Characterization of the Targeting, Binding, and Phosphorylation Site Domains of an A Kinase Anchor Protein and a Myristoylated Alanine-rich C Kinase Substrate-like Analog That Are Encoded by a Single Gene*

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A novel Drosophila A kinase anchor protein, Drosophila A kinase anchor protein 200 (DAKAP200), is predicted to be involved in routing, mediating, and integrating signals carried by cAMP, Ca\(^{2+}\), and diacylglycerol (Li, Z., Rossi, E. A., Hoheisel, J. D., Kalderon, D., and Rubin, C. S. (1999) J. Biol. Chem. 274, 27191–27200). Experiments designed to assess this hypothesis now (a) establish the function, boundaries and identity of critical amino acids of the protein kinase AII (PKAII) tethering site of DAKAP200; (b) demonstrate that residues 119–148 mediate binding with Ca\(^{2+}\)-calmodulin and F-actin; (c) show that a polybasic region of DAKAP200 is a substrate for protein kinase C; (d) reveal that phosphorylation of the polybasic domain regulates affinity for F-actin and Ca\(^{2+}\)-calmodulin; and (e) indicate that DAKAP200 is myristoylated and that this modification promotes targeting of DAKAP200 to plasma membrane. ∆DAKAP200, a second product of the DAKAP200 gene, cannot tether PKAII. However, ∆DAKAP200 is myristoylated and contains a phosphorylation site domain that binds Ca\(^{2+}\)-calmodulin and F-actin. An atypical amino acid composition, a high level of negative charge, exceptional thermostability, unusual hydrodynamic properties, properties of the phosphorylation site domain, and a calculated M\(_{r}\) of 38,000 suggest that ∆DAKAP200 is a new member of the myristoylated alanine-rich C kinase substrate protein family. DAKAP200 is a potentially mobile, chimeric A kinase anchor protein-myristoylated alanine-rich C kinase substrate protein that may facilitate localized reception and targeted transmission of signals carried by cAMP, Ca\(^{2+}\), and diacylglycerol.

Signals carried by different second messenger molecules often converge on a common effector protein in a discrete cell compartment (e.g. an ion channel in plasma membrane) (1–5). This enables coordinated and integrated physiological control of cell functions by combinations of stimulatory and inhibitory hormones and/or neurotransmitters. The discovery (6) of a novel cDNA and unique gene that encode Drosophila A kinase anchor protein 200 (DAKAP200)

provide an opportunity to investigate molecular mechanisms underlying integrated responses to multiple second messengers. DAKAP200 avidly binds regulatory subunits (RI) of protein kinase AII (PKAII) isoforms in vitro and in cells (6). The amino acid sequence of this 753-residue PKAII anchor protein also includes an N-terminal myristoylation motif and a potential “phosphorylation site domain” (PSD) (6). PSD modules (~25–30 residues) of vertebrate myristoylated alanine rich C kinase substrate (MARCKS) proteins bind calcium-calmodulin, phospholipids, and actin; cooperate with N-terminal myristate to attach proteins to cell membranes; constitute specific target sites for protein kinase C (PKC)-catalyzed phosphorylation; and participate in dynamic regulation of the actin cytoskeleton that is associated with such fundamental cellular processes as motility and differentiation (7–12). Thus, predicted properties of DAKAP200 suggest that this polypeptide is multifunctional, differentially targeted to membranes, and involved in routing and integrating signals propagated by three important second messengers: cAMP, diacylglycerol, and calcium (6).

Numerous caveats preclude acceptance of these ideas solely on the basis of information obtained from the amino acid sequence of DAKAP200. For example, incorporation of myristate at the N terminus is not an accurate predictor of membrane association. The myristoylated catalytic subunit of PKA isoforms and B subunit of calcineurin are not recruited to membranes (13, 14). Moreover, alignment of the DAKAP200 PSD-like domain with established vertebrate PSDs yields only a minimal level of sequence identity, despite similarities in amino acid composition and net charge. Thus, a comprehensive assessment of possible roles for DAKAP200 in signal transduction and integration must be preceded by systematic characterization of targeting, protein binding, and regulatory properties of predicted functional domains in the anchor protein. A series of studies addressing these topics and the properties of ∆DAKAP200, a second product of the DAKAP200 gene, are presented in this paper.

**Experimental Procedures**

**Assay for RII Binding Activity**—A Western blot was probed with \(^{32}\)P-labeled RII\(\beta\) (using a subunit concentration of 0.3 nM and \(2 \times 10^{5}\) cpm \(^{32}\)P radioactivity/ml), and RII\(\beta\)-binding proteins were visualized by autoradiography as described by Bregman et al. (15, 16). Results were quantified by laser densitometry or PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA) as described previously (17).

**Expression and Purification of DAKAP200 Fusion Proteins**—Segments of cDNA encoding partial DAKAP200 proteins were synthesized.
coverslips were washed with 10 mM sodium phosphate buffer, pH 7.4, protein is generated.

Subsequently, cells were incubated with either anti-DAKAP200 serum (1:1000 in PBS) or murine anti-FLAG IgG (30 

20 °C. After washing three additional times in PBS, fixed/permeabilized 4% paraformaldehyde in PBS for 10 min at 20 °C. After fixation and incubation in standard medium supplemented with 0.7 mM CuSO4, 60-mm culture dishes (6) and transfected with 1 

by the isopropyl-1-thio-D-galactopyranoside-insoluble T7 RNA polymerase. In pGEX6, expression is driven by an isopropyl-1-thio-β-galactopyranoside-regulated lac promoter, and a glutathione S-transferase (GST) fusion protein is generated. *Escherichia coli* BL21 (DE3) transformed with recombinant expression plasmid was grown and induced with isopropyl-1-thio-β-galactopyranoside as previously reported (18). Bacteria were harvested, disrupted, and separated into soluble and particulate fractions as described previously (18). Partial DAKAP200 fusion proteins were recovered in the soluble fraction and were purified to near homogeneity using IMAC columns.

Expression and Purification of His-tagged DAKAP200—BamHI and XhoI restriction sites were appended to the 5'- and 3'-ends of the DAKAP cDNA, respectively. After cloning the cDNA into the expression plasmid pRSETB (Invitrogen), His-tagged DAKAP200 was expressed in *E. coli* and purified to near homogeneity by using the strategy described above for pET14b.

