reduce PKAc activation loop phosphorylation and PKAc stability in cells (9). Thus, targeting PKA phosphatase sensitivity via these molecular motifs may offer more selective PKA targeting strategies to enhance or to reduce PKA activity in cells. The FXXF motif at the PKAc C terminus bears resemblance to a hydrophobic motif that occupies a solvent-exposed hydrophobic pocket conserved among AGC kinases (Fig. 1B). Dynamic occupation of this pocket (known as the PIF pocket) in other AGC kinases regulates protein ATP binding, activity, and interaction with substrates (22). We have shown that peptide binding in this pocket regulates phosphatase sensitivity in the AGC kinase member Akt (13). Also, phosphomimic allostERIC small molecules have been developed to target the AGC kinase member PDK1 that act as activators (38) or inhibitors (39).

Based on our mutation data showing that mutating at least two of three motifs (myristoylation site, FXXW, and FXXF) is required to affect phosphatase sensitivity, we speculate that a combination of small molecules targeting these sites could decrease PKAc phosphorylation. Encouraging such an approach, small molecules that bind PDK engaged in bidirectional allostERIC communication affected the PIF pocket of AGC kinase family members (40). Also encouraging is the recent clinical success of triple-combination therapies using exel-acafitor/ivacaftor/tezacaftor to treat cystic fibrosis (41, 42). These small molecules each target separate and non-redundant motifs on cystic fibrosis transmembrane conductance regulator to restore its function in a cooperative fashion.

In summary, we describe cooperation of three allosteric motifs contained in N-terminal and C-terminal PKAc sequences that restrain PKAc activation loop dephosphorylation. Given the importance of and interest in developing therapeutic compounds targeting AGC kinases, these observations raise the intriguing possibility that structural features that regulate overall kinase conformation, located outside of canonical ATP binding, are amenable to development of novel small-molecule modulators of kinase conformational states.

**Experimental procedures**

**Plasmids**

Human PROTEIN KINASE Aα was fused at the N terminus with three copies of the HA epitope in the pcDNA3 vector. A green fluorescent protein vector (pEGFP-N1, Clontech) was used in cotransfections to monitor transfection efficiency. All mutant constructs were under control of the cytomegalovirus promoter and generated using standard molecular biology strategies. We mutated the myristoylation site (G1A), Phe356 and Trp350 (FXXW motif, F26A/W30A), Phe357 and Phe350 (FXXF motif, F347A/F350A), and the Thr197 activation loop (T197A) of human PKA catalytic subunit α.
**Protein Kinase A phosphatase sensitivity**

**Chemicals**

A-443654, an ATP-competitive AGC kinase inhibitor (PKAc $K_i = 6.3 \text{ nm}$, Akt kinase $K_i = 0.16 \text{ nm}$) (43), was a kind gift from Abbott Laboratories. The phosphatase inhibitor Per-VO$_4$ was freshly prepared by mixing equal molar amounts of hydrogen peroxide and NaVO$_4$ (44). Forskolin (Sigma) stock solution was prepared in ethanol.

**Cell culture and transfection**

HASM cells cultures were established from human tracheae and primary bronchi as described previously (45). Third- to sixth-passage cells were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum. HEK293 cells were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. Cells were transfected using FuGENE 6 HD (Roche) according to the manufacturer’s protocols. Cells were homogenized on ice using NP40 lysis buffer (25 mM Tris-HCl (pH 7.6), 137 mM NaCl, 10% glycerol, 1% NP40, and 10 mM NaF) freshly supplemented with 1 mM sodium pyrophosphate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM EDTA, 10 mM PMSF, 1 mM NaVO$_4$, and 1 mM DTT. For immunoprecipitation, clarified cellular lysates were diluted with equal volumes of phosphatase assay buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 10 mM NaF) supplemented with 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 mM PMSF, 1 mM DTT, 0.05% NP40, and 0.05% Triton X-100.

HA-tagged PKAc WT and mutants were immunopurified by incubation with anti-HA agarose affinity gel (Sigma) for 3 h at 4°C. The immunoprecipitates were washed sequentially at 4°C, three times with an equal mixture of NP40 lysis buffer and phosphatase assay buffer, three times with phosphatase assay buffer and once with λ phosphatase buffer (New England Biolabs; 50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35 (pH 7.5), and 1 mM MnCl$_2$). After washing, immunoprecipitates were aliquoted and suspended in 36 μl of λ phosphatase buffer. The assays were initiated by adding 4 μl of recombinant phosphatase (50 ng of recombinant PP2A catalytic subunit or 60 units of λ phosphatase for 20 min at 30°C). Assays were stopped by adding an equal volume (40 μl) of 2× protein elution buffer (0.5% SDS, 150 mM DTT, 50 mM Tris-HCl (pH 7.5, 95°C), and 100 mM calycin) and heated at 95°C for 5 min. For analysis with the Wes imaging system, samples were diluted three times with NP40 lysis buffer and probed for PKAc pThr$^{197}$ and HA tag expression.

