Androcam Is a Tissue-specific Light Chain for Myosin VI in the Drosophila Testis*

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Myosin VI, a ubiquitously expressed unconventional myosin, has roles in a broad array of biological processes. Unusual for this motor family, myosin VI moves toward the minus (pointed) end of actin filaments. Myosin VI has two light chain binding sites that can both bind calmodulin (CaM). However unconventional myosins could use tissue-specific light chains to modify their activity. In the Drosophila testis, myosin VI is important for maintenance of moving actin structures, called actin cones, which mediate spermatid individualization. A CaM-related protein, Androcam (Acam), is abundantly expressed in the testis and like myosin VI, accumulates on these cones. We have investigated the possibility that Acam is a testis-specific light chain of Drosophila myosin VI. We find that Acam and myosin VI precisely colocalize at the leading edge of the actin cones and that myosin VI is necessary for this Acam localization. Further, myosin VI and Acam co-immunoprecipitate from the testis and interact in yeast two-hybrid assays. Finally Acam binds with high affinity to peptide versions of both myosin VI light chain binding sites. In contrast, although Drosophila CaM also shows high affinity interactions with these peptides, we cannot detect a CaM/myosin VI interaction in the testis. We conclude that Acam and not CaM acts as a myosin VI light chain in the Drosophila testis and hypothesize that it may alter the regulation of myosin VI in this tissue.

The myosins constitute a superfamily of 18 classes (1 conventional and 17 unconventional) (1), all of which share sequence similarity in the motor domain. It is thought that they also share the ability to translocate along actin filaments. Myosins play roles in numerous cellular activities, such as division, endocytosis, cell movement, and organelle trafficking. In addition to the conserved motor domain, all myosins contain one or more conserved IQ motifs (consensus sequence IQXXRGXXX, where X is any amino acid) that bind calmodulin (CaM), a ubiquitous calcium sensor protein, or CaM-like light chains (2–4). The ATPase activity and motility of unconventional myosins can be regulated by calcium binding to CaM or a CaM-like protein (5).

Members of the myosin VI class appear to be unique among the unconventional myosins in their direction of translocation along actin. They move toward the pointed, or minus, end of actin filaments (6) whereas all other myosins tested move toward the barbed, or plus, end. Recent structural studies of pig myosin VI (7) indicate that this reversed directionality is associated with the two unique inserts in myosin VI, termed insert 2. This sequence is located between the converter region of the motor domain and the IQ motif, and its converter-proximal sequences are proposed to redirect the lever arm in the opposite direction relative to other myosins.

Insert 2 is also proposed to perform a second, structural, role in myosin VI. In the pig crystal structure, its more C-terminal sequences form a continuous helix with the IQ motif, thus potentially extending the redirected lever arm. A CaM molecule (fully Ca2⁺ saturated) is bound to the helical region of insert 2, in addition to the CaM molecule bound at the IQ motif. Hydrophobic residues spaced in a 1–6–14 configuration (7), a variant of the 1–8–14 spacing found in other CaM-binding proteins (8), act to anchor the insert 2 helix to CaM. The conformation imposed by this interaction positions CaM to make significant contacts not only with insert 2 but also with the adjacent converter domain. Thus, binding to insert 2 in essence converts CaM into a structural component of the myosin VI head region.

As in other myosins, the CaM or CaM-like molecule bound to the single lever arm IQ motif is thought to provide calcium regulation of myosin VI function. In at least one case, myosin X, a protein other than CaM, termed calmodulin-like protein, is used as a light chain to interact with the IQ motif (9). Intriguingly, a peptide version of the IQ motif of pig myosin VI is able to bind either CaM or a human myosin II essential light chain (10), suggesting that myosin VI might also use light chains other than CaM.

Numerous in vivo functions have been assigned to myosin VI in different biological contexts. It is involved in endocytosis in

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¶ This abbreviations used are: CaM, calmodulin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Acam, Androcam; GST, glutathione S-transferase; C-Acam, C-terminal-half of Acam.

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mammalian cells (11, 12), protein localization and stabilization in neuroblasts and migrating ovarian cells in Drosophila melanogaster (13, 14), maintenance of stereocilia in the mammalian inner ear (15, 16), and creation or preservation of an actin barrier during spermatogenesis in the nematode Caenorhabditis elegans (17). It is also required for the stability and continued movement of an actin structure used during spermatid individualization in Drosophila (18, 19). Although these seem like very diverse roles, they may all involve a common mechanism based on the ability of myosin VI to serve as an anchor (20, 21).