Directed Site-directed Mutagenesis—Deletion mutagenesis was performed via polymerase chain reaction as described for AKAP75 and AKAP-KL (19, 21). Amino acid substitutions were introduced into the RII binding site and PSD by site-directed mutagenesis, as described previously (19–22).

Immunofluorescence Analysis—Full-length DAKAP200 cDNA and DAKAP200 cDNA lacking codons 1–7 were cloned into the pCMV-Tag1 expression plasmid (InVitrogen) to append eight codons for the FLAG epitope (DYKDDDDK) and a translation termination signal at the 3'-ends of the mRNA transcripts. Subsequently, the chimeric cDNAs were excised by digestion with *Not*I and *Eco*RV and cloned in the copper-inducible *Drosophila* expression vector pMK33 HS, which was cleaved with *Not*I and *Stu*I. Properties of the pMK33 HS plasmid are given in an accompanying paper (6). Drosophila S2 cells were grown in 60-mm culture dishes (6) and transfected with 1 μg of recombinant pMK33 HS plasmid by using the CaCl2 method (18–20) according to the manufacturer’s instructions. Twenty-four hours after transfection, S2 cells were resuspended in culture medium, washed, and then plated on 20-mm glass coverslips that were coated with poly-L-lysine. After a 16-h incubation in standard medium supplemented with 0.7 mM CuSO4, coverslips were washed with 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Next, cells were fixed by incubation with 4% paraformaldehyde in PBS for 10 min at 20 °C. After fixation, three washes with PBS, cells were permeabilized by incubation in acetone at −20 °C for 10 min. Further manipulations were performed at 20 °C. After washing three additional times in PBS, fixed/permeabilized cells were incubated in PBS containing 3% (*w/v*) albumin for 1 h. Subsequently, cells were incubated with either anti-DAKAP200 serum (1:1000 in PBS) or murine anti-FLAG IgG (30 μg/ml Sigma). Following five washes with PBS containing 0.1% (*w/v*) Tween 20, coverslips were incubated with 10 μg/ml fluorescein isothiocyanate-conjugated, goat IgGs directed against rabbit or mouse immunoglobulins. Samples were incubated for 1 h with secondary antibodies. Next, cells were washed three times in PBS containing 0.1% Tween 20. After air drying, 15 μl of 50% glycerol in PBS, containing 1 mg/ml p-phenylene diamine (anti-bleaching agent), was placed on the specimens, and the coverslips were mounted on slides.

Fluorescence signals corresponding to antigen-antibody complexes were collected with a Bio-Rad MRC 600 laser scanning confocal microscope (Image Analysis Facility, Albert Einstein College of Medicine) as described previously (23). For each series of immunofluorescence experiments, replicate samples of fixed/permeabilized cells were treated as described above except that antibodies directed against DAKAP200 were used with saturating amounts of purified recombinant antigen. No fluorescence signals were obtained from these samples.

The homogenate was centrifuged at 40,000 × *g* for 15 min at 4 °C. The supernatant solution was collected and applied to a 1.5 × 6-cm column of DEAE-Sepharose Fast Flow, which was equilibrated with buffer A containing 75 mM NaCl. After washing with 10 volumes of buffer A containing 0.1 mM NaCl, DAKAP200 and ΔDAKAP200 were eluted with buffer B containing 0.5 mM NaCl in buffer A and applied to a 0.9 × 2-cm column of SP Sepharose Fast Flow. After washing with 20 volumes of buffer B plus 0.1 mM NaCl, DAKAP200 and ΔDAKAP200 were eluted with 0.35 mM NaCl in buffer A. Partially purified proteins were dialyzed against PBS and stored in liquid N2. Phosphorylation of DAKAP200 and GST-PD Fusion Protein by PKA—Full-length DAKAP200 cDNA was cloned into a GST fusion protein (GST-PD) that contains residues 103–153 from DAKAP200 (see Fig. 1) were incubated with highly purified PKC (3–5 nM) (Calbiochem) in 25 mM Tris-HCl, pH 7.4, containing 5 mM MgCl2, 1 mM dithiothreitol, 0.2 mM CaCl2, 0.1 mM ATP, and sonicated lipids (20 μg/ml phosphatidylserine plus 5 μg/ml diacylglycerol). After 15 min at 30 °C, GST-PD and ΔDAKAP200 were separated from components of the reaction mixture by affinity chromatography on GSH-Sepharose 4B and Nε-cysteine chelate resin, respectively, as described above. When desired, 32P-labeled proteins were generated by substituting 50 μM [γ-32P]ATP (3,000 cpm/ pmol) for 0.1 mM nonradioactive ATP.

**Phosphorylation of GST-PD by Protein Kinase A—cDNA encoding the PD (residues 103–153) of DAKAP200 was cloned into the bacterial plasmid pGEM3Z (Amersham Pharmacia Biotech). This enables synthesis and purification of a GST fusion protein that contains a consensus PKA phosphorylation site between GST and the C-terminal PSD. Purified fusion protein was prepared as described above. 32P-labeled GST-PD (5 × 105 cpm/pmol) was produced by incubation with Mg-γ-[32P]ATP and the purified catalytic subunit of PKA, using conditions described by Bregman et al. (15). PKA does not phosphorylate Ser or Thr residues in the PD.**

**F-Actin Binding Assay—Actin (Cytoskeleton Inc.) was dissolved in 5 mM Tris-HCl, 0.2 mM ATP, 0.5 mM dithiothreitol, 0.2 mM CaCl2 to yield a final concentration of 0.4 mg/ml. Polymerization to F-actin was induced by adding 2% (*v/v*) solution B (2.5 mM KCl, 0.1 mM MgCl2, 50 mM ATP) and incubating for 30 min at 22 °C. Test ligands (ΔDAKAP200, GST-PD) were incubated with 5 μM F-actin in 5 mM Tris-HCl, 1.2 mM ATP, 2 mM MgCl2, 0.2 mM CaCl2, 0.5 mM dithiothreitol, 50 mM KCl, 10 mg/ml bovine serum albumin for 30 min at 22 °C in a 100-μl total volume. Subsequently, samples were centrifuged at 157,000 × *g* for 1 h (4 °C) in a Beckman Ti 42.2 rotor. The supernatant solution (containing free ligand) was collected, and 200 μl of incubation buffer was added to the pellet. After centrifugation at 157,000 × *g* for 30 min, the supernatant was discarded, and the pellet (containing bound ligand) was dissolved in gel loading buffer. F-actin-bound and free GST-PD or ΔDAKAP200 were size-fractionated by denaturing electrophoresis and detected by either Western immunoblotting or autoradiography (when proteins were labeled with 32P; see above). Gel lanes received 10% of the total supernatant or pellet fractions. Ligands did not pellet in the absence of F-actin.

