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AKAP-Lbc Nucleates a Protein Kinase D Activation Scaffold

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Summary

The transmission of cellular signals often proceeds through multiprotein complexes where enzymes are positioned in proximity to their upstream activators and downstream substrates. In this report we demonstrate that the A-kinase anchoring protein AKAP-Lbc assembles an activation complex for the lipid-dependent enzyme protein kinase D (PKD). Using a combination of biochemical, enzymatic, and immunofluorescence techniques, we show that the anchoring protein contributes to PKD activation in two ways: it recruits an upstream kinase PKCη and coordinates PKA phosphorylation events that release activated protein kinase D. Thus, AKAP-Lbc synchronizes PKA and PKC activities in a manner that leads to the activation of a third kinase. This configuration illustrates the utility of kinase anchoring as a mechanism to constrain the action of broad-spectrum enzymes.

Introduction

Efficient transduction of cellular signals often requires the positioning of signaling proteins in proximity to their upstream activators and downstream targets. The clustering of receptors, protein kinases, and phosphatases with their substrates contributes to the spatiotemporal regulation of signaling events (Hunter, 2000). This sophisticated degree of organization may also help to prevent the indiscriminate activation of related signaling complexes in the same vicinity (Bauman and Scott, 2002). This is of particular importance for second messenger-dependent signaling pathways that lead to the activation of broad specificity enzymes, such as the cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and a variety of other kinases and phosphatases (Carnegie and Scott, 2003). Compartmentalization of these enzymes is often achieved through their association with anchoring and scaffolding proteins that simultaneously coordinate the location of several enzymes (Pawson and Nash, 2003; Pawson and Scott, 1997).

A-Kinase Anchoring Proteins (AKAPs) are a family of scaffolding proteins that target PKA and other signaling enzymes to specified subcellular locations (Colledge and Scott, 1999; Tasken and Aandahl, 2004). Each AKAP contains a conserved amphipathic helix that binds to the R subunit dimer with high affinity (Carr et al., 1991; Newlon et al., 2001) and a targeting domain that directs the PKA-AKAP complex to specific subcellular compartments (Alto et al., 2002; Dell'Acqua et al., 1998; Kapiloff et al., 1999; Westphal et al., 1999, 2000). An important feature of AKAPs is their ability to interact with several signaling proteins. By simultaneously tethering PKA with enzymes such as phosphatases, phosphodiesterases, G proteins, and other protein kinases, these multivalent anchoring proteins provide focal points for the processing and integration of distinct intracellular messages (Dodge et al., 2001; Schillace and Scott, 1999; Tasken et al., 2001).

For over a decade, a PKA binding fragment called Ht31 has served as a model for the structural elucidation of how the PKA holoenzyme interacts with AKAPs (Carr et al., 1992). Recently, we and others have demonstrated that the original Ht31 fragment is part of a larger molecule, now called AKAP-Lbc, which possesses Rho-specific guanine nucleotide exchange activity (Diviani et al., 2001; Klussmann et al., 2001). Cell-based experiments demonstrated that AKAP-Lbc nucleates a $G\alpha_{12/13}$-mediated pathway that leads to activation of the small molecular weight GTPase Rho. Rho regulates a number of diverse cellular processes such as remodeling of the actin cytoskeleton, stimulation of gene transcription, and activation of certain protein kinases (Hall, 1998; Hill et al., 1995; Olson et al., 1995). It has been demonstrated with the Rho-specific inhibitor C3 toxin that a $G\alpha_{12/13}$-mediated activation of Rho stimulates PKD activity (Valverde et al., 1994; Yuan et al., 2001). Here, we report that AKAP-Lbc forms a multiprotein complex that mediates PKD activation.

Protein kinase D requires phosphorylation of serines 744 and 748 in the activation loop of the enzyme to obtain full activity (Waldron et al., 1999; Zugaza et al., 1996). This initial activation step is catalyzed by the PKC isozymes PKCδ, PKCε, PKCθ, or PKCη (Storz et al., 2004; Waldron et al., 1999; Yuan et al., 2002). These events must occur within a microenvironment where PKC can access its lipid activator diacylglycerol and be located in close proximity to its substrate PKD. Here, we demonstrate that AKAP-Lbc tethers PKCη and protein kinase D to favor the activation of the latter enzyme. We have also defined a second phase in the activation process that requires PKA phosphorylation of AKAP-Lbc to release protein kinase D.

Results

AKAP-Lbc Binds to PKD

AKAP-Lbc binds protein kinase A (PKA) and functions as a guanine nucleotide exchange factor (GEF) that couples $G\alpha_{12/13}$ signals to Rho activation (Diviani et al., 2001). Rho and PKC are also implicated in the activation of protein kinase D (PKD), a lipid-dependent enzyme that functions at the plasma membrane and Golgi (Liljedahl et al., 2001; Matthews et al., 2000; Yuan et al., 2001). Therefore, it seemed logical to determine whether PKD was recruited into the AKAP-Lbc complex. Five lines of

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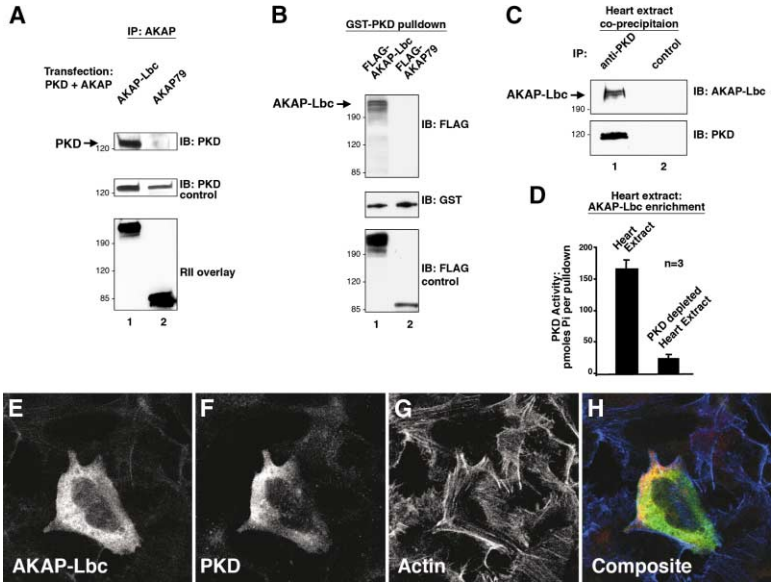

Figure 1. Interaction of AKAP-Lbc with PKD

(A) Coimmunoprecipitation of PKD with AKAP-Lbc from HEK293 cells. Top, immunoblot detection of PKD with anti-GST antibodies. Middle, immunoblot detection of PKD levels in the HEK293 cell lysates. Bottom, detection of AKAPs was performed by RII overlay.

(B) Reciprocal coimmunoprecipitation of AKAP-Lbc with PKD. Top, immunoblot detection of AKAPs with anti-FLAG antibodies. Middle, immunoblot detection of PKD. Bottom, immunoblot detection of FLAG-tagged AKAPs in HEK293 cell lysates.

