The rôle of the proline-rich region in A1-type myosin essential light chains: implications for information transmission in the actomyosin complex

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Abstract The proline-rich region of A1-type myosin essential light chains functions as a spacer arm separating an actin binding site at the extreme N-terminus from the remainder of the protein. Alteration of the length of this region leaving the actin binding site intact results in altered actin-activated MgATPase kinetics when these light chains are hybridised into myosin subfragment-1. In the case of a mutant in which the length of the proline-rich region was doubled, actin binding by the light chain was uncoupled from kinetic modulation. The implications of this result for information transmission in the actomyosin complex are discussed.

Key words: Muscle contraction; Actin binding; Proline; Protein engineering; ATPase; Protein-protein interaction

1. Introduction

It has now been shown unequivocally that A1-type myosin essential light chains (ELC) bind actin and that this actin binding event is directly correlated to modulation of the actin activated MgATPase kinetics of the myosin motor [1]. A1-type ELCs of vertebrate striated muscle are distinguished by the presence of 40 or so amino acids at the N-terminus which are not present in the alternative isoform – A2-type [2]. In vertebrate fast skeletal muscle, the two proteins result from differential splicing of the same mRNA transcript [3].

The crystal structure of myosin subfragment 1 (S1, a soluble, proteolytic fragment of myosin which retains the essential motor properties of the intact molecule [4]) shows that the light chains (there are two per S1 molecule: one ELC and one regulatory light chain, RLC) are far (>8 nm) from the site of MgATP hydrolysis and the main sites of actin binding which are located on the heavy chain of S1 [5–7]. Nevertheless, events in this light chain binding domain can have profound effects on the behaviour of the myosin motor. In scallop myosin, calcium ion binding by the ELC switches the motor on [8] and in smooth muscle myosin, phosphorylation of the RLC results in the switching on of the motor (reviewed in [9]). In both cases, a message must pass from the light chain to the distant motor domain.

Although the light chains are not responsible for regulating myosin from striated muscle, they can modulate the kinetics of the motor. A1-type ELCs typically endow S1 carrying this light chain (S1A1) with a lower apparent K_m for actin and a lower k_{cat} for MgATP turnover in the presence of F-actin than A2-type ELCs [10,11]. This suggests that A1-type ELCs increase the affinity of S1 for actin (a result supported by F-actin affinity chromatography studies[12,13]) but slow down the molecular motor. We have recently demonstrated that this increase in affinity results from a direct ELC-actin interaction involving the first 11 residues of the human atrial ELC (HmAtELC, an A1-type ELC [14]) and that removal of these residues results not only in loss of actin binding by the light chain but also in loss of kinetic modulation in S1 molecules carrying it [1]. However, these studies did not address the problem of how the ELC – which is some 8 nm from the actin filament in models of the acto-S1 complex [6,7] – can contact actin. In order to explain the apparent contradiction between our work and the structural work, it is necessary to explain how this gap can be bridged.

The remaining 30-odd residues in the extension on A1-type ELCs is rich in (Xxx-Pro) repeats (where Xxx represents any amino acid; lysine and alanine are most commonly found, Fig. 1). Such sequences form rigid extended structures [15–18] and we tested the hypothesis that this sequence is responsible for bridging the gap between the ELC and actin which is apparent from the models of acto-S1 derived from the crystal structures. To do this, we constructed mutants of the HmAtELC (which we have previously expressed in *E. coli*) in which the length of this region was altered. The effect of these changes was assessed by hybridising the mutant light chains into rabbit skeletal S1 and testing whether the MgATPase kinetics were S1A1- or S1A2-like. The ability of the mutants to bind actin – both in complex with the heavy chain and 'free' in solution – was tested by chemical cross-linking. We found that a mutant with this region deleted (HmAtELCΔXP) and one in which the length was doubled (HmAtELC2XP) endowed kinetics on their respective S1 hybrids which were significantly different from the wild-type HmAtELC. The actin binding ability of both mutants is not compromised by altering the length of the putative spacer arm: both can be cross-linked to actin when in free solution. However, only HmAtELC2XP and the wild-type HmAtELC can cross-link when hybridised into S1.

