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RIC, a calmodulin-binding Ras-like GTPase

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Neuronal activity dramatically increases the concentration of cytosolic Ca^{2+}, which then serves as a second messenger to direct diverse cellular responses. Calmodulin is a primary mediator of Ca^{2+} signals in the nervous system. In a screen for calmodulin-binding proteins, we identified RIC, a protein related to the Ras subfamily of small GTPases. In addition to the ability to bind calmodulin, a number of unique features distinguished RIC from other Ras-like GTPases, including the absence of a signal for prenylation and a distinct effector (G2) domain. Furthermore, we describe two human proteins, RIN and RIT, which were 71% and 66% identical to RIC respectively, shared related G2 domains with RIC, and lacked prenylation signals, suggesting that the RIC family is conserved from flies to humans. While *Ric* and *RIT* were widely expressed, expression of *RIN* was confined to the neuron system.

Keywords: calcium/calmodulin/*Drosophila*/GTPase/Ras

Introduction

Calcium ions mediate signal transduction events in cells ranging from bacteria to neurons (reviewed in Clapham, 1995). In metazoans, Ca^{2+} relays information from diverse signals, including activation of neurotransmitter receptors, membrane depolarization and activation of phospholipase C-dependent signaling cascades (reviewed in Clapham, 1995; Ghosh and Greenberg, 1995). In neurons, dramatic increases in the concentration of cytosolic Ca^{2+} mediate many aspects of nervous system function, including synaptic transmission (reviewed in Jessell and Kandel, 1993; Kelly, 1993), neurite outgrowth (reviewed in Kater and Lipton, 1995; Finkbeiner and Greenberg, 1996), axon pathfinding (reviewed in Kater *et al.*, 1988; Kater and Mills, 1991; Cypher and Letourneau, 1992), synaptic development (reviewed in Hall and Sanes, 1993), synaptic plasticity (reviewed in Berridge, 1993; Bourne and Nicoll, 1993; Hall and Sanes, 1993; Bear and Malenka, 1994; Malenka, 1994; Collingridge and Bliss, 1995; Ghosh and Greenberg, 1995), sensory perception (reviewed in Fain and Matthews, 1990; Jaramillo, 1995; Ranganathan *et al.*, 1995; Ronnett and Payne, 1995) and neuronal survival (reviewed in Franklin and Johnson, 1994; Spitzer, 1994; Choi, 1995; Finkbeiner and Greenberg, 1996).

The ubiquitous, highly-conserved Ca^{2+}-sensing regulatory protein, calmodulin, is a primary mediator of Ca^{2+}-dependent signaling in eukaryotic cells (reviewed in James *et al.*, 1995). Calmodulin regulates a wide range of proteins intimately involved in the cell biology of the neuron, including ion channels and pumps, protein kinases and phosphatases, cyclic nucleotide metabolisers, cytoskeletal proteins, synaptic vesicle fusion machinery and molecular motors (M.Yu and C.Montell, unpublished results; reviewed in O'Neil and DeGrado, 1990; James *et al.*, 1995). Since the action of calmodulin can be modulated by Ca^{2+}-independent events, such as protein phosphorylation, calmodulin provides an extra level of regulation in Ca^{2+} signal transduction (reviewed in James *et al.*, 1995).

Like Ca^{2+}, the superfamily of Ras-related small GTPases regulates many processes that occur within the cell (reviewed in Bourne *et al.*, 1990; Boguski and McCormick, 1993). The Ras superfamily consists of a growing number of subfamilies which include the Ras, Rho, Rab, Ran and Arf subfamilies (reviewed in Bourne *et al.*, 1990; Boguski and McCormick, 1993). These proteins share the ability to act as binary molecular switches, oscillating between an active GTP-bound form and an inactive GDP-bound form (reviewed in Bourne *et al.*, 1991). Progression through this GTPase cycle is modulated by various regulators, including factors that stimulate guanine nucleotide exchange or GTP hydrolysis, which may themselves execute a variety of effector functions (reviewed in Boguski and McCormick, 1993). The targets of small GTPases are determined largely by the G2 effector domain. A wide repertoire of G2 regions provides the Ras superfamily with an expansive range of effector pathways (reviewed in Bourne *et al.*, 1991). Thus, small GTPases are extremely versatile, mediating such diverse processes as signal transduction, vesicle trafficking, nuclear import, cell cycle progression and cytoskeletal organization (reviewed in Boguski and McCormick, 1993).

Since Ca^{2+} and small GTPases both direct a wide scope of cellular events, it is possible that a variety of Ras-related GTPases are regulated directly or indirectly by Ca^{2+} fluxes. Recent reports have provided evidence for crosstalk between Ca^{2+} and Ras signal transduction in neurons (reviewed in Ghosh and Greenberg, 1995; Finkbeiner and Greenberg, 1996). In rat pheochromocytoma PC12 cells, which respond to Ca^{2+} signaling much as neurons do, Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and Ca^{2+} release from internal stores result in activation of Ras, which in turn activates the serine/threonine protein kinase, Raf (Rosen *et al.*, 1994; Lev *et al.*, 1995; Rusanescu *et al.*, 1995; Rosen and Greenberg, 1996). The mode of Ca^{2+} action in this system is mediated at least in part by phosphorylation-induced activation of the non-receptor tyrosine kinases, Src (Rusanescu *et al.*, 1995) and PYK2 (Lev *et al.*, 1995), as well as ligand-independent activation of the epidermal growth factor receptor (Rosen and Greenberg, 1996). In central nervous

system (CNS) neurons, Ca^{2+} signaling also activates Raf (reviewed in Ghosh and Greenberg, 1995; Finkbeiner and Greenberg, 1996). Although the Ca^{2+}-dependent tyrosine phosphorylation cascades observed in PC12 cells may contribute to activation of Raf in the CNS, an alternative route involving the Ras guanine nucleotide exchange factor, RasGRF, has been detected (Cen *et al.*, 1992; Shou *et al.*, 1992; Wei *et al.*, 1992). RasGRF, which is expressed in neurons but not in PC12 cells, has recently been shown to become activated in response to Ca^{2+} via direct binding of calmodulin (Farnsworth *et al.*, 1995). In the fetal and adult nervous system, Ca^{2+}-dependent activation of Ras pathways may account for activity-dependent modulation of neuronal differentiation, survival and synaptic strength (reviewed in Finkbeiner and Greenberg, 1996). Thus, Ca^{2+}–Ras crosstalk may provide the molecular machinery for refining the developing nervous system and/or for learning and memory (reviewed in Finkbeiner and Greenberg, 1996).