We have focused on the role of myosin VI during sperm individualization in Drosophila. In this final stage of spermatogenesis, 64 syncytial spermatids are separated into individual cells via actin cones that move down the axonemes and reorganize the membrane around them. Myosin VI is found at the flat, leading edges of these cones and its presence is required for their stability and continuous movement. Thus in myosin VI mutants, the actin cones are severely disrupted and individualization fails (18).

How myosin VI activity is controlled in this context is unknown. However, one clue comes from the discovery that a testis-specific CaM-like protein, Androcam (Acam), also localizes to the fronts of the actin cones in individualizing spermatids (22). Acam is 68% identical in overall sequence to Drosophila CaM (with the C-terminal domain being closely similar) but Acam transcripts are only detectable in the testis (22–24). In vitro Acam has different calcium affinities than CaM: the affinities of the strong and weak calcium binding sites are ~0.04 μM and 100 μM, respectively, compared with 0.15 μM and 15 μM for CaM (25). Further, the calcium-induced conformational changes in the protein clearly differ from those in CaM (25). Thus in the testis, Acam might play roles distinct from those of CaM, perhaps acting as a calcium sensor at calcium concentrations outside the response range for CaM. Here we investigate whether Acam might be a tissue-specific light chain for myosin VI. We report that both in vivo and in vitro experiments support this hypothesis, and indicate that Acam could interact with both insert 2 and the IQ motif of myosin VI in vivo. In contrast, although in vitro experiments also demonstrate strong binding of CaM to the relevant myosin VI peptides, we cannot detect an interaction between these two proteins in the testis.

**EXPERIMENTAL PROCEDURES**

Immunolocalization—Testis preparations were performed as previously described (19). Antibodies used were mouse monoclonal anti-myosin VI (3C7) (37) at 1:20 dilution and rabbit polyclonal anti-Acam (RU158) (22) at 1:1000 dilution. Secondary antibodies were Alexa-488 or -568 anti-mouse or anti-rabbit (Invitrogen, Carlsbad, CA). Actin was visualized by incubating antibody 3C7 culture supernatant with protein G-agarose beads (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 0.1 M sodium phosphate, 0.15 M sodium borate, pH 7.2 for 2 h at 4 °C while rotating. Beads were then rinsed twice in testis IP buffer (100 mM NaCl, 1% Nonidet P-40, 20 mM Hepes pH 7.5, 2 mM ATP, 1 mM protease inhibitor mixture (Sigma)). For extracts made with Ca^{2+}, 2 mM CaCl_2, and 5 mM MgCl_2 were also added. Ca^{2+}-free extracts were made by including 2 mM EGTA. Thirty-five males of each genotype were collected within 24 h after eclosion and dissected as described (19); testes were kept on ice until all samples were prepared. They were rinsed twice in buffer (+2 mM Ca^{2+} or 2 mM EGTA), allowing the testes to settle by gravity. Testes were crushed in 100 μl of the same buffer using a pestle designed for a 1.5-ml microcentrifuge tube. The extract was spun at 9,000 rpm for 10 min in an SS34 rotor, and supernatant was diluted to 200 μl, added to antibody-bound beads, and incubated at 4 °C for 4 h with rotation. A sample of the supernatant was collected, and the beads were washed five times in 1 ml of testis IP buffer; at each wash, beads were transferred to a new tube and pelleted by spinning at 1000 rpm in an SS34 rotor. Antibody-bound beads were resuspended in sample buffer, boiled, and loaded onto a 15% polyacrylamide gel. 10 μl each of crude extract and supernatant (super in Fig. 2) were also run on the gel. Ovary extracts were made in the same manner using ovaries from 10 females that were fed yeast for 3 days. Myosin VI mutant testes or ovaries were isolated from jar322/ Df(3R)S87.5 (14) flies that have no myosin VI expression in the ovaries and testes. Following electrophoresis, gels were blotted to nitrocellulose using a semi-dry blot apparatus. Blots were stained with India Ink in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween), blocked in TBST + 5% nonfat dried milk, and probed with anti-CaM antibody RC13 (a high affinity anti-Drosophila CaM rabbit polyclonal antibody previously characterized in the Beckingham laboratory, Ref. 39) at 1:1000 dilution at 4 °C overnight. Blots were then washed in TBST, probed with horseradish peroxidase-conjugated anti-rabbit secondary at 1:20,000 dilution, washed, and signal was detected using the SuperSignal West Pico chemiluminescent substrate kit from Pierce. Testis blots were then stripped and re-probed with anti-Acam antibody RU-158 at 1:1000 dilution, and signal was detected as described above.