These results argue strongly for the rôle of the (Xxx-Pro)-

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Abbreviations: S1, myosin subfragment-1; S1A1, S1A2, S1 containing alkali 1 (A1) or alkali 2 (A2) essential light chain; RLC, myosin regulatory light chain; ELC, myosin essential light chain; HmAtELC, human atrial ELC; HmAtELCΔXP, HmAtELC lacking the proline-rich region; HmAtELC2XP, HmAtELC with a proline-rich region twice the length of the wild type; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide; F-actin, filamentous actin; Xxx, any amino acid; TEA-HCl, triethanolamine hydrochloride; $A_{280,1\ mg/ml}$, absorbance at 280 nm of a 1 mg ml^{-1} solution.

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rich region being to position the actin binding site correctly on the surface of actin. Furthermore, the results from the HmAtELC2XP mutant hybrid give rise to speculation on the pathways of information transfer within the acto-S1 complex.

2. Materials and methods

2.1. Construction of plasmids carrying genes encoding mutant HmAtELCs

HmAtELCΔXP (consisting of residues 1–11 of the wild-type sequence [14] fused directly to residue 45 (Fig. 1)) was expressed from a DNA sequence constructed by PCR [19] amplification the wild-type HmAtELC cDNA (a gift from Dr Anna Starzinski-Powitz, University of Cologne, Germany) using a 3′-primer (5′-AAGGATCCA-GACTCTGCTTCACCCTG-3′) which complements the 3′ end of the gene and introduces a *Bam*HI site 3′ to the stop codon along with a 5′-primer (5′-AAACATATGGCTCCCAAGAAGCCT-GAGCCTAAGAAGGAGGCAGACTTCACTGCCG-3′) which complements bases corresponding to amino acid residues 44–50 and introduces a sequence coding for the first 11 amino acids, an initiation (methionine) codon and an *Nde*I restriction site 5′ to this. The identity of this PCR product was checked by exploiting the fact that an *Nde*I-*Apa*I restriction fragment which spans the deletion is reduced from 150 bp in the wild type to 49 bp in the mutant. The PCR product was then subjected to a double restriction enzyme digest using the enzymes *Nde*I/*Bam*HI and ligated into *Nde*I/*Bam*HI cut pET-11c expression vector [20] using standard molecular biology techniques [21].

HmAtELC2XP (consisting of residues 1–32 of the wild-type sequence followed by a repeat of residues 15–32 and the wild-type sequence thereafter) was constructed by cutting pET-11c with the wild-type HmAtELC sequence inserted between the *Nde*I/*Bam*HI sites in the vector with the restriction enzyme *Bsu*36I which cuts uniquely at nucleotide position 92 (which forms part of the codon coding for amino acid 30). This permits the insertion of pre-annealed, synthetic oligonucleotides with sequence corresponding to amino acid residues 15–32 with ends which complement the overhanging ends resulting from the restriction enzyme digest (5′-TGAGCCAGCTCCAGC-TCCAGCTCCAGCTCCTGCACCAGCCCCTGCCCCAGCTCC-3′ and 5′-TCAGGAGCTGGGGCAGGGGCTGGTGCAGGGGCTG-GAGCTGGAGCTGGAGCTGGC-3′) into the *Bsu*36I cut pET-11c-HmAtELC construct.

Both mutant sequences were verified by double stranded DNA sequencing [22].

2.2. Protein preparations

All protein manipulations were carried out at 4°C. Actin [23] and chymotryptic myosin S1 isoenzymes [10] were prepared according to published procedures. Note that chymotrypsin cuts myosin between the ELC and RLC binding sites on the heavy chain resulting in an S1 that has only the ELC associated with it. The wild-type HmAtELC was prepared from a recombinant source. Briefly, the gene sequence was inserted between the *Nde*I and *Bam*HI sites of the expression vector pET-11c [20] and *E. coli* BL21 (DE3) cells transformed with this construct were grown until $A_{600} = 1.0$ in NZCYM medium [21], then induced with 0.4 M IPTG and grown for a further 3 h. Cells were collected by centrifugation and lysed by sonication. The lysate was subjected to ammonium sulphate precipitation and the 40–60% fraction collected and applied to a DEAE-fast flow column (Pharmacia Biotech). Purified ELC was eluted with a 0–0.4 M sodium chloride gradient and dialysed extensively against ammonium hydrogencarbonate prior to freeze-drying. Mutant ELCs were prepared in the same manner. The molecular mass of each protein was verified by matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS).

