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Spatial Restriction of PDK1 Activation Cascades by Anchoring to mAKAPα

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Summary

The muscle A-kinase anchoring protein (mAKAP) tethers cAMP-dependent enzymes to perinuclear membranes of cardiomyocytes. We now demonstrate that two alternatively spliced forms of mAKAP are expressed: mAKAPα and mAKAPβ. The longer form, mAKAPα, is preferentially expressed in the brain. mAKAPβ is a shorter form of the anchoring protein that lacks the first 244 amino acids and is preferentially expressed in the heart. The unique amino terminus of mAKAPα can spatially restrict the activity of 3-phosphoinositide-dependent kinase-1 (PDK1). Biochemical and genetic analyses demonstrate that simultaneous recruitment of PDK1 and ERK onto mAKAPα facilitates activation and release of the downstream target p90RSK. The assembly of tissue-specific signaling complexes provides an efficient mechanism to integrate and relay lipid-mediated and mitogenic activated signals to the nucleus.

Introduction

Transduction of extracellular signals from the plasma membrane to the nucleus requires the synchronized action of several signaling molecules. Many signal transduction cascades utilize broad specificity kinases and phosphatases that reversibly catalyze protein phosphorylation events (Cohen, 2002; Hunter, 2000; Krebs, 1985). Scaffolding proteins provide the cell with a mechanism to control the spatial and temporal activation of these signaling enzymes (Pawson and Scott, 1997). AKAPs are such scaffolds, defined by their ability to anchor the PKA holoenzyme to discrete locations within the cell where local pulses of the second-messenger cAMP regulate phosphorylation of nearby substrates (Wong and Scott, 2004). For example, mAKAP can interact with a variety of cAMP-dependent signaling molecules to orchestrate second messenger-mediated signaling events at nuclear membranes (Michel and Scott, 2002). In this study, we report that two forms of mAKAP are expressed by tissue-specific alternative splicing: mAKAPα and mAKAPβ. Further, we demonstrate that the unique amino terminus of mAKAPα can recruit PDK1.

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PDK1 is an upstream element of numerous pathways that catalyzes the activation of AGC serine/threonine kinases. These enzymes signal a variety of physiological processes, including cell survival, proliferation, cell cycle regulation, and insulin response (Maller et al., 2001; Toker and Newton, 2000). Because PDK1 activity is constitutive, its specificity is achieved by placing the enzyme in close proximity to its downstream targets (Casamayor et al., 1999). This can occur through a pleckstrin homology (PH) domain found in the carboxyl terminus of the enzyme that interacts with PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Alessi et al., 1997; Currie et al., 1999). When these lipids are generated by the stimulation of phosphoinositide 3-kinase (PI3K), PDK1 is recruited to the plasma membrane where it activates Akt/PKB (Anderson et al., 1998; Klippel et al., 1997; Vanhaesebroeck and Alessi, 2000). However, other mechanisms of PDK1 targeting must exist, as numerous reports have implicated the enzyme in the activation of cytoplasmic kinases such as SGK, p70S6K, and p90RSK (Toker and Newton, 2000).

p90RSK is a growth factor-responsive serine/threonine kinase with an unusual structure. It contains two distinct kinase domains: an amino-terminal catalytic core that phosphorylates substrates and a carboxy-terminal catalytic core that is involved in regulating its activity (Fisher and Blenis, 1996). Though the sequence of events that activates p90RSK is not fully understood, it is clear that the synergistic activities of PDK1 and ERK1/2 are required ([Gavin and Nebreda, 1999; Jensen et al., 1999]; reviewed in Frodin and Gammeltoft [1999]). Inactive RSKs can be found complexed with ERK1/2 in the cell, providing a mechanism for efficient p90RSK phosphorylation upon stimulation of ERK1/2 (Roux et al., 2003; Smith et al., 1999). Although there is evidence that phosphorylation of p90RSK creates a PDK1 docking site (Frodin et al., 2000), it is unclear how the activities of PDK1 and ERK are coordinated. In this study, we provide evidence that mAKAPα could be a platform for both activation events to take place.

Results

Two Forms of mAKAP Protein

We had previously noted that the rat brain form of mAKAP migrates with a slower mobility on SDS gels than the rat heart form (Kapiloff et al., 1999). We investigated this difference further in mouse, and immunoblot analysis detected mAKAP species of slightly different molecular weights from mouse brain and mouse heart extracts (Figure 1A). Though there was no existing evidence for alternative splicing of the mAKAP gene, we considered the possibility because other AKAP genes use this mechanism to generate distinct variants. A search of the EST database with the first 1761 bp of the mAKAP cDNA detected ESTs that contained a 5′ untranslated sequence unique from the published mAKAP sequence (gi:4257094 and gi:26277742). This implicated the existence of two distinct start sites for the mAKAP gene.

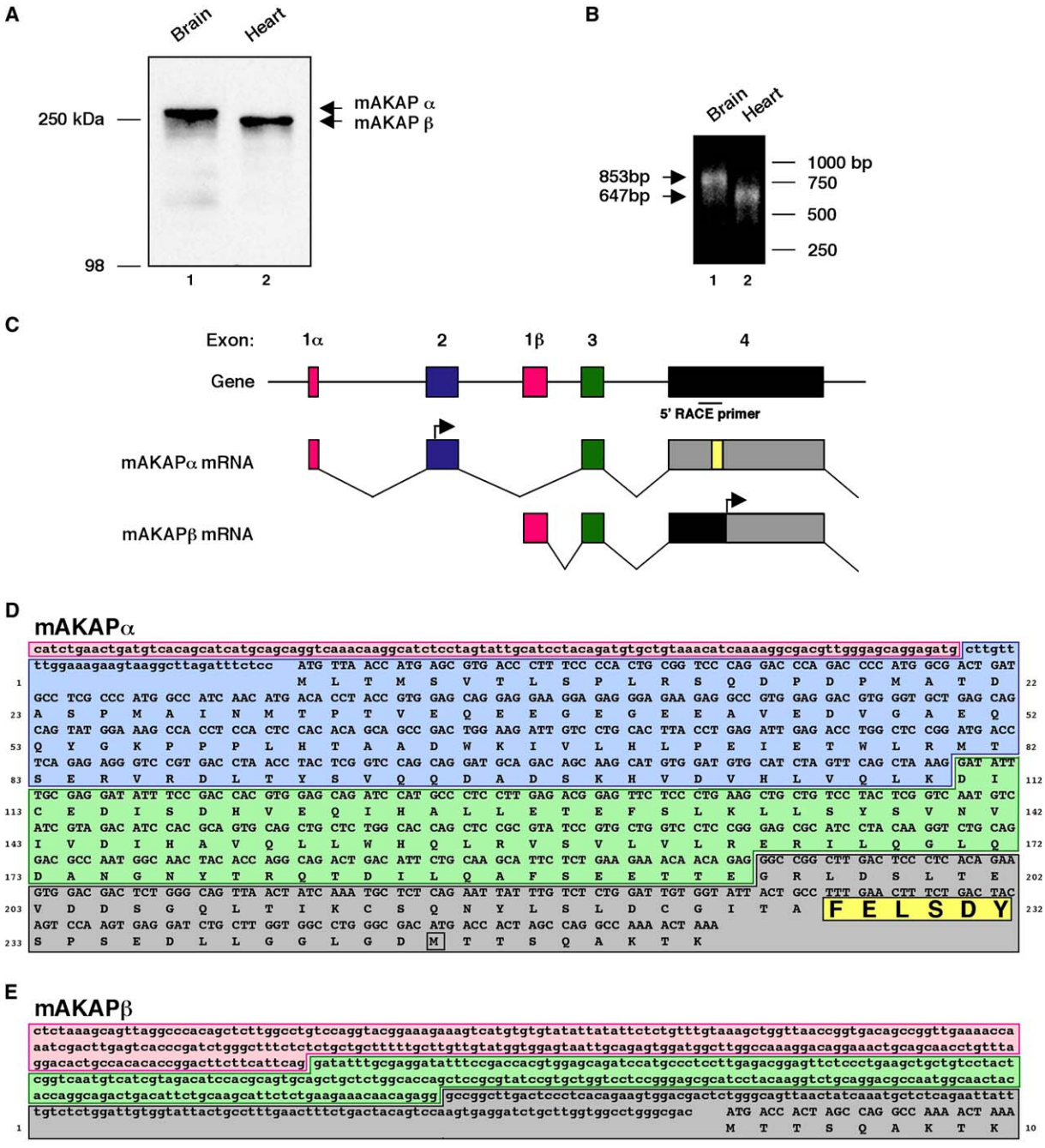

Figure 1. mAKAPα and mAKAPβ Are Tissue-Specific Proteins Expressed from Alternative Initiation Sites

(A) Western analysis of mAKAP immunoprecipitates from mouse brain (lane 1) and heart (lane 2). Arrows indicate mAKAPα and mAKAPβ.

