

Molecular Characterization of a Novel A Kinase Anchor Protein from *Drosophila melanogaster**

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Activation of protein kinase A (PKA) at discrete intracellular sites facilitates oogenesis and development in *Drosophila*. Thus, PKA-anchor protein complexes may be involved in controlling these crucial biological processes. Evaluation of this proposition requires knowledge of PKA binding/targeting proteins in the fly. We now report the discovery and characterization of cDNAs encoding a novel, *Drosophila* A kinase anchor protein, DAKAP550. DAKAP550 is a large (>2300 amino acids) acidic protein that is maximally expressed in anterior tissues. It binds regulatory subunits (RII) of both mammalian and *Drosophila* PKAII isoforms. The tethering region of DAKAP550 includes two proximal, but non-contiguous RII-binding sites (B1 and B2). The B1 domain (residues 1406–1425) binds RII ~20-fold more avidly than B2 (amino acids 1350–1369). Affinity-purified anti-DAKAP550 IgGs were exploited to demonstrate that the anchor protein is expressed in many cells in nearly all tissues throughout the lifespan of the fly. However, DAKAP550 is highly enriched and asymmetrically positioned in subpopulations of neurons and in apical portions of cells in gut and trachea. The combination of RII (PKAII) binding activity with differential expression and polarized localization is consistent with a role for DAKAP550 in creating target loci for the reception of signals carried by cAMP. The DAKAP550 gene was mapped to the 4F1.2 region of the X chromosome; flies that carry a deletion for this portion of the X chromosome lack DAKAP550 protein.

Cyclic AMP-dependent protein kinase (PKA)¹ is the principal mediator of actions of hormones and neurotransmitters that activate adenylate cyclase (1–4). Signals borne by cAMP are often targeted to effectors that accumulate at discrete intracellular locations (5–7). In part, targeting is accomplished by establishing a non-uniform distribution of PKA molecules

within cells. Such asymmetry is evident in cells where PKAII α and PKAII β isoforms² are attached to cytoskeleton and/or organelles via A kinase anchor proteins (AKAPs) (5, 6). Prototypic neuronal anchor proteins (AKAPs 75, 79, and 150) possess a binding site for regulatory subunits (RII) of PKAII isoforms and distinct domains that non-covalently link AKAP-PKAII complexes to the microtubule-based dendritic cytoskeleton of neurons and the cortical actin cytoskeleton of non-neuronal cells (5–13). Both cytoskeletal locations are closely apposed to the plasma membrane. Thus, anchored PKAII is placed in proximity with a signal generator (adenylate cyclase) and multiple PKA substrate/effector proteins (e.g. myosin light chain kinase, microtubule-associated protein-2, ion channels, serpentine receptors that couple with Gs and adenylate cyclase). This arrangement generates target sites for cAMP action (5, 6, 14). A distinct group of RII-binding proteins mediates association of PKAII isoforms with mitochondria, Golgi membranes, peroxisomes, centrioles, and other organelles in numerous cell types (5, 6, 15, 16). Thus, anchored PKAs appear to be involved in the regulation multiple cell functions.

Although substantial progress has been made in identifying and characterizing AKAPs, knowledge of anchor protein functions *in vivo* is limited. One approach toward linking AKAP structure with physiological function is to complement studies on PKA anchoring in mammalian systems with investigations on a lower organism that is amenable to concerted biochemical, cellular, molecular, and genetic analysis. *Drosophila melanogaster* provides an attractive model system because classical and molecular genetics have implicated PKA in signaling pathways that (a) generate cell or tissue polarity and/or (b) require accumulation of PKA at a discrete intracellular location (17–21). For example, localized activation of PKA negatively modulates *hedgehog* signaling during *Drosophila* development. *Hedgehog* is a secreted protein that binds with serpentine receptors in plasma membrane. Receptor occupancy activates a signaling pathway that elicits expression of genes (*dpp*, *wg* etc.) that control anterior-posterior patterning in developing tissues. PKA accumulates selectively along segments of plasma membrane (or underlying cytoskeleton) that constitute adhesive junctions between oocytes and supporting nurse cells (20). Localized PKA activity appears to be essential for assembly and stabilization of intercellular bridges at these junctions. The bridges enable the flow of critical proteins and RNAs from nurse cells to developing oocytes. Finally, PKA-mediated phosphorylation controls a re-orientation of microtubule organizing centers in germ cells during oogenesis (19). Remodeling of the microtubule-based cytoskeleton generates a polarized structure that enables differential segregation of specific mRNAs to

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¹ The abbreviations used are: PKA, cAMP-dependent protein kinase; R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; AKAP, A kinase anchor protein; DAKAP550, *Drosophila* A kinase anchor protein with an apparent M_r of 550,000; bp, base pair(s); kbp, kilobase pair(s); GST, glutathione S-transferase; BLP, human beige-like protein.

² PKA isoforms are named according to their homodimeric R subunits. Distinct genes encode the RII α , RII β , RI α , and RI β proteins.

anterior or posterior locations via kinesin motors. Subsequent translation of spatially-segregated mRNAs establishes gradients of proteins (e.g. *biocoid*, *oskar*) that are essential for normal morphogenesis and development. It has been suggested that a subset of PKA molecules, that is anchored at the microtubule organizing center, governs the polarity and positioning of microtubules via the phosphorylation of microtubule-associated proteins (19).

The cited studies suggest that PKA-anchor protein complexes may be intimately involved in controlling critical aspects of *Drosophila* reproduction and development. No information is currently available concerning RII (PKAI)-binding proteins in the fly. Thus, initial steps in evaluation of the PKA anchoring model entail the identification and basic characterization of *Drosophila* AKAPs (DAKAPs). We now report the discovery of a novel *Drosophila* AKAP (DAKAP550), the cloning and sequencing of DAKAP550 cDNA, the differential expression of DAKAP550 protein during embryogenesis, the chromosomal location of the DAKAP550 gene, and the mapping of a binding site in DAKAP550 that complexes both mammalian and *Drosophila* RII subunits.

EXPERIMENTAL PROCEDURES

Screening of cDNA and Genomic DNA Libraries—Expression libraries of *D. melanogaster* (Canton S strain) cDNAs were searched for inserts encoding A kinase anchor proteins by both functional (RII binding) assays and classical DNA hybridization. cDNAs in the libraries were generated by reverse transcription of template mRNAs isolated from embryos 0–24 h after fertilization. Initially, cDNA libraries in bacteriophages λ ZAP (provided by Dr. R. Reinke, Department of Developmental Molecular Biology, Albert Einstein College of Medicine, Bronx, NY) and λ gt11 (CLONTECH) were screened by the procedure of Bregman *et al.* (8, 9). This method detects AKAP- β -galactosidase fusion proteins in phage plaques by their ability to bind 32 P-labeled RII β . Six recombinant phage clones (see Fig. 2, below) that contain overlapping cDNA sequences were retrieved from an initial population of 10^6 plaques and were characterized. A 5' *EcoRI*-*NcoI* fragment (300 bp) from clone Z cDNA (Fig. 2) was employed as a template to synthesize a random-primed 32 P-labeled probe for further screening. Screening via DNA hybridization (22) yielded 13 independent cDNA clones from a 5' stretched cDNA library in bacteriophage λ gt11 (CLONTECH). Four of these cDNAs were characterized as described under "Results."

A filter, which contains a gridded array of DNA fragments (in cosmids) that span the *Drosophila* genome (23), was provided by Dr. J. Hoheisel (University of Heidelberg, Heidelberg, Germany). Two overlapping genomic DNA clones (named 64G6 and 81C9) hybridized strongly with each of three 32 P-labeled cDNA probes: the cDNA insert from clone II-1 and the 2- and 1-kbp cDNA fragments released from clone number 8 by *EcoRI* digestion (see Fig. 2, below). Collectively, these cDNAs encode a segment of DAKAP550 that includes >2000 amino acids. Cosmids 64G6 and 81C9 were obtained from Dr. J. Hoheisel.

DNA Sequence Analysis—Complementary DNA inserts from recombinant λ ZAP phage were isolated in the plasmid pBluescript SK (Stratagene) via M13 phage-promoted, plasmid excision in *Escherichia coli* (24). cDNA inserts obtained from λ gt11 phage clones were subcloned into the plasmid pGEM7Z (Promega). Nested deletions were prepared by exonuclease III digestion according to the procedure of Henikoff (25). Intact and truncated cDNAs and genomic DNA fragments were sequenced by a dideoxynucleotide chain termination procedure (26) using T7, T3, SP6, and custom oligonucleotide primers. *Taq* Dye Deoxy Terminator Cycle Sequencing Kits (Applied Biosystems) were used according to the manufacturer's instructions. DNA products were separated and analyzed in a model 377 automated DNA Sequencer (Applied Biosystems) in the DNA Analysis Facility of Albert Einstein College of Medicine.