F-actin was prepared by reconstituting freeze dried G-actin in buffer A (5 mM triethanolamine hydrochloride (TEA-HCl), pH 7.6, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.25 mM DTT) in the presence of 1 mg solid DTT. This solution was dialysed vigorously overnight against buffer A, insoluble matter removed by centrifugation at $80\,000 \times g$ for 30 min and polymerisation to F-actin initiated by the addition of $MgCl_2$ (2 mM, final concentration). This method of actin polymerisation was chosen so as to minimise the ionic strength of the resulting solutions: kinetic differences between S1 isoenzymes are more pronounced (and thus more easily quantified) at lower salt concentrations.

2.3. Discontinuous actin activated MgATPase assays

Prior to analysis, the HmAtELC mutants were hybridised into rabbit skeletal S1A2 using the ammonium chloride dissociation method [11] as modified in [24] and the pure hybrid isolated from free light chains and residual S1A2 by ion exchange chromatography on SP-Trisacryl (IBF Biotechnics, France) [25]. Pure hybrid was collected and concentrated by precipitation in 70% (w/v) ammonium sulphate. Precipitates were taken up in 5 mM TEA-HCl, pH 7.5, 0.25 mM DTT and dialysed against this buffer overnight.

Assays were performed in 5 mM TEA-HCl at 25°C as follows. S1 hybrids were mixed with F-actin prepared as described above. S1 concentrations varied between 0.06 and 0.16 μM. Reactions were initiated by the addition of $MgCl_2$ to a final concentration of 2.5 mM and ATP to a concentration of 2.0 mM and allowed to proceed for 10 min after which time the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 6.7% (w/v). The amount of phosphate released in this time was quantified spectrophotometrically [26]. Rates of phosphate release (in μmol P_i released s^{-1}) were normalised by dividing by the S1 concentration. These activities were plotted against actin concentration and the kinetic parameters (apparent K_m for actin and k_{cat} for MgATP turnover) were derived by non-linear curve fitting to the equation activity = $(k_{cat} \cdot [actin])/(K_m + [actin])$ using the program Fig-P for Windows (Biosoft, Cambridge, UK). All points were weighted equally.

2.4. Cross-linking

Cross-linking was carried out using the zero-length cross-linker EDC. Proteins were mixed in the concentration ratios indicated below and incubated at room temperature prior to cross-linking. Protein concentrations ranged between 10 and 40 μM. EDC was then added to a final concentration of 66 mM and the reaction mixture left for a further 1 h before addition of 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol to terminate the reaction before analysis by SDS-PAGE. Bands were identified by reference to molecular mass standards and the work of others (e.g. [29–31]).

2.5. Analytical methods

SDS-PAGE analysis was performed using 12% slab gels run in 0.1 M Tris-Bicine, 0.1% (w/v) SDS at 50 mA (constant current) [27]. Bands were visualised by staining with Coomassie blue. MALDI-MS [28] was carried out by Mr. P. Ashton, School of Chemistry, The University of Birmingham, UK. Concentrations were estimated by UV spectroscopy using the following values: $A_{280,1\ mg/ml}$ (S1) = 0.80 mg^{-1} ml; $A_{280,1\ mg/ml}$ (ELC) = 0.22 mg^{-1} ml; $A_{290,1\ mg/ml}$ (actin) = 0.63 mg^{-1} ml and molecular masses: HmAtELC, 21 kDa; HmAtELCΔXP, 18 kDa; HmAtELC2XP, 24 kDa; actin, 42 kDa.

3. Results

3.1. Kinetic assays show that neither mutant behaves like the wild type

The results of steady-state kinetic analysis of both mutant hybrids are shown in Fig. 2 and the kinetic constants derived from this analysis are listed in Table 1. The kinetic parameters for rabbit skeletal S1A1 and S1A2 and the S1 (HmAtELC) hybrid under the same conditions are also given. The S1 (HmAtELCΔXP) hybrid clearly has A2-like kinetics, showing that the actin binding site, although intact, is unable to influence the myosin motor. The results with the S1 (HmAtELC2XP) hybrid are more interesting. The hybrid shows a high (i.e. S1A2-like) k_{cat} but a low (i.e. S1A1-like) apparent K_m for actin, suggesting that this hybrid interacts with actin with similar affinity to the wild-type hybrid, yet it turns MgATP over at an S1A2-like rate.