(B) 5′ RACE of mRNA from wild-type (wt) mouse brain (lane 1) and heart (lane 2) using an exon 4-specific reverse primer. The size of each product is indicated with an arrow.

(C) Structure of the first five exons of the mAKAP gene (top), structure of the 5′ region of mAKAPα mRNA (middle), and structure of the 5′ region of mAKAPβ mRNA (bottom). The reverse primer used for 5′ RACE is indicated by the bar below exon 4. Initiating methionines for each mAKAP protein form are indicated by the arrows. The yellow box indicates the hydrophobic motif within the unique amino terminal 1–244 residues of mAKAPα predicted to bind to PDK1.

(D) Unique mAKAPα DNA and protein sequences as determined by 5′ RACE (GenBank accession number DQ233645). Colored boxes refer to the corresponding exon structure depicted in (C). The capitalized residues boxed in yellow constitute the hydrophobic motif predicted to bind to PDK1 (aa 227–232). The boxed "M" indicates the initiating methionine of mAKAPβ.

(E) Unique mAKAPβ DNA and protein sequences as determined by 5′ RACE (GenBank accession number DQ233646). Colored boxes refer to corresponding exon structure depicted in (C).

To further investigate these observations, we performed 5′ RACE on mRNA harvested from brains and hearts of mice. From mouse brain, the expected 853 bp product that encoded the published mAKAP sequence was yielded (Figure 1B, lane 1), hereafter called mAKAPα (Figures 1C and 1D). In contrast, a 647 bp

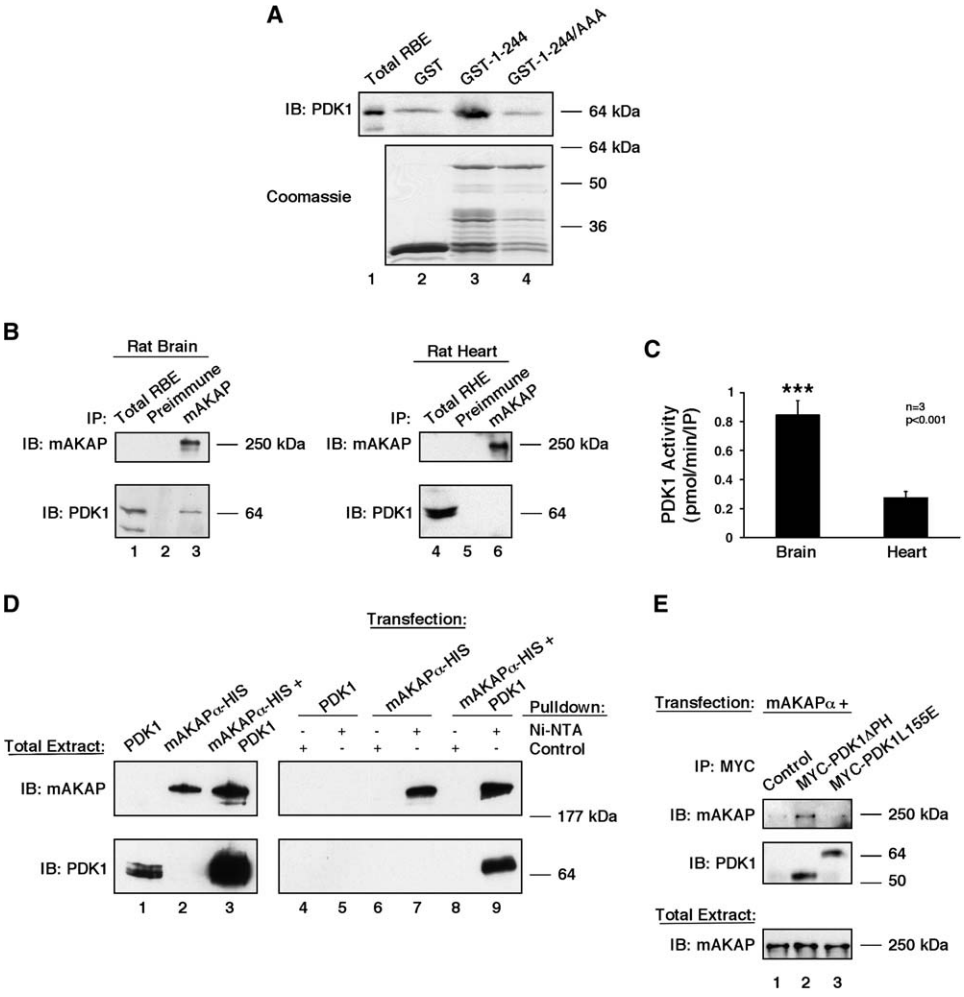

Figure 2. Active PDK1 Associates with the First 244 Amino Acids of mAKAPα

(A) Rat brain extract (lane 1) was precipitated with GST (lane 2), GST-rmAKAP-1–244 (lane 3), or GST-rmAKAP-1–244/AAA (lane 4) and analyzed by anti-PDK1 immunoblot (top). Coomassie staining of GST-fusion inputs (bottom).

(B) Rat brain extract (left) and rat heart extract (right) were immunoprecipitated with preimmune antibody (lanes 2 and 5) or mAKAP antibody (lanes 3 and 6). Total extracts (lanes 1 and 4) and immunoprecipitates were analyzed for the presence of mAKAP (top) and PDK1 (bottom).

(C) PDK1 activity was measured in mAKAP immunoprecipitates from rat brain extract (left bar) and from rat heart extract (right bar). Error bars represent the SEM from three independent experiments. The p value < 0.001 is indicated by asterisks.

(D) HEK293 cells were transfected with plasmids to express PDK1 and mAKAPα-HIS as indicated. Lysates were precipitated with control beads (lanes 4, 6, and 8) or Ni-NTA beads (lanes 5, 7, and 9). Total extracts (lanes 1–3) and precipitates were analyzed by Western blot for the presence of mAKAP (top) and PDK1 (bottom).

(E) HEK293 cells were transfected with plasmids to express mAKAPα and either control (lane 1), MYC-PDK1ΔPH (lane 2), or MYC-PDK1-L155E (lane 3). Lysates were immunoprecipitated with anti-MYC antibody and analyzed by Western blot for the presence of mAKAP (top) and MYC-PDK1 (middle). Total extracts were probed for mAKAP to demonstrate equal expression in all transfections (bottom).

cDNA amplified from mouse heart (Figure 1B, lane 2) encoded a unique 5′ untranslated region that spliced to exon 3 of mAKAP, hereafter called mAKAPβ (Figure 1C and 1E). Nucleotide sequencing identified that the first initiation codon for mAKAPβ is in exon 4, predicting a protein of 2070 amino acids (Figure 1C). Thus, mAKAPβ is a shorter form of the anchoring protein that is transcribed from a different start site and lacks the first 244 amino acids.

mAKAP Anchors PDK1 Activity

On the basis of our genomic analysis, we proposed that the unique amino-terminal portion of mAKAPα (residues 1–244) contains enzyme binding sites that could permit the assembly of distinct signaling complexes. In support of this notion, high stringency ScanSite analysis of the mAKAP protein predicted a consensus PDK1 hydrophobic motif binding sequence (FxxF[D/E/S][Y/F]) between residues 227 and 232 of mAKAPα (Figure 1D). In vitro binding studies confirmed this observation by showing that PDK1 could be pulled down from rat brain extract by using a GST-mAKAP 1–244 fusion protein, but not control GST protein (Figure 2A, lanes 2 and 3). Further, this interaction was significantly decreased to control levels after mutating several residues within the hydrophobic motif to alanines (GST-mAKAP 1–244/AAA,

lane 4). Three other consensus PDK1 binding sites were predicted in the remainder of the mAKAP sequence (data not shown).