Computer Analysis—Analysis of sequence data, sequence comparisons, and data base searches were performed using PCGENE-IntelliGenetics software (IntelliGenetics, Mountainview, CA) and BLAST programs (27) provided by the NCBI server (National Institutes of Health).

Assay for RII Binding Activity—Overlay binding assays have been described in several papers (8, 9). In brief, a Western blot is probed with 32 P-labeled RII β (using a subunit concentration of 0.3 nM and 2×10^5 cpm of 32 P-radioactivity/ml) and RII β -binding proteins are visualized by

autoradiography. Results were quantified by scanning laser densitometry (Pharmacia LKB Ultrosan XL laser densitometer) or PhosphorImager analysis (Molecular Dynamics) as described previously (28).

Determination of the Chromosomal Location of the DAKAP550 Gene—Polytene chromosomes were isolated from salivary glands of second instar *Drosophila* larvae as described by Ashburner (29). DAKAP550 cDNAs were labeled with biotinyl-16-dUTP (Boehringer Mannheim) via DNA polymerase I-catalyzed nick translation. Chromosomes were hybridized with the biotinylated cDNAs indicated under "Results" for 16 h at 58 °C, using conditions described by Ashburner (29). Gene-cDNA complexes were visualized by using a Detek I-horse-radish peroxidase kit (Enzo Biochemicals) according to the manufacturer's instructions. The basis for detection of the gene is that biotinylated DAKAP550 cDNA hybridized with complementary sequences in DAKAP550 exons will avidly bind streptavidin-conjugated peroxidase. Subsequent incubation with diaminobenzidine and H_2O_2 results in peroxidase-catalyzed synthesis of an insoluble precipitate at the site of cDNA-genomic DNA hybridization. Photographs of hybridized, peroxidase-stained chromosomes were obtained with a Zeiss axioplan microscope, using phase-contrast optics. The peroxidase-generated precipitate appears as an intense black stripe on the chromosome (see Fig. 4, below).

Expression and Purification of Recombinant DAKAP550 Fusion Proteins—A cDNA fragment encoding amino acids 1472–1676 in DAKAP550 (see Fig. 3 below) was excised from clone number 8 (Fig. 2) by digestion with *Bam*HI and *Eco*RI and was subcloned into the expression plasmid pGEX2T (Pharmacia), which was cleaved with the same enzymes. This places cDNA encoding the partial DAKAP550 polypeptide downstream from and in-frame with the 3' terminus of a glutathione S-transferase (GST) gene in the vector. Transcription of the GST-fusion gene is driven by an inducible *tac* promoter. A high level of chimeric GST-partial DAKAP550 polypeptide (designated fu-A) was produced when *E. coli* DH5 α was transformed with recombinant pGEX2T plasmid and then induced with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside as described previously (30). After induction, bacteria were disrupted in a French press and the soluble fu-A protein (~5 mg) was purified to homogeneity by affinity chromatography on glutathione-Sepharose 4B beads (Pharmacia) (30).

A cDNA fragment encoding residues 1001–1676 in DAKAP550 (Fig. 3) was cloned into the expression plasmid pET14b as described previously (15, 31). This enables isopropyl-1-thio- β -D-galactopyranoside-induced synthesis of a (His) $_6$ -tagged partial DAKAP550 fusion protein (designated fu-B) in *E. coli* BL21 (DE3) transformed with recombinant pET14b. The fusion protein is described in more detail under "Results." Induced *E. coli* BL21 were harvested, disrupted, and separated into soluble and particulate fractions (30). fu-B protein was recovered in the soluble fraction and purified to near-homogeneity by nickel-chelate chromatography as previously reported (31). Several batches of purified fu-B (~6 mg of protein from 1 liter of *E. coli*) were coupled to CNBr-activated Sepharose 4B as described previously (9).

Production and Affinity Purification of IgGs Directed against DAKAP550—Purified fu-A protein (see above) was injected into rabbits (0.35 mg of initial injection; 0.2 mg for each of three booster injections) at Covance Laboratories (Vienna, VA) to generate antisera. Serum was collected at 3-week intervals. Antibodies that bind GST were eliminated by passing serum over a column of GST-Sepharose 4B (30). Next, adsorbed serum was applied to a column of fu-B Sepharose 4B (see above). The fu-B protein includes the complete sequence of fu-A (and therefore, all fu-A epitopes). The resin was extensively washed and anti-DAKAP550 IgGs were then isolated by successive elutions at pH 2.5 and 11.8, as described previously (30).

Mutagenesis and Expression of Partial DAKAP550 Proteins in E. coli—Deletion mutagenesis was performed via exonuclease III digestion or polymerase chain reaction as described previously (11, 31). Mutants were verified by DNA sequencing. Amplified cDNAs were cloned in pET14b and fusion proteins were expressed in *E. coli* and purified, as described previously (31).

Preparation of Cytosolic and Particulate Proteins from Drosophila—Intact *Drosophila* or separated fly heads and bodies were suspended in 4 volumes of buffer A (20 mM sodium phosphate, pH 7.4, 20 mM NaCl, 0.2 mM dithiothreitol, 1 mM EDTA, 0.2 mM EGTA, 10 μ g/ml soybean trypsin inhibitor, 40 μ g/ml aprotinin, 10 μ g/ml pepstatin A, and 40 μ g/ml leupeptin) and disrupted in a Polytron homogenizer (two 30-s cycles of homogenization at the maximum setting). All operations were performed at 0–4 °C. The homogenate was centrifuged at $12,000 \times g$ for 20 min and the supernatant solution (cytosol) was collected. The pellet was resuspended in the original volume of buffer A and then was homogenized and centrifuged as described above. The resulting super-

natant solution (designated "wash") was collected; the pelleted, particulate fraction of *Drosophila* homogenates was dispersed in the starting volume of buffer A by a final round of homogenization. The concentration of protein in subcellular fractions from *Drosophila* and in purified samples of partial DAKAP550 polypeptides (see above) was determined by the method of Bradford (32).

Electrophoresis of Proteins—Proteins were denatured in gel loading buffer and subjected to electrophoresis in 5.5, 10, or 12% polyacrylamide gels containing 0.1% SDS as described previously (8, 9). Phosphorylase *b* ($M_r = 97,000$), transferrin (77,000), albumin (68,000), ovalbumin (45,000), carbonic anhydrase (29,000), and cross-linked phosphorylase (195,000–584,000) were used as standards for the estimation of M_r values.

Western Immunoblot Assays—Size-fractionated proteins were transferred from denaturing polyacrylamide gels to an Immobilon P membrane (Millipore Corp.) as described previously (8). Blots were blocked, incubated with affinity-purified IgGs directed against DAKAP550 (1:2000, relative to serum), and washed as described previously (11, 33). Antigen-IgG complexes were visualized by an indirect chemiluminescence procedure (28, 33). Signals were recorded on Kodak XAR-5 x-ray film. No signals were observed when Western blots were probed with antibodies in the presence of 5 μ g/ml fu-A or fu-B protein (see above).

Drosophila Stocks—The Canton S strain was used for all experiments. A mutant line (DfC1) JC70, which is deficient in the X chromosome region 4C15-16; 5A1-2, was obtained from the Bowling Green *Drosophila* Stock Center (Bowling Green, OH). *Drosophila* carrying ethyl methanesulfonate-induced mutations in the 4F1.2 region of the X chromosome (fs(1)456v24, fs(1) M60) were generously provided by Dr. N. Perrimon (Department of Genetics, Harvard Medical School) and Dr. R. Nagoshi (Department of Biology, University of Iowa).

Immunostaining of Embryos and Imaginal Discs—Embryos (0–24 h) were collected, dechorionated, and fixed according to the procedure of Mitchison and Sedat (34). Specimens were blocked, washed, and then incubated with anti-DAKAP550 IgGs for 16 h at 4 °C in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (phosphate-buffered saline), 20% fetal bovine serum, and 0.1% (v/v) Tween 20 as described previously (12). Affinity-purified anti-DAKAP IgGs were used at 1:160 dilution (relative to serum). After extensive washing with phosphate-buffered saline, 0.1% Tween 20, samples were incubated for 2 h at 20 °C with horseradish peroxidase-coupled sheep IgGs directed against rabbit immunoglobulins (Bio-Rad) in the buffer specified above. DAKAP550-IgG complexes were detected by incubation of the specimens with diaminobenzidine and H_2O_2 (12). In the presence of these substrates, peroxidase catalyzes the synthesis of an insoluble reaction product that is observed as a dark precipitate by light microscopy.