3.2. The actin binding site of both mutant light chains is functional

It was important to ascertain that changing the length of the (Xxx-Pro)-rich region, while leaving the actin binding site, intact does not affect the functionality of this site. The wild-

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             1          11                  31                                  46
HmAtELC      MAPKKPEPKK EAAKPAPAPA PAPAPAPAPA PE..... ..... APKEPAFDPK SVKIDFTA
HmAtELCΔXP   MAPKKPEPKK EA..... ..... ..... ..... ..... ....DFTA
HmAtELC2XP   MAPKKPEPKK EAAKPAPAPA PAPAPAPAPA PEPAPAPAPA PAPAPAPAPE APKEPAFDPK SVKIDFTA

             50                                                  99
HmAtELC      DQIEEFKEAF SLFDRTPTGE MKITYGQCGD VLRALGQNPT NAEVLRVLGK
HmAtELCΔXP   DQIEEFKEAF SLFDRTPTGE MKITYGQCGD VLRALGQNPT NAEVLRVLGK
HmAtELC2XP   DQIEEFKEAF SLFDRTPTGE MKITYGQCGD VLRALGQNPT NAEVLRVLGK
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Fig. 1. Sequences of the wild-type and mutant HmAtELCs. Gaps have been introduced to maximise sequence identity. Numbering is based on the wild-type HmAtELC sequence [14], neglecting the N-terminal methionine residue. Only the first 99 residues are shown; the sequences are identical thereafter.

type protein can be cross-linked to F-actin [1] and we tested the ability of both mutants to bind in the same way. Both clearly do (Fig. 3), showing that the actin binding site is fully functional and unaffected by changes occurring C-terminal to it.

3.3. *The length of the (Xxx-Pro)-rich arm controls actin binding by the mutants in the hybrids*

Rabbit skeletal A1-type ELC can be cross-linked to actin when in complex with the heavy chain [29–31], as can HmAtELC in S1 (HmAtELC) (Fig. 4A). However, if the (Xxx-Pro)-rich region is responsible for positioning the actin binding site then testing for this cross-linking will be a sensitive probe for whether this site is correctly located in the actohybrid S1 complex. HmAtELCΔXP in S1 (HmAtELCΔXP) cannot be cross-linked to F-actin (Fig. 4B), despite our success in cross-linking the free mutant light chain (Section 3.2). Thus, we conclude that this actin binding site is too far from actin to interact.

On the other hand, HmAtELC2XP in complex with rabbit skeletal heavy chain can be cross-linked to F-actin (Fig. 4C). This result is in agreement with the kinetic data on this hybrid which suggests that it binds to F-actin as strongly as the wild type does. This mutant hybrid is particularly interesting because although actin binding (as indicated by positive cross-linking results and low apparent K_m for actin) is correlated to modulation of k_{cat} in the wild type, these two activities of the ELC have become uncoupled in this case.

4. Discussion

4.1. *General*

The (Xxx-Pro)-rich region of A1-type ELCs does function as a spacer arm in S1 hybrids: reduction of its length prevents the actin binding site at the N-terminus of the light chain making contact with actin and this results in S1A2-like actin activated MgATPase kinetics. Increasing the length of the arm also affects the kinetics of the resulting hybrid.

4.2. *Why a rigid spacer arm?*

It is clear from models of acto-S1 based on the crystal structures [6,7] that a large gap needs to be bridged between the ELC and the actin filament if the two are to make contact. None of the residues in the N-terminal extension of the A1-type ELC are observed in this structure [5], because of the enhanced segmental mobility of this region of S1 [15], and it is entirely feasible that the 'missing' residues could bridge the gap. However, to do so they would have to be in an extended chain.