To determine whether mAKAPα and PDK1 interact in rat brain, mAKAPα immune complexes were probed for coimmunoprecipitation of PDK1. Western blot analysis confirmed that PDK1 was detected in mAKAPα immune complexes, but not in controls (Figure 2B, lanes 1–3). In contrast, we did not observe coimmunoprecipitation of PDK1 with mAKAPβ in experiments performed from rat heart extracts (Figure 2B, lanes 4–6). In parallel experiments, we measured coprecipitating PDK1 kinase activity. PDK1 activity toward its substrate PDKtide was significantly enriched in anti-mAKAP immunoprecipitates from rat brain (0.85 ± 0.10 pmol/min/IP, Figure 2C, n = 3, $p < 0.001$) and rat heart (0.27 ± 0.04 pmol/min/IP, Figure 2C, n = 3, $p < 0.0001$) as compared to preimmune controls. Importantly, 3-fold more PDK1 activity coprecipitated with mAKAPα as compared to mAKAPβ (n = 3, $p < 0.001$). Collectively, these data imply that PDK1 preferentially associates with mAKAPα. For this reason, we focused our subsequent analyses on the mAKAPα-PDK1 interaction in brain.

By using a heterologous transfection system, we tested for interaction between recombinant mAKAPα-HIS and PDK1 in HEK293 cells. Lysates were precipitated with Ni-NTA beads to specifically pull down mAKAPα-HIS (Figure 2D, lanes 5, 7, and 9), while protein G beads served as a negative control (Figure 2D, lanes 4, 6, and 8). PDK1 coprecipitated with mAKAPα-HIS when both plasmids were expressed (Figure 2D, bottom blot, lane 9), but not when mAKAPα-HIS was omitted from the transfection (Figure 2D, bottom blot, lane 5). Thus, recombinant mAKAPα and PDK1 interact inside cells.

Previous studies have indicated that the PH domain of PDK1 is required to direct the subcellular localization of the enzyme (Alessi et al., 1997; Currie et al., 1999). Therefore, we tested whether this region of the enzyme was required for interaction with mAKAPα. HEK293 cells were transfected with mAKAPα and MYC-PDK1ΔPH, a mutant form of the enzyme that lacks the PH domain. Immunoprecipitation of MYC-PDK1ΔPH resulted in copurification of mAKAPα (Figure 2E, lane 2). From this result, we conclude that the PH domain of PDK1 is not essential for the interaction with mAKAPα. Importantly, however, the results demonstrate reciprocal isolation of this protein complex. Alternatively, to test whether the hydrophobic motif on PDK1 was required for interaction with mAKAPα, HEK293 cells were transfected with mAKAPα and MYC-PDK1-L155E, a mutant form of the enzyme that abolishes its hydrophobic motif. Immunoprecipitation of this mutant PDK1 form did not result in copurification of mAKAPα, although equal amounts of enzyme were pulled down (Figure 2E, center panel, lane 3) and equivalent levels of mAKAPα were expressed in all samples (bottom panel). This confirms that the hydrophobic motif of PDK1 is essential for the interaction with mAKAPα and, thus, provides a means to anchor this enzyme to the nuclear membrane.

mAKAPα Coordinates PDK1 and p90RSK into a Complex

The function of PDK1 is to serve as an upstream activator for selected AGC serine/threonine kinase family members (Toker and Newton, 2000). In an effort to understand the physiological reason behind mAKAPα-anchored PDK1, we screened for the presence of AGC kinases, including p90RSK, p70S6K, SGK, PKC, and PKB, within the mAKAPα complex by using a heterologous transfection system. A plasmid directing the expression of mAKAPα was transfected into HEK293 cells individually and in tandem with HA-tagged AGC kinases (Figure 3A and data not shown). Western analysis of mAKAPα immune complexes demonstrated the presence of HA-RSK3 when mAKAPα, but not control plasmid, was included in the transfection (Figure 3A, center blot, lanes 1 and 3). Thus, mAKAPα can interact with the PDK1 substrate RSK3 in an overexpression system. We were precluded from establishing whether other members of the p90RSK family specifically interact with mAKAP due to nonspecific binding of the proteins to the protein A beads used to collect the immunoprecipitates (data not shown).

To determine whether p90RSK interacted with mAKAPα in rat brain extract, we probed mAKAPα immunoprecipitates for p90RSK by using an antibody that predominantly recognizes p90RSK1 and p90RSK2. A significant amount of p90RSK coimmunoprecipitated with mAKAPα as compared to controls (Figure 3B, bottom). Likewise, RSK activity toward an S6 peptide substrate was enriched 4-fold in anti-mAKAP immunoprecipitates (1.41 ± 0.10 pmol/min/IP) as compared to controls (Figure 3B, graph, n = 4, $p < 0.0001$). Further experiments were performed to confirm that p70S6K, an enzyme with similar substrate specificity, did not contribute to the mAKAPα-associated RSK activity. Western blot analysis on anti-mAKAP immune complexes using an anti-p70S6K antibody was negative (Figure 3C, bottom, lane 3). Thus, we conclude that the RSK activity detected in the mAKAP immunocomplex was due to p90RSK.

Immunofluorescence techniques were used to detect colocalization of all three proteins in cultured primary rat hippocampal neurons. As expected, the mAKAPα signal was concentrated around the perinuclear region (Figure 3D). Likewise, the PDK1 signal was localized to this region (Figure 3E). Although the p90RSK staining pattern was more equally distributed throughout the cytoplasm, a significant proportion of the signal was also concentrated at the perinuclear membrane (Figure 3F). As can be seen in the composite image, some areas of colocalization of mAKAPα, PDK1, and p90RSK occur at the perinuclear regions of hippocampal neurons (Figure 3G, overlap of all three appears white). Taken together, the immunofluorescence and biochemical data suggest that all three proteins can exist in a localized signaling complex.

ERK Upstream Activators of p90RSK Are Also in the mAKAPα Complex

Full activation of p90RSK requires hierarchical phosphorylation by ERK1/2 and by PDK1 ([Gavin and Nebreda, 1999; Jensen et al., 1999]; reviewed in Frodin and Gammeltoft [1999]). We have recently shown that ERK5 and MEK5 specifically associate with mAKAPβ in cardiomyocytes (Dodge-Kafka et al., 2005). There are conflicting reports as to whether the ERK5 pathway is a bona fide activator of RSK (Pearson et al., 2001; Mody et al., 2001; Watson et al., 2001). Therefore, we