RESULTS

Cloning and Sequence Analysis of cDNAs Encoding a *Drosophila* AKAP—To determine whether *Drosophila* expresses AKAPs, cytosolic and particulate proteins from fly embryos were size-fractionated by denaturing electrophoresis, transferred to a membrane, and incubated with 32 P-labeled bovine RII β . In this "overlay assay" binding sites in immobilized AKAPs renature and sequester radiolabeled RII subunits (8, 9). Autoradiography revealed a group of cytosolic RII-binding proteins with molecular weights ranging from 70,000 to >200,000 (Fig. 1, lane 2). Candidate cDNA clones for *Drosophila* AKAPs (DAKAPs) were retrieved from an embryo expression library (in bacteriophage λ ZAP) by using a modification of the overlay assay (8). Five cross-hybridizing clones were isolated (Fig. 2). After recombinant pBluescript phagemids were excised from λ ZAP (24), nested deletions of the cDNAs were prepared by exonuclease III digestion and religation. Sequencing of these overlapping cDNAs yielded an open reading frame of 3.2 kbp. To obtain contiguous flanking cDNA, a 5'-stretched embryonic cDNA expression library (λ gt11) was screened for β -galactosidase fusion proteins that bound 32 P-RII β with high affinity. A cDNA named clone Z was obtained (Fig. 2). Subsequently, a 5'-terminal *Eco*RI-*Nco*I fragment (330 bp) from clone Z was employed as a template for synthesis of random-primed, 32 P-labeled cDNA. This probe was used to re-screen the λ gt11 library via DNA hybridization. Four of 13 positive recombinant phage contained cDNAs that extended the 5' end of the open

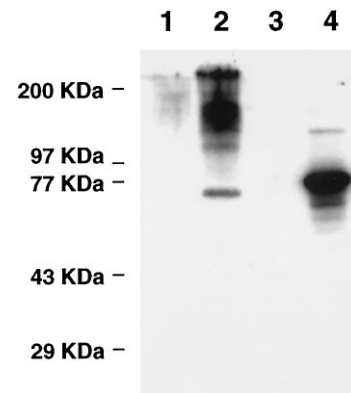


FIG. 1. Identification of RII-binding proteins in *Drosophila*. Cytosol and particulate fractions were prepared from *Drosophila* embryos as described under "Experimental Procedures." Proteins (100 μ g/lane) were size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon P membrane, and incubated with 32 P-RII β . Lane 1 contained particulate proteins from *Drosophila* embryos; lane 2 received *Drosophila* embryo cytosol; lane 3 contained the wash from the particulate fraction of *Drosophila* embryos; lane 4 received total lysate from HEK293 cells that overexpress bovine AKAP75 (approximate $M_r = 75,000$), which serves as a positive control. An autoradiogram is presented.

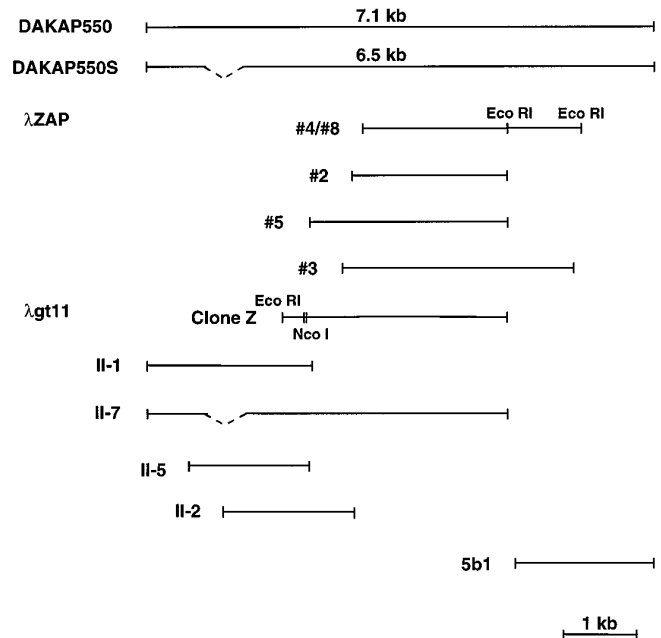


FIG. 2. Schematic diagram of cDNA clones encoding DAKAP550. Clones 2–5 and 8 were isolated from a *Drosophila* embryo cDNA library in λ ZAP phage by screening for β -galactosidase fusion proteins that bind 32 P-labeled RII β . Clone Z was obtained from a *Drosophila* embryo cDNA library in bacteriophage λ gt11 by the same approach. Clones II-1, II-2, II-5, and II-7 were isolated from the λ gt11 library by hybridization with 32 P-labeled cDNA corresponding to the 5' end of clone Z. Clones II-1 and II-7 are alternatively spliced transcripts. Clone II-7 lacks 618 bp that are present in II-1. Solid lines indicate the lengths of cDNA inserts; dashed lines show the segment of cDNA that is alternatively excised from some transcripts; single tick marks indicate the positions of intrinsic or linker-derived *Eco*RI sites; a double tick marks the site of *Nco*I cleavage in clone Z. Clone 5b1 corresponds to a fragment of the DAKAP550 gene. The 7.1-kbp DAKAP550 cDNA sequence was assembled from analyses of overlapping clones. DAKAP550S is identical with DAKAP550 except for the excision of the indicated exon.

reading frame (clones II-1, II-2, II-5, and II-7, Fig. 2). Comparison of sequences for clones II-1 and II-7 revealed a 618-bp deletion in the latter cDNA (Fig. 2). This is probably due to alternative retention/excision of an exon because sequences at