**Yeast Two-hybrid Assay**—Plasmid pMyo-head was constructed as follows: N terminus of myosin VI from the start codon through amino acid 911 was PCR-amplified using primers that introduced an N-terminal NcoI site and a C-terminal Xmal site, sequenced in Topo- TA vector (Invitrogen), and ligated in-frame into bait plasmid pAS1-CYH2 (indicated pAS in Fig. 3) cut with NcoI and Xmal. Plasmid pMutIQ was derived from pMyo-head using mutagenic PCR to change the IQ motif sequence from VIJAQRIARGF to VLAARIAAGF; the amplified region was sequenced to confirm that there were no unintended base changes. Plasmids plns-tail, pIQ-tail, and pCC-tail were generated by PCR amplifying the coding region of myosin VI from amino acid 755, 804, and 833, respectively, to the C-terminus using primers that introduced an N-terminal Ndel site and a C-terminal BamHI site. Resulting PCR products were cloned into Topo-TA vector, sequenced, and ligated into pAS1-CYH2 cut with Ndel and BamHI. Plasmid pAcam was generated by ligating a Ncol-EcoRI fragment encoding Acam in-frame into pACTII (Clontech) cut with Ncol and EcoRI.

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5 J. K. Morrison and K. G. Miller, unpublished data.
Plasmid pCam was constructed by PCR amplifying the CaM coding sequence using primers that introduced an N-terminal Ncol site and a C-terminal Xhol site. The PCR product was sequenced in Topo-TA vector and ligated into pACTII cut with Ncol and Sall. Preparation of yeast media, transformations, and assays were all performed as described in the Matchmaker Two-Hybrid System (Clontech) manual. Yeast strain Y-190 was used throughout. In each experiment, 6–8 transformant colonies of each plasmid pair were patched onto plates lacking tryptophan (to select for the bait plasmid) and leucine (to select for the prey plasmid) and after 1 day were replica-plated onto plates lacking tryptophan, leucine, and histidine and containing 25 mM 3-aminotriazole. Two days later the original patch plates were used for X-gal assays. Significant growth relative to controls on plates lacking histidine and dark blue in the X-gal assay indicated a strong interaction (+ +), whereas weaker growth and lighter blue indicated weaker interaction (+) and no growth and no blue-ness indicated no interaction (−). Each plasmid pair was tested in at least three separate transformations.

Peptides and Proteins—The following peptides were prepared. IQ peptide, YRNKCVLIAQRIARGFLARKQHRPRYQ; mutIQ peptide, YRNKCVLAARIAAGFLKQRHPRPYQ; insert2 peptide, AKVKKWLIRSRWVKSALGALCVIKLRNRI. Peptides were synthesized with an N-terminal acetyl group and a C-terminal amide group. The mutIQ peptide and the insert2 peptide were synthesized by the Protein Chemistry Core Laboratory of Baylor College of Medicine (Houston, TX) and were characterized by HPLC and amino acid analysis. The IQ peptide was synthesized by the University of Bristol Peptide Synthesis Facility. Other peptides described previously (28) and used here were; the CaM binding region of neuromodulin, ATKWQASFRGHITRKKLKG; the chick myosin VI IQ motif, YRASACIKIQKTIRMWLCKRKHKPRIDGL; and a high affinity CaM-binding peptide CBP1, LKLLKCLKKLKCLLKG (29). The IQ peptide and the mutIQ peptide were dansylated at their position 5 cysteines by incubating with excess dansyl maleimide in 10 mM Hepes (pH 7.3) and separating the labeled peptide from the free dye on a NAP-5 column. Concentrations of the labeled peptides were determined using 𝜀330 = 4300 m M⁻¹ cm⁻¹.

Drosophila CaM and Acam were expressed in Escherichia coli and purified essentially as reported earlier (25, 36). To generate a construct expressing the C-terminal-half of Acam (C-Acam), a PCR fragment, cloned into vector pCR2.1 (Invitrogen), was used that contained residues 79–147 of Acam preceded by a Xhol site and an ATG codon. The Xhol site was positioned so as to permit transfer of the Acam fragment as a Xhol fragment into the Sall site of vector pGEX-6P-3 (GE Healthcare Life Sciences). This generated a glutathione S-transferase (GST) fusion construct that was expressed in E. coli. The fusion protein was affinity-purified on glutathione-Sepharose, and the C-Acam was cleaved free from the bound GST using PreScission protease as recommended by the manufacturer (GE Healthcare Life Sciences). The C-Acam was then further purified using phenyl-Sepharose, as for the full-length protein.

Peptide Binding Studies—Protocols for the peptide binding experiments and calculations for the 𝐽𝐩 determinations were essentially as described previously (27). All titrations were performed at 20°C. For assays of holoproteins, titrations were performed in 25 mM Tris, 100 mM KCl, 1 mM CaCl₂, pH 8.0. For assay of apoproteins, titrations were performed in 25 mM Tris, 100 mM KCl, 1 mM EGTA, pH 8.0. For titrations involving fluorescence of the dansyl moiety, excitation was at 330 nm and emission was measured at 500 nm. For monitoring fluorescence from tryptophan in Acam or the insert2 peptide, excitation was at 290 nm and emission was measured at 370 nm.