**Calmodulin Binding Assay—Calmodulin Sepharose 6B beads (1 mg/ml calmodulin) were equilibrated in 20 mM Hepes-KOH, pH 7.2, 0.15 mM NaCl, 5 mg/ml bovine serum albumin, 0.02% Triton X-100 (*v/v*) (buffer C), and either 2.5 mM CaCl2 or 5 mM EGTA. Samples of GST-PD or ΔDAKAP200 were incubated with 5 μl of calmodulin-Sepharose 6B beads for 3 h at 22 °C in 0.5 ml of buffer C in the presence or absence (5 mM EGTA) of 2.5 mM CaCl2. Subsequently, beads were pelleted by centrifugation at 1000 × *g* and washed five times (1.5 ml each wash) with modified buffer C (Triton X-100 concentration was increased to 0.1% (*v/v*) and albumin was omitted), by resuspension and centrifugation at 1000 × *g*. Washed beads containing bound ligand were incubated in 0.1 ml of SDS gel loading buffer for 3 min at 95 °C. Bound GST-PD or ΔDAKAP200 was visualized by Western immunoblotting. Ten percent of each sample was assayed.

**Other Experimental Procedures**—Descriptions of DNA sequence analysis, production and affinity purification of antibodies, preparation of cytosolic and particulate fractions of *Drosophila* S2 cells and hamster AV12 cells, electrophoresis of proteins, immunoprecipitations, cell culture and transfections, protein determination, and Western immunoblot analysis are provided in an accompanying paper (6).

**RESULTS**

**Large N-terminal and Small C-terminal Modules of an A Kinase Anchor Protein (DAKAP200) Are Combined in**

H. Feng and C. S. Rubin, unpublished observation.
DAKAP200

DAKAP200, a Candidate cAMP-independent Signaling Protein—The amino acid sequence (753 residues) of DAKAP200 is presented in Fig. 1. DAKAP200 comprises only 372 amino acids. Residues 1–344 in DAKAP200 and DAKAP200 (Fig. 1) are identical. However, an exon encoding amino acids 345–725 in DAKAP200 is excised from DAKAP200 mRNA (6). Thus, DAKAP200 lacks the underlined portion of DAKAP200 (Fig. 1). The deleted segment includes the PKAII binding site (see below). Sequences of 28 amino acids at the C termini of the two proteins are identical. DAKAP200 and DAKAP200 have apparent Mₚ values of 200,000 and 95,000, respectively, in SDS-polyacrylamide gels (6).

DAKAP200 and DAKAP200 Are Modified by Myristoylation—Amino acid sequences at the N termini of DAKAP200 and DAKAP200 constitute optimal acceptor sites (24) for protein N-myristoyltransferase. N-Myristoylation is catalyzed by the C₁₄ saturated fatty acyl moiety from myristoyl-CoA in the amino group of N-terminal Gly within a specific sequence context that includes Ser and Lys at positions 5 and 6, respectively, relative to Gly. Methionylaminopeptidase eliminates the initiator Met residue during translation, thereby generating a free Gly amino group that forms an amide linkage with myristate. Since myristoylation occurs co-translationally and is a relatively rare, site-specific (N terminus only), and stable protein modification, it is amenable to study by metabolic labeling with [³H]myristic acid (24).

Drosophila S2 cells were incubated with [³H]myristate for 16 h and then fractionated into cytosol and total particulate proteins. Denaturing electrophoresis and fluorography revealed that only 7–10 polypeptides in these cell fractions incorporated [³H]myristate (Fig. 2A, lanes 1 and 3). Moreover, three [³H]-labeled cytosolic and three [³H]-labeled pellet proteins exhibited apparent Mₚ values of either 95,000 or 200,000 (Fig. 2A, lanes 1 and 3). These proteins were precipitated with anti-DAKAP200 IgGs (Fig. 2A, lanes 2 and 4). Thus, both DAKAP200 and DAKAP200 are myristoylated in intact cells.

The site of modification in DAKAP200 was probed by comparing [³H]myristate labeling of DAKAP200 and a mutant DAKAP200 that lacks residues 1–7 (Δ 1–7) in stably transfected (hamster) AV12 cells. Control AV12 cells do not (a) contain proteins that react with anti-DAKAP200 IgGs or (b) synthesize myristoylated proteins with a Mₚ of 200,000. DAKAP200 and DAKAP200 (and thereby identify) a minor [³H]-labeled 200-kDa protein by immunoprecipitation with antibodies directed against DAKAP200 failed (Fig. 2, lane 6). In contrast, several [³H]-labeled proteins with apparent Mₚ values of ~200,000 were evident in cells expressing DAKAP200 (Fig. 2A, lane 7). Moreover, the cluster of large myristoylated polypeptides was specifically precipitated with anti-DAKAP200 IgGs (Fig. 2A, lane 8). Thus, residues at the N terminus are essential for myristoylation of the anchor protein. The isolation of four protein species in the immunoprecipitate (Fig. 2A, lane 8) suggests that DAKAP200 undergoes multiple types of post-translational modification.

The DAKAP200 polypeptide sequence (Fig. 1) includes two Cys residues whose side chains can serve as acceptors for a second type of acylation, palmitoylation (25, 26). Although neither Cys residue is embedded within a classical palmitoylation context, the enzymology of palmitoyl transfer is not understood, and utilization of novel sites must be excluded experimentally. S2 cells that were labeled with [¹⁴C]palmitate contained six proteins that incorporated the C₁₆ fatty acyl moiety (data not shown). None of these proteins had an apparent Mₚ of 200,000 or 95,000, and none were precipitated by anti-DAKAP200 IgGs. Thus, palmitoylation does not appear to play a role in targeting/anchoring DAKAP200 and DAKAP200.