LLFNIALVVK FELLIIAFRS HFRFRFTVQ AMVLPPLAKW PYENGFTFTT 50
 WCRLDPINSV NIEREKPYLY SFKTSKGVGY TAHFVGNCLV LTSMKVKGKG 100
 FQHCVKYEFQ PRKWYIMIAV YIYNRWTKSE IKCLVNGQLA SSTEMAWFVS 150
 TNDPFDKCYI GATPELDEER VFCGQMSAIY LFSEALTTQQ ICAMHRLGPG 200
 YKSQRFDFNE CYLNLDPNHK RVSHFQLLPA TLGASALSGG SGSGAGSGSG 250
 NDASAAAAA VAAGQQOQLQ LQFOILAAEQ EARAIDWSDE KLDLNAAFVK 300
 IRAVLRTARNA VTLAGSSSTG TTAVATAAAA AAAAGAGAGT TAAATSAAAA 350
 AAATONENDA AVGOQQHATH HHATAATGSA DDPLGDLPTG NASSSSSSFE 400
 QLRRMSSVSS LNSMVGSADE EEVDQLKAVL YDGKLSNAIV FMYNPFVATDG 450
 QLCLQSSPKG NVSYFVHTPH ALMLQDVKAV VTHSIHCTLN SIGGIQVLPF 500
 LFSQLDMAHE GLGDIKRDP LCCKLGFIC ELVETSQTQV QHMIQNRGFL 550
 VISFMLQRSS REHLTLEVLG SFLNLTKEYL TCLSANSDDL KQLLDHVLV 600
 NPALWIYTPA NVQARLYSYL ATEFLSDTQI YSNVRRVSTV LQTVHTLKYY 650
 YWVNPRAKS GIIPKGLDGP RPAQKDILAI RAYILLFLKQ LIMIGNGVKE 700
 DELQSILNYL TTMHEDENLH DVLQMLISLM SEHPSSMVPA FDKVHGVRSI 750
 FKLLAAESQL IRLQALKLLG FFLSRSTHKK KYDVMSPHNL YTLAERLLL 800
 YEESLSLPTY NVLYEIMTEH ISQILYTRH PEPESHYRLE NPMILKVVAT 850
 LIRQSKQTES LIDVKKLFLQ DMTLLCNSNR ENRRTVLQMS VWQEWLIAMA 900
 YIHPKSSEEQ KISDMVSYLF RMLLHHAHKH EYGGWRVWVD TLAIHVSXVS 950
 YEEFKLQFAQ MYEHYERQRT DNITDPALRQ ARPISTISGW EREELHQQN 1000
 GGSAAAVATN QTAAVKGSVS IASLEDVPPV VEEVEEEL ELVEIEQEGPI 1050
 ← Fusion B N-terminus
 TEETEQKSVI ANISDVINEQ LKTDATCNGN LEDVKEEPEV QQQIGDLEKQ 1100
 PEPSTPLGAL RETLQLGDDM DVEEELATA KDALNAEQHV SRVLQASEAA 1150
 LNDCKMAVDV [LQESSVLK DEEIEELAVNE VVQGVLNNEK KTQSQDNKDN 1200
 D5'
 KEQPGQDVN VSLNLSKNLL NNNNNNNNS PSPTPTTATA TAETEAETEV 1250
 NANEIVSSTE APKAETETS APEVETPETA KPSPIVPSV LATNQKTEDA 1300
 ANKLNNEKL AEISASPEPP IVVETPEAIL LQLSDSETPK NKETEAEDSV 1350
 D5'
 [ALAVDIVEQ LIDKVIDATE AESASETKTE TNNNEIPKKE KOTSEEDV 1400
 D4 D3 D2-3
 ETAETLAAAA KETVORVFA ALVMVQEEST QEKPEKANS EEEKNEIGKE 1450
 D2 D1
 EILLQLEEK ASTEVQETKI EGDLLKPEDP KGHSSVEPKT PNLEPKPQE 1500
 ← Fusion A N-terminus
 TEQKSKQEVA EELPKQPEEQ VVAIVTQVLD TLVDDTVKAV AAEQTTQTSP 1550
 APEEQSPQIL AMESPATSVR VKPTEVDSTT QTPKNEAGS SLLVEQVQV 1600
 LQEDDAQQA GMTIEDEDYS NQAAAAVEN ANSSQLDANH YGPNPESKQ 1650
 QQQRKSGST RPFSPGPTR PPFRIPEFKW SYIHQRLLSD VLFSELTDIQ 1700
 Fusion A and B C-terminus
 VWRSHSTKSV LDFVNSSENA IFVNTVHLI SQLADNLIIA CGGLLPLLAS 1750
 ATSPNSELVD LEPTQGMPLV VAVSLQRLV NMADVLI FAT SLNFGELEAE 1800
 KNMSSGGILR QCLRLVCTCA VRNCLCKER TRYNVGALAR DVPGAHLQA 1850
 LIRGAQASPK NIVESITGQL SPVKDPEKLL QMDVNLRLA VIYRDVEETK 1900
 QAQFLSLAIV YFISVLMVSK YRDILEPPAE PQIQRQSPVL QRTAGGEAAS 1950
 ARPLFPQWSH HVYPQLPES HQNHNSNMQH QQQQQQQQQQ QQQQHYQQQ 2000
 QQQQVVHNHS HHMTAAYYQ QQQHQVVAAG QQQHSPTPLA THSTSSASS 2050
 TATSPASS SLSSLASQSQ QQSHRQLHKQ QQQQQQQQQQ QQPHYHPQP 2100
 HYGLINGHQ HPQLNGKHYA ENGSTAGYHP HSHPHPHGY MRNGDTSTLQ 2150
 QNGMPDYQAV GLMNGHSGG TGNMNNSSLM NNMRLNLRNGG GANSSSPPG 2200
 SHQVIQGVAT AGAVNANA AVGVGMGAVGV GMGGVAGGLD GGVIYKTS AI 2250
 NNNYRYNGRN ASAGTGRQI QDSDEIIVV DENNPSVLAD NDSHSGPPS 2150
 IKSVDSDVGS LNMNSTGKTK CPRWSHPSEI LIDDHKPSHS NDESWTDVNL 2200
 NEDAAVQAA

FIG. 3. Derived amino acid sequence of DAKAP550. The underlined region is absent in the short form (DAKAP550S, Fig. 2) of the DAKAP550 polypeptide. The RII-binding site is shaded. The region

the 5' and 3' ends of the DNA fragment deleted in clone II-7 correspond to consensus splice donor and acceptor sites. Alternative splicing was not detected at other sites in DAKAP cDNAs. Exhaustive screening of available *Drosophila* cDNA libraries failed to yield clones containing contiguous segments of upstream or downstream cDNA. Sequencing of fragments of the DAKAP gene enabled extension of the coding region ~900 bp downstream. Unfortunately, the presence of very long introns at the 5' and 3' ends of the DAKAP gene have impeded determination of the translation initiation and termination codons. Nevertheless, a composite 7.1-kbp DNA coding region has been established for a novel, invertebrate PKA-binding protein by assembling overlapping DNA sequences. Each segment of DNA was sequenced at least twice from both strands. The DNA sequence has been deposited in the GenBank data base (accession number AF003622). Only one of three possible reading frames for the 7.1-kbp cDNA yields an uninterrupted polypeptide (shown in Fig. 3). Moreover, this reading frame encodes (a) a high affinity RII-binding domain and (b) contiguous epitopes that appear in a *Drosophila* high molecular weight, RII-binding protein (see Figs. 5–7, below). The alternative reading frames contain either 80 or 81 translation termination codons. The largest polypeptides predicted for the alternate reading frames contain only 121 residues (M_r ~13,500) or 165 residues (M_r ~18,500) and are insufficient to account for any of the *Drosophila* RII-binding proteins (Figs. 1 and 7). It is probable that the continuous 7.1-kbp open reading frame accounts for nearly the entire amino acid sequence of the anchor protein (see "Discussion"). The *Drosophila* RII-binding protein was named DAKAP550 for its apparent M_r of 550,000 in denaturing electrophoresis (see below).

The predicted partial DAKAP550 polypeptide sequence includes >2,350 amino acids (Fig. 3). Like mammalian anchor proteins, partial DAKAP550 is acidic (predicted pI = 4.96) and will bear a substantial net negative charge (approximately -100) at neutral pH. DAKAP550 is not related to previously characterized proteins. However, substantial portions of the fly anchor protein are homologous with segments of (a) a predicted *Caenorhabditis elegans* protein³ designated F10F2.1 (35) and (b) a derived polypeptide encoded by a recently discovered human gene named "beige-like" (36) (Table I). Sequences homologous with portions of *C. elegans* F10F2.1 appear as discrete modules that are inserted at several points within the DAKAP550 polypeptide chain. For example, long (>175 amino acid residues), sharply demarcated N-terminal, central and near C-terminal segments of DAKAP550 are 40–50% identical with portions of the *C. elegans* protein (Table I). Central and C-terminal regions of DAKAP550 also have counterparts in the human beige-like protein. A segment of DAKAP550 bounded by amino acids 1693 and 1929 is highly conserved in both the human (69% identity) and *C. elegans* (42% identity) proteins.

Determination of the Chromosomal Location of the

³ The *C. elegans* homolog of DAKAP550 has not been studied experimentally. Its sequence is derived by linking a series of consecutive exons from chromosome III, that were identified in the *C. elegans* genome sequencing project.

between arrows marked "Fusion A N-term" and "Fusion A and B, C-term" was expressed as a GST-fusion protein and injected into rabbits to produce polyclonal anti-DAKAP550 IgGs. The region between arrows marked Fusion B N-term and Fusion A and B, C-term indicates a segment of anchor protein that was expressed as a (His)₆-tagged fusion protein and used for affinity purification of anti-DAKAP550 IgGs (see "Experimental Procedures"). The C termini of deletion mutants that were created to map RII-binding sites are marked with brackets. All mutants had the same N terminus, Val¹¹⁶² (D5').

TABLE I
Sequence identities shared among DAKAP550, *C. elegans* F10F2.1, and human BLP

The GenBank™ accession numbers for F10F2.1 and BLP are Z35598 and M83822, respectively.

Segment of DAKAP550	Related segment of F10F2.1	% Identity	Related segment of BLP	% Identity
Residues 33–210	372–500	51		
Residues 431–959	512–1042	45	9–155 ^a	55
Residues 1693–1929	1066–1314	42	530–780	69

^a The portion of BLP is homologous only with residues 892–955 at the C terminus of the segment indicated for DAKAP550.