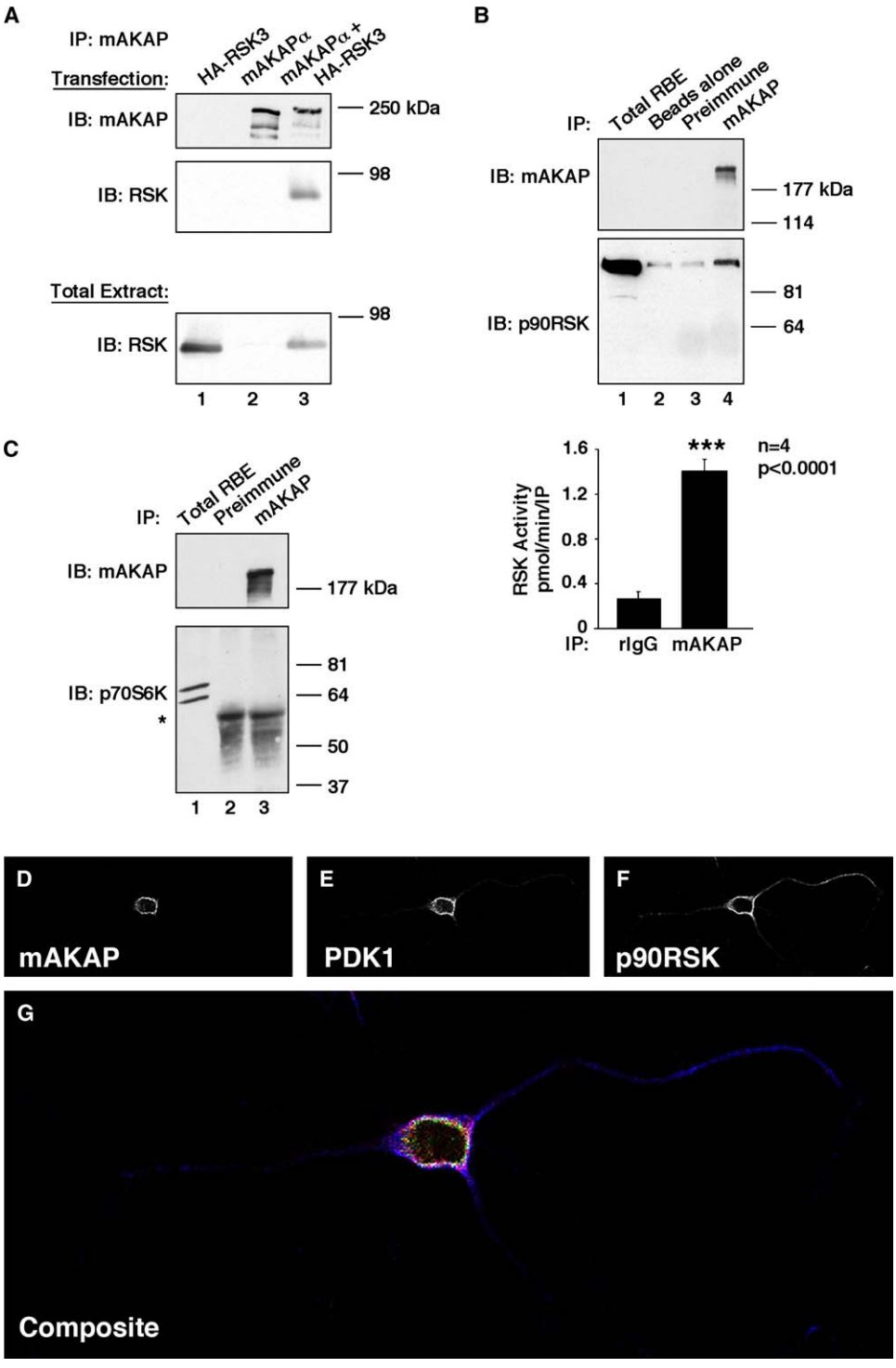

Figure 3. Active RSK Is Assembled into the mAKAPα Complex

(A) HEK293 cells were transfected with plasmids to express HA-RSK3 (lane 1), mAKAPα (lane 2), or both proteins (lane 3). Lysates were immunoprecipitated with anti-mAKAP antibody and analyzed by Western blot for mAKAP (top) and HA-RSK3 (middle). Total extracts were probed for HA-RSK3 to show specificity of the interaction (bottom).

(B) Rat brain extract was immunoprecipitated with the indicated antibodies and analyzed by Western blot for the presence of mAKAP (top) and p90RSK (bottom). RSK activity was measured in control (graph, left bar) and mAKAP (right bar) immunoprecipitates from rat brain extract. Error bars represent the SEM from four independent experiments. The p value < 0.0001 is indicated by asterisks.

(C) Rat brain extract (lane 1) was immunoprecipitated with preimmune (lane 2) or anti-mAKAP (lane 3) antibodies. Total extract and immunoprecipitates were analyzed by Western blot for mAKAP (top) and p70S6K (bottom). Asterisk indicates the IgG heavy chain.

(D–G) Primary rat hippocampal neurons were immunostained for mAKAP (D), PDK1 (E), and p90RSK (F). The composite image is shown in (G). Overlap of all three proteins appears white.

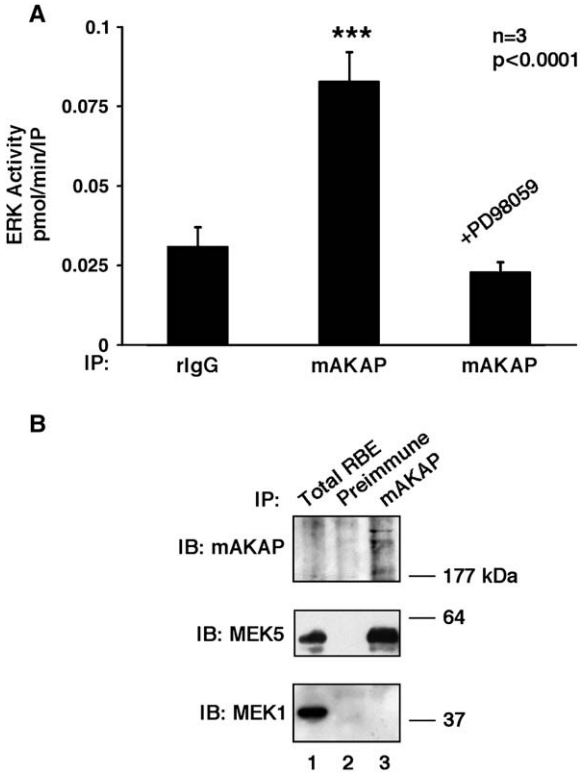

Figure 4. Active ERKs Associate with the mAKAP Complex

(A) ERK activity was measured in control immunoprecipitates (left bar), mAKAP immunoprecipitates (middle bar), and mAKAP immunoprecipitates in the presence of 2 μM PD98059 (right bar) from rat brain extract. Error bars represent the SEM from three independent experiments. The p value < 0.0001 is indicated by asterisks.

(B) Rat brain extract (lane 1) was immunoprecipitated with preimmune (lane 2) or anti-mAKAP (lane 3) antibodies. Total extract and immunoprecipitates were analyzed by Western blot for mAKAP (top), MEK5 (middle), or MEK1 (bottom).

looked for evidence that mAKAPα could serve as a scaffold to facilitate activation of p90RSK by recruiting ERK and PDK1 activities in brain. We observed 2-fold more ERK activity in anti-mAKAPα immunoprecipitates (0.083 ± 0.009 pmol/min/IP) from rat brain extract as compared to controls (Figure 4A, n = 3, p < 0.0001). Because this mAKAPα-associated ERK activity was blocked in the presence of the MEK inhibitor PD98059, we would argue in light of our previous studies that MEK activity was present in this complex. Western analysis confirmed that MEK5 was present in mAKAPα immune complexes (Figure 4B, lane 3). Under the same conditions, MEK1/2 was not detected (Figure 4B, lane 3 and data not shown), whereas ERK1/2 association was inconclusive (J.J.C.M. and J.D.S., unpublished data). These results demonstrate that mAKAPα has the capacity to assemble an activation cascade that permits the hierarchical phosphorylation of p90RSK.

Generation of mAKAPα Null Mice

To explore the mAKAPα complex and its relationship to PDK1 and p90RSK in a broader context, we generated mice lacking this form of the anchoring protein. This was achieved by targeted deletion of exon 2 (Figure 5A) to produce mAKAPα null mice that express mAKAPβ in the brain. Of note, mAKAPα null mice had reduced viability (Table S1 available in the Supplemental Data with this article online, Mendelian ratio = 1.04:1.97:0.45) and a smaller body size as compared to wild-type (wt) and heterozygous littermates (Figure 5B and Figure S1).

