FEBS Letters 567 (2004) 166–170 FEBS 28422

Calmodulin bridging of IQ motifs in myosin-V

Stephen R. Martin, Peter M. Bayley*

Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

Received 12 March 2004; revised 14 April 2004; accepted 23 April 2004

Available online 3 May 2004

Edited by Michael R. Bubb

Abstract **Ca^{2+}-saturated calmodulin binds to double-length IQ lever-arm sequences from murine myosin-V, forming a 1:1 "bridging" complex with very high affinity, ($K_d < 10$ pM for double motifs, IQ34, IQ45 and IQ56). Such a 1:1 complex involves interaction of one calmodulin (CaM) molecule with two adjacent IQ-motifs, providing a molecular mechanism for the observed Ca^{2+}-dependent CaM dissociation from the IQ-region. Structural considerations suggest that formation of the 1:1 complex requires a severe distortion of the lever-arm, potentially regulating functional motility. This would be consistent with a recent report of diverse, irregular shapes of the lever arm of myosin-V induced by the presence of Ca^{2+}.**

Keywords: Calmodulin; IQ motif; Myosin-V; Motility; Calcium regulation; Peptides

1. Introduction

Myosin-V is a double-headed processive motor protein [1], involved in a variety of intracellular vesicle transport processes [2]. As with other unconventional myosins, Ca^{2+}-free calmodulin (apo-CaM) molecules serve as light-chains, bound to the myosin-V lever-arm which comprises six concatenated IQ-motifs [3]. The IQ motifs, Table 1, are almost completely conserved in terms of the consensus IQ sequence, but they also show significant diversity [3]. Ca^{2+} has multiple effects on the function of myosin-V, with different effects on natural myosin-V and recombinant constructs [2]. Thus, at micromolar concentrations, Ca^{2+}stimulates the actin-dependent ATPase of myosin-V, and also stabilises the interaction of (chick brain) ATP-myosin-V with actin [4]. At higher concentration, Ca^{2+} is also observed to inhibit the actin–based motility of recombinant myosin-V in a flow-cell [5]. Such inhibition was associated with dissociation and loss of one or more calmodulin (CaM) molecules, since motility was restored in the absence of Ca^{2+} only by adding exogenous CaM [5]. Similar inhibition/restoration was observed for chicken brush-border myosin-I [6]. In both cases, the interpretation was that binding of Ca^{2+} to the CaM light chain weakened the interaction with an IQ sequence, causing dissociation.

In order to clarify further the complicated effects of Ca^{2+} on this motor protein, we have quantitated the interaction of CaM and its two isolated half-molecule domains with the individual IQ sequences of myosin-V, both with and without Ca^{2+}. Previous work showed unexpectedly that Ca^{2+} *enhances* the affinity of CaM for peptides IQ3 and IQ4 [7]. We have now examined the complete set of peptides IQ1 to IQ6, and further characterised the interactions of double-length peptide IQ34, as well as IQ45 and IQ56. We relate these results to possible regulatory effects of Ca^{2+} on the myosin-V motor protein, and discuss the structural and regulatory implications of formation of the complex in which one molecule of Ca^{2+}-saturated calmodulin (Ca_4CaM) interacts with two adjacent IQ motifs in the lever arm of myosin-V.

2. Materials and methods

2.1. Proteins and peptides

Drosophila CaM and its tryptic fragments were prepared as described [8,9]. The peptides (Table 1) with N-terminal acetylation and C-terminal amidation were purchased from the University of Bristol, and characterised by HPLC and mass spectrometry. Concentration determination used calculated extinction coefficients for peptides [10] and published values for CaM [11].

2.2. Determination of peptide affinities

Titrations of peptides with CaM were performed at 20 °C in 25 mM Tris, 100 mM KCl, and 1 mM DTT (pH 8) with 1 mM $CaCl_2$ or 0.2 mM EDTA, using a SPEX FluoroMax fluorimeter with $\lambda_{ex} = 290$ or 295 nm and $\lambda_{em} = 323$ or 335 nm [7]. Dissociation constants (K_d) for Trp-containing peptides were determined by direct titration, and those for non-Trp-peptides were determined by fluorescence competition assays against a Trp-containing peptide, with analysis as in [7].