DAKAP550 Gene—Polytene chromosomes were isolated from salivary glands of third instar larvae and fixed (29). The 2.2-kbp cDNA from clone II-1 and 2- and 1-kbp fragments released from clone 8 by *Eco*RI (Fig. 2) were tagged with biotinylated dUMP by nick-translation and hybridized with the chromosomes. The cDNA probes hybridized with the 4F1.2 region on the X chromosome (Fig. 4, A and B). Only one locus was detected, indicating that the DAKAP550 gene is unique. This result was confirmed by Southern analysis of cloned fragments of *Drosophila* genomic DNA (data not shown). All three cDNA probes hybridized exclusively with two overlapping cosmids, 64G6 and 81C9, on filter grids that contain arrays of cloned DNA fragments that span the *Drosophila* genome (23). Inserts from both cosmids contain exons that encode the DAKAP550 polypeptide shown in Fig. 3.

Mapping the RII-binding Site in DAKAP550—The apparent K_D for the DAKAP550-RII β complex was estimated to be ~10 nM by overlay binding assays. This value is similar to that observed for the binding of RII β with AKAPs 75 and 79 (10, 31). The size and sequence of the RII-binding domain of DAKAP550 was characterized by a combination of mutagenesis, expression in *E. coli*, and RII-binding assays. All DAKAP550 cDNAs isolated from bacteriophage expression libraries by functional screening (*i.e.* ³²P-RII β overlay binding assays) contain a common 2-kbp fragment (see clones Z, 2–5, and 8 in Fig. 2 and text above). Thus, this segment of DAKAP550 cDNA must encode amino acids that constitute an RII tethering site in the anchor protein. The shared 2-kbp cDNA fragment was excised from clone 8 (Fig. 2) via digestion with *Eco*RI and subcloned in pBluescript. Subsequently, the location of the RII tethering site was defined more precisely by applying exonuclease III digestion or polymerase chain reaction methodology to systematically delete 5' and/or 3' portions of the target cDNA. The 2-kbp cDNA fragment and its truncated derivatives were subcloned into the isopropyl-1-thio- β -D-galactopyranoside-inducible *E. coli* expression plasmid pET14b. Upstream plasmid DNA encodes a 20-residue peptide that is appended at the N termini of the partial DAKAP550 proteins. The fusion peptide contains six consecutive His residues that enables purification of recombinant, chimeric proteins to near-homogeneity by affinity chromatography on a Ni²⁺-chelate resin. The shared 2-kbp cDNA encodes a partial protein (amino acids 1001–1676 in DAKAP550, Fig. 3) that avidly binds RII β (Fig. 5, A, lane 1, and B, lane 1). This protein is designated fu-B in Fig. 5. (Several proteolytic fragments of fu-B also exhibit RII β binding activity.) Elimination of 161 N-terminal residues and 240 C-terminal residues from fu-B yielded the D1 protein (residues 1162–1436 in DAKAP550). The RII β binding activities of D1 and fu-B are very similar (Fig. 5A, lanes 1 and 2). However, further deletion of 26 amino acids from the C terminus of D1 generated a protein (D2) whose RII binding activity was diminished by $\geq 90\%$ (Fig. 5, lane 3). Residual RII β tethering capacity was evident in the D2–3 (residues 1162–1397) and D3 (residues 1162–1381) recombinant proteins (Fig. 5A, lanes 4 and 5). Elimination of all RII binding activity was observed when the C terminus was further truncated to produce the D4 protein (residues 1162–1355, Fig. 5A, lane 6). The recombinant, partial

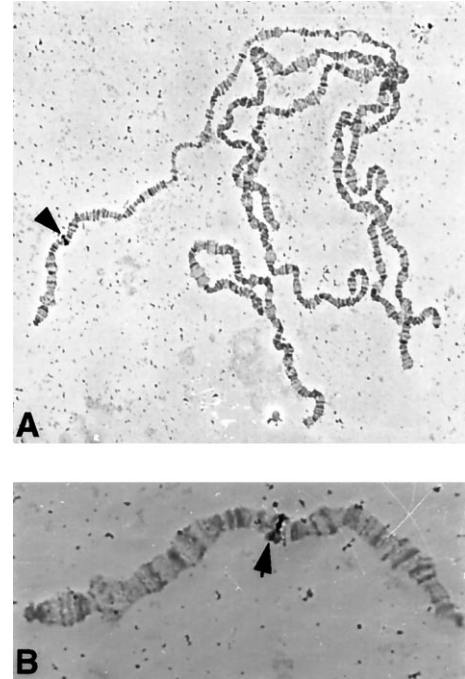


FIG. 4. **Determination of the chromosomal locus for the DAKAP550 gene.** Several DAKAP550 cDNA fragments were biotinylated and hybridized with polytene chromosomes (see "Experimental Procedures"). Complexes were visualized by incubation with streptavidin-coupled horseradish peroxidase and diaminobenzidine, a chromogenic substrate. **Panel A** shows a complete chromosome squash (312 \times) on which a discrete (black) hybridization signal (arrowhead) is evident. **Panel B** (1250 \times) shows the banding pattern near the site of hybridization (arrowhead). Chromosomes in panels A and B were hybridized with the cDNA insert from clone II-1 (Fig. 2). The same locus was detected with the same intensity by hybridization with cDNA probes prepared from the 2- and 1-kbp *Eco*RI fragments of clone 8. All of the probes hybridize with the 4F1.2 region on the X chromosome.

DAKAP550 proteins exhibited the same pattern of binding activity when mouse RII α and *Drosophila* RII⁴ were used as the radiolabeled ligands. The tethering region defined by truncation analysis includes two stretches of 20 amino acids (designated B1 and B2 in Figs. 5C and 6A and *shading* in Fig. 3) which contain arrangements of large, aliphatic amino acids (Leu, Ile, and Val) that are similar to each other and to patterns observed for previously characterized RII-binding sites (Fig. 6A). Residues comprising such binding sites are predicted to be assembled as an amphipathic helix by various algorithms (37, 38). Moreover, mutagenesis/transfection experiments indicate that the creation of a large hydrophobic surface, rather than conservation of a specific primary sequence, governs high-affinity binding of RII isoforms at tethering sites in AKAPs (11). Computer-based predictions suggest that both the B1 and B2 regions fold into amphipathic helices that contain extended

⁴ As our studies neared completion, a *Drosophila* cDNA encoding a novel fly RII was cloned by D. Kalderon and colleagues at Columbia University. D. Kalderon generously supplied a *Drosophila* cDNA that enabled us to express and phosphorylate fly RII for binding studies.

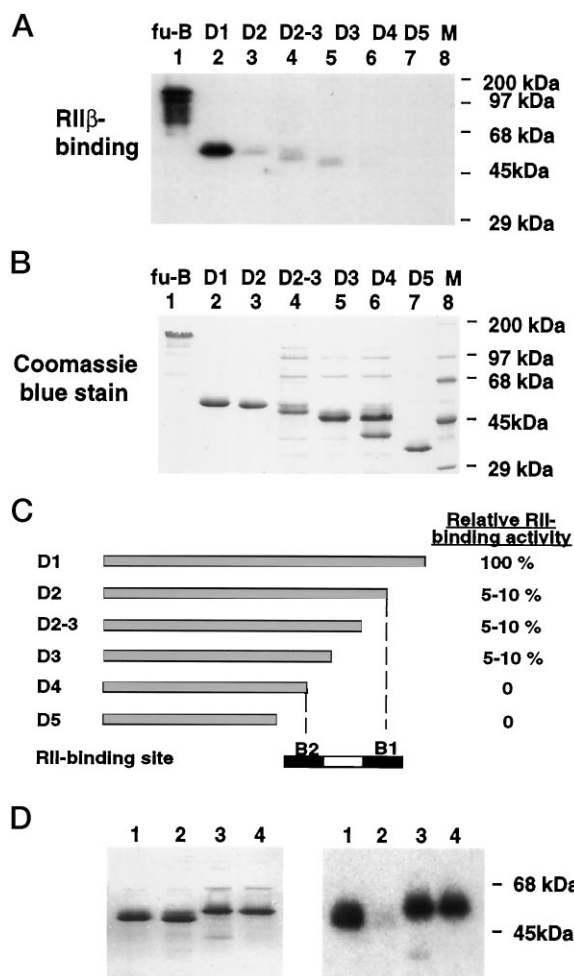


FIG. 5. Mapping RII-binding sites in DAKAP550. The partial DAKAP550 polypeptide named fu-B (residues 1001–1676, Fig. 1) and truncated proteins (D1–D5) derived from fu-B were produced and purified as described in the text and under “Experimental Procedures.” Sequences of D1–D5 proteins are given in Fig. 3. In *panel A*, each lane of an SDS-12% polyacrylamide gel received 35 ng of purified recombinant protein. After size-fractionation, proteins were transferred to an Immobilon P membrane and incubated with 32 P-RII β in an overlay assay. An autoradiogram is shown. *Lane M* contained M_r markers. *Panel B* shows recombinant proteins (0.7 μ g/lane) visualized by staining with Coomassie Blue. *Panel C* presents a diagram of the C-terminally truncated proteins and lists their relative RII binding capacities. *Panel D*, left, shows recombinant proteins (0.9 μ g/lane) visualized by staining with Coomassie Blue. *Lanes 1* and *2* received samples of truncated proteins D1 and D2, respectively; *lanes 3* and *4* contained recombinant (His) $_6$ -DAKAP550 fusion proteins (corresponding to amino acids 1371–1589, in Fig. 3) that include B1, but lack B2 (see *panel C* above). Proteins in *lanes 3* and *4* were encoded by two independently isolated cDNA clones. *Panel D*, right, presents an autoradiogram showing the results of an overlay binding assay performed with 32 P-RII β . Purified recombinant proteins loaded in *lanes 1–4* were the same as those described in *panel D*, left. The sample size was 150 ng. The sequences of B1 and B2 are shown in Fig. 6.