(C) Coimmunoprecipitation of AKAP-Lbc with PKD from heart extract. Top, immunoblot detection of the anchoring protein with anti-AKAP-Lbc antibodies. Bottom, immunoblot detection of the kinase with anti-PKD antibodies. Rabbit IgG was used for control immunoprecipitates in place of anti-PKD antibodies.

(D) Copurification of PKD activity from heart extract. AKAP-Lbc complexes were isolated from heart extract using cAMP-agarose, and copurification of PKD activity was measured by kinase assay. Control experiments were performed with immunodepleted heart extract. Data are presented from three independent experiments.

(E–H) Co-distribution of AKAP-Lbc and PKD in HeLa cells. (E) Fluorescence detection of GFP-AKAP-Lbc. (F) Immunodetection of PKD with anti-HA antibodies, followed by antimouse Texas red. (G) Actin was counterstained with Alexa-Fluor 660 phalloidin. (H) Composite image.

evidence support this hypothesis. First, PKD copurified with the anchoring protein when both proteins were expressed in HEK293 cells. Cells were transfected with plasmids encoding the protein kinase D1 isoform (all studies were performed with this isoform, accession number gi:2499575) and full-length AKAP-Lbc (AKAP13, gi:15986728) or AKAP79 (AKAP5, gi:399022). Cell lysates were prepared, and the anchoring proteins were immunoprecipitated with specific antisera. Copurification of protein kinase D was detected by immunoblot. PKD copurified with AKAP-Lbc (Figure 1A, top, lane 1) but was not detected in AKAP79 immune complexes (Figure 1A, top, lane 2). Control experiments confirmed that similar amounts of PKD were expressed in cell lysates (Figure 1A, middle) and that equivalent levels of AKAP-Lbc or AKAP79 were immunoprecipitated (Figure 1A, bottom). Next, reciprocal pull-down experiments demonstrated that AKAP-Lbc specifically bound to GST-PKD when both proteins were expressed in HEK293 cells (Figure 1B, top, lane 1). Control experiments confirmed that GST-PKD did not interact with AKAP79 (Figure 1B, top, lane 2). Western blots confirmed that equivalent amounts of the kinase were immunopurified, and both anchoring proteins were expressed similarly (Figure 1B, middle and bottom panels). Additionally, immunoblot analysis of PKD immune complexes isolated from rat heart detected AKAP-Lbc, further suggesting that the anchoring protein interacts with this kinase (Figure 1C, lane 1). Control experiments with rabbit IgG were negative (Figure 1C, lane 2). Antibody limitations prevented clear detection of PKD by Western blot in reciprocal AKAP-Lbc immunoprecipitation experiments. However, endogenous PKD activity was measured in samples enriched in AKAP-Lbc from mouse heart extracts by assaying the syntide-2 substrate peptide phosphorylation (Figure 1D). PKD activity was measured in the presence of selective PKA, PKC, and CaMKII inhibitors to eliminate potential syntide-2 phosphorylation by other AKAP-associated serine/threonine kinases. Finally, fluorescent detection of GFP-tagged AKAP-Lbc (Figure 1E, green in 1H), PKD (Figure 1F, red in 1H), and actin (Figure 1G, blue in 1H) demonstrated that the kinase and the anchoring protein exhibited distinct but overlapping subcellular distributions. A composite image illustrates significant overlap of the AKAP-Lbc and PKD signals (Figure 1H, yellow). Together, these imaging, biochemical, and enzymology studies show that PKD is recruited into the AKAP-Lbc signaling complex.

Mapping the Protein Kinase Binding Sites on AKAP-Lbc

PKD is activated via a signaling pathway that involves the generation of diacylglycerol and the stimulation of novel PKC isoforms. Working on the assumption that AKAP-Lbc might orient PKD toward upstream elements in this activation pathway, we reasoned that certain PKC isoforms might be present in the signaling complex. Therefore, GST fusion proteins encompassing defined regions of the anchoring protein were generated to map binding sites for each enzyme (Figure 2A). Each purified fragment (Figure 2B) was incubated with RII subunit of PKA (Figures 2C and 2D), PKD (Figures 2E and 2F), or rat brain extracts containing a mixture of PKC isoforms (Figures 2G and 2H). As expected, RII associates with the AKAP-Lbc 1001-1387 fragment (Figure 2C, lane 3). PKD associates with a region in the carboxyl terminus of the anchoring protein between residues 2337–2817 (Figure 2E, lanes 5 and 6). This finding was confirmed in HEK293 cells when we demonstrated that PKD coprecipitated with a GFP-tagged AKAP-Lbc 2339-2817 fragment (data not shown). PKC bound to the AKAP-Lbc 1923-2817 fragment (Figure 2G, lane 5). Further studies

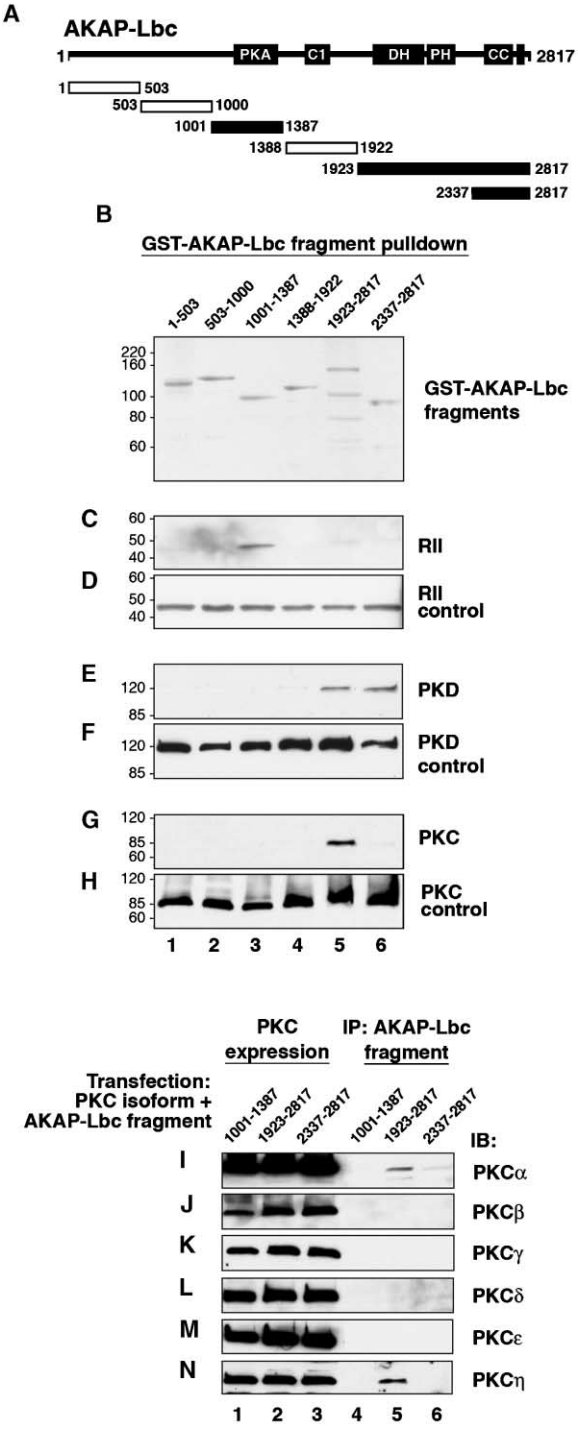

Figure 2. Mapping Kinase Interaction Sites on AKAP-Lbc

(A) Schematic diagram of AKAP-Lbc fragments used to map the kinase binding regions on AKAP-Lbc. The first and last amino acid residues of each fragment are indicated. Also shown are identified domains in AKAP-Lbc.