For most sequences this would not be the natural state and actin binding would be accompanied by a transition from a folded to an extended structure by the 'bridging' sequence. This transition would be energetically unfavourable for not only would it involve the breaking of many intra-molecular contacts (enthalpically unfavourable) it would also require the imposition of greater order onto the system (entropically unfavourable). Both these unfavourable influences on the free energy of binding can be overcome by having a sequence (such as the one found in A1-type light chains) which is elongated and rigid prior to binding [15–18]: the enthalpic costs need never be paid and the entropic costs will already have been (largely) paid [32].

There is an alternative (but equivalent) kinetic way of approaching this problem. If the actin binding site were on a flexible arm, its search for its target site on actin would (to a first approximation) be one through three-dimensional space. By fixing the actin binding site on the end of a rigid arm this is reduced, essentially, to a two-dimensional search over a limited area. (In other words we would expect the principal benefits of having a rigid arm would be an increased association rate constant; the dissociation rate constant should be relatively unaffected.)

4.3. *Pathways of communication in the actomyosin complex*

The site of interaction between the ELC and actin is some 8 nm from the site of MgATP hydrolysis [6,33] and furthermore, the ELC and the myosin heavy chain interact with different actin monomers – most likely the ELC interacts

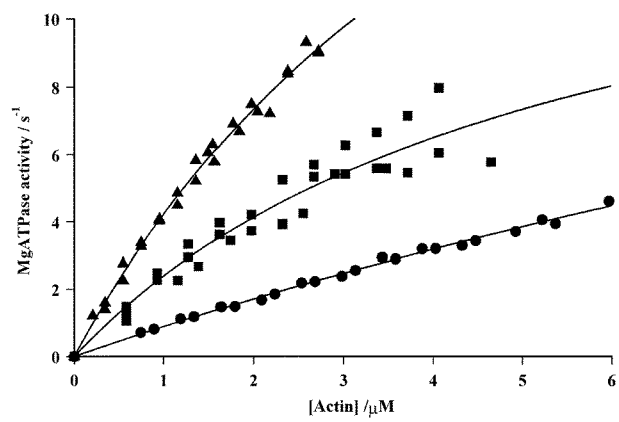

Fig. 2. Actin activated MgATPase kinetics of the wild-type and mutant hybrids. (■) S1 (HmAtELC); (●) S1 (HmAtELCΔXP); (▲) S1 (HmAtELC2XP). The lines were fitted to each data set as described in the text.