N Terminal Myristate Is Involved in Intracellular Targeting of DAKAP200—Immunofluorescence microscopy revealed the intracellular disposition of endogenous, myristoylated DAKAP200 and DAKAP200 in Drosophila S2 cells (Fig. 3C). A modest level of antigen is dispersed in the cytoplasm. How-
Properties of Domains in DAKAP200 and a MARCKS Analog

Fig. 2. DAKAP200 and ΔDAKAP200 are myristoylated at the N terminus. A, S2 cells were incubated with 0.5 mCi/ml [3H]myristic acid for 16 h prior to lysis. Cytosolic proteins and membrane proteins solubilized with 2% Triton X-100 were isolated from metabolically labeled S2 cells that were grown to confluence in a 30-mm culture dish. Samples (30% of total) of the cytosolic (lane 1) and detergent-solubilized (lane 3) proteins were size-fractionated by denaturing electrophoresis (7.5% polyacrylamide gel). Anti-DAKAP200 serum (2 μl) and protein A-Sepharose 6B were sequentially added to the remainder of the samples. Samples were processed as indicated under “Experimental Procedures.” Cytosolic (lane 2) and membrane (lane 4) proteins precipitated by the antibodies were analyzed by denaturing electrophoresis. Myristoylation of the Drosophila anchor protein was also investigated in AV12 cells stably transfected with transgenes encoding DAKAP200 or DAKAP200 lacking residues 1–7 (Δ1–7 DAKAP200). Cells were labeled with [3H]myristate, and cytosol was prepared, analyzed, and immunoprecipitated as described above. For SDS-polyacrylamide gel analysis, lanes 5 and 7 received total cytosolic proteins from AV12 cells expressing the Δ1–7 DAKAP200 and wild type DAKAP200 transgenes, respectively. Proteins immunoprecipitated from cells containing the Δ1–7 DAKAP transgene were applied to lane 6. Immunoprecipitated proteins from cells expressing wild type DAKAP200 were loaded in lane 8. After electrophoresis, the gels were stained with Coomassie Blue, destained, impregnated with Enhance (fluorochrome; NEN Life Science Products) and air-dried. Proteins that were labeled with [3H]myristate were visualized by fluorography on x-ray film (48-h exposure) at –75 °C. The fluorogram is shown. Stably transfected AV12 cells that express DAKAP200 (WT) or Δ1–7 DAKAP200 (Δ Myristate) were lysed in either buffer lacking detergent (Control) or buffer supplemented with 2% Triton X-100 (TX-100) as indicated under “Experimental Procedures.” Supernatant (S) and pellet (P) fractions were then resolved by centrifugation at 150,000 × g for 30 min. Samples (30 μg of protein) of soluble and particulate proteins from cells expressing DAKAP200 (B) and Δ1–7 DAKAP200 (C) were size-fractionated by denaturing electrophoresis (7.5% gel) and transferred to an Immobilon P membrane. The distribution of DAKAP200 and Δ1–7 DAKAP200 between soluble and particulate fractions was determined by Western immunoblot analysis as described under “Experimental Procedures.” Chemiluminescence signals recorded on x-ray film are shown. Only the relevant portion of the blot (200-kDa region) is shown.

However, a high proportion of DAKAP200 and/or ΔDAKAP200 is associated with plasma membrane in round embryo-derived cells. The role of N-terminal myristoylation in routing and anchoring DAKAP200 in situ was investigated by expressing wild type and mutant DAKAP200 proteins that were tagged at the C terminus with a FLAG epitope (DYKDDDDK). Like the endogenous anchor protein, wild type DAKAP200-FLAG is targeted to the surface of S2 cells (Fig. 3A). In contrast, anchor protein lacking myristate (Δ1–7 DAKAP200-FLAG) is distributed uniformly throughout S2 cell cytoplasm (Fig. 3B). Thus, co-translational myristoylation of DAKAP200 apparently promotes differential accumulation of the anchor protein at the cell periphery.

A Candidate PSD from DAKAP200/ΔDAKAP200 Mediates Binding with F-actin—DAKAP200 and ΔDAKAP200 are predicted to bear high levels of negative charge (–116 and –36, respectively) at neutral pH (6). However, both proteins include a compact region (residues 119–148, Fig. 1) that is positively charged (+12) and contains clusters of three types of amino acids: 13 Lys (plus one Arg), five Ser, and five residues with large hydrophobic side chains. These properties match characteristic features of PSDs in vertebrate MARCKS and MARCKS-related proteins (7, 8). PSDs mediate binding of vertebrate MARCKS with F-actin and calmodulin (7–11). PKC-catalyzed phosphorylation of Ser residues in PSDs regulates these binding interactions and enables MARCKS to participate in dynamic regulation of cell shape (7–12).

Despite similarities in charge, size, and composition, the analogy between the basic region of DAKAP200/ΔDAKAP200 and vertebrate PSDs is incomplete. Alignment of the basic region with conserved mouse and chicken PSD sequences reveals only a modest level (30%) of scattered identical residues (Fig. 4A). To determine the significance of the basic region, we assayed DAKAP200/ΔDAKAP200 proteins for a critical PSD-linked function, the ability to bind F-actin. Full-length DAKAP200 was expressed as a His-tagged fusion protein in E. coli and purified to near homogeneity by affinity chromatography. Although protein yields were low (<50 μg per 500-ml culture of E. coli), this approach yielded sufficient material to perform F-actin binding assays in the absence of competing actin-binding proteins, actin-severing proteins, proteases etc., thereby enabling straightforward analysis of data. DAKAP200 was quantitatively recovered in the supernatant fraction after incubation in actin binding buffer and centrifugation at 157,000 × g for 1 h (Fig. 4B, lanes 1 and 3). In contrast, a substantial portion of ΔDAKAP200 pelleted when the binding buffer was supplemented with polymerized actin (5 μM) (Fig. 4B, lanes 2 and 4), thereby indicating formation of ΔDAKAP200/F-actin complexes. Similar results were obtained using DAKAP200 and ΔDAKAP200 proteins that were partially purified (via anion and cation exchange chromatography) from S2 cells (data not shown). Thus, ligation of F-actin by ΔDAKAP200 appears to be governed by a sequence(s) shared with DAKAP200; binding was not altered by the presence or absence of myristate. Using a fixed concentration (5 μM) of F-actin, binding of the variable ligand (ΔDAKAP200) was readily detected at a concentration of 0.1 μM (Fig. 4C). Binding increased as a function ΔDAKAP200 concentration and reached saturation near 10 μM. Half-maximal binding with F-actin was observed at ~1 μM ΔDAKAP200. A more precise measurement of the binding affinity for F-actin was obtained in studies on a fragment of ΔDAKAP200 that corresponds to the PSD (see below).