RESULTS

In Vivo Interactions of Acam with Myosin VI—During Drosophila spermatogenesis, myosin VI localizes to the fronts of actin cones, structures that are crucial to the individualization step in forming mature sperm (18). Although Acam has a wider range of localization sites in the testis, previous studies indicated that one of its sites of accumulation is also on the actin cone fronts (22). To begin to ask if Acam and myosin VI function together at this site, we examined whether they show precise colocalization at this position. We co-labeled wild-type testes with antibodies specific for myosin VI and Acam. The Acam antibody used, RU158, has a ~100-fold higher specificity for Acam than CaM (22). We found that in individualizing spermatids, myosin VI and Acam showed perfect co-localization in a series of discrete, tight bands (Fig. 1A). Co-labeling with anti-Acam antibody and phalloidin to visualize actin, we established that these bands are located at the extreme leading edges of the actin cones (Fig. 1B). If Acam is functioning as a light chain for myosin VI at this site, then its localization there should be dependent on the presence of myosin VI. To test this possibility, we used the anti-Acam antibody to label testes from myosin VI mutant flies (jar¹/jar¹), which have extremely reduced myosin VI levels in the testes (18). The enrichment of Acam at the fronts of actin cones seen in wild type was not observed in the myosin VI mutant. Acam was instead dispersed along the cones (Fig. 1B). Thus, Acam localization at the fronts of actin cones in individualizing spermatids is myosin VI-dependent.

If Acam functions as a light chain for myosin VI during spermatogenesis, then the two proteins should form a complex. To search for such complexes, we made extracts from wild-type testes and performed immunoprecipitations using a myosin VI-specific antibody. Immunoblot analysis revealed that Acam was present in the precipitates in the presence of calcium but not in its absence (+ EGTA) (Fig. 2). As a control, we performed the same experiment using extracts made from the testes of a myosin VI mutant combination that has no detectable myosin VI expression (see “Experimental Procedures”). No Acam was immunoprecipitated (Fig. 2), indicating that the interaction of Acam with the anti-myosin VI antibody beads is dependent on myosin VI. The above results indicate that myosin VI and Acam colocalize and are part of the same complex in the testis. But they do not prove that Acam and myosin VI bind to one another directly, as required if Acam is acting as a myosin VI light chain. Further, if Acam does bind directly to myosin VI, additional questions arise; how does its binding compare with that of CaM, a known myosin VI light chain, and to which of the two light chain binding sites (insert 2, the IQ motif, or both) does it bind?
To address these issues, we first determined whether CaM behaved similarly to Acam in terms of association with myosin VI in the testis. In previous immunolocalization experiments (22) we failed to detect any concentration of CaM at the actin cone fronts, but given that this could reflect epitope masking we attempted co-immunoprecipitation as a more definitive assay for an interaction. Both in the presence and absence of calcium, we could not co-immunoprecipitate CaM from the testis with myosin VI antibodies (Fig. 2). In contrast, we could co-immunoprecipitate CaM with myosin VI from ovarian extracts. Although this interaction was detectable in the presence of calcium (data not shown), much stronger co-immunoprecipitation was achieved in the presence of EGTA (see Fig. 2). These findings eliminate the possibility that some technical problem might underlie our failure with testes extracts and indicate that in the testis, specific conditions exist that prevent CaM from interacting with myosin VI.

As our studies represent the first molecular investigations of potential light chain interactions for *Drosophila* myosin VI, we also used the yeast two-hybrid system (26) to examine CaM and Acam interactions further. Our initial bait construct consisted of a Gal4 DNA binding domain fused to a truncated version of *Drosophila* myosin VI that began at the N terminus and extended to just before the coiled-coil domain (amino acids 1–911) (Fig. 3). We used two prey constructs, consisting of a Gal4 activation domain fused to either full-length Acam or full-length *Drosophila* CaM. Using two assays (expression of His3 or a lacZ reporter), we found that both Acam and CaM interacted with this myosin VI construct (Fig. 3).

![Figure 1](https://example.com/fig1.png)

**FIGURE 1.** Acam colocalizes with myosin VI at the fronts of actin cones and this localization is dependent on myosin VI expression. A, wild-type testis stained with both anti-myosin VI and anti-Acam antibodies. B, wild-type and myosin VI mutant (jar1/jar1) testes stained with fluorescent phalloidin and anti-Acam antibody. Arrows indicate the fronts of representative actin cones. Scale bar is 8 μm in A and 4 μm in B.