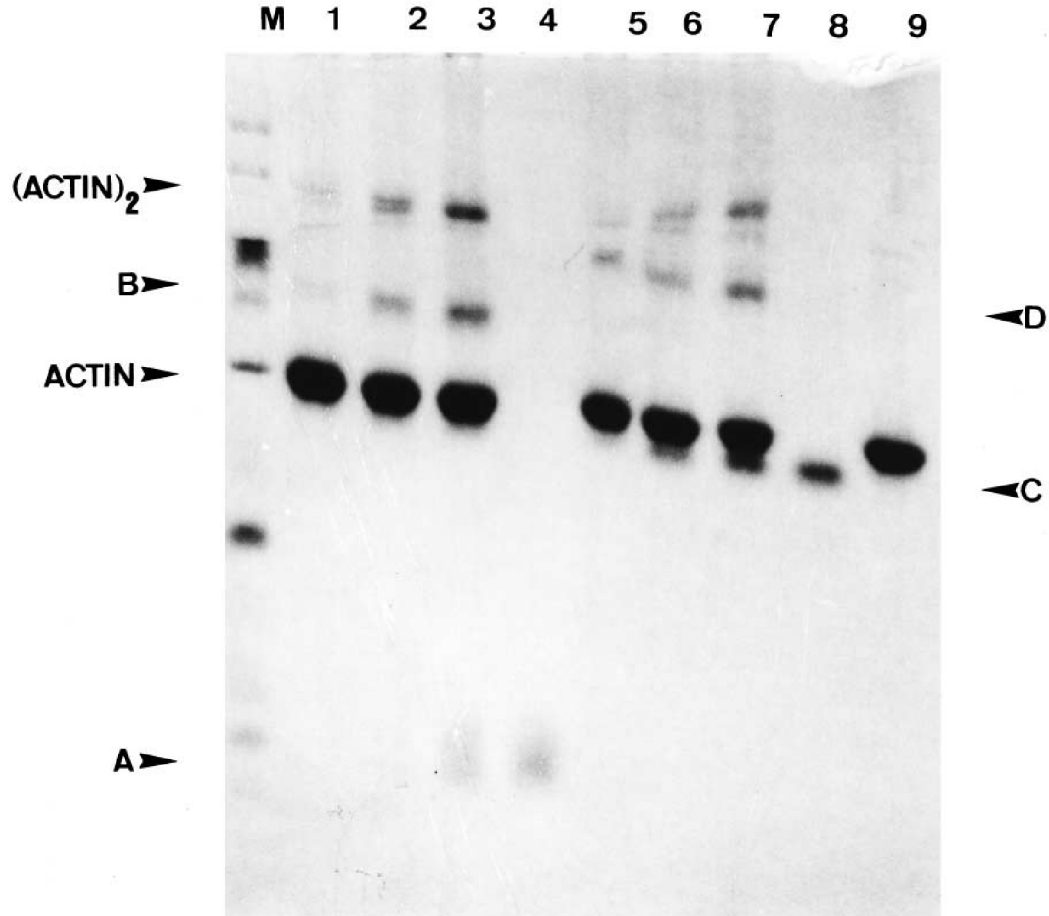

Fig. 3. Cross-linking of mutant HmAtELCs in free solution to F-actin. M, molecular mass markers; lanes 1–3, HmAtELCΔXP:actin 1:2, 1:1, 2:1, respectively; lane 4, HmAtELCΔXP; lanes 5–7, HmAtELC2XP:actin 1:2, 1:1, 2:1, respectively; lane 8 HmAtELC2XP; lane 9; actin. EDC was added in all cases. A, HmAtELCΔXP; B, HmAtELCΔXP-actin; C, HmAtELC2XP; D, HmAtELC2XP-actin.

with the monomer immediately adjacent to the one the heavy chain contacts [31]. There are, therefore, two pathways by which information could be transmitted from the ELC-actin interaction site to the active site: one proceeding via S1 and the other passing through the actin filament.

The results with S1 (HmAtELC2XP) hybrid favour one pathway over the other. In this hybrid, the ELC interacts with actin in a similar manner to the wild type (hence the S1A1-like apparent K_m for actin and the success in cross-linking the light chain to actin in the hybrid) and yet, the message fails to reach the active site. This argues against the 'through S1' pathway – since it is unlikely that this pathway could distinguish between different modes of actin binding: it would be an on/off switch triggered by the binding event. On the other hand, if the additional length of the arm means that the ELC contacts a monomer yet more distant from the sites of heavy chain contact with actin than the wild type (and this seems likely on account of the considerably increased length of the mutant arm) then it is conceivable that this message might 'fade out' as it travels that extra distance within the actin filament.

This would suggest that information transmission in this system proceeds by a different pathway than in thick filament regulated smooth muscle myosin. Since there is no opportunity for light chain-actin interaction in this system, information must pass through the myosin molecule. Although smooth muscle RLC is phosphorylated near the N-terminus, the C-terminus appears to be most important in initiating information transmission to the active site [34]. The pathway does not require the ELC [35] suggesting that the initial stages

Table 1
Kinetic parameters of the mutant hybrids

Hybrid	Apparent K_m for actin (μM)	k_{cat} (s^{-1})	n
RbSk S1A1	4.2±0.6	13.7±1.1	20
RbSk S1A2	31.9±9.9	34.6±9.5	24
S1 (HmAtELC)	5.4±1.5	15.3±2.8	32
S1 (HmAtELCΔXP)	26.6±4.8	24.3±3.8	24
S1 (HmAtELC2XP)	5.7±0.7	28.3±2.7	28

Rabbit skeletal (RbSk) S1A1 and S1A2 and the wild-type HmAtELC hybrid under the same conditions are given for comparison. n, number of determinations.