We analyzed mRNA and protein products to verify the removal of the mAKAPα specific exon. These experiments were performed in three stages. First, 5′ RACE specifically generated a 488 bp product from RNA isolated from mAKAPα null brains (Figure 5C, lane 2). Sequencing analysis established the deletion of exon 2. Expression of mAKAPβ was confirmed by detection of a 647 bp product from RNA isolated from wt and null hearts followed by sequence analysis (Figure 5C, lanes 3 and 4).

Second, RT-PCR confirmed the tissue-specific expression of the modified transcript. Primers designed to amplify exon 1α through a portion of exon 4 of mAKAPα yielded a band of 830 bp only from RNA isolated from wt and heterozygous brains (Figure 5D, top, lanes 1 and 2, top band). Homologous recombination was confirmed by detection of a 465 bp band from RNA isolated from heterozygous and null brains (Figure 5D, top, lanes 2 and 3, bottom band). Amplification of exon 13 served as a control (Figure 5D, bottom). Primers designed to amplify exon 1β through a portion of exon 4 of mAKAPβ produced bands of the expected size from RNA isolated from hearts and brains of all genotypes, though at a much lower level in brains (Figure 5D, middle).

Third, Western analysis of protein extracts harvested from wt and mAKAPα null littermates verified the tissue-specific expression of mAKAPα and mAKAPβ. The predominant mAKAP form in the brains of wt mice was 280 kDa, whereas a protein product of 250 kDa was the predominant form in brain extracts from null mice (Figure 5E, lanes 1 and 2). This 250 kDa protein that corresponds to mAKAPβ was also the predominant form in heart extracts isolated from wt and mAKAPα null mice (Figure 5E, lanes 3 and 4). From these studies, we conclude that a preferred form of mAKAP is expressed in brain and in heart.

mAKAP-Associated PDK1 Activity Is Reduced in mAKAPα Null Mice

The difference between mAKAPα and mAKAPβ is the presence of the first 244 amino acids. We show above that this region contains a PDK1 consensus binding site (Figure 1D). To determine whether genetic manipulation of mAKAPα resulted in changes in the levels of associated PDK1, we performed PDK1 kinase assays on mAKAP immunoprecipitates from brain by using PDKtide as a substrate peptide. Although equal amounts of mAKAP protein were immunoprecipitated from wt and null brains (Figure 6A, top), there was 29.1% ± 7.1% less PDK1 activity associated with mAKAP from null mice as compared to wt (Figure 6A, graph, n = 3, p < 0.02). Qualitatively similar results were obtained by immunoblot, suggesting subtle differences in PDK1 binding between wt and null mice (Figure 6B, lanes 3 and 6). Based upon this data, we conclude that the larger mAKAPα contains an additional PDK1 binding site that recruits more active enzyme to the signaling complex.

mAKAPα-Anchored PDK1 Regulates p90RSK Release

Because PDK1 is required for full activation of p90RSK (Jensen et al., 1999), we determined the role of these two kinases within the mAKAP complex. The potential utilization of additional PDK1 sites in mAKAP precluded us from utilizing the mAKAPα null mice for these assays. Therefore, we moved to a transfection system where we could selectively express mutant proteins and subsequently examine complex formation. HEK293 cells were cotransfected with plasmids encoding mAKAPα and HA-RSK3 in combination with wt or catalytically inactive PDK1. As expected, RSK copurified with mAKAPα (Figure 6C, second panel, lane 1). However, the level of RSK in mAKAPα complexes was reduced 2-fold upon overexpression of wt PDK1 (Figure 6C, second panel, lane 2 and bottom graph, $n = 3$, $p < 0.02$). Control experiments confirmed that approximately equal levels of RSK were expressed (Figure 6C, bottom). PDK1 kinase activity was required for the release of RSK, as control experiments performed with catalytically inactive PDK1 were unable to disrupt the RSK-mAKAPα complex (Figure 6C, second panel, lane 3 and bottom graph). Control experiments verified that catalytically inactive PDK1 bound to mAKAP as well as or even better than wt PDK1 (Figure 6C, third panel).

The mechanism of p90RSK activation by PDK1 requires phosphorylation of the T loop within the RSK amino-terminal kinase domain. In RSK3, this corresponds to serine 218. Therefore, we generated a phosphorylation-resistant mutant, HA-RSK3-S218A, that is insensitive to PDK1. This form of RSK, but not wt RSK, remained associated with the anchoring protein even in the presence of active PDK1 as assessed by Western analysis of mAKAPα complexes (Figure 6D, middle, lanes 3 and 4, and graph, $n = 3$, $p < 0.02$). Control experiments confirmed that approximately equal amounts of RSK protein were expressed (Figure 6D, bottom). Thus, PDK1 phosphorylation of RSK3 on serine 218 releases the enzyme from the mAKAP complex.

Discussion

In this report, we have shown that brain-specific mAKAPα participates in the assembly of a signaling complex that includes PDK1 and its downstream target p90RSK. These genetic and biochemical studies also indicate that two mAKAP forms are transcribed from the same gene and that tissue-specific expression of these proteins from alternate initiation sites can influence the assembly of distinct multiprotein signaling complexes. Both processes represent previously unappreciated mechanisms that drive the assembly of tissue-specific mAKAP complexes.

Some AKAPs undergo alternative splicing to achieve compartment-specific targeting or tissue-specific expression. For example, multiple transcripts from the *AKAP350/450/CG-NAP/yotiao* gene on chromosome 7q21 generate a plethora of compartment-specific AKAP splice variants that anchor PKA and other enzymes to the Golgi, the centrosome, or the plasma membrane (Shanks et al., 2002; Westphal et al., 1999; Witczak et al., 1999). A variation on this theme is the anchoring proteins D-AKAP1, AKAP121, and AKAP149, unique splice variants derived from the same gene that interact with distinct combinations of binding partners. D-AKAP1 can bind to type I PKA (Huang et al., 1997), AKAP121 contains a KH domain that binds to RNA (Ginsberg et al., 2003), and the longest splice form, AKAP149, binds to PP1 (Steen et al., 2000). All of the above examples highlight genomic mechanisms used to expand the repertoire of AKAPs that can be transcribed from a limited number of genes. Intriguingly, the genomic structure of mAKAP suggests yet another mechanism to create diversity. Our evidence demonstrating the two forms of mAKAP, α and β, provides the first example of alternative initiation sites used to express tissue-specific AKAPs. This was confirmed by the generation of exon 2 deleted mice that express mAKAPβ in brain, instead of mAKAPα. The mice display a phenotype characterized by partial embryonic lethality as determined by an altered Mendelian ratio. Of the survivors, we observed some postnatal lethality, reduced growth, and craniofacial defects (J.J.C.M. and J.D.S., unpublished data). Furthermore, loss of mAKAPα caused a reduction in anchored PDK1 activity (Figure 6). These findings are intriguing when considering that PDK1 has a role in regulating cell size and development in mice (Lawlor et al., 2002). Likewise, several functional studies have suggested that RSK certainly plays a role in cell growth and differentiation (Gross et al., 2001; Torres et al., 1999; Wong et al., 1996). Thus, we postulate that switching the tissue-specific expression pattern of mAKAP forms may have critical and as yet unappreciated effects on the formation of brain signaling complexes maintained by this anchoring protein. Continuing work will focus on the precise roles of these brain-specific mAKAP signaling complexes in postnatal growth control.