Given the versatility of Ras-like GTPases, it is possible that other Ca^{2+}-dependent cellular events are mediated by small GTPases. In this report, we describe a *Drosophila* Ras-related GTPase, RIC, which bound to calmodulin. In addition to calmodulin-binding, RIC displayed unique characteristics not observed in other Ras-like GTPases, including a distinct effector (G2) domain and the absence of a C-terminal prenylation motif. Furthermore, we describe two human proteins, RIN and RIT, which were 71% and 66% identical to RIC, respectively. Expression of *RIN*, which also bound to calmodulin, was restricted to the developing and adult nervous system. We propose that the two human proteins and the *Drosophila* protein comprise a new subgroup of the Ras subfamily.

Results

Molecular characterization of the gene encoding a calmodulin-binding Ras-related GTPase

To identify calmodulin-binding proteins expressed in the *Drosophila* retina, we probed a retinal cDNA expression library with ^{125}I-labeled calmodulin. One positive cDNA clone, dm197 (nucleotides 404–2000 of the composite cDNA sequence), contained an open reading frame with a deduced amino acid sequence most similar to the Ras subfamily of the Ras superfamily (see below). In order to obtain the complete coding region, we rescreened the retinal cDNA library by DNA hybridization using the dm197 clone as a probe, and isolated several additional cDNAs, the longest of which were c-*Ric*-1 (nucleotides 109–2236) and c-*Ric*-2 (nucleotides 1–1509). Both of these cDNAs contained a single open reading frame (nucleotides 123–914) predicted to encode a protein of 264 amino acids. The combined sequences of these cDNAs indicated that the mRNA was a minimum of 2.24 kb. We named the gene *Ric* because it encoded a Ras-related protein which interacted with calmodulin (see below).

The deduced amino acid sequence included three distinct domains. The N-terminal 57 amino acids and the C-terminal 41 amino acids did not show significant homology with any protein in the databanks. However, amino acids 58–223 shared high sequence similarity with the Ras subfamily (Figure 1), with 59% identity to its closest relative, *Mucor racemosus* RAS3 (Casale *et al.*, 1990) and 56% identity to *Drosophila* RAS1. The G2 effector domain of RIC (amino acids 88–96) was closely related to that of other Ras subfamily members except for a histidine rather than a tyrosine residue at the first position. In contrast to most Ras subfamily members, however, the predicted RIC protein contained neither a C-terminal CaaX motif, which is required for isoprenyl lipid addition (reviewed in Clarke, 1992; Marshall, 1993; Glomset and Farnsworth, 1994), nor any cysteine residue near the C terminus that could potentially serve as a site for lipid modification. The N-terminal extension contained a number of putative protein kinase A and protein kinase C phosphorylation sites (residue 16 and residues 15 and 29, respectively). In addition, amino acids 44–51, KPPPVPPQ, were similar to the SH3-binding domain, VPPPVPPR, of the Ras guanine nucleotide exchange factor, SOS (see Figures 1 and 2; reviewed in Cohen *et al.*, 1995).

To map the *Ric* gene to the *Drosophila* genome, polytene chromosome squashes from third instar larval salivary glands were probed with a biotinylated λ phage genomic clone, λroc-1. A signal was observed on the right arm of chromosome 2 at 52E (data not shown). To confirm this chromosomal localization, Yeast Artificial Chromosomes (YACs) and cosmid clones that cover 52D-F were screened by PCR and colony hybridization. One cosmid, 39C11, which maps to 52E, included *Ric* (Siden-Kiamos *et al.*, 1990; Madueno *et al.*, 1995). Several YACs (DY517, DY510, DYR17–20, DY773, DYN03–74, DYR15–76 and DY506) that cover part or all of 52E also hybridized to *Ric* (Ajioka *et al.*, 1991; Cai *et al.*, 1994). The genomic region encompassing the open reading frame of *Ric* was sequenced (see Materials and methods), revealing two introns of 198 and 97 nucleotides after nucleotide positions 342 and 399 in the cDNA, respectively.

To determine the developmental stages during which *Ric* was expressed, we probed an RNA blot containing poly(A)$^+$ RNA prepared from different developmental stages. *Ric* was expressed throughout development as a 2.3 kb RNA, a size similar to that predicted by the composite cDNA sequence (Figure 2). Comparison of the intensity of the *RIC* signal with the intensity of the signal for *RAS1* on the same RNA blot suggests that *Ric* is expressed at similar levels at each stage of development (data not shown).

RIC bound to calmodulin in several independent assays

To confirm the ability of RIC to bind calmodulin, we expressed a fusion protein in bacteria which consisted of β-galactosidase fused to the entire RIC protein. Total bacterial extracts were resolved by SDS–PAGE, transferred to membranes and probed with biotinylated calmodulin. A signal was observed at the precise position of the β-galactosidase–RIC fusion protein (Figure 3A and B). No other signals were observed in extracts containing the RIC fusion protein nor in extracts which did not express RIC but which did express β-galactosidase (Figure 3A and B). Binding of calmodulin to the RIC fusion protein in the overlay required the addition of Ca^{2+} (Figure 3C). The Ras-like GTPases Rac, Rho and Cdc42 expressed in bacteria did not bind calmodulin in the overlay (data not shown).