(B) Coomassie blue-stained SDS-PAGE gel of purified GST-AKAP-Lbc fragments.

(C–H) Mapping binding sites for PKA, PKC, and PKD. Each GST-AKAP-Lbc fragment was incubated with RII (C and D). (E and F) Recombinant PKD. (G and H) Rat brain extract. Proteins in the pull downs (C, E, and G) and supernatants (D, F, and H) were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against (C and D) the RII subunit of PKA, (E and F) PKD, and (G and H) antibodies that recognize all PKC isoforms.

(I–N) Identification of specific PKC isoforms that bind to AKAP-Lbc. PKC isoforms were detected with specific antibodies against (I) PKCα, (J) PKCβ, (K) PKCγ, (L) PKCδ, (M) PKCε, and (N) PKCη.

demonstrated that only the PKCα and PKCη isoforms bound to this region (Figures 2I and 2N, lane 5). Thus AKAP-Lbc maintains a kinase scaffold of PKA, PKC, and PKD.

Anchored PKC Facilitates Activation of Protein Kinase D

Next, we wanted to determine whether tethered PKC catalyzes the phosphorylation and activation of PKD. To test this hypothesis, we monitored the activity state of protein kinase D from HEK293 cells stimulated with agonists for PKC (PDBu). Activation of PKD was detected by immunoblot with phosphorylation-state specific antibodies against Ser744 and Ser748 in the activation loop of the kinase and by measuring changes in enzyme activity with the syntide-2 peptide substrate.

HEK293 cells endogenously express low levels of AKAP-Lbc. In these cells, PDBu treatment had a modest effect on Ser744/48 phosphorylation (Figure 3A, histogram, columns one and two) and enzyme activity (Figure 3B, columns one and two). In contrast, ectopic expression of AKAP-Lbc enhanced PDBu-dependent Ser744/48 phosphorylation 3.2 $\pm$ 1 fold (n = 3) and increased PKD activity 2.2 $\pm$ 0.7 fold (n = 6) (Figure 3A, histogram, and Figure 3B, columns three and four). Phosphorylation of syntide-2 was at background levels in immunoprecipitates from PDBu-stimulated HEK293 lysates expressing the kinase dead mutant of PKD (data not shown). Control immunoblots confirmed that equivalent levels of PKD and AKAP-Lbc were expressed in these samples (Figure 3A, middle and bottom). These data suggest that a pool of PKC associated with AKAP-Lbc is a principal upstream element in PKD activation.

To examine this phenomenon via a more physiological agonist, we performed additional experiments in HEK293 cells expressing the M1 muscarinic receptor. The muscarinic agonist oxotremorine-M (Oxo-M) engages a Gq/11-coupled signaling pathway that involves calcium mobilization and diacylglycerol (DAG) synthesis and leads to the activation of PKC isoforms (Suh and Hille, 2002). Oxo-M-stimulated PKD activation (Figure 3C, top, and histogram, lane 2), but this activation was further augmented in the presence of AKAP-Lbc (Figure 3C, top, and histogram, lane 4). Control immunoblots confirmed that equivalent amounts of PKD (Figure 3C, second panel) and AKAP-Lbc (Figure 3C, bottom) were expressed in these cells.

More detailed mapping studies indicate that PKCη binds to a region of AKAP-Lbc encompassing amino acids 2225-2340 (Figure 3D). This region corresponds to the PH domain and is distinct from the PKA and PKD binding sites (Figure 3D, top, lane 1). Therefore, we tested whether the soluble PH domain of AKAP-Lbc antagonized PKD activation. Reconstitution of the AKAP-Lbc-signaling scaffold in HEK293 cells permitted phorbol ester-dependent activation of PKD as assessed

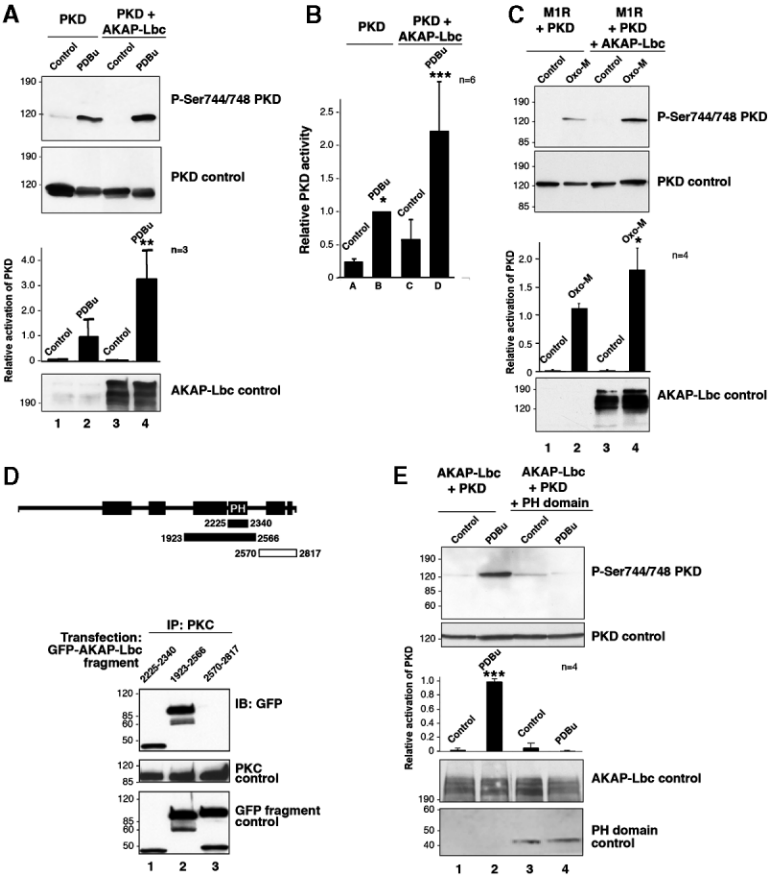

Figure 3. Activation of Protein Dinase D by Anchored PKC

(A) PKD activation upon stimulation with phorbol ester. Top, immunoblot detection of active PKD with p-Ser744/748 antibodies. Middle, immunoblot detection of PKD levels in each lysate. Histogram, PKD activation was quantitated by densitometry. Data from three experiments is presented. Bottom, immunoblot confirming AKAP-Lbc expression. The statistical significance of each experiment was calculated with the one-way analysis of variance test. Each data point is indicated above the column. Asterisk corresponds to a significant p value of 0.01 to 0.05; double asterisk, a very significant p value of 0.001 to 0.01; triple asterisk, an extremely significant p value of <0.001.

(B) PKD activity is stimulated by PKC in the presence of AKAP-Lbc. PKD activity was measured by incorporation of [γ-^{32}P]-phosphate into the peptide substrate syntide-2. Results were quantitated from six independent experiments.