Subsequent experiments were focused on the candidate PSD and were designed to distinguish among the possibilities that residues 119–148 constitute (a) a novel, multifunctional PSD, (b) a region that confers a single function, or (c) a fortuitous grouping of residues that resembles a PSD but lacks the proper alignment (or secondary structure) to perform PSD-mediated functions. A fragment of DAKAP200 cDNA that encodes amino
acids 103–153 (Fig. 1) in the anchor protein was cloned downstream from a tac promoter and the GST gene in the expression plasmid pGEX6. This enables high level, isopropyl-1-thio-
galactopyranoside-inducible synthesis of a soluble GST-PSD chimeric protein in E. coli. Large amounts (–2 mg) of highly purified PSD fusion protein were obtained by affinity chromatography on GSH-Sepharose 4B. The GST-PSD fusion protein avidly complexes F-actin in binding assays, whereas GST alone was isolated in the actin-free supernatant fraction (Fig. 4D). Moreover, a 10-fold excess of PSD fusion protein proved to be a potent inhibitor of complex formation between intact ∆DAKAP200 protein and F-actin (Fig. 4E). Thus, a short segment of DAKAP200/∆DAKAP200 that includes the basic region (residues 119–148, Fig. 4A) and only 16 N-terminal and five C-terminal residues mediated association with F-actin. Ligation of the PSD evidently occurs at a site in F-actin that couples with intact ∆DAKAP200 protein.

Radiolabeled GST-PSD was needed to perform more precise F-actin binding studies. This was obtained by cloning the cDNA insert described above (encoding amino acids 103–155 in DAKAP200) in the pGEX2T plasmid. Recombinant plasmid direct synthesis of a GST-PSD fusion protein that differs from the chimera described above only by the insertion of a short PKA phosphorylation peptide between the C terminus of GST and the N terminus of the PSD. Purified, recombinant fusion protein was radiolabeled by incubation with [γ-32P]ATP and the C subunit of PKA. (C does not phosphorylate any Ser residues in the PSD.) Various amounts of 32P-labeled GST-PSD were incubated in equilibrium with F-actin, and then free and bound ligand were separated by centrifugation at 157,000 × g. The resulting data best fit a single site binding model and yield an apparent Kd value of 0.6 μM (Fig. 4F). Moreover, the Bmax value yields a 1:3 ratio of GST-PSD molecules to actin monomers. Thus, a ∆DAKAP200 dimer might associate with one turn of an actin filament α-helix (~7 monomers/turn).

PKC Phosphorylates the PSD of DAKAP200/∆DAKAP200 and Regulates Its Functions—Endogenous DAKAP200 and ∆DAKAP200 were purified ~20-fold (via anion and cation exchange chromatography) to eliminate endogenous kinases and phosphatases. Brief incubation of this preparation with [γ-32P]ATP and highly purified, Ca2+ - and diacylglycerol-dependent PKC (a mixture of α, β, and γ isoforms) resulted in incorporation of 32P radioactivity into the DAKAP200 and DAKAP200 poly peptides (Fig. 5A, lane 1). Omission of diacylglycerol or the addition of a specific PKC inhibitor suppressed PKC-mediated phosphorylation of the two proteins by ≥98% (Fig. 5A, lanes 2 and 3). No phosphorylation was observed when incubations were performed without added PKC (data not shown). Thus, native DAKAP200/∆DAKAP200 are PKC substrates.

Comparison of the arrangement of Ser residues and hydrophobic amino acids in the DAKAP200 PSD (Fig. 1) with established PKC phosphorylation sites in the MARCKS protein (7, 8) indicated that Ser135, Ser137, and Ser139 in the anchor protein are potential targets for PKC. Subsequent assays demonstrated that GST-PSD (residues 103–153) is an excellent substrate for PKC (Fig. 5B, lane 1). Purified PKC phosphorylated GST-PSD according to classical Michaelis-Menten kinetics and had an apparent Kms of ~2 μM (ATP was used at 0.1 mM) and a Vmax of 5 μmol of phosphate incorporated/min/mg of enzyme at 30 °C. A similar Km value was obtained for vertebrate MARCKS (27).

In order to determine the optimal PKC target site within the DAKAP200 PSD region, Ser residues were mutated to Ala, and phosphotransferase reactions were terminated when wild type GST-PSD incorporated ~1 mol of phosphate/mol of protein (as in Fig. 5B, lane 1). Under these conditions, replacement of Ser132 with Ala (Fig. 5B, lane 2) had the most deleterious effect, reducing incorporation of 32P radioactivity by 70%. In contrast, conversion of both Ser135 and Ser137 (Fig. 4A) to Ala reduced PSD phosphorylation by ~50% (Fig. 5B, lane 3). Substitution of all three Ser residues with Ala virtually eliminated PKC-catalyzed phosphorylation (>95% decrease in 32P incorporation; Fig. 5B, lane 4). Thus, the optimal site for PKC-mediated phosphorylation is embedded in the sequence KEWSF314. Serines at positions 135 and/or 137 (Fig. 4A) are also phosphorylated. However, they are either used less efficiently by the kinase, or their phosphorylation is enhanced by prior phosphorylation of the neighboring Ser132.

Preincubation with PKC and MgATP abolished the ability of DAKAP200 to form stable complexes with F-actin (Fig. 5C, upper panel). Likewise, PKC-catalyzed phosphorylation of GST-PSD abrogated its sequestration by polymerized actin (Fig. 5C, lower panel). Therefore, hormonal activation of phospholipases β and γ and the consequent diacylglycerol-mediated activation of PKCs may promote the disengagement of anchored DAKAP200/∆DAKAP200 (and their protein ligands) from sites in cytoskeleton.

