Pericentrin anchors protein kinase A at the centrosome through a newly identified RII-binding domain

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Centrosomes orchestrate microtubule nucleation and spindle assembly during cell division [1,2] and have long been recognized as major anchoring sites for cAMP-dependent protein kinase (PKA) [3,4]. Subcellular compartmentalization of PKA is achieved through the association of the PKA holoenzyme with A-kinase anchoring proteins (AKAPs) [5,6]. AKAPs have been shown to contain a conserved helical motif, responsible for binding to the type II regulatory subunit (RII) of PKA, and a specific targeting motif unique to each anchoring protein that directs the kinase to specific intracellular locations. Here, we show that pericentrin, an integral component of the pericentriolar matrix of the centrosome that has been shown to regulate centrosome assembly and organization, directly interacts with PKA through a newly identified binding domain. We demonstrate that both RII and the catalytic subunit of PKA coimmunoprecipitate with pericentrin isolated from HEK-293 cell extracts and that PKA catalytic activity is enriched in pericentrin immunoprecipitates. The interaction of pericentrin with RII is mediated through a binding domain of 100 amino acids which does not exhibit the structural characteristics of similar regions on conventional AKAPs. Collectively, these results provide strong evidence that pericentrin is an AKAP *in vivo*.

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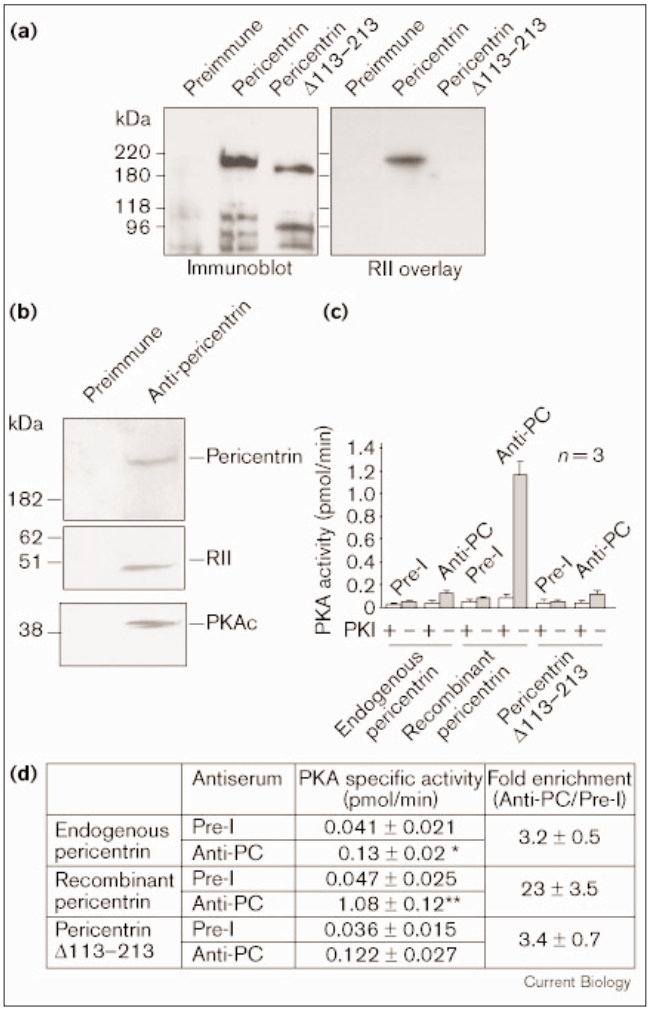
Results and discussion