![Figure 2](https://example.com/fig2.png)

**FIGURE 2.** Acam, but not CaM, co-immunoprecipitates with myosin VI in testis extracts; CaM does co-immunoprecipitate with myosin VI in ovary extracts. Anti-myosin VI antibody-bound beads were exposed to extracts made from wild-type or myosin VI mutant (jar322/DF(3R)S87.5) testes or ovaries, with (Ca) or without (EGTA) calcium. Immunoblots of the immunoprecipitates were then probed with anti-CaM antibody, stripped, and then reprobed with anti-Acam antibody. In all panels, size markers are 29.8 and 20.0 kDa.

To investigate Acam and CaM binding further, we created construct pMutIQ, which contains both insert 2 and the IQ motif but in which the three residues of the IQ motif considered critical for CaM binding (3) were mutated to alanine (that is VLIAQRIARGF was changed to VLAAARIAAGF). As shown
in Fig. 3, CaM showed no binding to the MutIQ fusion protein. This result confirms the importance of the three critical residues of the IQ motif for CaM binding but also indicates that CaM is not binding to the insert 2 sequence in these myosin VI two hybrid fusion constructs. The result for the pIQ-tail construct (see above and Fig. 3) corroborates this interpretation, since absence of the insert 2 sequence in the IQ-tail fusion product did not affect interaction with CaM. Given that CaM is known to bind to the insert 2 region of mammalian myosin VI, these are surprising findings. One possibility is that, in these yeast two-hybrid experiments where CaM is fused to a transcriptional activation domain of approximately its own molecular size, CaM cannot gain access to the insert 2 binding site.

In contrast to CaM, Acam did bind to the Mut IQ construct fusion protein (Fig. 3). This could indicate that despite being part of a larger fusion protein like CaM in these experiments, Acam can gain access to the insert 2 sequence and bind there. However, an alternative explanation is possible. The mutations made to the IQ sequence were designed to prevent CaM binding to this motif. Given that Acam and CaM do not have identical conformations, it is possible that these mutations do not prevent Acam binding to this region.

In Vitro Peptide Binding Studies—To address these ambiguities and to define the binding characteristics of Acam and CaM in more detail, we used peptide binding studies comparable to those described previously (25, 27). Peptides representing the IQ motif (IQ peptide) and the insert 2 region (insert2 peptide) of Drosophila myosin VI were prepared together with a mutant version of the IQ peptide carrying the three alanine mutations used in the yeast two-hybrid experiments (mutIQ peptide). An alignment of the insert 2-IQ motif region for myosin VI from several vertebrates and from Drosophila is shown in Fig. 4, with the peptide sequences indicated. All three peptides were assayed for binding to Acam or CaM in their holo (calcium-bound) and apo (calcium-free) forms.

**Calcium-dependent Binding of CaM and Acam to the IQ and mutIQ Peptides**—As the IQ peptide contains no tryptophan residues, the fluorometric assay that depends on the blue shifted and intensified tryptophan fluorescence upon binding to CaM (27) could not be used. We therefore introduced a dansyl group onto the position 5 cysteine of the peptides and monitored binding using changes in dansyl fluorescence (Fig. 5A). The affinities of the dansylated IQ peptide (d-IQ peptide) for CaM and Acam were determined by direct titration and then the affinities of the non-dansylated peptides were determined by competition titrations in which they were used to displace the dansylated peptide from its complex with either CaM or Acam. This approach was successful for binding assays in the presence of calcium and representative titrations for holoAcam and the IQ peptide are shown in Fig. 5, B and C. The $K_d$ determined for the interaction of the d-IQ peptide with Acam was 9 nM and that for the unmodified peptide, 4 nM (Table 1). The corresponding values for holoCaM were 5 nM and 28 nM, respectively.

Affinities for the mutIQ peptide were determined in the same way. The three amino acid changes in the mutant sequence altered binding affinity for both proteins markedly. Nevertheless, the effect on CaM binding was much more dramatic than on Acam binding. That is, $K_d$ was increased by ~20-fold (to 75 nM) for Acam and by ~500-fold (to 2.5 μM) for CaM (Table 1).

To confirm the validity of the approach we also used competition titrations in which the unmodified IQ peptide was used to displace a tryptophan-containing peptide derived from neuromodulin (Ac-ATKWQ-ASFRGHITRKKLKG-NH$_2$) from its complex with holoCaM ($K_d$, 34 nM, Ref. 28). As shown in Table 1, the $K_d$...
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FIGURE 5. Determination of the affinity of holoAcam for the IQ motif peptide. A, fluorescence emission spectra for free dansylated IQ motif peptide (curve a) and its complex with Acam (curve b). Fluorescence is indicated in arbitrary units (a.u.). B, direct fluorescence titration of dansylated IQ peptide (0.5 μM) with Acam. C, competition titration of dansylated IQ (0.5 μM) + Acam (1.03 μM) with unlabeled IQ peptide. In 8 and C, fluorescence signals were normalized so that the total change in intensity is scaled to run from 0–1.