The PSD of DAKAP200 Avidly Complexes Calmodulin; PKC-catalyzed Phosphorylation of the PSD Region Ablates Calmodulin Binding Activity—Calmodulin, a centrally important mediator of Ca2+ signaling, is recruited to polybasic regulatory domains that also serve as PKC substrates in several proteins (7–11). If this activity were associated with the polybasic, PKC-phosphorylated DAKAP200 PSD region, it would diversify and
significantly enhance signal reception and integrative capacities of the PKAII anchor protein and ΔDAKAP200. A 5-fold molar excess of Ca\(^{2+}\)-calmodulin effectively inhibited binding of recombinant GST-PSD fusion protein to F-actin (−85% decrease) (Fig. 6A). Moreover, immobilized calmodulin directly sequestered both intact DAKAP200 and the PSD fragment (Fig. 6B, lanes 1 and 2). Substantial binding activity was evident at low concentrations (10–50 nM) of these ligands. Formation of PSD-calmodulin and ΔDAKAP200-calmodulin complexes was abrogated by either chelation of free Ca\(^{2+}\) or PKC-mediated phosphorylation of the PSD (Fig. 6B, lanes 3–6).

**Fig. 4. ΔDAKAP200 and a candidate PSD fragment bind with F-actin.** A, the candidate PSD from DAKAP200 and ΔDAKAP200 is aligned with established PSDs from chicken and mouse MARCKS (8). B, recombinant ΔDAKAP200 (1 μM) was incubated with actin binding buffer in the presence and absence of 5 μM F-actin (see “Experimental Procedures”). Free and F-actin-bound ΔDAKAP200 were separated by centrifugation at 157,000 × g. Samples (10% of total) of actin-depleted supernatant solutions and the F-actin pellet (which contains bound ligand) were assayed by denaturing electrophoresis and Western immunoblot analysis. Blots are probed with antisera directed against ΔDAKAP200 (1:2000). After incubating with secondary antibodies coupled to peroxidase, ΔDAKAP200/IgG complexes were visualized by an enhanced chemiluminescence procedure (ECL; NEN Life Science Products). Signals were recorded on x-ray film. Only the relevant portion of the blot is shown. C, the indicated amounts of ΔDAKAP200 were incubated with F-actin (5 μM). ΔDAKAP200 that complexed with actin was detected by Western immunoblotting. The methodology is described in B. ECL signals are shown. D, GST (1 μM) or a GST fusion protein (1 μM) that contains residues 103–153 from DAKAP200 (designated GST-PSD) were assayed for F-actin binding activity as described in B. Western blots were probed with affinity-purified anti-GST IgGs (1:1000 relative to serum). ECL signals are shown. E, ΔDAKAP200 (0.6 μM) was incubated with F-actin (5 μM) in the presence or absence of 6 μM GST-PSD fusion protein. Binding of ΔDAKAP200 with polymerized actin (pellet) was assessed as described in B. The blot was probed with anti-ΔDAKAP200 serum (1:2000) as described above. F, equilibrium binding of the PSD of DAKAP200/ΔDAKAP200 to F-actin. The GST-PSD (residues 103–153) fusion protein was labeled with \(^{32}\)P by incubation with the C subunit of PKA and [γ-\(^{32}\)P]ATP as described under “Results” and “Experimental Procedures.” F-actin binding assays were performed as described above. Amounts of \(^{32}\)P-labeled GST-PSD bound to the pelleted actin were measured in a scintillation counter. Nonspecific binding, determined by centrifuging \(^{32}\)P-labeled GST-PSD in the absence of F-actin, was less than 1% of specific binding for all points. Data were best fit by a single-site binding equation.

A | Amino acids
---|---
PSD DAKAP200 | KSKKKKVRKKKKKWSFRSISFGERKDRKQPAK
PSD CHICKEN | KKKRRFRRKKKKKSGREKDSG
PSD MOUSE | KKKRRFRRKKKKKSGREKDSG

| ΔDAKAP200 (μM) |
---|---
10 | +
5  | +
2  | +
1  | +
0.6 | +
0.4 | +
0.2 | +
0.1 | +

**Properties of Domains in DAKAP200 and a MARCKS Analog**
mammalian AKAPs and secondary structure predictions suggested that residues 511–530 constitute the high affinity RII (PKAII) binding site. This region is predicted to fold as an amphipathic α-helix. On one face of the helix, a group of amino acids with large aliphatic side chains, including Ile511, Ile518, Val519, Thr523, and Val530 in DAKAP200, are thought to cooperate to create an extensive hydrophobic surface with a precise size and configuration (19). This surface then binds with a complementary apolar region near the N terminus of RII subunits to generate stable DAKAP200-RII (PKAII) complexes (22, 28–30). However, neither the precise boundaries of the tethering domain nor the identity of critical residues within the binding site were established by biochemical analysis.

Fig. 5. PKC-catalyzed phosphorylation of the DAKAP200/ΔDAKAP200 PSD disrupts binding with F-actin. A, a partially purified sample (10 μg protein) of Drosophila DAKAP200 and ΔDAKAP200 proteins was incubated with highly purified Ca2+-diacylglycerol-dependent PKC and [γ-32P]ATP as described under “Experimental Procedures.” Subsequently, DAKAP200 and ΔDAKAP200 were immunoprecipitated with specific antiserum and protein A-Sepharose 4B (6). After extensive washing, precipitated 32P proteins were dissolved in gel loading buffer and separated by size in a denaturing (7.5%) polyacrylamide gel. 32P-Labeled DAKAP200 and ΔDAKAP200 were detected by autoradiography, using XAR-5 film. Amounts of 32P incorporated were determined by excision of the relevant portion of the gel and scintillation counting. The sample applied to lane 2 was phosphorylated in the absence of diacylglycerol and phosphatidylserine. Other samples were incubated in complete reaction mixtures in the absence (lane 1) or presence (lane 3) of 1 μM GF109203X (BIOMOL), a potent inhibitor of PKC. B, samples (1 μg of protein) of purified wild type and mutant GST-PSD fusion proteins were phosphorylated by incubation with [γ-32P]ATP and PKC, as described in A. Reactions were terminated by the addition of gel loading buffer, and labeled proteins were subjected to denaturing electrophoresis (12% polyacrylamide gel), autoradiography, and scintillation counting as described in A. An autoradiogram is shown in the upper panel; the lower panel shows the gel after staining with Coomassie Blue to reveal the amounts of GST-PSD (or GST) protein in the assays. The PSD fusion proteins contained residues 103–153 from DAKAP200. Serines 132, 135, and 137 were mutated to Ala as indicated. C, recombinant (His-tagged) ΔDAKAP and wild type GST-PSD fusion protein (residues 103–153) were incubated with PKC and nonradioactive MgATP to phosphorylate Ser residues in the basic region (see “Experimental Procedures”). Control samples were incubated in the absence of ATP. After incubation, phosphorylated and nonphosphorylated proteins were purified by affinity chromatography on nickel-chelate or GSH-Sepharose 4B resins. The abilities of phospho- and dephospho-ΔDAKAP200 or GST-PSD proteins to bind F-actin were assayed as described under “Experimental Procedures” and Fig. 4. ECL signals were recorded on x-ray film.