A key finding of this study with broad implications is that protein-protein interactions participate in the subcellular localization of the constitutively active PDK1 to restrict its activity. The PH domain of PDK1 classically functions as a plasma membrane targeting signal to position the enzyme in a location where it can activate Akt/PKB (Anderson et al., 1998; Klippel et al., 1997; Vanhaesebroeck and Alessi, 2000). Also, nuclear import of PDK1 requires phosphorylation of S396 via a mechanism that is dependent upon the PH domain (Scheid et al., 2005). One important distinction of our findings is that the PDK1-mAKAPα interaction occurs independent of the PH domain as a mutant lacking the PH domain still bound to mAKAP (Figure 2E). Thus, the mAKAPα-PDK1 interaction must occur through an alternative mechanism. Interestingly, the unique region of mAKAPα contains a hydrophobic motif similar to those that can recruit PDK1. Previous studies have indicated that these are sites for phosphorylation-dependent recruitment of PDK1. This occurs through interaction with a specialized region in the amino terminal lobe of PDK1 (Biondi et al., 2000). However, a closer inspection of the sequence in mAKAPα suggests that its motif is phosphorylation independent. Mutation of this hydrophobic motif abolished the mAKAPα-PDK1 interaction (Figure 2A). Reciprocal studies show that the hydrophobic motif on PDK1 is required for interaction with mAKAPα (Figure 2E). Thus, we envision a model for the mAKAPα-PDK1 interaction where hydrophobic surfaces on both proteins form a docking site. To our knowledge, this is the first

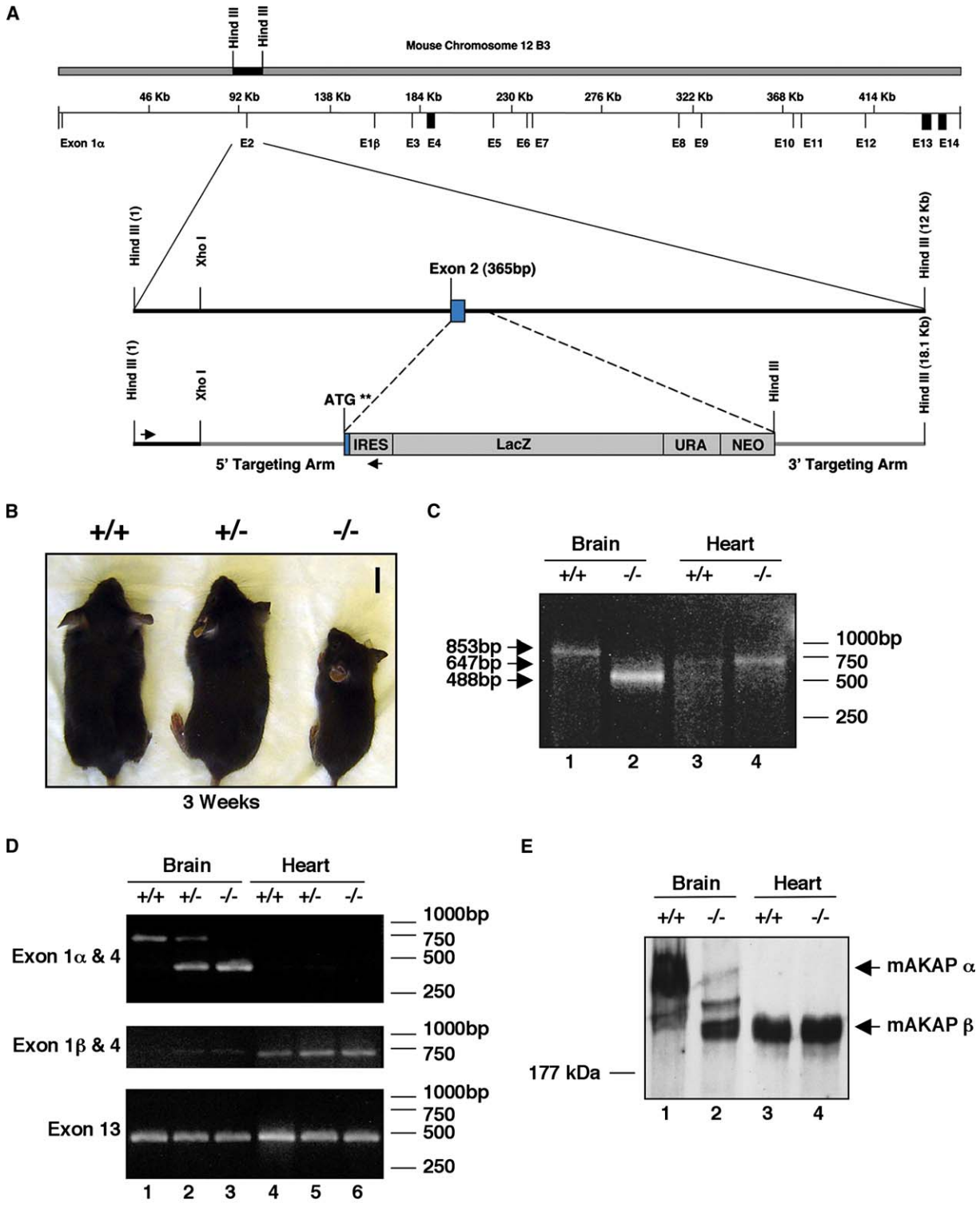

Figure 5. mAKAPα Null Mice Have Reduced Viability and Growth

(A) Schematic diagram of the mouse mAKAP gene (top) on chromosome 12B3. The locations of relevant enzyme restriction sites and exons for the mAKAP coding regions are indicated, including alternative exons 1α and 1β. The HindIII fragment used to generate the construct is indicated in black. The site of homologous recombination (bottom) that inserted the Ura/Neo markers and deleted the majority of exon 2 and its splice junction is highlighted. The arrows indicate the primer set used to verify homologous recombination in the correctly targeted mice.

(B) Representative photographs of wt (+/+), heterozygous (+/−), and mAKAPα null (−/−) mice at week three (scale bar, 1.0 cm).

(C) 5′ RACE cDNA products from experiments performed on mRNA isolated from wt mouse brain (+/+, lane 1), mAKAPα null mouse brain (−/−, lane 2), wt mouse heart (lane 3), and mAKAPα null mouse heart (lane 4). Sizes of products are indicated with arrows.

instance of PDK1 interacting with a protein other than one of its kinase substrates via its hydrophobic motif. Further, this is the first demonstration that PDK1 can incorporate into a multiprotein signaling complex. Recruitment into AKAP complexes is an appealing idea, as the role of PDK1 is to phosphorylate and activate other AGC kinases, including PKCs (Balendran et al., 2000) and RSKs (this study), which are known AKAP binding partners. Furthermore, our immunohistochemical analysis demonstrating a ternary complex of mAKAPα, PDK1, and p90RSK at the perinuclear membrane of rat hippocampal neurons is consistent with the notion that the anchoring protein tethers both kinases to sites where they could be efficiently moved into the nucleus.

The p90RSK activation sequence involves the concerted action of two exogenous enzymes: ERK1/2 and PDK1 (reviewed in Frodin and Gammeltoft [1999]). The current model proposes three phases of phosphorylation: (1) ERK docking and phosphorylation of the RSK C-terminal kinase domain, (2) autophosphorylation by this domain to create a binding site for PDK1, and (3) PDK1 phosphorylation of the RSK N-terminal kinase domain. Our biochemical and enzymatic data argue that mAKAP facilitates this process by providing the platform to synchronize these events. By analogy to previously published reports (Roux et al., 2003; Smith et al., 1999), we propose that RSK is recruited to this complex through its interactions with ERK. Although RSK3 was used in the transfection studies, Western analysis from brain extracts suggests all RSK isoforms may have the capacity to interact with mAKAPα. The coimmunoprecipitation studies presented in Figure 4 and published studies (Dodge-Kafka et al., 2005) confirm the presence of ERK activity within the mAKAPα complex. Although our data suggest that ERK5 activity promotes p90RSK activation in the context of the mAKAPα complex, conflicting reports exist as to whether the ERK5 pathway is a bona fide activator of RSK (Pearson et al., 2001; Mody et al., 2001; Watson et al., 2001). Further, we cannot rule out activation of RSK by the ERK1/2 pathway, because association with phosphodiesterase 4D3, another component of the mAKAP complex, can recruit all ERKs to mAKAP (Hoffmann, et al., 1999; Dodge et al., 2001). Therefore, additional studies are warranted to delineate the individual contributions of the ERK5 and the ERK1/2 pathways to mAKAPα-anchored RSK activation. Finally, we demonstrate that PDK1 phosphorylation of anchored p90RSK promotes its dissociation from the mAKAPα signaling complex (Figure 6). Surprisingly, the level of activatable RSK activity in vitro was similar in the mAKAPα and mAKAPβ immune complexes. One explanation for this finding is that loss of PDK1 resulted in more dormant RSK protein remaining associated with mAKAPβ. Results from our transfection studies (Figures 6C and 6D), where we selectively expressed mutant proteins and examined complex formation, support this hypothesis. Consequently, the high degree of spatial resolution provided by mAKAPα favors p90RSK activation in two ways. First, anchoring of ERK and PDK1 generates a cellular focal point for the convergence of phospholipid dependent and mitogenic activated signaling pathways. Second, both pathways must be switched on before the perinuclear pool of p90RSK is activated. This configuration would allow the selective and efficient translocation of p90RSK into the nucleus where it could phosphorylate physiologically relevant downstream targets such as the transcription factors CREB and c-*fos*, the chromatin-associated protein histone H3, and the $p34^{cdc2}$ inihibitory kinase Myt1 (Chen et al., 1993; Palmer et al., 1998; Sassone-Corsi et al., 1999; Xing et al., 1996).