hydrophobic surfaces (Fig. 6B). However, the two tethering domains do not contribute equally to overall RII binding activity. A partial DAKAP550 protein that contains B1, but lacks B2, complexes 32 P-RII subunits nearly as avidly as a polypeptide (D1) that includes both B1 and B2 (Fig. 5D, lanes 1, 3, and 4). This result and the 90–95% decrease in RII binding activity associated with selective disruption of B1 in the D2 protein (Fig. 5A, lane 3) demonstrate that amino acids 1406–1425 constitute the high-affinity tethering site in the *Drosophila* anchor protein.

Expression of DAKAP550 during *Drosophila* Development—Antibodies directed against epitopes located between residues

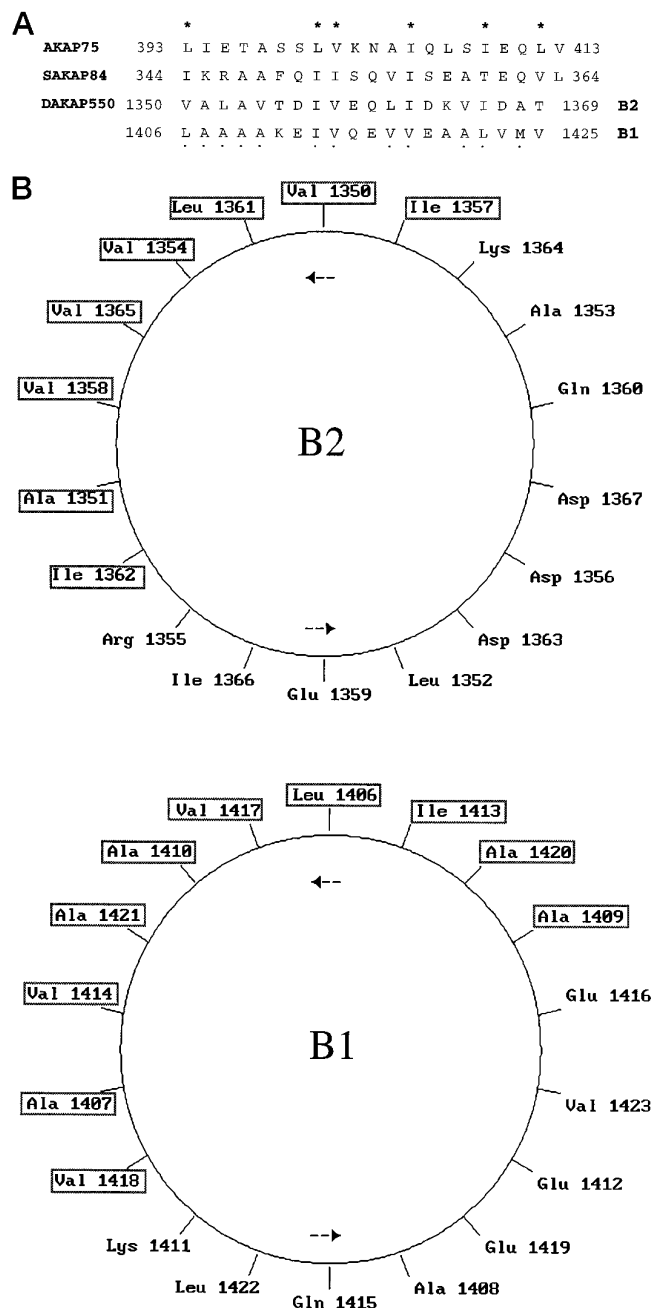


FIG. 6. Conservation of hydrophobic amino acids and predicted secondary structure of RII binding sites in DAKAP550. In *panel A*, RII tethering sites (B2, B1) from the fly anchor protein are aligned with the high-affinity binding regions of AKAP75 (11) and the germ cell anchor protein named SAKAP84 (15). Asterisks mark the positions of conserved amino acids with long-aliphatic side chains. Single dots indicate positions of hydrophobic residues in the two DAKAP550 RII-binding sites. *Panel B* shows a helical wheel presentation of the orientation of hydrophobic and hydrophilic residues in the RII-binding sites of DAKAP550. Residues that constitute a large, uninterrupted hydrophobic surface are in rectangular boxes.

1472 and 1673 in DAKAP550 (Fig. 3) were produced in rabbits and purified by affinity chromatography. Western immunoblot analyses revealed that the affinity-purified IgGs bound a 550-kDa cytosolic protein at each stage of *Drosophila* development. Typical results, obtained with extracts derived from adult flies, are presented in Fig. 7, lanes 1–4 (data not shown for embryos and pupae). Parallel Western blot analyses performed in the presence of excess recombinant antigen yielded no signals, thereby verifying the specificity of the antibodies. In adult *Drosophila*, DAKAP550 accumulates preferentially in anterior

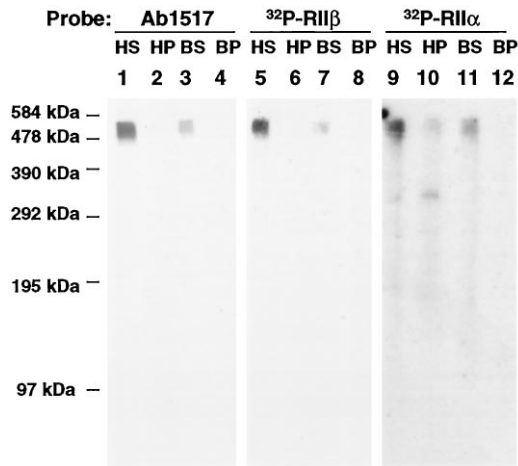


FIG. 7. Expression of DAKAP550 in adult *Drosophila*. *Drosophila* heads and bodies were disrupted in a Polytron homogenizer. Homogenates were centrifuged at $40,000 \times g$ for 20 min to separate particulate and soluble, cytosolic proteins. Samples (50 μ g) of soluble and particulate proteins were denatured in gel loading buffer and fractionated by electrophoresis in a 0.1% SDS-5.5% polyacrylamide gel. Resolved polypeptides were transferred to an Immobilon P membrane for further analysis. Lanes 1, 5, and 9 received samples of soluble cytosolic proteins from fly heads (HS); lanes 2, 6, and 10 contained particulate proteins from fly heads (HP); lanes 3, 7, and 11 received soluble proteins from fly bodies (BS); lanes 4, 8, and 12 contained particulate proteins from fly bodies (BP). One Western blot (lanes 1–4) was incubated sequentially with rabbit IgGs directed against DAKAP550 (Ab1517) and peroxidase-coupled goat IgGs directed against rabbit immunoglobulins. Antigen-antibody complexes were visualized by an enhanced chemiluminescence procedure, recorded on x-ray film, and quantified as described previously (15, 28). Two replicate Western blots (lanes 5–8 and lanes 9–12) were assayed for 32 P-RII β and/or 32 P-RII α -binding proteins, respectively, via the standard overlay procedure (see “Experimental Procedures” and Refs. 10 and 11). Autoradiograms are presented for lanes 5–12.

tissues. The concentration of DAKAP550 in cytosol derived from fly heads is ~ 10 -fold higher than the level of anchor protein in extracts of fly bodies (Fig. 7, lanes 1 and 3). Binding assays also detected a 550-kDa protein, which accounts for a very high proportion of total RII tethering activity in adult *Drosophila* (Fig. 7, lanes 5–12). Like the immunoreactive DAKAP550 polypeptide (Fig. 7, lanes 1 and 3), the 550-kDa RII β /RII α -binding protein is isolated in cytosol and enriched ~ 8 – 12 -fold in anterior tissues (head) (Fig. 7, lanes 5, 7, 9, and 11). Thus, DAKAP550 appears to be the principal PKA anchoring protein in mature flies. A 330-kDa polypeptide in the particulate fraction from fly heads exhibited a modest level of RII α binding activity (Fig. 7, lane 10). Since the RII-binding domain of DAKAP550 is not isoform-selective⁵ and the 330-kDa protein fails to react with anti-DAKAP550 IgGs (Fig. 7), it is probable that the smaller, putative AKAP is encoded by a distinct gene. No further information is currently available regarding the 330-kDa RII α -binding protein.