We isolated a cDNA encoding a 436 amino-acid fragment of pericentrin using an interaction cloning strategy to identify AKAPs [7]. Pericentrin is a highly conserved component of the centrosomal matrix that plays a critical role in centrosome assembly and spindle integrity [8]. Evidence that pericentrin binds the PKA holoenzyme was provided by the transient transfection of the haemagglutinin (HA)-tagged protein in HEK-293 cells (Figure 1a, left panel). A 220 kDa RII-binding protein was detected after immunoprecipitation of recombinant pericentrin with anti-pericentrin antibodies (Figure 1a, right panel). In pericentrin immunoprecipitates, the specific activity of PKA was enriched 23 ± 3.5 fold ($n = 3$) over that of the preimmune control (Figure 1c,d). Immunoprecipitation of endogenous pericentrin from untransfected cells significantly enriched kinase activity by 3.2 ± 0.5 fold ($n = 3$) (Figure 1c,d). These values are similar to those obtained after the immunoprecipitation of other AKAP–PKA complexes from tissues or cell extracts [9,10]. Additional experiments demonstrated the presence of both the regulatory (RII) and catalytic subunits of the PKA holoenzyme when endogenous pericentrin was immunoprecipitated from HEK-293 cells (Figure 1b). Collectively, these data demonstrate that pericentrin functions as an AKAP inside cells and fulfil recent criteria that have been proposed to more stringently define AKAPs [6].

The RII-binding determinants on all AKAPs identified to date are contained within a stretch of approximately 20 amino acids that forms an amphipathic helix [11–13]. Therefore, a family of human pericentrin fragments were screened to map the RII-binding domain (Figure 2a). Surprisingly, an entire region of 100 amino acids between residues 220 and 320 was necessary for RII binding (Figure 2b). This finding was confirmed by the generation of a mouse pericentrin deletion mutant (pericentrin Δ113–213) that lacked these residues (Figure 1a, left panel). The truncated recombinant pericentrin did not bind RII in the overlay assay (Figure 1a, right panel) or coimmunoprecipitate PKA catalytic activity over endogenous levels when overexpressed in HEK-293 cells (Figure 1c,d). These results infer that pericentrin is a nonconventional AKAP, which binds RII through a newly identified binding domain of 100 amino acids.

Structural studies have shown that the AKAP-binding determinants are located within the first 45 residues of the RII molecule [13]. Moreover, mutation of isoleucine residues at positions 3 and 5 of RIIα completely inhibits the interaction with AKAPs [14]. In order to demonstrate that pericentrin interacts with the same AKAP-binding determinants, a series of overlays were performed with various RII forms as competitors. A fragment containing the first 45 amino acids of RIIα competitively antagonized the interaction between RII and the pericentrin fragment encompassing the first 436 residues of the protein (Figure 2c). In



(d)

	Antiserum	PKA specific activity (pmol/min)	Fold enrichment (Anti-PC/Pre-I)
Endogenous pericentrin	Pre-I	0.041 ± 0.021	3.2 ± 0.5
	Anti-PC	0.13 ± 0.02 *	
Recombinant pericentrin	Pre-I	0.047 ± 0.025	23 ± 3.5
	Anti-PC	1.08 ± 0.12**	
Pericentrin Δ113–213	Pre-I	0.036 ± 0.015	3.4 ± 0.7
	Anti-PC	0.122 ± 0.027	