3. Results

3.1. Interactions with single IQ sequences

The complexes of peptides IQ1 and IQ5 with CaM and its separate domains have fluorescence properties similar to those of IQ3 [7]; unlike IQ3, simple 1:1 complexes are formed with these peptides. Typical direct titrations with IQ5 are shown in Fig. 1A and B. Competition titrations of the corresponding complexes with IQ5 using IQ6 are shown in Fig. 1C and D. Complexes of CaM with IQ1 titrated with IQ2, IQ4 or IQ6 as competitor are shown in Fig. 1E (+Ca^{2+}) and 1F (Ca^{2+}-free conditions). The competition curves were analysed using the values of the affinities for IQ1 or IQ5 determined as above. Fig. 2 shows the resulting K_d values, which include values for

* Corresponding author. Fax: +44-20-8906-4477.
E-mail address: pbayley@nimr.mrc.ac.uk (P.M. Bayley).

Abbreviations: IQ peptides, see Table 1; CBP1, LKLKKLLKLLKKLLKKLLKLG; CaM, calmodulin; apo-CaM, Ca^{2+}-free calmodulin; holo-CaM; Ca_4CaM, Ca^{2+}-saturated calmodulin; Tr1C, calmodulin N-domain, residues 1–77; Tr2C, calmodulin C-domain, residues 78–148

0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.053

Table 1
IQ peptide sequences of murine myosin-V

IQ1	K L R A A C I R **I Q** K T I **R G** W L L **R** K R Y L
IQ2	C M Q R A A I T **V Q** R Y V **R G** Y Q A **R** C Y A K F L
IQ3[a]	*R R T K* A A T T **I Q** K Y W **R M** Y V V **R** R R Y K
IQ4	I R R A A T I V **I Q** S Y L **R G** Y L T **R** N R Y R *K I*
IQ5	L R E Y K A V I **I Q** K R V **R G** W L A **R** T H Y K
IQ6[a]	R T M K A I V Y **L Q** C C F **R R** M M A **K** R D V K K L

Consensus residues are in bold; tryptophan residues are underlined.
[a] Peptides IQ3 and IQ4 omits the italicised *wt* residues, and peptide IQ6 has S rather than wild-type C at position 11.

IQ3 and IQ4 taken from [7], plus additional values for holo-CaM with IQ3 or IQ4 of 1.2 and 3.5 nM, respectively, derived from direct and competition titrations of holo-CaM with IQ3, performed at low initial concentrations in order to minimise formation of the 2:1 peptide:CaM complex [7].

3.2. Interactions with double-length IQ sequences

Titrations of the double-length peptide IQ34 with holo-CaM (up to a ratio of 1:1.8) show the formation of a 1:1 complex with very high affinity [7]. Similar titrations performed with IQ56 (Fig. 3A) and IQ45 (Fig. 3B) show comparable high affinity, but with an additional subsequent binding step. In both cases, the fluorescence increases linearly with added CaM up to a [CaM]/[peptide] ratio of 1:1 but with a further significant increase at higher ratios. This additional binding saturates at a lower [CaM]/[peptide] ratio when the initial peptide concentration is high. Under these conditions, at high [CaM]/[peptide] ratios additional binding with IQ34 is indeed seen, although it is less pronounced than with IQ45 or IQ56 (Fig. 3C).

Apparent dissociation constants for 1:1 mixtures of holo-CaM with these double-length peptides have been determined using competition assays with the peptide CBP1, which binds to holo-CaM with very high affinity ($K_d \sim 5$ pM [12]). Analysis of the curves shown in Fig. 4 gave K_d values of ~0.4 pM for holo-CaM–IQ34, 0.25 pM for holo-CaM–IQ56, and 12.5 pM for holo-CaM–IQ45.