To demonstrate that RIC expressed in flies could bind

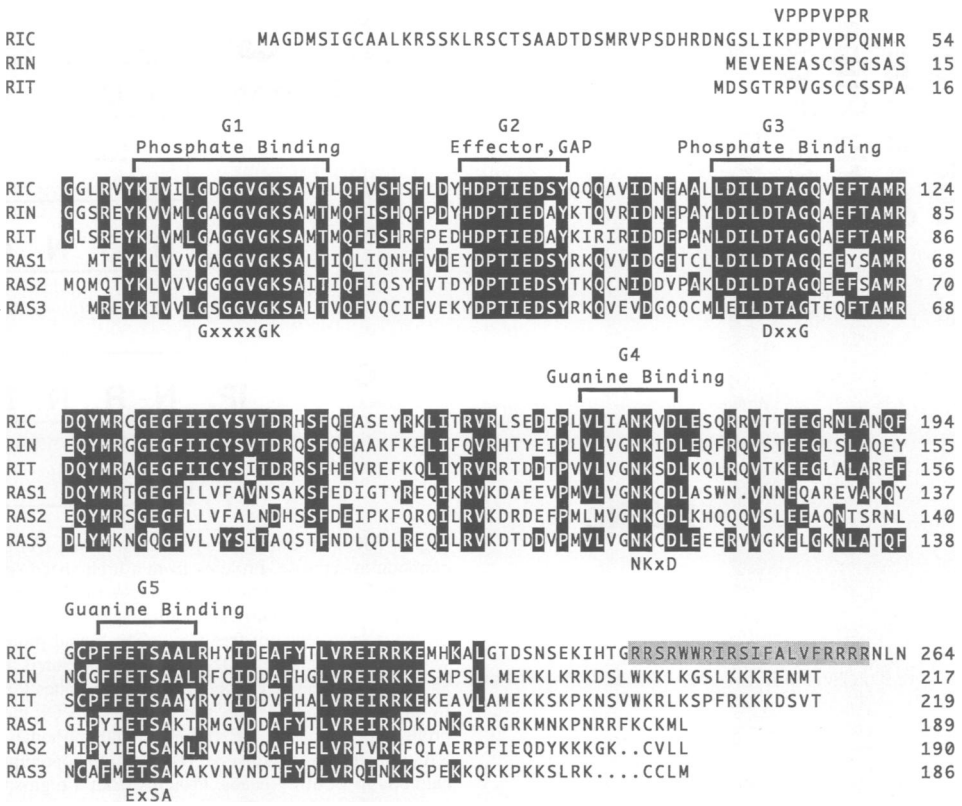

Fig. 1. Amino acid sequence alignment of RIC, RIN, RIT, *Drosophila* RAS1, RAS2 and RAS3. Residues that are identical between RIC and at least two other proteins in the alignment are indicated with white lettering on a black background. The G1–G5 domains (reviewed in Bourne *et al.*, 1991) are indicated. Consensus sequences for GTP-binding regions (reviewed in Bourne *et al.*, 1991; Pai *et al.*, 1990) are shown below the alignment. x, any amino acid. The region of RIC sufficient to bind calmodulin (amino acids 239–264) is shaded gray. The SH3-binding domain of SOS is shown above amino acids 44–51 of RIC to indicate RIC's putative SH3-binding domain. The accession numbers for the *Ric*, *RIN* and *RIT* cDNA sequences are Y07564, Y07565 and Y07566, respectively.

calmodulin in solution, we generated a line of transgenic flies, P[*MycRic*], which expressed a Myc-tagged version of RIC exclusively in the adult eye. Protein extracts from heads of these flies were incubated with gelatin-agarose beads or agarose beads conjugated with calmodulin. RIC bound to the calmodulin–agarose beads but not to the gelatin-agarose beads, as determined by Western blot analysis utilizing an anti-Myc antibody for detection (Figure 4A). Moreover, binding of RIC to calmodulin–agarose did not require the presence of Ca^{2+}, and the extent of binding was not significantly different in the presence or absence of free Ca^{2+}.

In a distinct assay, calmodulin was immunoprecipitated from P[*MycRic*] head extracts, and the immunoprecipitated proteins were fractionated by SDS–PAGE and probed with anti-Myc antibodies. A band of the predicted size for MycRIC was detected, indicating that RIC coimmunoprecipitated with calmodulin (Figure 4B). To confirm these results, RIC was immunoprecipitated from P[*MycRic*] head extracts with anti-Myc antibodies, the immunoprecipitate was fractionated by SDS–PAGE and probed with anti-calmodulin antibodies. A band of the predicted molecular weight for calmodulin was detected, indicating that calmodulin coimmunoprecipitated with RIC (Figure 4C). RIC and calmodulin coimmunoprecipitated in the presence and absence of free Ca^{2+} with no detectable differences in stoichiometry.

A 20 residue domain was necessary and sufficient to bind calmodulin in an overlay

Some calmodulin-binding sites consist of basic amphiphilic helices (reviewed in James *et al.*, 1995) or IQ motifs (Chapman *et al.*, 1991; Mercer *et al.*, 1991; Espreafico *et al.*, 1992; Swanljung-Collins and Collins, 1992; Xie *et al.*, 1994; Farnsworth *et al.*, 1995; Porter *et al.*, 1995; reviewed in Cheney and Mooseker, 1992). However, inspection of the RIC amino acid sequence did not reveal any such candidate calmodulin-binding motifs. To map the calmodulin-binding site of RIC, we generated a series of fusion proteins (Figure 5) and analyzed them for the ability to bind calmodulin in a calmodulin overlay. The smallest fusion that bound calmodulin consisted of amino acids 242–261 (shaded gray in Figure 1), whereas the largest fusion that did not bind calmodulin included the entire RIC protein except the C-terminal 10 amino acids (Figure 5). Thus, 20 residues, amino acids 242–261, were necessary and sufficient to bind calmodulin in an overlay.

Identification of human RIC-related proteins, RIN and RIT

To identify human proteins related to RIC, we queried the NCBI Expressed Sequence Tag (EST) database, dbEST, with the BLAST local multiple alignment algorithm (Altschul and Lipman, 1990; Altschul *et al.*, 1990; Gish and States, 1993). Several ESTs representing two distinct

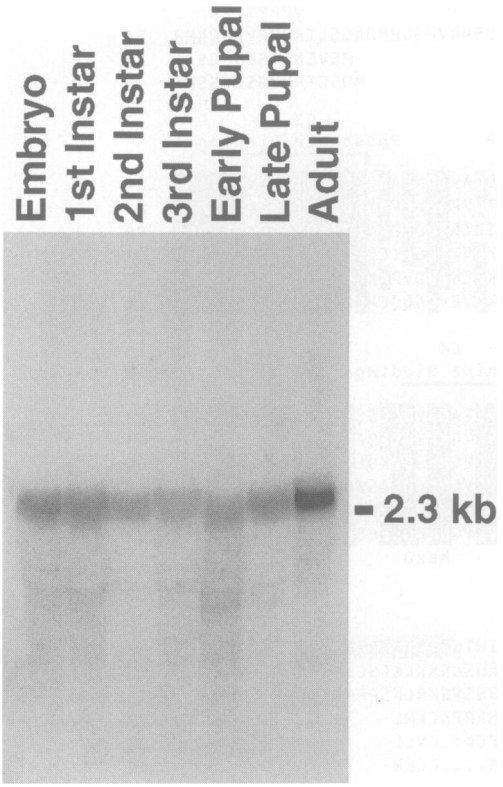

Fig. 2. RNA blot analysis of *Ric* expression. *Drosophila* developmental RNA blot. The signal migrated at 2.3 kb.