(C) AKAP-Lbc enhances PKD activation via stimulation of the muscarinic receptor. Top, activation of PKD assessed by immunoblot using p-Ser744/748 antibodies. Second panel, lysates were probed to determine the amount of PKD expression under each condition. Histogram, PKD activation was quantitated by densitometry. Amalgamated data from four experiments is presented. Bottom, immunoblot confirming expression of AKAP-Lbc.

(D) PKC binds to the PH domain of AKAP-Lbc. Top, anti-GFP antibodies to detect AKAP-Lbc fragments. Middle, PKC immunoblot. Bottom, control immunoblot showing expression of AKAP-Lbc fragments in the cell lysates.

(E) The soluble PH domain of AKAP-Lbc disrupts PKD activation. Top, activation of PKD was assessed by immunoblot with p-Ser744/748 antibodies. Second panel, lysates were probed to determine the level of PKD in each sample. Histogram, PKD activation was quantitated by densitometry. Amalgamated data from four experiments is presented. Third panel, immunoblot confirming expression of AKAP-Lbc. Bottom, immunoblot confirming expression of AKAP-Lbc-PH domain.

by immunoblot assay (Figure 3E, top, lanes 1 and 2 and histogram). In contrast, ectopic expression of the PH domain from AKAP-Lbc abolished PBDu-dependent activation of PKD (Figure 3E, top, lanes three and four, and histogram). Immunoblots confirmed that equivalent levels of PKD (Figure 3E, second panel), AKAP-Lbc, and the isolated PH domain were expressed in the appropriate samples (Figure 3E, lower panels). Control experiments confirmed that ectopic expression of the PH domain reduced the levels of PKC associated with the AKAP-Lbc complex (data not shown). Thus, disruption of the PKC/AKAP-Lbc interaction suppresses agonist-mediated activation of protein kinase D.

Anchored PKA Phosphorylates Serine 2737 of AKAP-Lbc

It was unclear whether anchored PKA also plays a role in PKD signaling. Consensus PKA sites were identified throughout AKAP-Lbc with the Scansite program (http://scansite.mit.edu), yet only C-terminal regions of the anchoring protein were efficiently phosphorylated by the catalytic subunit of PKA in vitro (Figure 4A, lanes 4–6). Interestingly, the principal PKA phosphorylation site(s) lie within the protein kinase D binding fragment (Figure 4B, lanes 1–4) and are located within the last 129 amino acids of the anchoring protein (Figure 4C). As this region (residues 2689–2817) contains several potential PKA substrate sequences, we screened an immobilized array of overlapping 20-mer peptides each displaced by three residues (Figure 4D). A single site at serine 2737 with the core motif KRNS was efficiently phosphorylated by PKA (Figure 4D). Additional analysis of peptide arrays demonstrated that substitution of Ser2737 for alanine abolished phosphorylation by PKA (Figure 4E). This finding was independently confirmed by demonstrating that replacement of Ser2737 with alanine abolishes PKA phosphorylation of the AKAP-Lbc 1923-2817 fragment in vitro (Figure 4B, lane 5).

A phospho-specific antibody was generated against Ser2737 to monitor the phosphorylation of AKAP-Lbc in situ. The specificity of this reagent was initially tested on recombinant AKAP-Lbc (Figure 5A). HEK293 cells expressing FLAG-tagged AKAP-Lbc were treated with forskolin/IBMX to favor PKA activation. The anchoring protein was immunoprecipitated and phosphorylation of Ser2737 was monitored by immunoblot (Figure 5A, top). PKA activation promoted a 13-fold $\pm$ 5-fold ($n = 4$) increase in antibody signal over control (Figure 4A, histogram). A low level of phospho-Ser2737 signal was consistently detected in AKAP-Lbc immune complexes from unstimulated cell extracts (Figure 5A, top, lane 1). This is likely to represent basal phosphorylation of the

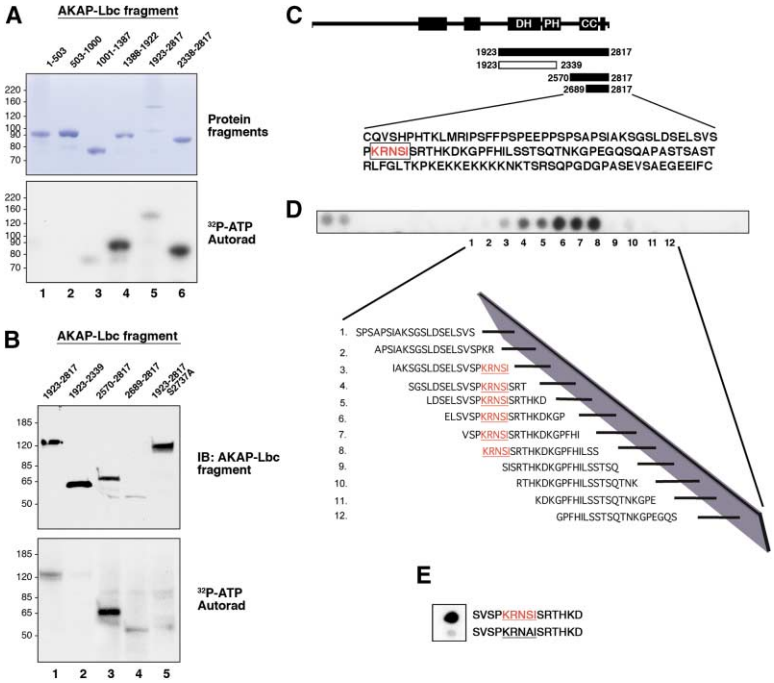

Figure 4. In Vitro Phosphorylation of AKAP-Lbc by PKA

(A) Purified GST-AKAP-Lbc fragments were incubated with the catalytic subunit of PKA and [γ-^{32}P]-ATP for 20 min at 30°C. Top, Coomassie blue-stained gel of GST-AKAP-Lbc fragments with the first to last amino acid residues of each fragment indicated above each lane. Bottom, incorporation of [γ-^{32}P]-phosphate was detected by autoradiography.

(B) Phosphorylation of AKAP-Lbc C-terminal fragments by PKA. Top, immunoblot detection of AKAP-Lbc fragments. Bottom, incorporation of [γ-^{32}P]-phosphate was detected by autoradiography.

(C) Schematic diagram depicting the location of PKA phosphorylation sites in AKAP-Lbc. The last 128 residues of the anchoring protein are indicated by a single letter amino acid code. A putative PKA phosphorylation site is indicated in red letters.

(D) Autospot peptide array mapping of PKA phosphorylation sites. Top, autoradiograph of [γ-^{32}P]-phosphate incorporation into 20-mer peptides encompassing the C terminus of AKAP-Lbc. Bottom, sequences of positive peptides. The major PKA phosphorylation site in this region is indicated in red letters.

(E) The PKA phosphorylation site was confirmed by replacing the phospho-acceptor serine with alanine.

anchoring protein as treatment with λ phosphatase abolished the signal (data not shown). Control immunoblots confirmed that equivalent amounts of AKAP-Lbc were immunoprecipitated (Figure 5A, middle).