Fragment analyses indicated various segments of the predicted RII binding site were amplified by polymerase chain reaction. Pairs of short DNA sequences corresponding to NdeI and BamHI or to EcoRI and BamHI restriction endonuclease sites were appended to 5'- and 3'-primers, respectively, to facilitate cloning of cDNA fragments into the bacterial expression plasmid pET14b or pGEX6P. Substantial amounts of soluble, His-tagged (pET14b) or GST (pGEX6P) partial DAKAP200 proteins were produced in E. coli and purified to near homogeneity by affinity chromatography (see “Experimental Procedures” and Refs. 6 and 18). His-tagged and GST fusion proteins containing the same fragments of ΔDAKAP200 yielded similar results in RII binding assays. Thus, the two types of fusion proteins were used interchangeably.

Initially, N and C-terminal deletion mutants were characterized in the RII binding overlay assay (15, 16) to establish boundaries of the tethering domain. Removal of an N-terminal fragment that contained residues 511–531 yielded a protein that was devoid of RII binding activity (Fig. 7A, lane 1). Less drastic changes, including deletion of only three (Fig. 7A, lane 2) or one (Fig. 7A, lane 3) predicted crucial hydrophobic amino acid, also ablated binding activity. In contrast, deletion of all amino acids preceding the first predicted critical hydrophobic residue (Ile511) did not significantly reduce RII binding activity (Fig. 7B, lane 3). C-terminal truncation analysis produced a similar pattern of results. Two informative results are shown in Fig. 7C. Elimination of all amino acids following the C terminus of a predicted amphipathic α-helix (i.e. beyond residue 531)
did not compromise the ligation of RII (Fig. 7C, lane 2). However, a deletion that omitted Thr263 and Val530 (Fig. 7C, compare lanes 1 and 2) had no tethering activity.

Site-directed mutagenesis was employed to identify critical amino acids within the RII tethering domain. Replacement of Ile518-Val519 with Ala518-Ala519 eliminated RII binding activity (Fig. 7D, lane 3). Substitution of either Thr263 or Ala515 with Ala and Ser, respectively, caused a 70–80% decrease in RII binding (Fig. 7, D, lane 2, and E, lane 2). Mutation of a residue predicted to be irrelevant to tethering activity (Glu521 to Ala) had no effect on RII binding activity (data not shown). Thus, the RII tethering domain of DAKAP200 encompasses residues 511–531. The presence of two large hydrophobic side chains on adjacent, centrally located residues (positions 518 and 519) appears to be essential for the generation of the PKAII binding site. Although the two substituted Ala residues (Ile518-Val519 to Ala518-Ala519) contain hydrophobic side chains and promote retention of α-helical structure, the resulting reduction in overall size of the hydrophobic surface at the tethering site apparently compromises interaction with a large, apolar surface on RII subunits (22, 28–30). Thr263 and Ala515 may be principally involved in controlling the affinity of the anchor protein for RII subunits.

**DAKAP200 and ΔDAKAP200 Are Heat-stable Proteins with Atypical Hydrodynamic Properties**—Samples of cytosol from S2 cells were incubated for a period of 20 min at temperatures ranging from 0 to 90 °C. Subsequently, each sample was centrifuged at 14,000 \( \times g \) to separate “stable” soluble proteins from denatured protein aggregates that pellet. A high proportion of the multiple DAKAP200 polypeptides and the 95-kDa ΔDAKAP200 protein were recovered in the soluble, stable fraction at all temperatures. Incubation at 90 °C caused aggregation and pelleting of >95% of total cytosolic proteins. However, 75–90% of the DAKAP200 and ΔDAKAP200 proteins were isolated in the supernatant solution after exposure to this nearly boiling temperature. Similar results were obtained for DAKAP200 and ΔDAKAP200 proteins that were solubilized from the organelle/cytoskeleton fraction of S2 homogenates by 1% Triton X-100 prior to heating. Thus, the 753-amino acid PKAII anchor protein and ΔDAKAP200 are exceptionally thermostable proteins.

Determination of the sedimentation coefficients of native DAKAP200 (753 amino acids) and ΔDAKAP200 (372 amino acids) via sucrose gradient centrifugation (22) yielded values of 2.4 and 2.0 S, respectively (data not shown). Typical globular proteins with similar sedimentation coefficients have molecular weights in the range of 12,000–20,000. The aberrant hydrodynamic behavior of the 38-kDa (calculated molecular mass) ΔDAKAP200 and 79-kDa DAKAP200 proteins indicates that they assume highly extended (perhaps rodlike) configurations in order to be retarded in the gradient in this manner.

**DISCUSSION**

The *Drosophila* DAKAP200 gene encodes two related but distinct signaling proteins, DAKAP200 and ΔDAKAP200. Primary structure analysis suggested that DAKAP200 is a multifunctional anchor protein that receives, integrates, and focuses signals propagated by cAMP, Ca\(^{2+}\), and diacylglycerol (6). ΔDAKAP200, which lacks a PKAII binding domain, apparently mediates reception and propagation of signals borne by Ca\(^{2+}\) and lipid-derived second messengers. These models were tested by determining biochemical properties and functions of key domains in the DAKAP200 and ΔDAKAP200 proteins. The observation that amino acids 2–7 (Fig. 1) constitute a potential myristoylation site resulted in identification of a targeting domain. The observation that amino acids 2–7 (Fig. 1) constitute a potential myristoylation site resulted in identification of a targeting domain. Metabolic labeling with [\(^3\)H]myristate and immunoprecipitation with anti-DAKAP200 IgGs revealed that endogenous DAKAP200 and ΔDAKAP200 are myristoylated in *Drosophila* S2 cells. Incorporated myristate provides a long alky chain that inserts into the interior of phospholipid bilayers, thereby promoting the targeting, anchoring, and enrichment of DAKAP200 at a membrane surface (9). Myristoylated anchor protein is also synthesized in mammalian (AV12) cells that contain a DAKAP200 transgene. A deleted version of
DAKAP200 that lacks residues 1–7 was expressed in AV12 cells, but the protein failed to incorporate [3H]myristate. Thus, the N-terminal region of DAKAP200 is the sole site of myristoylation and a probable targeting domain for the anchor protein. Several myristoylated signaling proteins contain proximal Cys residues that undergo palmitoylation (7–10, 25, 26). Acylation at multiple sites causes extremely stable membrane anchoring. However, DAKAP200 is not modified by palmitoylation.