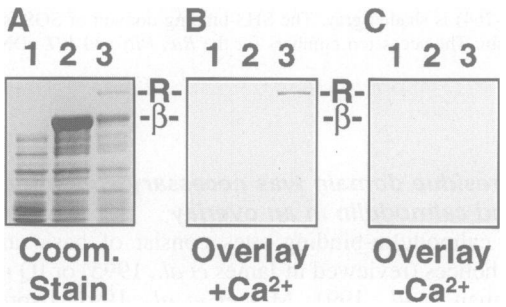

Fig. 3. RIC bound to calmodulin in a gel overlay. Total protein extracts from uninduced bacteria (lane 1), bacteria expressing β-galactosidase (lane 2) or a β-galactosidase–RIC fusion protein (lane 3) were fractionated by SDS–PAGE (6% gel), and either: (**A**) stained with Coomassie Brilliant Blue; (B and C) transferred to nitrocellulose, and probed with biotinylated calmodulin in the presence of 0.5 mM $CaCl_2$ (**B**) or in the presence of 5 mM EGTA (**C**). The positions of β-galactosidase (β) and the β-galactosidase–RIC fusion protein (R) are indicated.

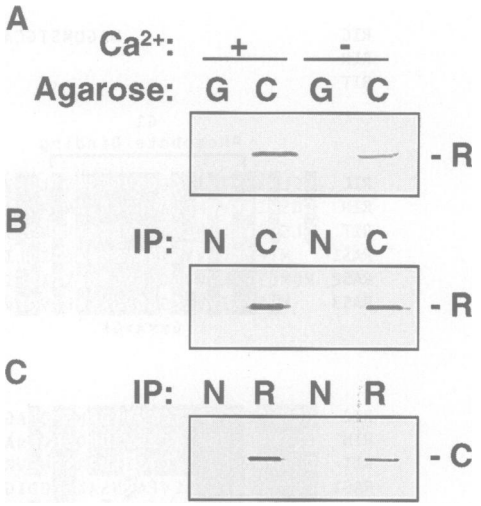

Fig. 4. RIC bound to calmodulin in solution. (**A**) Binding of RIC to calmodulin–agarose. Proteins from a high-speed supernatant, prepared from fly heads expressing a Myc-tagged RIC transgene, were incubated in batch with calmodulin–agarose or gelatin–agarose in the presence or absence of free Ca^{2+}. The bound proteins were eluted with 2× SDS sample buffer. Proteins that eluted from the gelatin-agarose (lanes G) or calmodulin–agarose (lanes C) were fractionated by SDS–PAGE (12% gel), transferred to PVDF membranes, and probed with anti-Myc 9E10 monoclonal antibodies. The position of the RIC protein (R) is indicated. (**B**) Coimmunoprecipitation of RIC and calmodulin from fly heads. Proteins from a high-speed supernatant, prepared from fly heads expressing a Myc-tagged RIC transgene, were incubated with a non-immune mouse serum (lanes N) or an anti-calmodulin monoclonal antibody (lanes C) in the presence or absence of free Ca^{2+}. Antibodies complexed with antigen and associated proteins were pelleted with a 50:50 mixture of protein-A and protein-G beads. Proteins were eluted from the beads, fractionated by SDS–PAGE (12% gel), transferred to PVDF membranes, and probed with an anti-Myc 9E10 monoclonal antibody. The position of the RIC protein (R) is indicated. (**C**) Coimmunoprecipitation of RIC and calmodulin from fly heads. The identical experiment as in (B), except that extracts were immunoprecipitated with an anti-Myc 9E10 antibody (lanes R) rather than an anti-calmodulin antibody and the membranes were probed with an anti-calmodulin polyclonal serum (a gift from Kate Beckingham) rather than the anti-Myc 9E10 antibody. The position of the calmodulin protein (c) is indicated.

uncharacterized human genes showed strong conservation with *Drosophila* RIC. The first gene, which we will refer to as *RIN* (*Ric*-related gene expressed in neural tissues; see below), was represented by clones 39507, 283954, 45534 and 59040 (EST accession numbers R52317, N53351, H08460 and Z41031), which were isolated from cDNA libraries made from human infant brain and human multiple sclerotic tissue. The second gene, *RIT* (*Ric*-related gene expressed throughout the organism; see below), was represented by clones 72286, 268332 and 146933 (EST accession numbers T58089, N36448, N25735, R81023, T58027 and R80818) from human fetal spleen, melanocyte and placenta cDNA libraries, respectively. The *RIT* clone 72286 has been mapped by the Genome Cross-Referencing Group (Bassett Jr *et al.*, 1995) to mouse chromosome 3 with a 64 cM offset on the Jackson Laboratory BSS Interspecific Backcross DNA panel and to human chromosome 1 pter-qter by hybridization to somatic cell hybrid DNA.

The composite sequences of the *RIN* and *RIT* cDNAs indicated that the mRNAs were a minimum of 1.2 kb and 1.1 kb, respectively. The deduced amino acid sequence of the RIN and RIT proteins were 53% and 51% identical to *Drosophila* RAS1, 71% and 66% identical to *Drosophila* RIC, and 76% identical to each other in the core GTPase domain (amino acids 58–223 of RIC; Figure 1). Moreover, the effector G2 region was closely related amongst all three RIC proteins, with only one conservative substitution between the human and *Drosophila* proteins. In addition to the core GTPase domain, RIN and RIT possessed 18 and 19 amino acid N-terminal extensions and 33 and 34 amino acid C-terminal extensions, respectively. The N-terminal domains of the three RIC proteins were not related to each other. In contrast, the C-terminal domains of RIN and RIT (residues 191–217 and 201–219, respect-

ively) were 52% identical and 81% similar to each other. Though the C-terminal domains of RIN and RIT were not related to the C-terminal domain of *Drosophila* RIC at the level of amino acid sequence, all three regions contained a high proportion of positively-charged residues. As with *Drosophila* RIC, RIN and RIT neither contained a CaaX motif for prenylation, nor did they possess any cysteine residue near the C terminus that could potentially serve as a site for lipidation. The same human ESTs and related mouse clones were simultaneously identified by Zack and colleagues (Lee *et al.*, 1996).