Analysis of the quantitative behaviour of CaM with the different double-length IQ peptides suggests that, when the double-length IQxy sequence is in excess, a simple 1:1 (bridged) complex is formed (with $K_{d(IQxy)}$); each domain of CaM is bound to one of the IQ motifs x,y. However, when CaM is in excess, a 2:1 CaM:peptide complex can be formed in which each of the constituent IQ motifs binds a single CaM, with affinities assumed identical to those for binding to the single IQ peptides, IQx and IQy. It is readily shown from equilibrium considerations that the absolute concentrations of the 1:1 complex CaM · IQxy and the 2:1 complex $(CaM)_2 \cdot (IQxy)$ will be equal when the *free* CaM concentration is equal to $K_{d(IQx)}K_{d(IQy)}/K_{d(IQxy)}$. This value is 11.4 μM in the case of IQ34, but only 0.41 μM for IQ56 and 0.24 μM for IQ45. Thus, the 1:1 complex persists to higher CaM concentrations for IQ34 than for IQ45 or IQ56. Also amplitude differences in the titrations (Fig. 3) result from IQ34 in the CaM.IQxy complex having ~85% of the fluorescence intensity of $(CaM)_2$IQxy, whereas this percentage is 68% for IQ56 and 62% for IQ45. This correlates with the different position of the Trp in IQ3 compared with that in IQ1 and IQ5 (see Table 1).

4. Discussion

The initial work with IQ3 and IQ4 established a number of generalities which are largely borne out for IQ5 and IQ6, but which illustrate significant differences with IQ1 and IQ2. Thus, (a) for all of the peptides IQ3, IQ4, IQ5, and IQ6, the presence of Ca^{2+} increases the affinity of CaM (or either of its domains), showing that this is a relatively general property, not limited to IQ3 and IQ4; (b) both IQ1 and IQ2 have higher affinity (<10-fold) for interaction with CaM or the C-domain in the presence of Ca^{2+}; (c) the affinities of IQ2 are substantially lower than all the other IQs, possibly due to the presence of the "VQ" motif, although V, I and L are generally considered as conserved hydrophobic residues; (d) all peptides bind more strongly to the N-domain in the presence of Ca^{2+}, though the absolute affinity of IQ1 and IQ2 with apo-N-domain is surprisingly weak, approaching a K_d of 1 mM; this may reflect a possible preference in IQ1 or IQ2 for a light chain other than CaM, as has been reported for chick myosin-V [13], but not found in mouse myosin-V [14]; it is also interesting that a truncated mouse myosin-V with only 2 IQ motifs (plus apo-CaM) shows significantly reduced processivity [15]; (e) the affinity of peptides IQ3–IQ6 for holo-CaM is mainly due to the C-domain interaction of CaM; and (f) the N-domain in Ca-free conditions showed only low affinity, particularly sensitive to ionic strength over the range 5 to 500 mM KCl [7].

The crystal structure of the complex of two molecules of apo-CaM bound to the IQ12 sequence of myosin-V has been described [16]. The apo-CaM/IQ interaction resembles the model structure for the complex of apo-CaM binding to IQ1 of brush border myosin, (1aji.pdb) based on the structure (1wdc.pdb) of the scallop myosin regulatory complex [3]. This shows that the C-domain interacts with several residues of the IQ motif, and the N-domain interacts just C-terminal to its partner C-domain, with relatively few close peptide contacts (A. Houdusse, personal communication). In all these structures, and those of the complexes of mlc1p (the yeast myosin light chain, which lacks Ca^{2+} binding sites), with IQ sequences of the yeast myosin-V homologue (myo2p) [17], it is always the light-chain C-domain which interacts directly with the IQ residues of the motif.

Fluorescence measurements show that binding of peptides IQ1, IQ3, or IQ5 to C-domain perturbs Trp residue emission, similarly to Ca_4CaM. Hence, the primary binding of Ca_4CaM is that of the C-domain to the sequence containing IQ and Trp residues. In vitro, peptides IQ2, IQ4 or IQ6 directly compete (with IQ1, IQ3 or IQ5, respectively) for binding at the same site. In peptide binding to the isolated N-domain, the principal interaction again involves the IQ and Trp residues of IQ1, IQ3 and IQ5. However, in the binding of peptides to the full Ca_4CaM, since the IQ site is preferentially occupied by C-domain, the N-domain must bind, with lowered affinity, to an adjacent site. Thus, as expected, both in the presence and absence of Ca^{2+}, the affinity of a given peptide for CaM is less than the sum of the affinities for the two domains measured separately. Most importantly, the large Ca^{2+}-dependent increase in affinity of the N-domain for the IQ motif previously observed for IQ3 or IQ4 is also shown (to somewhat smaller extent) with IQ5 or IQ6. This effect is essential in forming the 1:1 complex of holo-CaM with a double-length IQ motif (see below).