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Figure 1

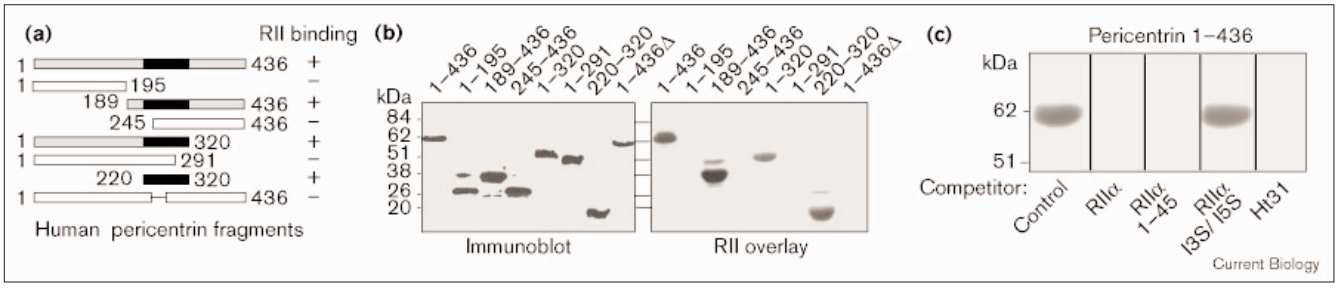
Pericentrin is an A-kinase anchoring protein. **(a)** HEK-293 cells grown in 100 mm dishes were transfected with 5 μg of HA–pericentrin/pcDNA1Amp [17] or HA–pericentrin Δ113–213/pcDNA1Amp construct. Cells were harvested 24 h after transfection and lysed in ice-cold hypotonic lysis buffer (20 mM HEPES pH 7.4, 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Triton X-100 (w/v), 1 mM benzamidine, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM AEBSF) and subsequently sonicated for 30 sec. Lysates were spun at 100,000 × *g* for 30 min and 500 μg of supernatant was then subjected to immunoprecipitation with preimmune or anti-pericentrin immune sera [19]. Immunoprecipitated complexes were separated by SDS–PAGE (4–15%) and electrotransferred to nitrocellulose. The recombinant proteins were detected with a polyclonal anti-HA antiserum (left panel) or subjected to an RII-overlay assay (right panel). For [^{32}P]RII overlays, the filters were blocked for 1 h with Blotto + 1% bovine serum albumin (BSA) in TBS buffer at room temperature and then incubated for 4–16 h with 100,000 cpm/ml of PKA-phosphorylated [^{32}P]RII in Blotto + 0.1% BSA. **(b)** Lysates prepared from untransfected HEK-293 cells were subjected to immunoprecipitation with preimmune or anti-pericentrin immune sera. Immunoprecipitates were separated by SDS–PAGE (4–15%) and electrotransferred to nitrocellulose. Pericentrin, RII and the C subunit of PKA (PKAc) were detected by immunoblotting using an affinity-purified rabbit polyclonal antibody (upper panel), a monoclonal anti-RII antibody (middle panel), and a monoclonal anti-C subunit antibody (lower panel), respectively. **(c,d)** Pericentrin was immunoprecipitated from HEK-293 cell extracts (endogenous pericentrin), cells transfected with the full-length protein (recombinant pericentrin), and cells expressing the pericentrin deletion mutant (pericentrin Δ113–213). Control immunoprecipitates were performed on each sample using non-immune IgG (Pre-I). Each immunoprecipitate (Anti-PC) was incubated with cAMP (1 mM) and assayed for kinase activity in the absence (–) and presence (+) of 4 μM PKA inhibitor (PKI). The results are expressed as mean ± standard error (SE) of three independent experiments. Statistical significance was analyzed by paired Student's t test: *$p < 0.05$ as compared to the PKA activity measured in control immunoprecipitates from untransfected cells; **$p < 0.01$ as compared to the PKA activity measured in control immunoprecipitates from pericentrin transfected cells.

contrast, a RIIα mutant in which the isoleucines at positions 3 and 5 were mutated to serines did not inhibit RII binding (Figure 2c). Identical results were obtained when competition experiments were performed using the 220–320 fragment of pericentrin (data not shown). These studies confirm that pericentrin and other conventional anchoring proteins recognize the same structural determinants on RII.

A striking feature of the RII-binding region in pericentrin is the presence of repeat sequences that are rich in leucine and valine (Figure 3a). Moreover, hydrophobic interactions have been shown to mediate the association between RII and AKAPs [13,14]. To test whether these side chains participate in RII binding, clusters of leucine and valine residues between positions 220 and 320 of the human pericentrin fragment were sequentially mutated (Figure 3b–j). Substitution of leucine residues with proline between residues 228 and 247 completely abolished RII binding, as assessed by the overlay assay (Figure 3b–d). Single proline substitutions of leucine 230, 235 or 245 also abolished interaction with RII (Figure 3e–g). Likewise, replacement of these residues with alanine also abolished RII binding, whereas alanine substitution of adjacent residues had no effect on anchoring, indicating that the leucine residues at positions 230, 235 and 245 are specific PKA-anchoring determinants (Figure 3h–j). This confirms the role of hydrophobic side-chains located between residues 228 and 247 of pericentrin as determinants for RII anchoring (Figure 3h–j). Unlike other AKAPs, however, this region is not predicted to form an amphipathic helix and additional determinants for interaction with RII lie outside this region. This is supported by evidence that proline substitutions at leucine clusters between residues 260 and 289 diminished RII binding by 80–90% (Figure 3b–d). These results suggest that leucine repeat sequences located between residues 228 and 288 of pericentrin form an interactive surface with the regulatory subunit dimer of PKA.