Affinity-purified IgGs directed against DAKAP550 were employed to monitor the expression and location of the anchor protein during *Drosophila* development (Fig. 8). Antigen-antibody complexes were visualized by indirect, immunoperoxidase-mediated staining. Specimens stained in the presence of excess recombinant antigen yielded no signals, thereby confirming the specificity of the antibodies.

During early embryogenesis DAKAP550 was evident in most cells (Fig. 8A). From gastrulation onwards the anchor protein seemed to be ubiquitous, but DAKAP550 levels differed markedly in distinct tissues. For example, at gastrulation anchor

protein content was elevated in the ventral furrow and adjacent mesectodermal cells (Fig. 8B). DAKAP550 immunoreactivity remained high in mesoderm for several hours (Fig. 8C), and was also elevated in neuroblasts delaminating from the ectoderm during stages 8–9 (Fig. 8E). Some DAKAP550 appeared to be in the nuclei of several neuroblasts (Fig. 8E). Late in embryogenesis DAKAP550 content was reduced in epidermis and skeletal muscle and became maximal in ventral nerve cord (Fig. 8, D and F). Peripheral neurons, subsets of central neurons, hindgut, the tracheal system and salivary gland (Fig. 8, D and F-I), also had substantial levels of DAKAP550 at this stage. Postembryonically, DAKAP550 was detected in imaginal discs and primordia for various adult tissues. The anchor protein is especially-enriched in neural cells such as photoreceptor neurons of the developing eye (Fig. 8J). In embryonic hindgut, trachea, and salivary gland, DAKAP550 is selectively concentrated in the apical portion of highly-polarized cells (Fig. 8, H and I). In the photoreceptor neurons DAKAP550 is also concentrated apically (Fig. 8K); in addition, a basal localization is noted for the anchor protein in cytoplasmic granules in the same cells (Fig. 8L). Granular staining was also seen in cells of the larval brain (data not shown).

***Drosophila* Mutants with Deficiencies in the 4F1.2 Region of the X Chromosome Lack DAKAP550**—Ethyl methanesulfonate-generated mutants fs(1)456v24 and fs(1)M60 map in the 4F1.2 region of the X chromosome. Proteins extracted from adult flies homozygous for these mutations were analyzed by Western immunoblot analysis. Neither mutant lacked the DAKAP550 protein (data not shown). Coupled anti-DAKAP550-immunoperoxidase staining of embryos was also consistent with normal DAKAP550 expression (data not shown). However, 25% (27/101) of embryos from a strain heterozygous for a deficiency, Df(1)JC70 (deleted for chromosome band 4C5,6-5A1,2), were not stained in the immunocytochemical assay (Fig. 8M), confirming that the antibody recognizes a protein encoded by the 4F1.2 region of the X chromosome.

DISCUSSION

We have discovered and characterized cDNAs that encode a novel *Drosophila* A kinase anchor protein (DAKAP550). Immunoblot analysis and immunocytochemistry performed with affinity-purified anti-DAKAP550 IgGs demonstrate that the anchor protein is expressed at all stages of *Drosophila* development. Both the intact protein and partial DAKAP550 fragments (expressed in *E. coli*) exhibit a hallmark property of AKAPs, high-affinity binding of RII subunits of type II PKA (Figs. 5 and 7). DAKAP550 is a very large protein that is composed of $>2,350$ amino acids (calculated $M_r > 260,000$). Although the extreme N and C termini of DAKAP550 remain to be characterized, the amino acid sequence reported in Fig. 3 appears to be close to completion. The *Drosophila* anchor protein is highly acidic and will bind SDS poorly. Thus, DAKAP550 will migrate aberrantly slowly during denaturing electrophoresis. For example, partial DAKAP550 polypeptides composed of only 676 and 275 amino acids had apparent M_r values of 150,000 and 53,000, respectively, in the denaturing gel shown in Fig. 5B (lanes 1 and 2). More generally, all partial DAKAP550 polypeptides tested to date have apparent molecular weights (in denaturing gels) that are approximately twice as large as their calculated M_r values. Thus, a protein with the sequence shown in Fig. 3 is likely to have an apparent $M_r \approx 520,000$ under denaturing conditions. This is in good agreement with the apparent size of DAKAP550 ($M_r \sim 550,000$) in fly extracts (Fig. 7). Similar physicochemical properties are observed for AKAPs from mammals (8, 9, 33).

DAKAP550 is organized in a mosaic fashion. Modules of conserved sequences, which presumably mediate similar func-

⁵ J. D. Han and C. S. Rubin, unpublished observations.