 TABLE 1

| Peptide protein | d-IQ | IQ | mutIQ | Insert2 |
|----------------|------|----|-------|---------|
| HoloAcam       | 9ⁿ  | 4ⁿ | 75 nmᵇ | 22 nmᵈ |
| ApoAcam        | NDⁿ | 38ⁿ | 700 nmᶜ | NDⁿ |
| HoloC-Acam     | 60ⁿ | 210ⁿ | NDⁿ | NDⁿ |
| HoloCaM        | 5ⁿ  | 28ⁿ | 2.5 μmᶜ | <0.5 μmᶜ |
| ApoCaM         | NDⁿ | 134ⁿ | >50 μmᶜ | NDⁿ |

ᵃ Kd was determined by direct titration monitored fluorescence of dansyl-IQ (d-IQ) peptide.
ᵇ Kd was determined by competition titration displacing d-IQ peptide.
ᶜ Kd was determined by direct titration monitored by tryptophan fluorescence of Acam.
ᵈ Not determined; complicated by formation of 2:1 peptide:protein complexes.
ᵉ Kd was determined by competition titration displacing the CPB1.
ᶠ Kd was determined by competition titration displacing a neomodulin peptide.
ᵍ Kd was determined by competition titration displacing a chick myosin VI IQ peptide.

Determined in this way for IQ peptide binding to CaM (19 nm), was very similar to that determined via competition experiments with the d-IQ peptide (28 nm).

Calcium-independent Binding of CaM and Acam to the IQ and mutIQ Peptides—Titrations of the dansylated IQ peptide with CaM or Acam in the absence of calcium were complicated by the formation of 2:1 peptide:protein complexes and thus it was not possible to determine the affinities of the apo proteins by this route. In contrast to CaM, Acam has a single tryptophan in its C-terminal domain, which has been shown previously to be conformation-sensitive (25). The fluorescence of this residue is affected by binding of the IQ peptides (Fig. 6A), and we used this change to determine a Kd of 38 nm for binding of the IQ peptide to apoAcam (Fig. 6B). Using the same technique, the affinity of apoAcam for the mut-IQ peptide was shown to be ~20-fold lower (Kd = 700 nm) than that of the normal sequence. The same fluorescence change is also observed in the presence of calcium and we used this to determine a Kd of 8 nm for Acam binding to the IQ peptide (Table 1) in good agreement with the value (9 nm) determined by competition experiments with the d-IQ peptide (see above).

In the case of apoCaM we used competition titrations with a peptide version of the chick myosin VI IQ motif. Using the previously determined affinity of this peptide for apoCaM (Kd 23 nm; SRM), we determined a Kd of 134 nm for the interaction of apoCaM with the IQ motif peptide, ~4 times weaker than that of the holoprotein. This same approach established that the interaction of apoCaM with the mutIQ peptide is at least 350-fold weaker than the IQ peptide with that of the normal sequence, with a Kd > 50 μM.

In summary, these data for the IQ peptide demonstrate that Acam has a higher affinity for this sequence than CaM in both the apo and holo state and that the alanine mutations present in the mutIQ peptide affect Acam binding considerably less than CaM binding. Altogether, these observations suggest that Acam binds to the IQ motif by a different mechanism than CaM, in both the apo and holo states.

In this context, the known differences in the calcium binding properties of Acam and CaM (25) could indicate that in Acam, the C-terminal domain plays a more dominant role in target binding than in CaM. The C-terminal sites of Acam have a higher affinity for calcium than the comparable sites of CaM, whereas in the N terminus, Acam has only one functional calcium binding site of very low affinity. We thus compared binding of the isolated Acam holo C-terminal domain to the IQ peptide with that of the intact protein. As shown in Table 1, the affinity of the holo C-terminal domain for the IQ peptide was 35-fold lower than that of the intact protein, indicating a significant contribution of the N-terminal domain to the interaction of the peptide with full-length Acam.