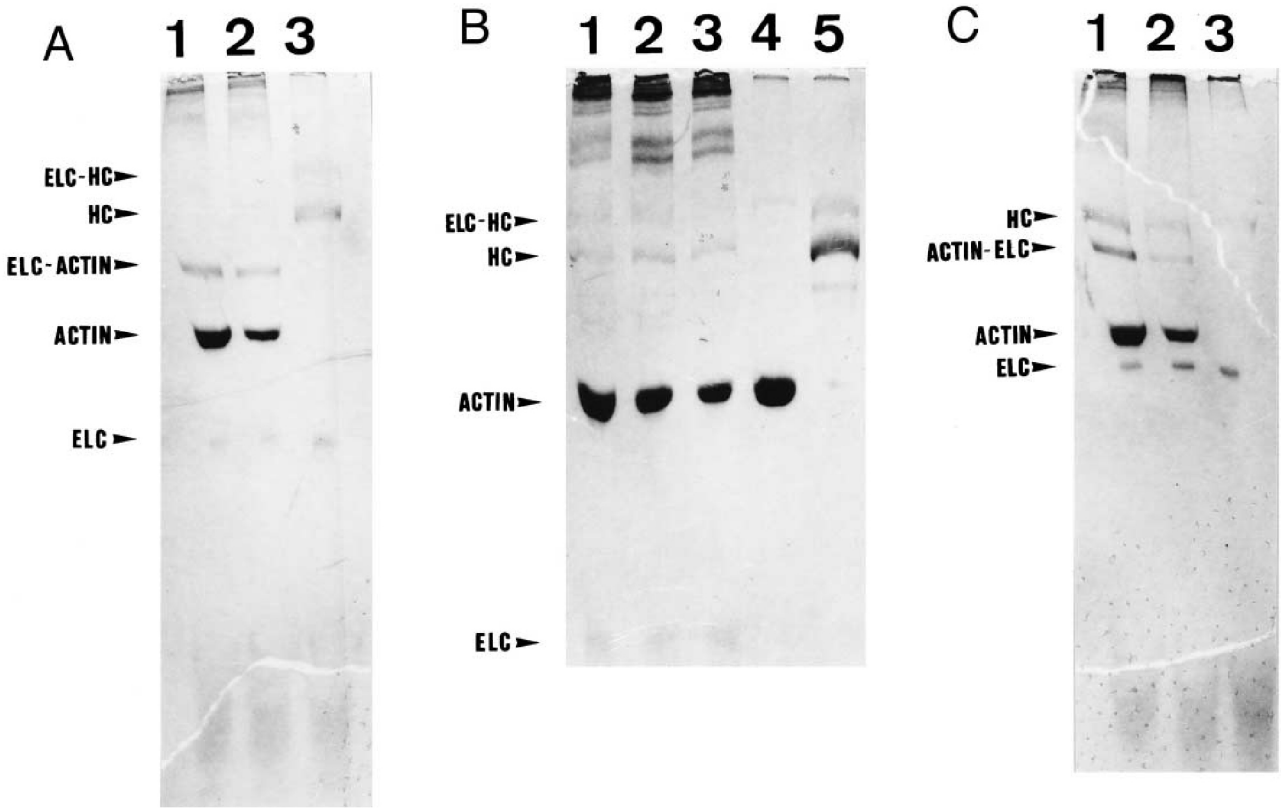

Fig. 4. Cross-linking of wild-type and mutant HmAtELC hybrids to F-actin. (A) Lanes 1,2, S1 (HmAtELC):actin 1:4 and 1:2, respectively; lane 3, S1 (HmAtELC). (B) Lanes 1–3, S1 (HmAtELCΔXP):actin 1:4, 1:2 and 1:1, respectively; lane 4, actin; lane 5, HmAtELCΔXP. (C) Lanes 1,2, S1 (HmAtELC2XP):actin 1:4 and 1:2, respectively; lane 3, S1 (HmAtELC2XP). EDC was added in all cases.

of information transfer involve the N-terminus of the RLC, then the C-terminus and thereafter the myosin heavy chain.

Clearly the acto-myosin system is a complex one – acted upon by many, interacting modulators. It seems likely that different pathways of communication are important in different systems. While determining the rôle of the proline-rich region of A1-type myosin essential light chains we have also been able to suggest what one important pathway in striated muscle myosin might be. A1-type myosin ELCs bind actin and modulate motor function; they do so by transmitting a message to the active site of myosin. Our results suggest that this message is unlikely to pass through S1 and may well be propagated through the actin filament. The possession of a mutant ELC which, when hybridised into S1, results in the uncoupling of the actin binding and kinetic modulation functions of the light chain should enable this important problem to be investigated further.

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