**Recombinant Akt and protein kinase A dephosphorylation in vitro**

Recombinant activated Akt1 (50 ng, Millipore) and recombinant active PKAc (50 ng, New England Biolabs) were dephosphorylated as described previously (13). Although the PKAc gene has a native myristoylation signal, recombinant PKAc was isolated from E. coli lacking the compatible vertebrate N-myristoylacyltransferases (14, 27). Thus, the purified PKAc enzyme was expected to be nonmyristoylated.

Brieﬂy, puriﬁed recombinant enzymes were suspended in 50 μl of optimized phosphatase assay buffer containing nonionic detergents and supplemented with metal ions (1 mM MnCl$_2$ or 5 mM MgCl$_2$) with or without 1 μM A-443654 ATP-competitive inhibitor. The assays were initiated by adding 60–70 ng of recombinant PP2A catalytic subunit (Leu$^{309}$ deletion, Cayman Chemicals) or 40 units of λ phosphatase (New England Biolabs).

**Immunoblotting**

Clariﬁed cellular lysates were immunoblotted with the automated ProteinSimple Wes digital Western system (ProteinSimple, San Jose, CA) or PAGE gel/LI-COR IR image western detection system (LI-COR Biosciences, Lincoln, NE).

**Optimized quantitative PKA pThr$^{197}$ detection with the Wes digital Western system**

When transfected in HASM cells, we found that the pThr$^{197}$/HA signal-to-noise ratio of PKAc constructs was relatively poor using the standard PAGE gel/LI-COR IR image detection system (Fig. S1A), whereas the automated ProteinSimple Wes digital Western system with the Abcam anti-
pThr\textsuperscript{197} antibody provided superior pThr\textsuperscript{197} detection sensitivity and consistency (Fig. S1, B and C). Thus, the Wes system was employed for detecting relatively weak PKAc phosphosite (Thr\textsuperscript{197}) signals. The samples, antibody diluent, primary antibodies, secondary antibodies, chemiluminescent substrate, and wash buffer were added to the designated wells in the manufacturer-provided microplate. After plate loading, separation electrophoresis and immunodetection were performed using instrument default settings. Data were analyzed using the manufacturer-provided Compass software.

For standard PAGE gel/LI-COR system detection, samples were boiled and separated by electrophoresis in a 4%–12% SDS-PAGE and transferred onto nitrocellulose membranes. For immunoblotting, membranes were blocked for 30 min with LI-COR blocking buffer and probed with antibodies at 4 °C overnight. The blots were subsequently incubated with either IRDye 700 or 800 secondary antibodies conjugated with IR fluorophores for 60 min. Bands were visualized and directly quantified using the Odyssey IR imaging system (LI-COR Biosciences).

The following antibodies were used at 1:1000 dilution for LI-COR and 1:50 for Wes: anti-phospho-PKAc (Thr\textsuperscript{197}, Cell Signaling Technology (5661) or Abcam (ab75991)), anti-total PKAc (Cell Signaling Technology, 4782), anti-phospho-Akt (Thr\textsuperscript{308}, Cell Signaling Technology, 4056), anti-total Akt (BD Biosciences, 610861), anti-GAPDH (Cell Signaling Technology (5174) or LifeTech (Am4300)), anti-COX (Cell Signaling Technology (5174) or LifeTech (Am4300)), anti-COX (Cell Signaling Technology, 4850), and anti-HA (Covance (MMS-101) or Cell Signaling Technology (3724)).

Cell adhesion assay

Cell adhesion and spreading were measured using the xCELLigence real-time cell analysis system (ACEA Biosciences) as described previously (46, 47). After 6 h of transfection, transfected HASM cells were detached by trypsinization and plated onto E-Plate 16 (ACEA Bioscience) at 6000 cells/well. The impedance of the plate was then monitored every 1 min for 10 h. Fluctuation of the impedance is calculated as the cell index over a period of time.

Migration assay

After 6 h of transfection, transfected HASM cells were detached by trypsinization, and 10,000 cells were plated into silicone insert molds with a 500-μm defined cell-free gap (Ibidi). 24 h after transfection, the mold barrier was removed. The gap spaces were imaged at (Ibidi). 24 h after transfection, the mold barrier was removed.

Modeling structure

Structural modeling was performed with our established methods (48). Figures were prepared based on structures of myristoylated PKAc (PDB code 4DFX) (14).

Calculation of relative free energy contributions

A Chemistry at HARvard Macromolecular Mechanics molecular simulation program-based method was utilized to approximate the free energy contribution of each residue side chain interaction with the kinase domain binding groove interface, as we detailed previously (49). The free energy contributions of side chains are approximated by first removing peptide backbone atoms, transforming the α carbon into a methyl group, and calculating the linear interaction energy difference between the scaled potential energies of the bound and free state (49). Calculations were performed utilizing the generalized-born with molecular volume implicit solvent model.

Statistical analysis

A commercial software package was used for statistical analysis (Prism, GraphPad Software, La Jolla, CA). Comparison of means ± S.E. was analyzed by nonparametric Mann–Whitney test or nonparametric analysis of variance (Kruskal–Wallis test with Dunn post-test). Nonparametric statistical tests were used to account for sample size and distribution limitations.

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