RIN is a calmodulin-binding protein highly enriched in the brain

To determine which human fetal and adult tissues expressed *RIN* and *RIT*, we probed RNA blots containing poly(A)$^+$ RNA prepared from various tissues. We found

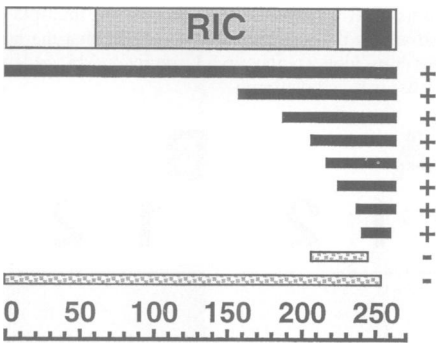

Fig. 5. Mapping of RIC's calmodulin-binding site. Shown are representations of bacterially expressed RIC–GST fusion proteins and their abilities to bind calmodulin in an overlay. The unique N- and C-terminal domains of RIC are depicted by open squares and the core GTPase domain depicted by shading. The calmodulin-binding domain is represented by the black box. The regions of RIC fused to GST are shown by the bars below the protein schematic. In order from top to bottom, these regions are amino acids 1–264, 158–264, 188–264, 207–264, 217–264, 226–264, 239–264, 242–261, 207–244 and 1–254. Those fusions that bound calmodulin are shown in black and are marked by a '+', while those that did not bind are stippled and are marked by a '–'. An amino acid scale is shown below.

that *RIN* was expressed as a single mRNA species which migrated at 1.4 kb, a size similar to that predicted by the composite cDNA sequence (Figure 6). During fetal development, *RIN* was expressed at high levels in the brain, but was not detected in the heart, lung or liver, even after extended exposures. In the adult, *RIN* was expressed at high levels in many regions of the brain, but a signal of the predicted size was not detected in any other tissue. However, a weak 2.0 kb signal was detected in mRNA prepared from the testes. *RIT* was expressed at high levels in most tissues (Figure 7). mRNA species of 1.35 kb, 2.9 kb and 3.9 kb were detected with the *RIT* probe.

Since *RIN* was expressed almost exclusively in the brain, we characterized the RIN protein further to determine whether it bound to calmodulin. We found that the C-terminal 74 residues of RIN fused to glutathione-*S*-transferase (GST) bound to calmodulin in a gel overlay experiment, while GST alone did not (Figure 8A). As with RIC, the ability of RIN to bind calmodulin in an overlay required the presence of calcium (Figure 8B).

Discussion

Ric encodes a calmodulin-binding Ras-like GTPase

We have identified a *Drosophila* calmodulin-binding protein, RIC, which shared 56% identity with *Drosophila* RAS1, defining RIC as a protein related to the Ras subfamily of small guanine nucleotide-binding proteins. Mutational analyses and crystallographic studies of Ras family members have defined the residues important for GTP-binding and hydrolysis. The phosphate-binding (GxxxxGK and DxxG in G1 and G3, respectively) and guanine specificity consensus sequences (NKxD and ExSA in G4 and G5, respectively) of monomeric GTPases are perfectly conserved in RIC (see Figure 1). Therefore, it is likely that RIC binds and hydrolyzes GTP specifically. The putative effector (G2) region of RIC was also conserved with Ras subfamily members (see Figure 1), except for the presence of a histidine instead of the conserved

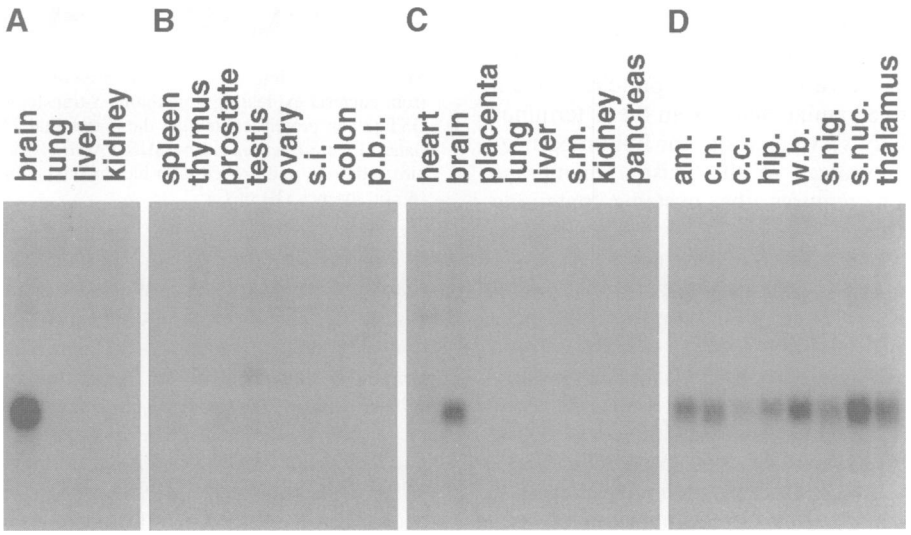

Fig. 6. RNA blot analysis of *RIN*. (**A**) Human fetal RNA blot II. (**B**) Human adult multiple tissue RNA blot I. (**C**) Human adult multiple tissue RNA blot II. (**D**) Human adult brain multiple tissue RNA blot III. Each filter was reprobed with a β-actin probe demonstrating that the lanes were evenly loaded (data not shown). am., amygdala; c.c., corpus callosum; c.n., caudate nucleus; hip., hippocampus; p.b.l.; peripheral blood leukocytes; s.i., small intestines; s.m., skeletal muscle; s.nig., substantia nigra; s.nuc., subthalamic nucleus; w.b., whole brain.