Similar results were obtained when the phosphorylation state of AKAP-Lbc was monitored in cardiomyocytes after stimulation with the β-agonist isoproterenol, which elevates intracellular cAMP (Figure 5B, top, lane 2). Immunofluorescence techniques were also used to establish the phosphorylation pattern of AKAP-Lbc in cardiomyocytes (Figures 5C–5H). Minimal phospho-AKAP-Lbc staining (green) was detected in untreated cells (Figure 5C), whereas the phospho-AKAP-Lbc was increased dramatically upon treatment with isoproterenol (Figure 5F). An antibody against α-actinin (Figures 5D and 5G) detected the striated cytoskeleton of the cardiomyocytes. Composite images illustrate that AKAP-Lbc phosphorylation is increased upon elevation of intracellular cAMP (Figures 5E and 5H).

More mechanistic experiments were performed to determine whether PKA anchored to AKAP-Lbc catalyzed the phosphorylation of Ser2737 (Figure 5I). HEK293 cells were transfected with plasmids that encode AKAP-Lbc or a PKA-anchoring defective mutant (AKAP-Lbc-PP) in which the RII binding helix is disrupted by proline mutations (Carr et al., 1991). Immune complexes of wild-type and mutant AKAP-Lbc were isolated and treated with cAMP to promote activation of PKA. Phosphorylation of Ser2337 was monitored by immunoblot (Figure 5I, top). Activation of PKA enhanced Ser2737 phosphorylation 2.3 $\pm$ 0.3 fold (n = 3) over background, whereas the phosphorylation state of the AKAP-Lbc-PP mutant was unchanged (Figure 5I, top and histogram). Control experiments confirmed that equivalent amounts of AKAP-Lbc were present in each sample (Figure 5I, second panel). These data suggest that Ser2737 of AKAP-Lbc is a bona fide target for the anchored pool of PKA in vivo.

The anti-p-Ser2737 antibody detects a PKA phosphorylation event in situ. Therefore, we were able to directly test a central tenet of our anchoring hypothesis: AKAP-associated pools of PKA are spatially restricted to favor the phosphorylation of selected substrates. Cultured rat cardiomyocytes were incubated with 50 μM stearated-Ht31, a cell-soluble peptide derived from AKAP-Lbc that disrupts PKA anchoring inside cells (Figures 5J–5O). Control cells were treated with the st-Ht31-PP control peptide (Figures 5P–5U). Cells from both groups were stimulated with isoproterenol to activate PKA, prior to fixation and staining with the anti-p-Ser2737 antibody to monitor AKAP-Lbc phosphorylation (Figures 5M and 5S). An antibody against α-actinin (red) labeled the striated cytoskeleton. Ht31-peptide-mediated disruption of PKA anchoring reduced the isoproterenol-stimulated phosphorylation of AKAP-Lbc (Figure 5M) as compared to control experiments performed with Ht31-PP peptide (Figure 5S). Likewise, pretreatment with st-Ht31 abolished the background phospho-Ser2737 signal in unstimulated cardiomyocytes (Figure 5J) as compared to the Ht31-PP controls (Figure 5P). Thus basal PKA phosphorylation of AKAP-Lbc was prevented upon displacement of the kinase. Collectively, the data presented in Figure 5 suggest that PKA anchoring to AKAP-Lbc is required to phosphorylate Ser2737 in situ.

Anchored PKA Regulates PKD Interaction with AKAP-Lbc

Next we examined the functional consequences of Ser2737 phosphorylation on PKD interaction with AKAP-

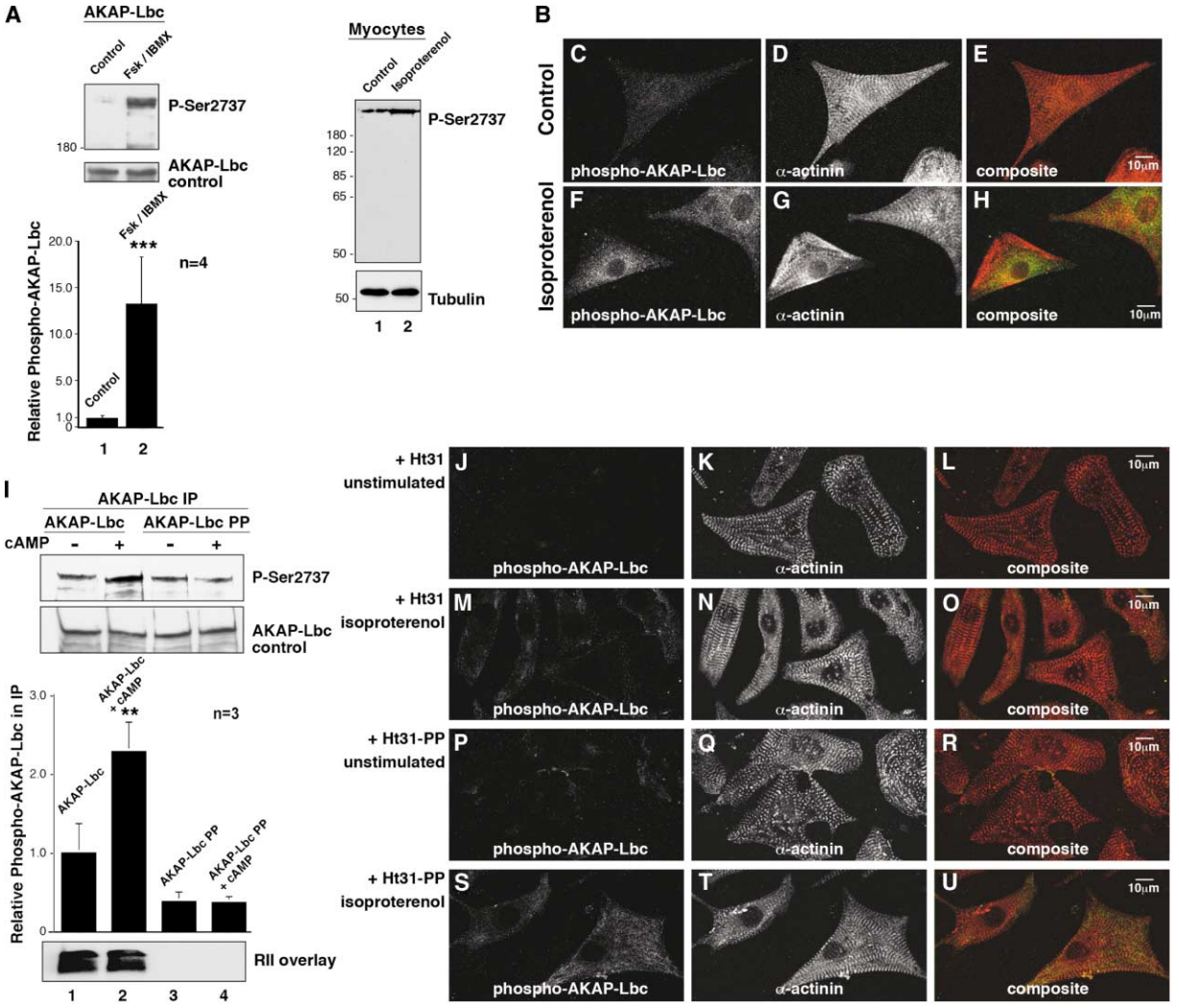

Figure 5. In Vivo Phosphorylation of AKAP-Lbc at S2737 upon Stimulation of PKA

(A) Characterization of the anti phospho-Ser2737 antibody. AKAP-Lbc immune complexes were isolated from unstimulated and forskolin/IBMX-treated lysates (indicated above each lane). Top, immunoblot with the anti-phospho-Ser2737 antibody. Bottom, AKAP-Lbc levels in each immune complex were assessed by immunoblot. Histogram, phosphorylation of AKAP-Lbc was quantitated over four experiments by densitometry with the NIH Image program.