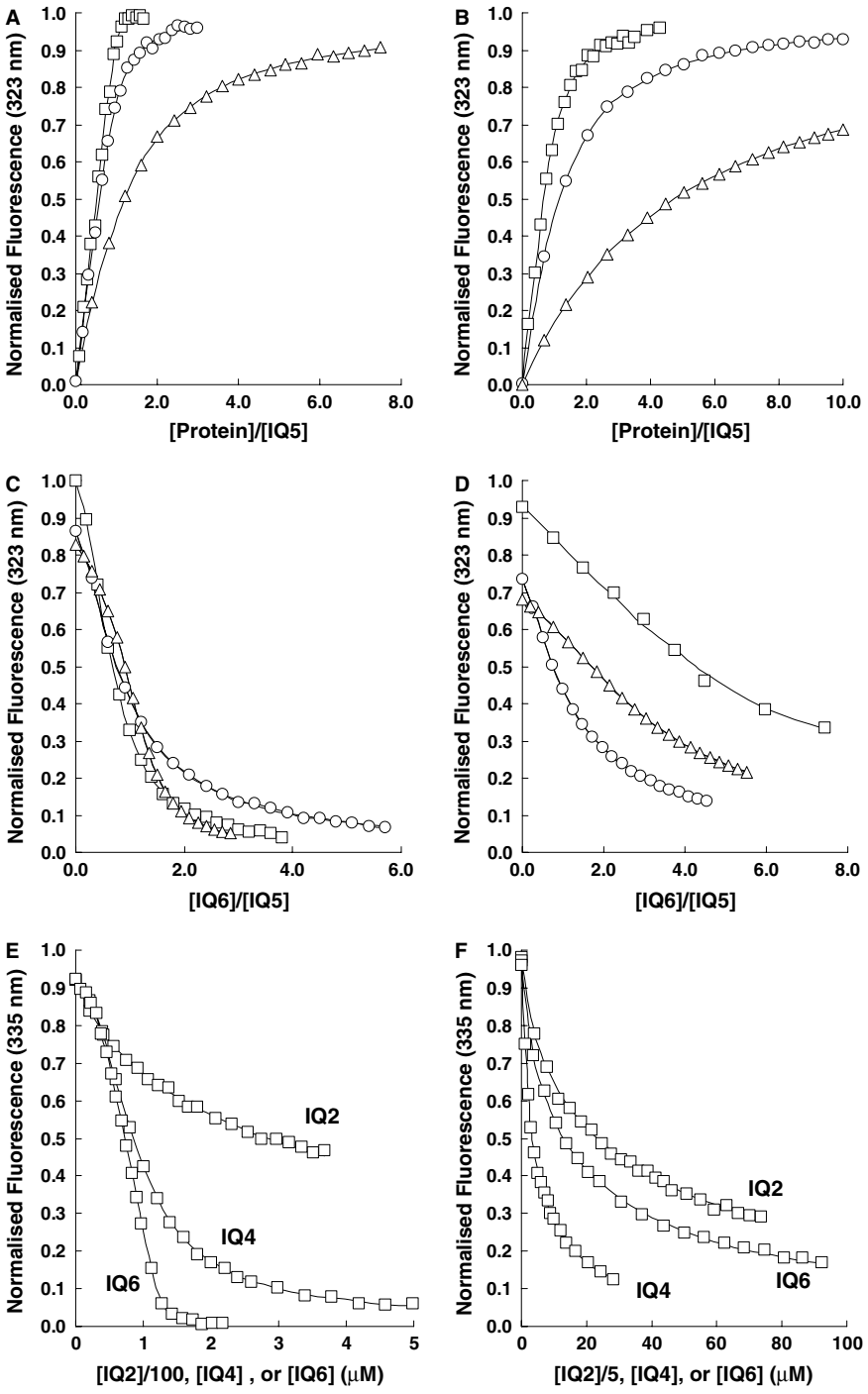

Fig. 1. Determination of dissociation constants for the binding of CaM (squares), Tr2C (circles) and Tr1C (triangles) to the single IQ motif peptides. Direct Trp fluorescence titrations of IQ5 with CaM, Tr2C and Tr1C (A) with Ca^{2+}: [IQ5] = 0.2, 0.5 and 2 μM; and (B) without Ca^{2+}: [IQ5] = 0.5, 2 and 20 μM, respectively. Competition titrations with IQ6 (C) of 1 μM IQ5 + 1.1 μM holo-CaM, 1 μM IQ5 + 1.1 μM holo-Tr2C, and 10 μM IQ5 + 15 μM holo-Tr1C; and (D) of 1 μM IQ5 + 2 μM apo-CaM, 10 μM IQ5 + 11 μM apo-Tr2C, and 100 μM IQ5 + 250 μM apo-Tr1C. Competition titrations of 1 μM IQ1 + 1.25 μM CaM with IQ2, IQ4, and IQ6 using (E) holo-CaM; and (F) apo-CaM. Solid lines are the computed best fits to the raw data. $\lambda_{ex} = 290$ (or 295) nm and $\lambda_{em} = 323$ (or 335) nm.

The most striking finding with peptide IQ34 was the formation of a very stable complex with Ca_4CaM, verified by analytical ultracentrifugation as containing one molecule of CaM and one IQ34 peptide [7]. In order to exclude the possibility that this behaviour was a specific property of the IQ34 sequence, similar measurements were made of the affinities of the equimolar complexes of IQ45 and IQ56 for holo-CaM. Equilibrium displacement reactions of the complexes with a strongly binding peptide (Fig. 4) gave K_d values of 12.5 and 0.25 pM, respectively. As with IQ34, the high affinities are consistent with those for the individual peptides binding to separate CaM domains. We conclude that the bridging of two

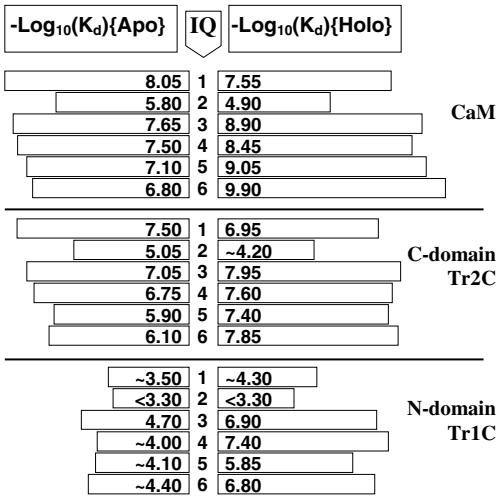

Fig. 2. Dissociation constants for single IQ motifs. The average value of the K_d (from three independent titrations) is reported as $-\mathrm{Log}_{10}(K_d)$ for individual IQ motifs with CaM, and its separate C-domain (Tr2C) or N-domain (Tr1C) in the presence of Ca^{2+} or EDTA (see Section 2).