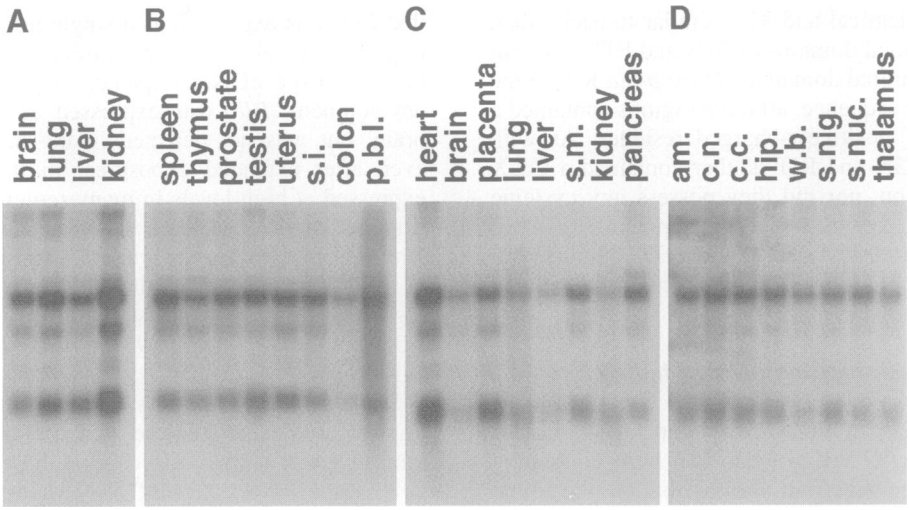

Fig. 7. RNA blot analysis of *RIT*. (**A**) Human fetal RNA blot II. (**B**) Human adult multiple tissue RNA blots I. (**C**) Human adult multiple tissue RNA blot IV. (**D**) Human adult brain multiple tissue RNA blot III. Each filter was reprobed with a β-actin probe demonstrating that the lanes were evenly loaded (data not shown). am., amygdala; c.c., corpus callosum; c.n., caudate nucleus; hip., hippocampus; p.b.l.; peripheral blood leukocytes; s.i., small intestines; s.m., skeletal muscle; s.nig., substantia nigra; s.nuc., subthalamic nucleus; w.b., whole brain.

tyrosine at the first position of the G2 domain. RIC is the first GTPase with the G2 domain sequence, HDPTIEDSY. This difference was significant because the G2 domains of GTPases are often perfectly conserved within a subfamily but show little, if any, similarities between subfamilies, reflecting their critical role in determining the repertoire of effectors (reviewed in Bourne *et al.*, 1991).

Strikingly, RIC lacked a C-terminal prenylation motif which is encountered in most Ras-like proteins and which is required for the attachment of Ras to the plasma membrane (reviewed in Lowy *et al.*, 1991; Clarke, 1992; Marshall, 1993; Glomset and Farnsworth, 1994). Specific plasma membrane localization of Ras proteins also requires a second targeting signal present in the C-terminal domain, either a stretch of basic amino acids or internal cysteines that are modified with the palmitoyl lipid moiety (Hancock *et al.*, 1990). While most Ras-like proteins contain prenylation signals, there have been reports of proteins closely related to Ras, Rad and Gem/Kir, which do not contain cysteine residues that fall within any known prenylation motifs (Reynet and Kahn, 1993; Cohen *et al.*, 1994; Maguire *et al.*, 1994). Nevertheless, both proteins contain a cysteine residue seven amino acids from the C terminus which could potentially serve as a site for lipid modification. Indeed, Gem localizes to the plasma membrane and this localization requires the cysteine-containing C-terminal domain (Maguire *et al.*, 1994). RIC is the first member of the Ras subfamily that does not possess any cysteine residue in the C-terminal domain that could potentially serve as a site for prenyl or palmitoyl modification. Therefore, RIC may not localize to the plasma membrane *in vivo*. Alternatively, RIC may be targeted to the plasma membrane, either constitutively or in a regulated manner, by interacting with membrane-associated proteins or by direct binding to plasma membrane lipids.

RIC had larger N- and C-terminal domains than most other Ras-related proteins. The N-terminal domain contained a putative protein–protein interaction motif, KPPPVPPQ, similar to the region of SOS, VPPPVPPR, that binds to the SH3 domain of the adaptor protein, Grb2 (see Figure 1; reviewed in Cohen *et al.*, 1995). The

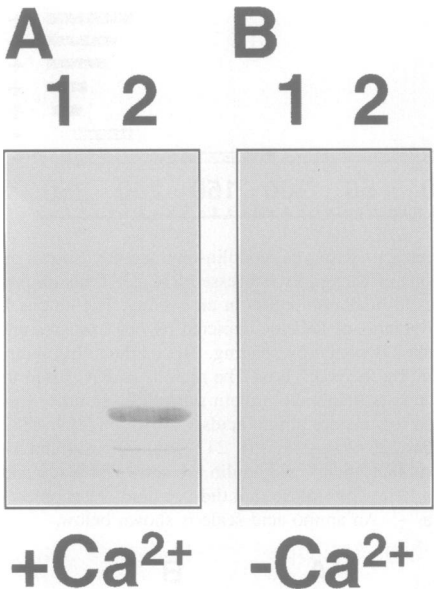

Fig. 8. RIN binding to calmodulin in an overlay. Total protein extracts from bacteria expressing glutathione-*S*-transferase (GST; lane 1) or a GST fusion protein containing the C-terminal 74 amino acids of RIN (lane 2) were fractionated by SDS–PAGE (12% gel), transferred to nitrocellulose, and probed with biotinylated calmodulin in the presence (**A**) or absence (**B**) of Ca^{2+}.

C-terminal region of RIC contained a 20 residue domain that was necessary and sufficient to bind calmodulin (see Figure 1). However, this calmodulin-binding site did not conform to the two canonical calmodulin-binding motifs, the basic amphiphilic helix (reviewed in James *et al.*, 1995) and the IQ motif (Chapman *et al.*, 1991; Mercer *et al.*, 1991; Espreafico *et al.*, 1992; Swanljung-Collins and Collins, 1992; Xie *et al.*, 1994; Farnsworth *et al.*, 1995; Porter *et al.*, 1995; reviewed in Cheney and Mooseker, 1992). Rather, the site was composed of patches of the basic amino acid, arginine, intermixed with patches of hydrophobic amino acids, as well as two serine residues (see Figure 1). Given the unconventional nature of the sequence, it was important to rule out artefactual binding

by demonstrating that calmodulin bound to RIC in a number of independent assays. This was a particular concern in the calmodulin overlay assay, where the proteins immobilized on membranes are partially denatured. In fact, several members of the EF-hand superfamily, including calmodulin, are known to bind hydrophobic moieties *in vitro* in the presence of calcium (Zozulya and Stryer, 1992; Eshel *et al.*, 1993). Nevertheless, we were able to demonstrate that RIC bound to calmodulin–agarose beads in solution and that calmodulin and RIC coimmunoprecipitated from fly head extracts, suggesting that RIC and calmodulin associate *in vivo*.