(B) Phosphorylation of AKAP-Lbc. Cultured rat cardiomyocyes were treated with isoproterenol (10 μM) for 10 min prior to lysis. Top, lysates were probed for Ser2737-phosphorylated AKAP-Lbc with the phospho-specific antibody. Bottom, immunoblot detection of tubulin as a loading control.

(C–H) Immunofluorescence detection of phospho-AKAP-Lbc in cardiomyocytes. Following isoproterenol treatment (10 μM for 10 min) cells were fixed and immunostained with (C and F) anti-p-Ser2737 antibodies (green) and (D and G) coimmunostained with anti-α-actinin antibodies (red). (E and H) Composite images of control and isoproterenol stimulated cells. Analysis was performed with a BioRad MRC 1024 confocal microscope.

(I) Phosphorylation of AKAP-Lbc by anchored PKA. AKAP-Lbc immune complexes were resuspended in kinase buffer, and PKA activity was stimulated with 100 mM cAMP and 100 μM ATP. Top, immunoblot detection of phospho-Ser2737 AKAP-Lbc. Middle, immunoblot detection of total AKAP-Lbc. Histogram, quantitation of phospho-AKAP-Lbc levels by densitometry. Amalgamated data from three experiments. Bottom, RII overlay.

(J–U) AKAP-Lbc phosphorylation is lost upon disruption of PKA anchoring in cardiomyocytes. (J–O) Cardiomyocytes were treated with cell-soluble anchoring inhibitor peptide st-Ht31 (50 μM) to displace anchored PKA or (P–U) control peptide st-Ht31-PP. Isoproterenol treated cells were fixed and stained with (J, M, P, and F) anti-p-Ser2737 antibodies (green). (K, N, Q, and T), α-actinin antibodies. (L, O, R, and U), composite images.

Lbc. Untreated or in vitro phosphorylated AKAP-Lbc 1923-2817 fragments were incubated with recombinant PKD for 1 hr at 4°C. Copurification of PKD was monitored by immunoblot (Figure 6A, top, lanes 1–3). PKA phosphorylation reduced protein kinase D interaction by 85% ± 5% (n = 3) compared with untreated samples (Figure 6A, histogram, lane 2), whereas dephosphorylation of the anchoring protein fragment increased PKD association by 22% ± 6% (n = 3) as compared to untreated samples (Figure 6A, top and histogram, lane 3). Additional controls confirmed PKA phosphorylation of Ser2737 and that equivalent amounts of the anchoring

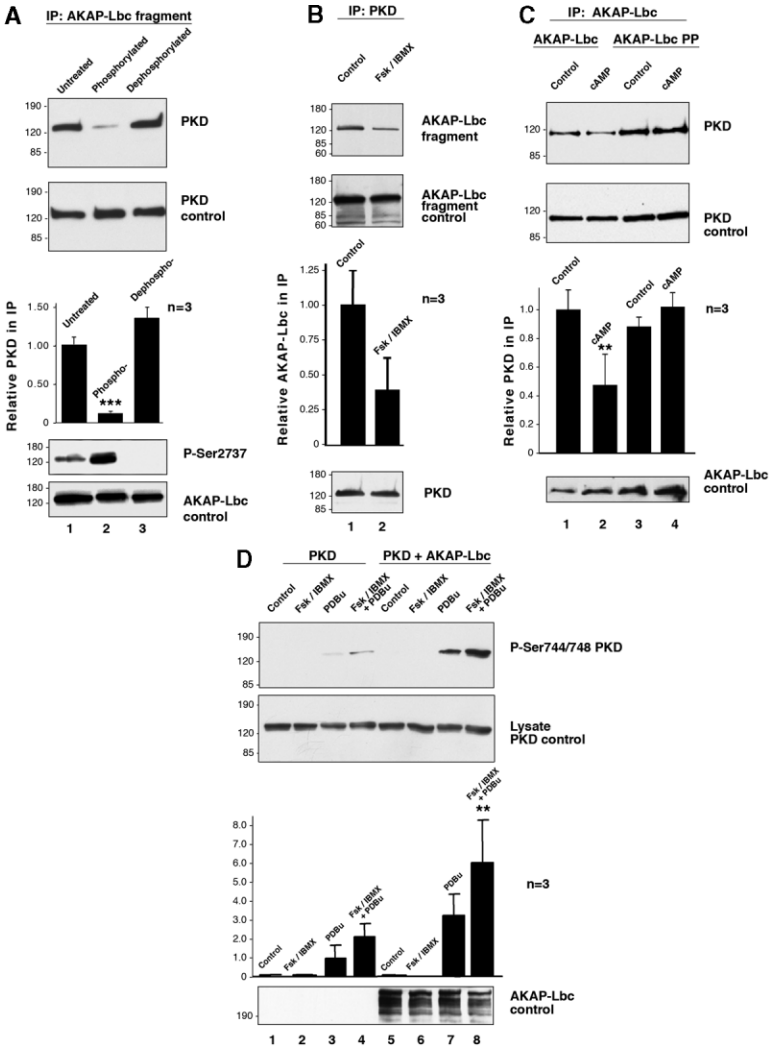

Figure 6. PKA Phosphorylation Releases Anchored Protein Kinase D

(A) AKAP-Lbc immune complexes were PKA phosphorylated (lane 2) or phosphatase treated (lane 3). PKD binding reactions were carried out using recombinant enzyme (0.5 μg). Top, immunoblot detection of bound PKD. Second panel, immunoblot detection of unbound PKD. Histogram, PKD binding to AKAP-Lbc was quantitated by densitometry. Graph of amalgamated data from three independent experiments. Third panel, immunoblot detection of phospho-Ser2737 AKAP-Lbc. Bottom, immunodetection of total AKAP-Lbc in each binding reaction.

(B) Regulation of AKAP-Lbc/PKD interaction inside cells. Top, immunoblot detection of AKAP-Lbc coprecipitating with PKD. Second panel, immunoblot detection of total AKAP-Lbc fragment expressed in cell lysates. Histogram, the amount of AKAP-Lbc coprecipitating with PKD upon PKA activation in HEK293 cells was quantitated by densitometry. AKAP-Lbc binding to PKD was normalized to a value of 1 for AKAP-Lbc bound to PKD immunoprecipitated from untreated cells. Graph represents the amalgamated data from three independent experiments.