adjacent IQ motifs by a single molecule of Ca_4CaM is a general property of the IQ3456 region of the lever arm. The high affinity of the interaction and the reduced stoichiometry of one CaM per IQ motif means that one molecule of CaM would be displaced for each bridged complex formed. Since IQ45 cannot engage in this interaction simultaneously with both IQ34 and IQ56, the presence of Ca^{2+} would cause the dissociation of only one or two CaMs, in agreement with experiment [5].

These results indicate a possible general mechanism for the interaction of Ca^{2+}-CaM with multiple sequences of IQ motifs. However, there is an important structural consideration, since the formation of the 1:1 complex poses a question of whether this is compatible with the normal structure of the lever arm, depicted as a continuous α-helix. The distance between the two Q residues of the adjacent IQ motifs, as measured along the α-helical heavy chain in the regulatory region of scallop myosin, is (40 Å) [18]. This is effectively double the 12-residue (20 Å) separation of two key hydrophobic target residues of an α-helical target sequence in typical "1:14" compact Ca_4CaM–peptide complexes [9,19]. In addition, the translational relationship of two light chain domains attached at adjacent IQ residues to the motifs in a continuous α-helix would further obviate double occupancy by a single calmodulin, owing to their disadvantageous relative orientation. We therefore postulate that the formation of the 1:1 complex at one or more sites in the C-terminal half of the lever arm generates a distorted or bent conformation of the myosin heavy chain, most likely in its short sequence between the appropriate IQ motifs. A possible topological arrangement is shown in [7]. Any expenditure of free energy to effect conformational distortion would be available from formation of a bridged 1:1 complex. $K_d = 1$ pM is equivalent to a free energy of ~16.5 kcal/mol (at 300 K).