Ca^{2+} was required for calmodulin to bind RIC in an overlay, but not for binding in solution. Different Ca^{2+} requirements for calmodulin-binding to an identical site under different conditions is not unprecedented: calmodulin does not require Ca^{2+} to bind to the intact γ subunit of phosphorylase kinase, but does require Ca^{2+} to bind to peptides that represent identical sites (Dasgupta *et al.*, 1989). These seemingly conflicting results may be due to long range interactions of the intact protein that stabilize binding. In an overlay, such interactions may be disrupted because the immobilized proteins are partially denatured. Therefore, binding characteristics in solution most likely represent the physiological requirements. We have not ruled out the possibility that the calmodulin-binding site we have defined is indeed Ca^{2+}-dependent, but that RIC possesses a second Ca^{2+}-independent calmodulin-binding site which fails to bind calmodulin in an overlay, but which binds to calmodulin in solution.

Ca^{2+}-independent calmodulin association has been observed in a variety of proteins that are known or are suspected to respond to Ca^{2+} signals, including unconventional myosins (Cheney and Mooseker, 1992), a bovine lung cyclic nucleotide phosphodiesterase (Sharma and Wang, 1986; Sonnenberg *et al.*, 1995), the γ subunit of phosphorylase kinase (Dasgupta *et al.*, 1989), adenylate cyclase (Ladant, 1988) and the TRPL cation channel (Warr and Kelly, 1996). Interestingly, as for RIC, the calmodulin-binding domains of the latter four proteins resemble neither basic amphiphilic α-helices nor IQ motifs. Therefore, RIC may belong to a distinct class of Ca^{2+}-sensing protein complexes that possess calmodulin as an integral regulatory subunit.

RIC represents the prototype of a new subgroup of the Ras subfamily

While RIC shared many of the features of Ras, it also displayed several unusual characteristics that set it apart from other members of the Ras subfamily. These included relatively large N- and C-terminal domains, a distinct G2 domain, the absence of a signal for lipidation and a calmodulin-binding domain. The two human proteins, RIN and RIT, which shared the highest percent identity with RIC, also displayed several of the unusual characteristics of RIC, suggesting the presence of a novel gene family conserved from flies to humans. The three RIC proteins were 66%–76% identical to each other, but 51%–56% identical to *Drosophila* RAS1. Given the high level of relatedness amongst the RIC proteins, both with respect to primary sequence and to shared structural features, we propose that the RIC family constitutes a distinct subgroup of the Ras subfamily.

RIN and RIT were more similar to each other than they were to RIC. The core GTPase domains of RIN and RIT were 76% identical to each other, but 71% and 66% identical to RIC, respectively. In addition, RIN and RIT had shorter N-terminal extensions than RIC (18 and 19 amino acids, respectively, compared with 57 amino acids for RIC), and neither possessed the putative SH3-binding site found in the RIC N-terminal domain (see Figure 1). The C-terminal domains of RIN and RIT were 52% identical and 81% similar to each other, and while they showed no sequence conservation with the C-terminal domain of *Drosophila* RIC, all three regions shared the common feature of possessing a high proportion of basic residues. The brain-specific expression of *RIN* was striking since many members of the Ras subfamily of GTPases demonstrate widespread expression patterns.

Small Ras-like GTPases are known to function in many cellular processes, including activation of catalytic enzymes, membrane turnover, cytoskeletal organization, modulation of gene expression and protein and vesicle trafficking. It is therefore possible that the RIC family of proteins mediates some of the Ca^{2+}-dependent events that require these cellular processes. In neurons, these events include synaptic transmission, growth cone behavior, synaptic development, synaptic plasticity, adaptation and neuronal survival.

Materials and methods

Molecular cloning

To screen for calmodulin-binding proteins, a λZAP *Drosophila* retinal expression library (gift from Charles Zuker) was probed with [^{125}I]calmodulin at 10^7 c.p.m./ml in the presence of 10 mM imidazole, pH 7.4, 150 mM KCl, 0.1 mM $CaCl_2$, 0.2% Tween 20 and 5% BSA. Positive plaques from a tertiary screen were purified and the pBluescript SK– clones containing the cDNAs were excised *in vivo* by coinfection with 10^7 p.f.u./ml of R408 helper phage according to the Stratagene protocol. The library was rescreened with the cDNA clone dm197 by DNA hybridization. Plasmid cDNA clones were rescued from positive plaques obtained in a secondary screen as described above. The composite cDNA sequence contained a single open reading frame (nucleotides 123–917). The large 3′ untranslated region (nucleotides 918–2236) displayed characteristics of an untranslated sequence since it was significantly more AT-rich than the open reading frame (63.9% compared with 40.2%).

Ric genomic DNA was isolated from a Charon 4A genomic library (Maniatis *et al.*, 1978) by probing with the dm197 cDNA clone. The *Eco*RI fragments from one genomic clone, λroc-1, was subcloned into pBluescript SK–. One of the genomic subclones from λroc-1, groc1.9, contained a 5.5 kb insert which encompassed the entire coding region and began 1.2 kb upstream of nucleotide 1 of the composite cDNA sequence. The first 2.6 kb of groc1.9 were sequenced.

DNA sequencing

Oligonucleotides were synthesized on a Perkin Elmer, Applied Biosystems Division (PE/ABd) 394 DNA Synthesizer using standard 0.2 mM scale. All synthesis reagents were obtained from PE/ABd. The samples were sequenced using the fluorescent di-deoxy terminator method of cycle sequencing (McCombie *et al.*, 1992) on a Perkin Elmer, Applied Biosystems Division (PE/ABd) 373a automated DNA sequencer (Smith *et al.*, 1986) at the DNA Analysis Facility of the Johns Hopkins University, following ABd protocols.

Chromosomal in situ hybridizations

Salivary gland polytene chromosomes from late third instar larvae were prepared and hybridized with biotinylated genomic clone λroc-1 as described (Zuker *et al.*, 1985). The probe was detected using the Detek HRP Signal Generating System for Biotin Detection as described by the supplier (Enzo Diagnostics, Inc., New York).

RNA blots

The *Drosophila* developmental RNA blot was a gift from Lee Ann Leshko. Human RNA blots were purchased from Clonetech, Palo Alto, CA (human fetal MTN Blot II, Lots 52886 and 5Y404; human MTN Blot, Lots 51717 and 62701; human MTN Blot II, Lot 51637; human MTN Blot IV, Lot 61600; human brain MTN Blot III, Lots 4y936 and 62346). Fetal RNA was pooled from various developmental stages (brain, 21–26 weeks; lung, 22–23 weeks; liver 22–26 weeks; kidney 19–23 weeks).