(C) Regulation of PKD/AKAP-Lbc interaction by anchored PKA. Top, copurification of PKD with AKAP-Lbc was assessed by immunoblot. Second panel, immunoblot detection of PKD expression in cell lysates used for these experiments. Histogram, quantitation of data by densitometry. Graph represents the amalgamated data from three independent experiments. Bottom, immunoblot detection of total AKAP-Lbc.

(D) Activation of PKD in response to stimulation of cells with phorbol ester and forskolin/IBMX. Top, activation of PKD was assessed by immunodetection with p-Ser744/748 PKD antibodies. Second panel, lysates were probed to determine the total level of PKD expression in each sample. Histogram, quantitation of PKD activation from three experiments. Bottom, immunoblot confirming expression of AKAP-Lbc.

protein fragment were used in these studies (Figure 6A, bottom).

Cell-based experiments were performed in HEK293 cells transfected with plasmids that encode PKD and the AKAP-Lbc fragment. Cells were treated with vehicle or forskolin/IBMX to maximally activate PKA. The AKAP fragments were immunoprecipitated, and copurification of PKD was monitored by immunoblot (Figure 6B, top). Intracellular activation of PKA caused a 70% ± 15% ($n = 3$) reduction in the amount of PKD coprecipitating with the anchoring protein fragment as compared to untreated samples. (Figure 6B, top and histogram). Control immunoblots confirmed that equivalent amounts of AKAP-Lbc fragment and PKD were expressed in both samples (Figure 6B, second and bottom panels).

Other experiments tested whether phosphorylation of AKAP-Lbc by the anchored pool of PKA could reduce the amount PKD associated with the anchoring protein. Full-length AKAP-Lbc or AKAP-Lbc-PP was immunoprecipitated from HEK293 cells. Less PKD was bound to AKAP-Lbc after treatment with cAMP as compared to untreated samples (Figure 6C, top, lanes 1 and 2, and histogram). Importantly, PKD associated with the AKAP-Lbc PP mutant and was unaffected by cAMP treatment (Figure 6C, top, lanes 3 and 4, and histogram). Control experiments confirmed that equivalent levels of PKD and AKAP-Lbc were present in the cell lysates (Figure 6C, second and bottom panels). Collectively, these data suggest that phosphorylation on Ser2737 of AKAP-Lbc by an anchored pool of PKA negatively regulates the interaction between protein kinase D and AKAP-Lbc.

Finally, we tested whether the actions of anchored PKA and anchored PKC acted synergistically to favor activation of protein kinase D. These experiments were performed in HEK293 cells with the Ser744/748 phosphorylation assay as an index of PKD activity. Forskolin/IBMX treatment had no effect on PKD activation, regardless of the expression of AKAP-Lbc (Figure 6D, top, lanes 2 and 6). Stimulation with PDBu resulted in a minimal activation of PKD (Figure 6D, top, lane 3, and histo-

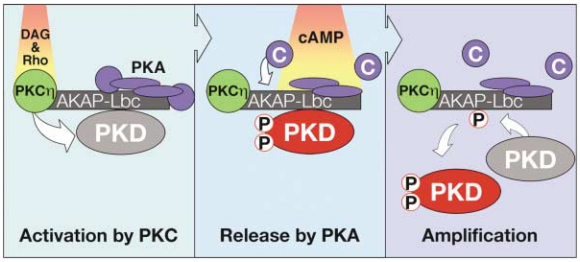

Figure 7. Model of the AKAP-Lbc Complex and the Synchronization of Enzymatic Activation of Protein Kinase D

See text for details.

gram). This effect was slightly more robust when combined with forskolin/IBMX treatment (Figure 6D, top, lane 4, and histogram). Importantly, PDBu-dependent activation of PKD was significantly enhanced in the presence of AKAP-Lbc (Figure 6D, top, lane 7).

Maximal protein kinase D activation was observed from cells expressing AKAP-Lbc treated with forskolin/IBMX and PDBu (Figure 6D, top, lane eight, and histogram). Control immunoblots confirmed that equivalent levels of PKD and AKAP-Lbc were expressed in each sample (Figure 6D, middle and bottom). Collectively, these experiments suggest that the anchored pools of PKA and PKC on AKAP-Lbc act synergistically to promote activation of protein kinase D.

Discussion

In this report we describe a previously unappreciated role for AKAP-Lbc in the coordination of an activation complex for PKD. The anchoring protein contributes to protein kinase D activation in two ways: it recruits the upstream kinase PKCη, and it directs PKA phosphorylation events to release the newly activated enzyme into the cytoplasm. Not only does this configuration provide an efficient means to synchronize the action of two upstream kinases, but the transient nature of the PKD interaction may permit the passage of multiple molecules through the activation complex. This model, which is presented in Figure 7, suggests that signal processing through AKAP-Lbc may amplify PKD activity. This model is consistent with current opinions that activated PKD is recruited to membranes and can then translocate to other cellular sites of action (Marklund et al., 2003; Matthews et al., 2000; Oancea et al., 2003). Thus, AKAP-Lbc organizes three enzymes into a protein kinase cascade. This is a function for AKAPs that is distinct from the role of anchoring proteins such as AKAP79/150 and AKAP350/CG-Nap, which spatially organize diverse combinations of enzymes that act autonomously (Colledge et al., 2000; Klauck et al., 1996; Takahashi et al., 2002).

AKAP-Lbc is a modular protein that is composed of several recognizable domains. Most notable of these are the consecutive DH and PH domains, a hallmark of guanine nucleotide exchange (GEF) factors (Olson et al., 1997; Zheng et al., 1995). The DH domain contains the nucleotide exchange activity, whereas the PH domain may participate in protein-lipid interactions that contribute to the compartmentalization of GEFs at the plasma membrane. Our binding studies suggest that the PH domain also participates in the recruitment of the PKCα and PKCη isoforms to the AKAP-Lbc complex. Although PKCη is recognized as an activating kinase for protein kinase D, a function for anchored PKCα is less evident. One possibility is that PKCα phosphorylates proteins in the AKAP-Lbc complex or substrates in close proximity to the signaling scaffold. Potential PKCα substrates include Rho, an AKAP-Lbc binding partner, or the Rho-guanine nucleotide dissociation inhibitor, Rho-GDI, an effector protein that controls Rho activation in response to $G\alpha_{13}$ (Meacci et al., 2000; Mehta et al., 2001).