The structural effect of Ca^{2+} on myosin-V has recently been shown by electron microscopy to produce marked changes in morphology, specifically an increase in flexibility, diversity of overall shape, and a loss of the typical linear structure of the lever-arm region [20]. Such changes are consistent with the

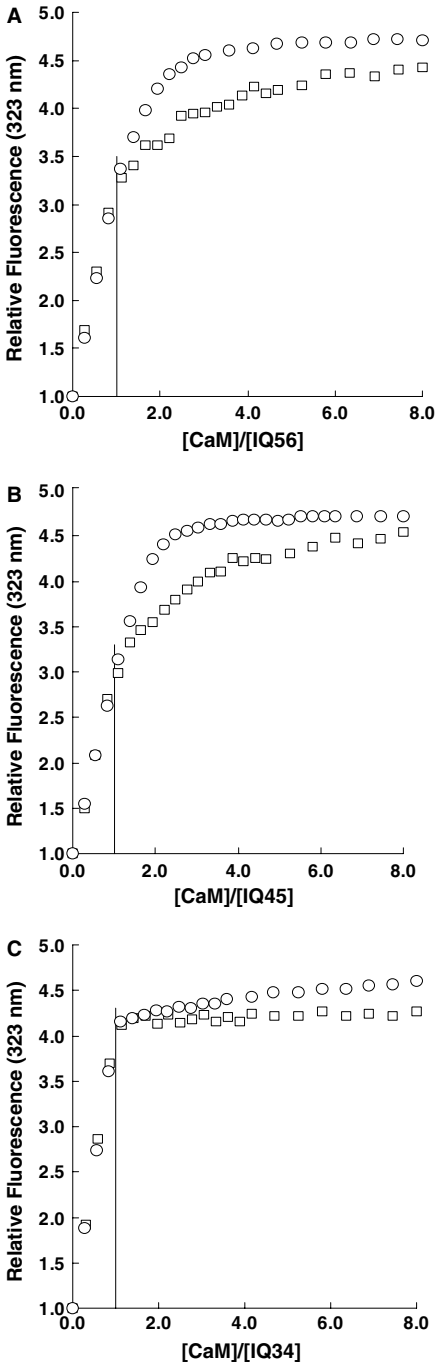

Fig. 3. Titrations of double-length IQ motifs with holo-CaM. Titrations were performed with initial peptide concentrations of 0.25 μM (squares) and 2.5 μM (circles) for IQ56 (A), IQ45 (B), and IQ34 (C) monitoring peptide Trp fluorescence as in Fig. 1. Vertical lines indicate the 1:1 equivalence points.

Ca^{2+}-induced change in the mode of interaction from a 1:1 to a 2:1 complex of Ca_4CaM with the C-terminal half of the IQ region of myosin-V, as observed in the work presented here and elsewhere [7].

Ca^{2+} has been shown to inhibit myosin-V motility at pCa ~ 6, without dissociation of CaM [5]. Since the apparent Ca^{2+} affinity of CaM is enhanced in the presence of targets binding preferentially to the holo form [21] and since the

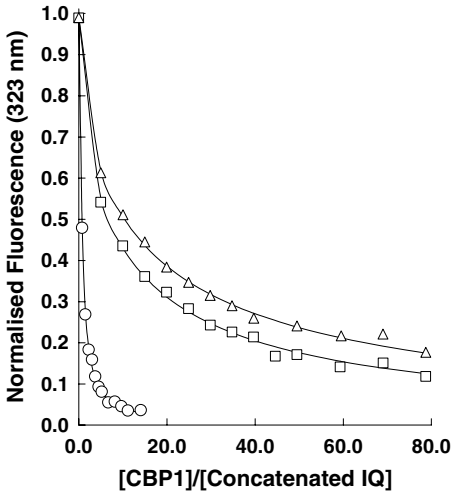

Fig. 4. Determination of the apparent dissociation constant for double-length IQ motifs from holo-CaM. Mixtures of 1 μM CaM and 1 μM peptide IQ34 (squares), IQ56 (triangles), or IQ45 (circles) were titrated with "silent" peptide CBP1, monitoring Trp fluorescence as in Fig. 1.