Over the past decade, AKAPs have emerged as a family of functionally related proteins that tether the PKA holoenzyme and other signaling enzymes to cellular organelles and membranes [5,6]. These proteins are classified on the basis of their ability to co-purify with the PKA holoenzyme from tissue or cell lysates. Our data clearly show that

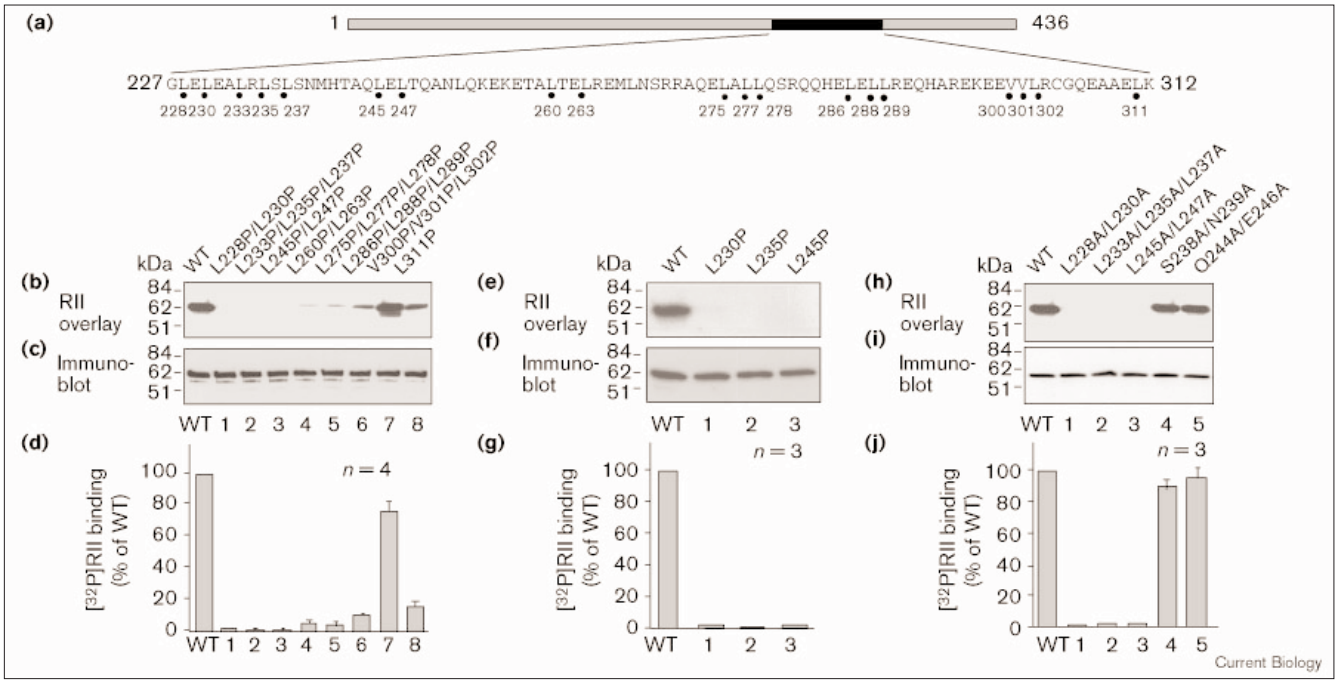
Figure 2



Pericentrin interacts with RII through a newly identified binding domain. **(a)** Schematic representation of the human pericentrin fragments used in mapping the RII-binding site. Interacting fragments are shaded and the RII-binding domain is indicated (black box). The first and the last residues of each fragment are indicated. **(b)** Recombinant pericentrin protein fragments were expressed from the pET30 (Novagen) vector as His_6-tagged fusion proteins, separated by electrophoresis on 4–15% SDS–PAGE gels and transferred to nitrocellulose membrane. Each fragment was detected with monoclonal antibodies against the His_6-tag epitope (left panel) and tested for solid-phase RII binding by overlay assay (right panel). **(c)** Overlay competition assay for the interaction between RII and pericentrin. Purified human pericentrin 1–436 fragments (1 μg) were separated by SDS–PAGE (4–15%), electrotransferred to nitrocellulose and subjected to RII-overlay assay in the absence or presence of 500 nM RIIα, RIIα1–45, RIIα I3S/I5S or anchoring inhibitor fragment Ht31 [16], as indicated. The results shown in (b,c) are representative of three independent experiments.

pericentrin fulfils this criterion. Interaction with the PKA holoenzyme is mediated by protein–protein interactions between a binding site formed by the regulatory subunit dimer of PKA and a specialized region on the anchoring

Figure 3



Characterization of the RII-binding surface of pericentrin. **(a)** Schematic representation of the RII-interacting surface of human pericentrin. The amino-acid sequence (in single-letter code) between residues 227 and 312 is indicated. Side chains that were changed by site-directed mutagenesis are indicated by bullet points. A series of mutants of the pericentrin 1–436 fragment were produced. The bacterially expressed His_6-tagged proteins were affinity purified on a nickel resin, separated by electrophoresis on 4–15% SDS–PAGE gels, electrotransferred to nitrocellulose and tested for RII binding by overlay assay. **(b,e,h)** RII-overlay assays. **(c,f,i)** Detection of the fragments with monoclonal antibodies against the His_6-tag epitope. **(d,g,j)** Quantitation of the binding by phosphoimage analysis. The results shown in (d,g,j) are expressed as mean ± SE of three independent experiments.