Activation of PKD requires the phosphorylation of two key serine residues, Ser744 and Ser748, within the activation loop of the catalytic domain of the kinase (Iglesias et al., 1998; Waldron et al., 2001; Waldron and Rozengurt, 2003). The novel protein kinase C isoforms catalyze these phosphorylation events, which lead to full activation of the enzyme (Storz et al., 2004; Waldron et al., 1999). An important conclusion of our study is that the recruitment of PKCη into the AKAP-Lbc signaling complex markedly enhances protein kinase D activation. Three protein-protein interactions act synergistically to favor this effect: (1) PKCη binding to the PH domain of AKAP-Lbc, (2) PKD tethering to the C-terminal region of the anchoring protein, and (3) intermolecular interactions between PKCη and protein kinase D (Waldron et al., 1999). The arrangement of both kinases in this configuration permits the linear transduction of signals from one anchored enzyme to the next. This is analogous to the sequential relay of signals through scaffolding proteins such as Ste-5, KSR-1, and JIP that organize the three-tiered MAP kinase cascades (Bauman and Scott, 2002). In each of these scaffolds, mislocalization or inhibition of an upstream kinase prevents activation of the terminal kinase (Kelkar et al., 2000; Sabbagh et al., 2001; Wartmann and Davis, 1994). Likewise, we show that mislocalization of PKCη upon disruption of its tethering to AKAP-Lbc interrupts signal flow through the scaffold and impairs activation of PKD (Figure 3E). However, one clear distinction between the AKAP-Lbc complex and these other scaffolds is the use of a broad specificity enzyme such as PKCη in the role of an upstream kinase. In this context anchored PKCη appears to be orientated toward a single substrate, protein kinase D, yet in other cellular contexts it is available to phosphorylate a variety of target proteins (Storz and Toker, 2003; Yeaman et al., 2004). This latter point clearly illustrates the utility of kinase anchoring as a mechanism to constrain the action of broad-spectrum enzymes.

Our original anchoring hypothesis postulated that spatial resolution of PKA phosphorylation events could provide an element of specificity to cAMP signaling. Consequently, we proposed that AKAPs should be PKA substrates or enzyme activities that were modulated by the kinase. In fact, surprisingly few of these anchoring proteins have proven to be physiological substrates for PKA (Carr et al., 1991). The only previous example is Gravin/AKAP250, which is phosphorylated by PKA to regulate binding to the β2-adrenergic receptor (Tao et al., 2003). Three pieces of evidence indicate that AKAP-Lbc is a physiological substrate for PKA and is phosphorylated by its own anchored pool of kinase: (1) eleva-

tion of intracellular cAMP promotes phosphorylation of Ser2737 on AKAP-Lbc in cardiomyocytes, (2) Ser2737 phosphorylation is reduced in PKA anchoring-defective AKAP-Lbc mutants, and (3) agonist-dependent release of PKD from the anchoring protein complex is impaired upon disruption of PKA anchoring. Consequently, we propose that one function of PKA anchoring to AKAP-Lbc is to catalyze the release of PKD through a mechanism that involves phosphorylation of Ser2737. However, it is possible that other kinases can also participate in this process as we can detect low levels of Ser2737 phosphorylation in samples from unstimulated cells. This scenario is reminiscent of phosphorylation on the LKB1 kinase, which regulates cell growth, where genetic and pharmacological evidence indicate that Ser431 is a target for PKA and the p90 ribosomal kinase (RSK) (Boudeau et al., 2003).

Another unanswered mechanistic question is how phosphorylation of Ser2737 mediates PKD release. Site-directed mutagenesis experiments indicate that the introduction of a negative charge at position 2737 with aspartic or glutamic acid residues is not sufficient to displace protein kinase D from the anchoring protein (F.D.S., unpublished data). Thus, the added bulk provided by a phosphate group or the phosphorylation-dependent recruitment of another protein may be required to induce the release of PKD. Attractive candidates for this latter process could be 14-3-3 proteins, which regulate cellular processes by binding to phosphorylated serine motifs on their targets. Two loosely conserved 14-3-3 binding motifs of R(S/X)XpSXP and RXXXpSXP have been defined that vaguely resemble the residues surrounding Ser2737 (Rubio et al., 2004; Yaffe and Cantley, 1999). Hence one could imagine that PKA catalyzes the phosphorylation-dependent recruitment of 14-3-3, leading to conformational changes in AKAP-Lbc that release PKD into the cytoplasm. Another reasonable assumption is that protein phosphatases are recruited to AKAP-Lbc, given that multiple phosphorylation events govern PKD activation and that most AKAPs seem to bind phosphatases (Klauck et al., 1996; Schillace and Scott, 1999; Westphal et al., 1999). Although we have not detected PP1, PP2A, or PP2B with the AKAP-Lbc complex, it is possible that other phosphatases may associate with this anchoring protein. Interestingly, $G\alpha_{12/13}$ stimulation promotes translocation of PP5 to the cell periphery where this enzyme could catalyze dephosphorylation events that counteract PKD activation or favor its reassociation with the AKAP-Lbc complex (Marklund et al., 2003; Yamaguchi et al., 2002).

In conclusion, AKAP-Lbc is a multifunctional anchoring protein that provides a platform for the processing and integration of various second messenger signals. We describe how the anchoring protein synchronizes PKA and PKC phosphorylation events that lead to the activation of protein kinase D. Previous reports have implicated its guanine nucleotide exchange activity in the relay of signals from $G\alpha_{12/13}$ to the small molecular weight GTPase Rho. In both cases AKAP-Lbc receives signals from G protein-coupled receptors, which are then dispatched to particular subcellular compartments. Consequently, information flow through AKAP-Lbc scaffolds could propagate such diverse signaling events such as PKD-mediated fission of cell surface-destined carriers from the trans-Golgi network or Rho-mediated remodeling of the actin cytoskeleton (Baron and Malhotra, 2002; Diviani et al., 2001; Yeaman et al., 2004). An important unanswered question that remains is whether the combinatorial assembly of unique AKAP-Lbc complexes mediates individual cellular events or, alternatively, that all signals are processed in parallel through the same AKAP network.

Experimental Procedures

Constructs

cDNA for wild-type GFP-tagged PKD was provided by Dr. Vivek Malhotra (UCSD). Plasmids to PKC isozymes were provided by Professor Peter J. Parker (ICRF, UK). Human M1 muscarinic receptor plasmid was supplied by Dr. Naoto Hoshi (Vollum Institute).

Coimmunoprecipitations and Pull-Downs

HEK293 cells were transfected with calcium phosphate as described (Dell'Acqua et al., 1998). Immunoprecipitates were washed 4 × 1 ml in lysis buffer, eluted in 2× Laemmli sample buffer and separated by SDS-PAGE. GST pull downs were performed similarly, except that protein complexes were isolated by incubation with glutathione-sepharose for 1 hr at 4°C. For co-IPs, mouse heart lysate was prepared as described (Diviani et al., 2001). Immunoprecipitations were performed with anti-PKD antisera or control rabbit IgG. Western blotting and RII overlays were performed as described (Colledge et al., 2000).

Immunocytochemistry

All cells were fixed and processed for immunofluorescence as described (Dodge et al., 2001).

In Vitro Phosphorylation

Immunocomplexes and GST fusion proteins were phosphorylated in vitro in kinase assay buffer (25 mM Tris (pH 7.5), 0.1 mM EGTA, 0.1 mM Na_3VO_4, 0.03% Brij-35, 10 mM $MgCl_2$, and 0.1 mM ATP) supplemented with PKA C-subunit (~0.2 μg) for 30 min at 30°C. Reactions were stopped by washing five times with fresh kinase buffer prior to resuspension in 2× Laemmli sample buffer and SDS-PAGE.

SPOT Synthesis

Peptide arrays were synthesized on cellulose membranes with an Auto-Spot Robot ASP 222 (AbiMed, Langenfeld, Germany) as described in Tegge and Frank (1998).

For more detailed Experimental Procedures, see Supplemental Data at http://www.molecule.org/cgi/content/full/15/6/889/DC1.

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