Acam and CaM Binding to the Insert2 Peptide—The Drosophila insert2 peptide region contains two tryptophans (Fig. 4), and there is major change in their fluorescence signal upon binding to holoCaM (not shown). Preliminary experiments with this peptide showed that its affinity for holoCaM is

⁶ S. R. Martin, unpublished data.
extremely high. Even at the lowest useable concentration of the peptide (80 nM) the direct fluorescence titration with holoCaM shows no sign of curvature at the end point (Fig. 7A). However, an upper limit for the value of the dissociation constant for this interaction could be defined by performing competition titrations using a synthetic peptide (CBP1) with very high affinity (Kₐ = 6 pm, Ref. 29) for holoCaM (Fig. 7B). By this approach, the Kₐ of holoCaM for the insert2 peptide was less than 0.5 pm. Problems with the formation of 2:1 peptide:protein complexes prevented the determination of a Kₐ for apoCaM with this peptide. However, the properties of the titration curves indicated that the peptide:protein interactions in these complexes are of high affinity.

In contrast to our findings for the IQ peptide, holoAcam proved to have a vastly lower affinity for the insert2 peptide than holoCaM. The Kₐ could thus be determined using competition titrations with the holoAcam: d-IQ peptide complex as in previous experiments (Fig. 7C). The Kₐ of 22 nM establishes that holoAcam has an affinity 4–5 orders of magnitude lower than that of holoCaM. As for apoCaM, formation of 2:1 peptide:protein complexes prevented determination of a Kₐ for apoAcam binding to this peptide but the data again indicated high affinity interactions.

**DISCUSSION**

Here we have presented four lines of evidence supporting the hypothesis that Acam serves as a light chain for myosin VI in the *Drosophila* testis. We have shown that 1) myosin VI and Acam colocalize on actin cones in individualizing spermatids and that this Acam localization is dependent upon the presence of myosin VI, 2) Acam, but not CaM, co-immunoprecipitates from the testis with myosin VI, 3) Acam interacts with myosin VI in the yeast two-hybrid assay, and 4) Acam binds to peptides corresponding to insert 2 and the IQ motif. Our findings thus strongly indicate that the *Drosophila* testis-specific CaM-like protein Acam is a light chain for myosin VI.

The idea that Acam is a tissue-specific light chain for myosin VI leads to many exciting possibilities. Binding of alternate light chains could allow myosin VI to respond to different ranges of calcium concentrations in diverse tissues. Even more speculatively, these alternate light chains could have different effects on the motility and anchoring capacity of myosin VI. In the testis, the ability of myosin VI to stabilize the actin cones during sperm individualization might depend upon the unique regulatory properties of Acam. In other tissues, which do not contain Acam, the actions of myosin VI could be modified by other light chains with alternative regulatory properties. Intriguingly, recent work has suggested that...
myosin VI functions in the salivary gland to promote large-scale secretion and that in this context it is regulated by yet another CaM-like protein, E63-1.7 The ability to co-express myosin VI along with CaM or CaM-like proteins in insect cell lines and to perform in vitro motility studies (for example, Refs. 30 and 31) will provide the opportunity to test such hypotheses.

Our results also indicate that CaM is not a light chain for myosin VI in the testis. Neither of the Drosophila CaM antibodies used previously for immunolocalization studies in other tissues (32) revealed specific staining for CaM on the actin cone fronts (22). Furthermore, immunoprecipitation experiments presented here showed that while CaM could be found in a complex with myosin VI in ovarian extracts, such an interaction was not detectable in testis extracts. While there are at least 20 Drosophila genes encoding additional CaM-like proteins that could regulate myosin VI, Acam could be the only light chain for myosin VI functioning during spermatogenesis.

If Acam is the only myosin VI light chain in the testis, it is reasonable to propose that it must occupy both the insert 2 sequence and the IQ motif, thus potentially altering both the head structure and calcium regulation of myosin VI. Many aspects of our data argue that Acam will occupy the IQ motif in the testis. Acam binds the IQ motif with high affinity (4–8 nM) in peptide form and also binds to this region in larger myosin VI constructs assayed by the yeast two hybrid system. Further, although CaM is three times more abundant in the testis than Acam (22), the affinity of Acam for the IQ peptide is 3–4-fold higher than that of CaM suggesting that it could effectively compete for binding to myosin VI.

Interestingly this higher affinity for the myosin VI IQ motif stands in contrast to the affinity of Acam for another class of CaM target binding regions (variants of the skeletal muscle myosin light chain kinase CaM binding region), to which Acam binds extremely weakly (25). Further, Acam appears to bind the myosin VI IQ motif in a different manner than CaM. In the yeast two-hybrid experiments, the MutIQ construct bound Acam but not CaM. The peptide work then established that the mutations present in the MutIQ construct affect CaM binding much more strongly than they affect Acam binding (see Table 1). Thus altogether, both our in vivo and in vitro data argue for a specific, tight interaction of Acam with the IQ motif in the testis that could allow Acam to regulate myosin VI differently than CaM.