In conclusion, we present evidence that anchoring by brain-specific mAKAPα regulates local PDK1 activity. Although the first 244 amino acids of mAKAPα participate in the recruitment of PDK1, future studies will investigate to what extent other regions of the anchoring protein contribute to the assembly and regulation of this tissue-specific signaling complex. Nonetheless, the simultaneous association of PDK1, ERK, and p90RSK with mAKAPα places the enzymes in a context that permits the rapid activation and release of p90RSK. Their proximity within the same complex provides an efficient mechanism to relay signals. Given the broad role of PDK1 in a variety of compartmentalized signaling events and the utility of AKAPs as specialized signal organizing molecules, it would not be surprising if other anchoring proteins recruit PDK1 activity.

Experimental Procedures

Antibodies

The following antibodies were used in this study: anti-HA 12CA5; anti-MYC9E10; from Santa Cruz, anti-p70S6 kinase H-9 (sc-8418), anti-MEK1 H-8 (sc-6250), and anti-PKB Kinase C-19 (sc-7686); from BD Transduction Laboratories, anti-RSK (610225), anti-PKB Kinase/PDK1 (611070), and anti-MEK5 (610956); anti-mAKAP VO54; and anti-mAKAP (VO145)-HRP conjugated. For indirect immunocytochemistry of neonatal rat hippocampal neurons, secondary antibodies were FITC-conjugated donkey anti-rabbit IgG, Texas Red-conjugated donkey anti-goat IgG, and Cy5-conjugated donkey anti-goat IgG (Jackson ImmunoResearch).

Plasmids

pcDNA3.1-rmAKAPα-MYC-HIS and pEGFP-N1-rmAKAPα were generated in a previous study (Kapiloff et al., 1999), pcDNA3-MYC-human PDK1 was a gift from Dr. Alexandra Newton, pKH3-HA-human RSK3 was a gift from Dr. John Blenis, and pCMV5-MYC-kinase dead PDK1 (D223A) and pCMV5-MYC-PDK1ΔPH (aa 1–449) were gifts from Dr. Dario Alessi. The pGEX-4T1-rmAKAP-1-244 construct for protein expression in bacteria was generated by PCR amplification. pKH3-HA-human RSK3 S218A, pcDNA3-MYC-PDK1-L155E, and pGEX-rmAKAP1-244 S230A/D231A/Y232A (AAA) were generated by site-directed mutagenesis per manufacturer's instructions (Stratagene) by using primers 5′-GAGAGCGTACGCCTTCTGC GGG-3′, 5′-CGACGAGAAGGAGTATTTCGGCC-3′, 5′-GCATTTGAAC TCGCGGCCGCCAGTCCAAGTGAG-3′, and their complements, respectively.

Cell Culture Transfections and Immunoprecipitations

HEK293 cells were transiently transfected by using the calcium phosphate method. For immunoprecipitation experiments, cells

(D) RT-PCR cDNA products from mouse brain (lanes 1–3) and mouse heart (lanes 4–6). Wt (+/+), heterozygous (+/−), and null (−/−) mice are indicated. Specific primers were used to amplify portions of exons 1α through 4 (top), exons 1β through 4 (middle), and exon 13 (bottom).
(E) mAKAP protein expression in brain (lanes 1 and 2) and heart (lanes 3 and 4) extracts from wt (+/+) and null (−/−) mice was assessed by immunoblot.

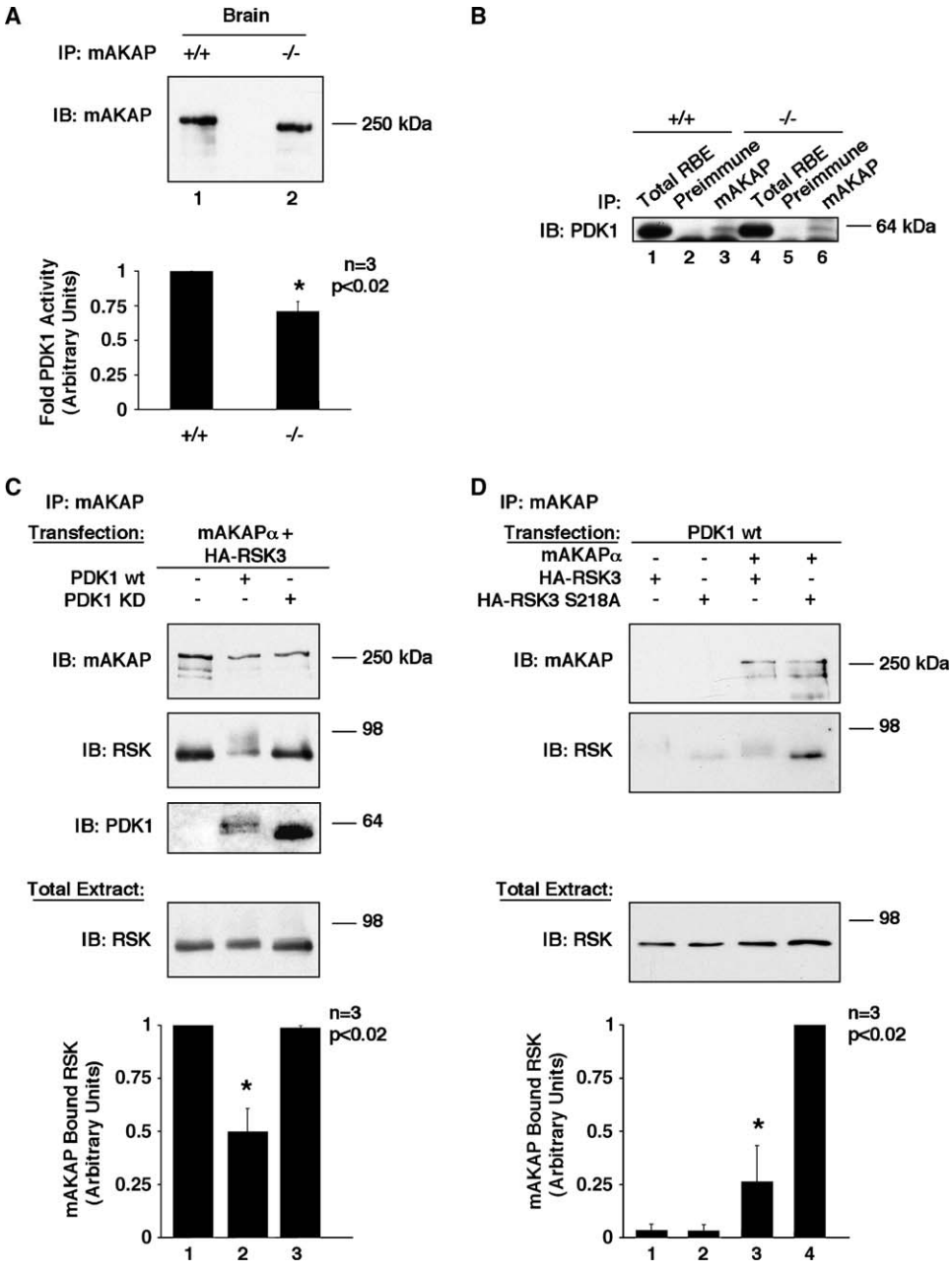

Figure 6. mAKAPα-Anchored PDK1 Activity Influences RSK Association with mAKAPα

(A) Brain extracts from wt (+/+) and null (−/−) mice were immunoprecipitated with anti-mAKAP antibody. Equal amounts of precipitated mAKAP protein were assessed by immunoblot (top). PDK1 activity was measured in mAKAP immunoprecipitates from wt (+/+) and null (−/−) mice (graph). Error bars represent the SEM from three independent experiments. The p value < 0.02 is indicated by an asterisk.