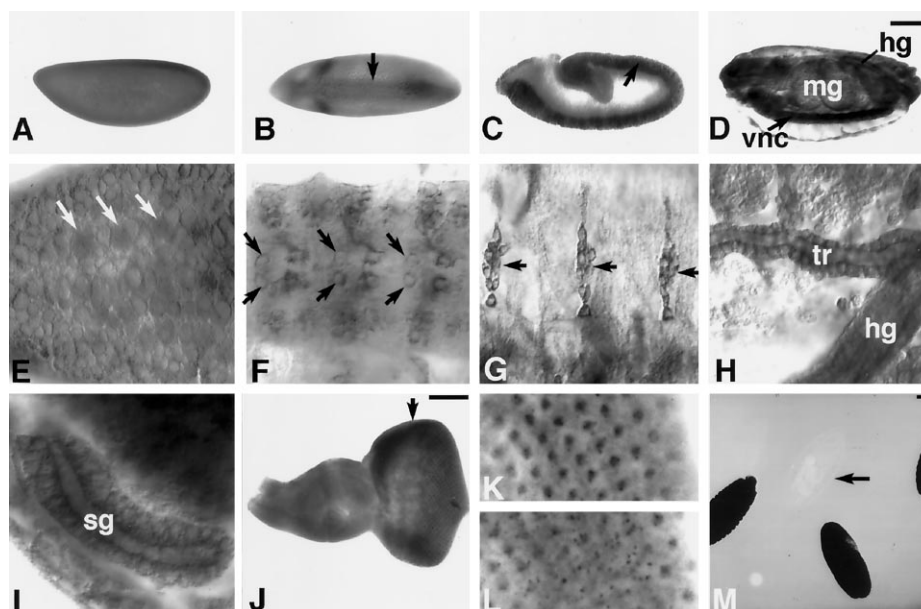


FIG. 8. Expression of DAKAP550 protein in *Drosophila* tissues at various developmental stages. Panels A-D show individual embryos and panels E-L present details of selected embryonic and imaginal tissues. Anterior is at the left in all cases. A, lateral aspect of blastoderm embryo (stage 5, ~3 h post-fertilization). DAKAP550 is detected in cytoplasm of all cells. B, ventral aspect of early gastrula (stage 6, ~3.5 h post-fertilization). DAKAP550 staining intensifies in the invaginating ventral furrow (arrow) and flanking mesectodermal cells. C, sagittal optical section of extended germband (stage 9, ~5 h post-fertilization). DAKAP550 expression is widespread, but is maximal in the mesodermal layer (arrow). D, sagittal optical section of larva at the end of embryogenesis (stage 16, ~15 h post-fertilization). DAKAP550 levels are elevated in the nervous system, gut endothelium, tracheal system, and salivary gland, and low in epidermis and skeletal muscle. The most intense signal for DAKAP550 is observed in ventral nerve cord (vnc), mg, midgut; hg, hindgut. E, ventral ectoderm (three abdominal segments) is shown during delamination of the first neuroblasts of the central nervous system (stage 9). DAKAP550 content is elevated in enlarged neuroblasts and may enter the nucleus in these cells (arrows). F, ventral nerve cord dissected from an embryo comparable to that in panel D (three abdominal segments are shown). High levels of DAKAP550 are seen in cell bodies of a segmentally-reiterated subset of central neurons (arrows), whereas other neural cells and the major axon tracts show little DAKAP550. G, Sagittal view of embryo comparable to that in panel D, showing higher DAKAP550 concentration in peripheral neurons than surrounding epidermis. Shown are neuron clusters from three abdominal segments (arrows). H, in cells lining the trachea (tr) DAKAP550 is concentrated in the apical cytoplasm (hg, hindgut). I, DAKAP550 is also predominantly apically localized in epithelium of developing salivary gland (sg). J, eye-antenna imaginal disc dissected from third instar larva. DAKAP550 is widely expressed in imaginal tissues, but anchor protein expression is especially elevated in many neural cells, such as the photoreceptor cells of the developing retina, that are differentiating posterior to the "morphogenetic furrow" (arrowhead). K and L, details of specimen from panel J showing photoreceptor cell clusters. Each cluster contains up to eight photoreceptor cells. DAKAP550 is enriched at the apical tips of photoreceptor cells (K), and is more basally concentrated in cytoplasmic granules (L). M, DAKAP550 expression by embryos derived from a *Drosophila* strain heterozygous for a chromosomal deficiency, Df(1)JC70:4C15-16;5A1-2. One in four embryos is homozygous for the deficiency and lacks DAKAP550 immunoreactivity. Normal embryos appear overstained because printing at high contrast was required for the unstained embryo to be visible (arrow). Panels A-D are magnified $\times 250$; E-H are at $\times 787.5$; and I is enlarged by $\times 1250$. Other magnifications are: J, $\times 312.5$; K and L, $\times 787.5$; and M, $\times 62.5$.

tions in mammals and nematodes, are interspersed with long segments of DAKAP550 polypeptide that have unique sequences (Table I). Divergent regions of DAKAP550 may perform widely shared functions that depend upon conservation of secondary or higher-order structure, rather than amino acid sequence. Alternatively, novel portions of DAKAP550 may be represented in proteins from other species, but are, as yet, undiscovered in vertebrates and nematodes. It is also possible that these sequences mediate physiological processes unique to *Drosophila*.

At present, physiological functions cannot be inferred for amino acid sequences conserved between *C. elegans* F10F2.1 and DAKAP550 (Table I). The F10F2.1 protein has not been characterized and no mutations have been mapped to the chromosomal locus for the F10F2.1 gene. However, we discovered that a short segment of apparently "divergent" sequence in the DAKAP550 polypeptide subserves a function established for several mammalian proteins: high-affinity binding of RII subunits (PKAII). Using deletion mutagenesis and overlay binding assays, the principal RII tethering domain was mapped to a region of DAKAP550 corresponding to amino acid residues 1406–1425 (B1, Figs. 3, 5, and 6). The arrangement of amino acids with long, aliphatic side chains (Leu, Ile, and Val) in B1 matches the distribution of critical, Leu, Ile, and Val residues in the RII tethering sites of AKAP75 and other mammalian AKAPs (Fig. 6, Refs. 11, 15, and 16). Substitution of any of

these residues with Ala eliminates or sharply reduces the ability of AKAP75 to sequester RII subunits (11). When the integrity of the B1 tethering site in DAKAP550 is disrupted by mutation, RII binding affinity declines by at least an order of magnitude (Fig. 5, A and C, and "Results"). In the absence of B1, a reduced, but detectable level of RII binding avidity is contributed by a proximal RII-binding site (residues 1350–1369) designated B2 (Figs. 5 and 6). In contrast, partial DAKAP polypeptides containing either B1 alone or both B1 and B2 bind similar amounts of the RII β ligand (Fig. 5D). Thus, under standard assay conditions the B1 tethering region plays a predominant role in sequestering RII subunits. The precise function of B2 remains to be determined. One speculative possibility is that in intact cells (where the PKAII concentration is higher than that used in overlay binding assays) B2 may provide a site for transient, rapidly reversible accumulation of RII subunits.

Secondary structure predictions suggest that residues 1350–1369 (B2) and 1406–1425 (B1) in DAKAP550 fold as amphipathic helices with large hydrophobic surfaces (Fig. 6B). Mutagenesis and binding studies on mammalian AKAPs 75 and 79 indicate that both a large hydrophobic surface at the tethering site and a complementary hydrophobic surface on RII β and RII α dimers are essential for formation of stable AKAP-PKAII complexes (11, 31, 39). Evidently, binding of DAKAP550 with *Drosophila* RII (and mammalian RII iso-

forms) is driven by similar hydrophobic interactions. The preferential binding of RII β with B1 (*versus* B2) indicates that the "core" ligand affinity contributed by the hydrophobic surface is further modulated by additional features in the structure of DAKAP550. Possible factors involved in modulating affinities of B1 and B2 for RII are: (a) substitution of conserved Val or Leu with Thr at the C terminus of B2 (Fig. 6A); (b) the presence of 10 hydrophobic residues on the surface of the predicted B1 amphipathic helix, as compared with 8 hydrophobic amino acids at the corresponding region of B2 (Fig. 6B); (c) differences in non-conserved amino acids in the 20-residue tethering domains; and (d) stabilization/destabilization of RII-tethering site interactions by flanking segments of the DAKAP550 polypeptide. Further systematic studies, employing mutagenesis, protein expression, and RII binding analyses, will be required to identify amino acid residues that enhance or diminish the affinity of the B1 and B2 domains for RII isoforms.

The RII-binding domain is inserted between two lengthy blocks of DAKAP550 sequence (residues 892–955 and 1693–1929) that are homologous with portions of both *C. elegans* F10F2.1 and the human beige-like protein (BLP) (Table I). F10F2.1 lacks a comparable tethering site for RII subunits. However, this is expected because *C. elegans* has a single R subunit gene, which encodes a cAMP-binding protein that is a homolog of mammalian RI, not RII (22). The inclusion of DAKAP550 homology regions in BLP raises the possibility that the previously uncharacterized human protein may (by analogy) be involved in cAMP-mediated signaling. BLP contains a partially conserved, candidate RII binding sequence that should be amenable to characterization by mutagenesis and overlay binding assays.

During the course of embryogenesis *Drosophila* expresses several RII-binding proteins with sizes ranging from 70,000 to >200,000 (*e.g.* see Fig. 1). As the fly matures the levels of the smaller binding proteins are diminished, thereby suggesting that these polypeptides play specialized roles in early development. In contrast, DAKAP550 is evident in many cells and nearly all tissues throughout development and becomes the principal anchor protein in adult flies (Fig. 7). Although DAKAP550 accumulates in multiple cell types, the content of the anchor protein is regulated. DAKAP550 is significantly enriched in subpopulations of peripheral and central neurons (*e.g.* photoreceptor neurons) as well as in cells of the gut and trachea. Moreover, immunocytochemical staining revealed that DAKAP550 accumulates asymmetrically (*e.g.* apical localization shown in Fig. 8, *H-L*) in certain neurons and portions of the gut and trachea. This pattern could potentially subserve polarized cAMP signaling and is consistent with the previous published observations that targeted PKA activity is essential for anterior/posterior patterning, oocyte development, and differential segregation of mRNAs (Refs. 17–21, reviewed in the Introduction).

Biochemical analysis documented that the content of DAKAP550 is markedly elevated in anterior tissues of *Drosophila*. A similar pattern of enrichment was reported for *Drosophila* RII (PKAII) (40). Moreover, the anchor protein was recovered in the cytosolic fraction of tissue homogenates. This result does not preclude a tight association of DAKAP550 with cytoskeleton. Many components of highly-organized cytoskeletal structures appear in cytosol (*e.g.* microtubule-associated protein-2, actin-binding proteins, and paxillin) when tissues are homogenized in standard, low ionic strength buffers (as used in our studies) in the absence of specific stabilizing agents.

Development of stabilizing conditions for *Drosophila* cytoskeleton will be essential to accurately monitor the distribution of the anchor protein with various subcellular fractions in future studies. Finally, it is also possible that DAKAP550 serves as a "cytoplasmic anchor" or "scaffold" to either (a) focus cAMP signaling to target proteins in cytoplasm, (b) inhibit signaling at certain membranes or cytoskeletal sites, or (c) co-assemble tethered PKAII with substrate/effector proteins directly bound to other domains of the anchor protein.

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