The high affinity of Acam for the insert 2 peptide (22 nM) indicates that Acam could also interact with the insert 2 region of myosin VI in the testis. In the crystal structure of pig myosin VI, the CaM bound to insert 2 has calcium occupying all four binding sites (7) and there are four points of contact between the holo N terminus and the insert 2 helix. Nevertheless, earlier studies demonstrated that the N-terminal calcium binding sites are not required for CaM binding to insert 2 (10), suggesting that Acam, which has only one very weak N-terminal calcium binding site (25), could occupy this site in the intact myosin VI protein.

However, one aspect of our findings is hard to reconcile with the idea that Acam occupies the insert 2 site in vivo: CaM has an overwhelmingly higher affinity (10,000 fold) for the insert 2 peptide than Acam suggesting that, based on simple competition alone, CaM would occupy this site. Our discovery that CaM does not appear to bind to myosin VI at all in the testis suggests that additional factors must favor an interaction with Acam over CaM in this tissue. Two alternative explanations are: (i) that there is no light chain occupying the insert 2 site in the testis or (ii) that a third, as yet unidentified protein, occupies this position.

Although CaM does not appear to be a light chain for myosin VI in the testis, our data for the ovary indicate that it does serve as a light chain for this motor in tissues that do not express Acam. Thus our results concerning CaM binding to the insert 2 and IQ motif peptides are important for understanding myosin VI regulation in other cellular contexts. Our finding that the CaM/myosin VI interaction in the ovary persists in the presence of EGTA is strikingly similar to an observation of Bahloul et al. (10) who found that EGTA will not remove the holoCaM molecule bound to insert 2 of mammalian myosin VI. By implication CaM may interact with the insert 2 region in the Drosophila ovary. In fact, the affinity determined here between Drosophila holoCaM and the Drosophila myosin VI insert 2 region (Kd < 0.5 pM) is one of the strongest interactions recorded for CaM and is much higher than that determined previously (Kd 29 nM) for the mammalian myosin VI: CaM pair (10). We have therefore examined the pig myosin VI:CaM crystal structure (7) with a view to understanding this difference. Strikingly, although the insert 2 binding motif is completely conserved in mammalian species (Fig. 4), half of the 14 residues of the 1-6-14 motif of insert 2 are not conserved in the Drosophila sequence. Most of these differences are clustered in the N-terminal region of insert 2, which interacts with the CaM C-terminal domain (Fig. 4). Given that there are three amino acid differences between mammalian CaM and Drosophila CaM, all within the C terminus (residues 99, 143, and 147), it is possible that some of the sequence differences in insert 2 are to accommodate these amino acid differences in Drosophila CaM. Perhaps as a consequence of these alterations, a much tighter interaction is forged. However, examination of the pig myosin VI crystal structure indicates that residues 99, 143, and 147 of CaM do not interact significantly with myosin VI, making this hypothesis unlikely unless the binding interaction between insert 2 and CaM is significantly different in Drosophila.

If, instead, a similar binding interaction is assumed, other sequence differences must be important. In fact, there are two differences between the Drosophila and mammalian myosin VI insert 2 sequences that are likely to affect the interaction with CaM. Lysine at position 2 and tryptophan at position 6 of the 1:6:14 motif are replaced by valine and leucine, respectively, in the Drosophila sequence. These two residues are the only residues of insert 2 that interact with both the N terminus and the C terminus of CaM, with tryptophan 6 being a key anchoring residue. Superficially then, this analysis would suggest a weaker interaction between Drosophila myosin VI and CaM, rather than a stronger one, as we have detected. Clearly, a structure determination of Drosophila myosin VI bound to CaM will be necessary to resolve these issues.

7 A. Andres, personal communication.
Androcam Is a Myosin VI Light Chain

For myosin VI, as for other unconventional myosins, calcium inhibits motility, although additional effects of calcium complicate interpretation of its regulatory role (30, 33). Calcium-induced inhibition has been most studied for myosins 1b and V, both of which contain lever arms composed of multiple, concatenated, IQ motifs. The mechanism whereby CaM–IQ motif interaction regulates these myosins must differ from that for myosin VI, given their additional IQ motifs and the finding that increased calcium levels cause dissociation of CaM molecules from some elements of their IQ motif arrays (34–36). In contrast, high calcium does not cause dissociation of CaM from mammalian myosin VI (33). Our peptide studies show that calcium-induced inhibition has been most studied for myosins 1b and V, given their additional IQ motifs and the finding that increased calcium levels cause dissociation of CaM from mammalian myosin VI (33). Our peptide studies show that calcium in fact increases (5–6-fold) the affinity of CaM and Acam and Dr. John Corrie and Acknowledgments—We thank Daniel McDonald for help with purification of recombinant Acam and C-Acam and Dr. John Corrie (NIMR) for providing us with dansyl maleimide.

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