(B) Wt (+/+) and null (−/−) mouse brain extracts (lanes 1 and 4) were immunoprecipitated with control (lanes 2 and 5) and mAKAP (lanes 3 and 6) antibodies and analyzed for the presence of PDK1.

(C) HEK293 cells were transfected with plasmids to express mAKAPα, HA-RSK3, and either control (lane 1), wt PDK1 (lane 2), or catalytically inactive PDK1 (KD, lane 3). Lysates were immunoprecipitated with anti-mAKAP antibody and analyzed by immunoblot for the presence of mAKAP (top) and HA-RSK3 (second panel). Catalytically inactive PDK1 binds to mAKAP as well as wt PDK1 (third panel). Total extracts were blotted for HA-RSK3 to show equivalent levels of expression in each sample (bottom). HA-RSK3 bands were quantitated by densitometry from three independent experiments by using NIH Image software, and the relative amount of mAKAP bound RSK was plotted (bottom graph). Error bars represent the SEM from three independent experiments. The p value < 0.02 is indicated by an asterisk.

(D) HEK293 cells were transfected with plasmids to express wt PDK1 in combination with mAKAPα (lanes 3 and 4), wt HA-RSK3 (lanes 1 and 3), or phosphorylation mutant HA-RSK3 S218A (lanes 2 and 4). Lysates were immunoprecipitated with anti-mAKAP antibody and analyzed by immunoblot for the presence of mAKAP (top) and HA-RSK3 (middle). Total extracts were blotted for HA-RSK3 to show equivalent levels of expression in each sample (bottom). HA-RSK3 bands were quantitated by densitometry from three independent experiments by using NIH Image software, and the relative amount of mAKAP bound RSK was plotted (bottom graph). Error bars represent the SEM from three independent experiments. The p value < 0.02 is indicated by an asterisk.

were lysed in 0.5 ml HSE buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1 mM DTT) plus an inhibitor cocktail (1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM AEBSF). For Ni-NTA agarose (Qiagen) precipitation experiments, cells were lysed in 0.5 ml buffer containing 50 mM Na_2HPO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 0.5% Tween-20 plus the inhibitor cocktail. Antibody and 10 μl prewashed protein A- or G-agarose beads (50% slurry, Upstate Biotechnology) or 50 μl prewashed Ni-NTA agarose (50% slurry) were added to extracts and incubated overnight with rocking at 4ºC. Precipitates were washed three times with lysis buffer, and bound proteins were analyzed by immunoblotting.

Densitometry of bands was measured by using National Institutes of Health (NIH) Image software. In-Stat statistical software was used to perform the two-tailed Student's t test to determine p values for statistical significance.

Preparation of Extract

Rat brain extract or rat heart extract was prepared by homogenizing one rat brain or heart (PelFreeze) in 10 ml HSE buffer by using a Polytron homogenizer. Extracts were cleared by centrifugation at 16,000 × g for 30 min. Soluble extracts were precleared with protein A-agarose for at least 1 hr before immunoprecipitation or GST-pull down.

Kinase Assays

PDK1 activity was assayed by using PDKtide as a substrate per manufacturer's instructions (Upstate Biotechnology). RSK activity was assayed by using the S6 Kinase Assay Kit from Upstate Biotechnology per manufacturer's instructions. ERK activity was assayed as previously described (Dodge-Kafka et al., 2005).

Neonatal Rat Hippocampal Neuron Culture and Immunocytochemistry

The procedure followed is as previously published (Westphal et al., 1999).

5′ RACE and RT-PCR

5′ RACE and RT-PCR were performed with RNA SMART kits (BD Biosciences) per manufacturer's instructions by using the following primers: exon 4 antisense, 5′-CACTTGGACTGTAGTCAGAAAGTTC AAAGGC-3′; exon 1α sense, 5′-CATGCAGCAGGTCAAACAAGGCAT CTCCTAGTATTGC-3′; exon 1β sense, 5′-CTCTAAAGCAGTTAGGCC CACAGCTCTTGG-3′; exon 13 sense, 5′-TGGGGACATAAGTGTGAG CAGTGGCTCGG-3′; and exon 13 antisense, 5′-GGTGAGAGTGGAG CTGGAGAGGAGGTCGCTG-3′.

mAKAP Genomic Cloning and Sequencing

A 12 Kb fragment isolated from a bacterial artificial chromosome (BAC; Incyte Genomics, St. Louis) that contained the first coding exon of mAKAPα (exon 2, 365 bp) was digested with HindIII, ligated into the vector Yplac22, and sequenced with the GPS-1 kit (New England Biolabs). Sequences (400 bp) from within exon 2 and intron 2 were subcloned 5′ to a cassette containing an internal ribosomal entry sequence (IRES), the *lacZ* gene, and URA- and NEO-selectable markers. This cassette was subcloned into the Yplac22-mAKAP BAC containing plasmid to generate a construct replacing 1.6 Kb of genomic DNA. The 5′ region of exon 2 and the native ATG start remained, but two stop codons were placed 30 bp into the coding sequence. The remaining 300 bp of exon 2 and 1.3 Kb of intron 2 were replaced by the selection cassette. After electroporation of the targeting vector, 268 neomycin-resistant mouse ES clones were screened for the altered mAKAP gene. A 6.1 Kb PCR product detected a homologous recombination event that was later reconfirmed in mAKAPα null mice (Figure S2A, lane 2). Samples containing a single copy of the targeted gene were detected by Southern blot (data not shown). ES cells from two positive, correctly targeted colonies were used to generate chimeric mice.

Unless noted, all mice and rats were housed in the Vollum Institute Animal Care Facility, and all procedures were approved by institutional animal care and use committees. Chimeric mice were generated at the University of Cincinnati according to established protocols. Successfully targeted mice were bred into the Black Swiss (Taconic) background for ten generations to establish isogeneity.

PCR Genotyping

Primers specific to the portion of exon 2 deleted by homologous recombination were used to generate a 171 bp band in wt and heterozygous progeny (Figure S2B, lanes 1 and 2): 5′-ATGTTAACCATGAG CGTG-3′ and 5′-TGGCTTTCCATACTGCTG-3′. Primers specific to the uracil-selectable marker amplified a 311 bp band from heterozygous and mAKAPα null mice (Figure S2B, lanes 2 and 3): 5′-CACGGT GTGGTGGGCCCAGGTATTG-3′ and 5′-GACCACATCATCCACGGT TCTATAC-3′. Primers to generate the 6.1Kb homologous recombination product in correctly targeted mice were 5′-ACTACCGTGGTC TCTGCGTGTCG-3′ and 5′-GCTGGCGACCTGCGTTTCAC-3′.

Body Weight Measurements

Female littermates of all genotypes (+/+, n = 9; +/−, n = 13; −/−, n = 10) were weighed from 2 to 15 weeks of age. Littermates showed no difference in their ability to nurse or eat solid food.

Supplemental Data

Supplemental Data include two figures and one table and are available with this article online at http://www.molecule.org/cgi/content/full/20/5/661/DC1/.

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Accession Numbers

Mouse sequences for mAKAPα (DQ233645) and mAKAPβ (DQ233646) have been deposited into the GenBank database.