N-terminal myristoylation is essential for routing various signaling proteins, including Src and MARCKS, to plasma membrane (7–10). Mutations that prevent myristoylation elicit mislocalization and loss of biological function. Myristoylated MARCKS accumulates at focal adhesions or at the junction of cortical cytoskeleton and plasma membrane (12, 31). MARCKS may simultaneously bind the lipid bilayer (via myristate) and the F-actin-based cytoskeleton. In this configuration, MARCKS mediates interactions between the membrane and actin filaments that stabilize the cytoskeleton. Nonmyristoylated MARCKS is restricted to cytoplasm, and its ability to modulate cytoskeleton organization is abrogated (7–10, 12, 31).

DAKAP200 and DΔAKAP200 are specifically enriched in the plasma membrane of Drosophila S2 cells. Epitope-tagged wild type DAKAP200 is also differentially targeted to plasma membrane in transfected S2 cells. Tagged nonmyristoylated DAKAP200 was evenly distributed throughout the cell cytoplasm. Thus, N-terminal myristoylation is essential for organelle-specific accumulation of DAKAP200-RII (PKAII) complexes. Mammalian AKAP18 contains a classical RII tethering site, but docking of this anchor protein with plasma membrane is guided by three acylated amino acids (32, 33). Gly1 is myristoylated, whereas Cys3 and Cys4 are palmitoylated. Deletion or mutation of Gly1 abrogates myristoylation but has no effect on AKAP18 localization. Likewise, mutation of Cys3 or Cys4 alone does not alter association of AKAP18 with plasma membrane. However, elimination of all acylation sites yields an AKAP18 variant that accumulates in cytoplasm (32, 33). Thus, myristoylation of AKAP18 supports, but is not essential for, targeting/anchoring of PKAII.

Deletion mutagenesis mapped the DAKAP200 RII binding domain to a region that includes amino acids 511–531. Activity and selectivity of this PKAII tethering site were not dependent on the context of flanking N- and C-terminal sequences. Conserved hydrophobic residues in the tethering region are predicted to generate an RII binding surface comparable in size and shape with that produced from the corresponding region of mammalian AKAP75 and AKAP79 (6, 22, 28–30). Substitution of Ile518 and Val519 or Thr523 with Ala sharply diminished RII ligation. This is interpreted as a consequence of the disruption of two parameters (size and shape) in a structure based on cooperative interactions among large, aliphatic side chains (22, 28–30). Substitution of Ala515 with Ser reduced RII binding activity by ~70%, thereby indicating that a hydrophobic side chain at this position is essential for a maximal ligand affinity. This mutation reveals evolutionary divergence in the fly AKAP. The analogous Ala to Ser mutation has no effect on RII sequestration by mammalian AKAP-KL (21).

A PSD (residues 119–148) from DAKAP200/DΔAKAP200 binds F-actin and Ca2+-calmodulin. Ser132 and other serines (Ser135 and/or Ser137) in the PSD region are phosphorylated by PKC. Both calmodulin and actin binding activities are lost as consequence of PSD-directed phosphorylation. This provides a mechanism for reversibly altering the location and Ca2+-calmodulin-related effector functions of DAKAP200 and DΔAKAP200. The physiological importance of multifunctional PSDs in vertebrate MARCKS and MARCKS-like proteins is paralleled by a high degree of sequence conservation across species (7, 8). However, alignment of PSD sequences (Fig. 4A) disclosed that the DAKAP200 PSD is a novel and rather divergent member of this domain family. A distinctive feature of the Drosophila PSD is the compact central cluster of large hydrophobic residues (Trp131, Phe133, Ile136, Phe138) that intermingle with three Ser residues that are PKC targets. This arrangement involves a contiguous block of only eight amino acids. Introduction of a high level of negative charge adjacent to sites where Trp, Phe, and Ile side chains are immersed in a lipid bilayer may negate membrane association by electrostatic repulsion from negatively charged phospholipid head groups. This “electrostatic switch mechanism” (9, 10) may be less efficient and require a higher level of phosphorylation in vertebrate PSDs where serines and hydrophobic amino acids are dispersed among a total of 14 residues. Incorporation of a novel
PSD into DAKAP200 produces a multifunctional anchor protein with enhanced capacities for intracellular targeting, regulation via phosphorylation, and integration of multiple second messenger signals.

MARCKS is a major PKC substrate in many mammalian cells, where it mediates aspects of cytoskeleton dynamics. Incorporation of an RII binding site within a PKC effector protein enables a novel mode of cross-talk between two signal transduction pathways. PKC-catalyzed phosphorylation of the DAKAP200 PSD could elicit translocation of anchor proteins to cytoplasm (or elsewhere). Consequently, hormones that stimulate synthesis of lipid second messengers could determine intracellular target sites at which the cAMP/PKA signaling pathway exerts its actions. Phosphorylation-dependent targeting of DAKAP200-RII complexes could constitute a novel, reversible mode of routing signals carried by cAMP. Previously characterized AKAPs appear to engage in static anchoring of PKA isoforms (1, 34, 35).

ΔDAKAP200 has the hallmark features of MARCKS proteins. It is myristoylated; highly acidic; heat-stable; enriched in Ala, Pro, Ser, and Thr; deficient in aromatic and sulfur-containing amino acids; contains a basic PSD region; and behaves like an extended rod upon sedimentation; and its molecular weight values of mammalian MARCKS proteins (30,000–33,000). Unlike the 79-kDa anchor protein DAKAP200, MARCKS and ΔDAKAP200 lack a discrete domain that binds RII subunits. Together, these properties make ΔDAKAP200 the first candidate analog of MARCKS in Drosophila.

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