N-domain shows a greater *relative* Ca^{2+}-dependent enhancement in affinity for a given peptide, the resultant apparent Ca^{2+} affinities will differ for these domains in an IQ-sequence-dependent manner. We suggest that micromolar [Ca^{2+}] could allow population of intermediate species of potentially different degrees of partial Ca^{2+}-saturation and structure. Such a mechanism could provide a further level of complexity of Ca^{2+}-dependent regulation of the activity of unconventional myosin-V.

Acknowledgements: We thank Prof. John Trinnick and Dr. Peter Knight (University of Leeds), and Dr. Jim Sellers (NIH) for helpful discussions.

References

[1] Mehta, A.D., Rock, R.S., Rief, M., Spudich, J.A., Mooseker, M.S. and Cheney, R.E. (1999) Nature 400, 590–593.
[2] Reck-Peterson, S.L., Provance, D.W., Mooseker, M.S. and Mercer, J.A. (2000) Biochim. Biophys. Acta 1496, 36–51.
[3] Houdusse, A., Silver, M. and Cohen, C. (1996) Structure 4, 1475–1490.
[4] Nascimento, A.A.C., Cheney, R.E., Tauhata, S.B., Larson, R.E. and Mooseker, M.S. (1996) J. Biol. Chem. 271, 17561–17569.
[5] Homma, K., Saito, J., Ikebe, R. and Ikebe, M. (2000) J. Biol. Chem. 275, 34766–34771.
[6] Collins, K., Sellers, J.R. and Matsudaira, P. (1990) J. Cell. Biol. 110, 1137–1147.
[7] Martin, S.R. and Bayley, P.M. (2002) Protein Sci. 11, 2909–2923.
[8] Browne, J.P., Strom, M., Martin, S.R. and Bayley, P.M. (1997) Biochemistry 36, 9550–9561.
[9] Barth, A., Martin, S.R. and Bayley, P.M. (1998) J. Biol. Chem. 273, 2174–2183.
[10] Pace, C.N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) Protein Sci. 4, 2411–2423.
[11] Maune, J.F., Beckingham, K., Martin, S.R. and Bayley, P.M. (1992) Biochemistry 31, 7779–7786.
[12] Brown, S.E., Martin, S.R. and Bayley, P.M. (1997) J. Biol. Chem. 272, 3389–3397.
[13] Espindola, F.S., Suter, D.M., Partata, L.B., Cao, T., Wolenski, J.S., Cheney, R.E., King, S.M. and Mooseker, M.S. (2000) Cell Motil. Cytoskel. 47, 269–281.
[14] Wang, F., Chen, L., Arcucci, O., Harvey, E.V., Bowers, B., Xu, Y., Hammer III, J.A. and Sellers, J.R. (2000) J. Biol. Chem. 275, 4329–4335.
[15] Sakamoto, T., Wang, F., Schmitz, S., Xu, Y., Xu, Q., Molloy, J.E., Veigel, C. and Sellers, J.R. (2003) J. Biol. Chem. 278, 29201–29207.
[16] Houdusse, A., Gaucher, J.-F., Mui, S., Krementsova, E., Trybus, K.M. and Cohen, C. (2000) Biophys. J. 78, 272A.
[17] Terrak, M., Wu, G., Stafford, W.F., Lu, R.C. and Dominguez, R. (2003) EMBO J. 22, 362–371.
[18] Houdusse, A. and Cohen, C. (1996) Structure 4, 21–32.
[19] Rhoads, A.R. and Friedberg, F. (1997) FASEB J. 11, 331–340.
[20] Wang, S., Thirumurugan, T., Stafford, W.F., Hammer, J.A., Knight, P.J. and Sellers, J.R. (2004) J. Biol. Chem. 279, 2333–2336.
[21] Bayley, P.M., Findlay, W.A. and Martin, S.R. (1996) Protein Sci. 5, 1215–1228.