RNA blots were prehybridized for 24 h at 65°C in 1× hybridization buffer (2× SSCP, 2× Denhardt's solution, 0.5% SDS) and 500 μg/ml salmon sperm DNA, and hybridized with 10^6 c.p.m./ml of probe in 1× hybridization solution and 250 μg/ml salmon sperm DNA at 65°C for 24 h. After hybridization, blots were washed three times for 30 min at 65°C with a buffer containing 0.5× SSC and 0.5% SDS. The *Drosophila* developmental RNA blot was probed with linearized c-*Ric-2* and reprobed with *Drosophila Ras1*. The human RNA blots were probed with the inserts of the human *RIN* clone, 39507, or the human *RIT* clone, 268332, and reprobed with a 2.0 kb β-actin cDNA supplied by Clontech.

RNA blots were stripped by heating sterile water containing 0.5% SDS to 95°C, placing wet blots into the solution with minimal exposure to air, incubating with shaking for 10 min at 65°C, and allowing to cool at room temperature for 10 min before removing the blots.

Construction of transgenic lines

The 2.4 kb *Bam*HI–*Hin*dIII fragment from pninac.1, containing the *ninaC* promoter (Porter *et al.*, 1992), was cloned into pHXK to create pRetinaTrap. A portion of the 3′ *ninaC* open reading frame and 2.3 kb of the *ninaC* 3′ untranslated region (Porter *et al.*, 1992) was subcloned into the *Eco*RI site of pRetinaTrap to create pRhabTrap.

The cDNA clone c-*Ric*-1 was digested with *Msc*I and *Eco*RV and religated to create the plasmid, pROC, thus removing 1166 nucleotides of 3′ untranslated sequence (nucleotides 1070–2236). The *Ric* open reading frame was amplified from pROC by PCR with the primers SK (CGC TCT AGA ACT AGT GGA TC) and o*Eco*RocStop (CAA AAA GAA TTC GGT CTA GTT AAG GTT TCG TC) and the PCR product was used to replace the *Eco*RI Ral fragment of pMT_3N-MycRal23V (Emkey *et al.*, 1991; Urano *et al.*, 1996) to create pMT_3MycRoc. The open reading frame from pMT_3MycRoc was amplified by PCR with the oligonucleotides oFixMyc (GAT ACC GTC GAC TCT AGA GGC AAA ATG GAA CAA AAG CTC ATT TCT GAA GAA GAC) and o*Hin*dRocStop (CAA AAA AAG CTT GGT CTA GTT AAG GTT TCG TC). The 5′ primer, oFixMyc, added a *Sal*I site, a consensus translation start sequence, CAAAATGG (Cavener, 1987), and destroyed a *Hin*dIII site present in the Myc-tag sequence. The 3′ primer, o*Hin*dRocStop added a *Hin*dIII site. The PCR product was digested with *Sal*I and *Hin*dIII and used to substitute the *Sal*I–*Hin*dIII insert of pRhabTrap to create pSMR. The *Not*I fragment containing the chimeric gene was cloned into the transformation vector pDM30 (Mismer and Rubin, 1987) to create pΠMR. All constructs generated by PCR were sequenced to ensure that no spurious mutations were present.

The chimeric gene carried by pΠMR was introduced into the germline of ry^{506} flies by P-element mediated germline transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Multiple transformant lines, designated P[*MycRic*], were isolated and the lines were made homozygous for the transgene.

Calmodulin-binding assays

The plasmid, pROC, was digested at the polylinker sites, *Bam*HI and *Sal*I, which flank the 5′ and 3′ ends of the *Ric* insert, and the insert was subcloned into the β-galactosidase expression vector, pUR278 (781), to create pZROC for expression of the full-length RIC fusion protein. All other fusion proteins were expressed as glutathione-*S*-transferase (GST) fusions using the vector, pGEX-5X-3 (Pharmacia Biotech Inc.). The pGEX-5X-3 clones pXR107C, pXR77C, pXR58C, pXR48C, pXR39C, pXR26C, pXR20, pXR38 and pXRΔ10C were used to express RIC fusion proteins containing amino acids 158–264, 188–264, 207–264, 217–264, 226–264, 239–264, 242–261, 207–244 and 1–254, respectively. The pGEX-5X-3 clone pXHROC1(74C) was used to express a fusion protein containing the C-terminal 74 amino acids of RIN. Induction of fusion protein expression was performed in freshly-transformed *Epicurian coli* BL21(DE3)pLysS cells (Stratagene) as described by the manufacturer.

The calmodulin overlay assay was performed with biotinylated calmodulin (Life Technologies) as described by the manufacturer. To perform overlays in the absence of free Ca^{2+}, 5 mM EGTA was included in all buffers and no $CaCl_2$ was added. Calmodulin–agarose binding and coimmunoprecipitations were performed as described (Porter *et al.*, 1995) in a buffer containing 10 mM imidazole, pH 7.35, 10% sucrose, 5 mM $MgCl_2$, 1 mM DTT, 500 mM KCl, 0.1% Igepal (Sigma), 1 mg/ml Pefabloc SC (Boehringer Mannheim), 2 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM EGTA and either no $CaCl_2$ (<1 nM free Ca^{2+}) or 5.5 mM $CaCl_2$ (~100 μM free Ca^{2+}). To reduce the increase in free Ca^{2+} upon the addition of antisera (which contains ~2 mM free Ca^{2+}) during immunoprecipitations, 2 mM EGTA was added to the antisera prior to use. In all solution assays, protein extracts were prepared from P[*MycRic*) transgenic flies, and RIC protein was immunoprecipitated or detected with an anti-Myc 9E10 monoclonal antibody (2.5 μg and a 1:10 dilution, respectively; Calbiochem). Protein blots were performed with an anti-*Drosophila* calmodulin polyclonal antibody (1:1000 dilution; gift from K.Beckingham), an anti-bovine calmodulin monoclonal antibody (1:1,000 dilution; UBI) or an anti-Myc 9E10 monoclonal antibody (1:10 dilution; Calbiochem) as previously described (Porter *et al.*, 1992, 1995).

Database searches

The NCBI Expressed Sequence Tag (EST) Database, dbEST, was searched with the BLAST heuristic local multiple alignment algorithm (Altschul and Lipman, 1990; Altschul *et al.*, 1990; Gish and States, 1993) using a BLOSUM-62 scoring matrix (Henikoff and Henikoff, 1993).

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