protein. A variety of structural studies have defined the RII-binding region of conventional AKAPs as a short helix which has a hydrophobic face that interacts with the regulatory subunit dimer [11,12,15,16]. These regions of approximately 24 residues contain clusters of branched hydrophobic side chains such as leucine or isoleucine [11,13]. The mapping and mutagenesis studies presented in Figures 2 and 3 suggest that pericentrin shares some but not all of the characteristics of a conventional AKAP. Leucine residues located between positions 228 and 268 are required for PKA anchoring; overlay competition experiments indicate that pericentrin binds to the same regions on RII as other AKAPs. Other characteristics of the RII-binding region are, however, unique. The RII-binding determinants are located over an extended region of 100 amino acids and secondary structure predictions do not indicate the probability of helices within this region. Thus, the RII-binding region on pericentrin is likely to adopt a conformation that is distinct from the corresponding regions on other AKAPs.

Pericentrin is an integral centrosomal protein that directs centrosome assembly by binding dynein complexes [17,18] and regulates microtubule nucleation through association with γ-tubulin [19]. We can now ascribe an additional function to the molecule, namely, to anchor the PKA holoenzyme at this site. As pericentrin is an integral component of the pericentriolar matrix and clearly plays a pivotal role in the organization of mitotic spindles [8,19], it is likely to be a viable candidate for directing PKA toward centrosomal substrates that are as yet undefined. Interestingly, another PKA-anchoring protein of unknown function called AKAP350/450/CG-NAP is detected at the centrosome and may participate in the regulation of PKA function [20–22]. AKAP350 is not exclusively a centrosomal protein, however, and is detected in the Golgi area and midbody of most cells [20–22]. This raises the intriguing possibility that pericentrin and AKAP350 might direct individual PKA pools to precise sites within the centrosome to modulate distinct centrosomal functions. Alternatively, both anchoring proteins might maintain unique complements of signaling enzymes, as biochemical studies have mapped distinct regions of CG-NAP that interact with PKA, the Rho-dependent kinase PKN and the phosphatases PP-1 and PP2A. Future studies will focus on elucidating a role for these individual pools of anchored PKA and other signaling enzymes at the centrosome.

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