Molecular basis for G-actin binding to RPEL motifs from the serum response factor coactivator MAL

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Serum response factor transcriptional activity is controlled through interactions with regulatory cofactors such as the coactivator MAL/MRTF-A (myocardin-related transcription factor A). MAL is itself regulated in vivo by changes in cellular actin dynamics, which alter its interaction with G-actin. The G-actin-sensing mechanism of MAL/MRTF-A resides in its N-terminal domain, which consists of three tandem RPEL repeats. We describe the first molecular insights into RPEL function obtained from structures of two independent RPELMAL peptide:G-actin complexes. Both RPEL peptides bind to the G-actin hydrophobic cleft and to subdomain 3. These RPELMAL-G-actin structures explain the sequence conservation defining the RPEL motif, including the invariant arginine. Characterisation of the RPELMAL-G-actin interaction by fluorescence anisotropy and cell reporter-based assays validates the significance of actin-binding residues for proper MAL localisation and regulation in vivo. We identify important differences in G-actin engagement between the two RPELMAL structures. Comparison with other actin-binding proteins reveals an unexpected similarity to the vitamin-D-binding protein, extending the G-actin-binding protein repertoire.

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Introduction

Actin is a major cytoskeletal constituent that can polymerise to form helical actin filaments (F-actin), the organisation of which contributes to cellular mechanical strength. Regulated assembly, rearrangement and disassembly of F-actin are critical in a wide variety of cellular processes, including cell morphology, cell motility and cellular interactions required for tissue formation and integrity (reviewed by Geiger and Bershadsky, 2001; Revenu et al, 2004; Chhabra and Higgs, 2007). Actin also participates in non-cytoskeletal processes, including transcription and chromatin remodelling, but here its functional roles and the molecular interactions involved are only poorly understood (Miralles and Visa, 2006; Chen and Shen, 2007; Su et al, 2007). One such system is actin-mediated control of the myocardin family transcriptional coactivators MAL/MRTF-A (myocardin-related transcription factor A) and MKL2/MRTF-B, which transduce Rho GTPase signals to the transcription factor serum response factor (SRF) (Cen et al, 2003; Miralles et al, 2003). Binding of unpolymerised actin (G-actin) to the MRTF N terminus inhibits MRTF activity by preventing their nuclear accumulation and repressing transcriptional activation by the MRTF–SRF complex (Miralles et al, 2003; Posern et al, 2004; Vartiainen et al, 2007).

The MRTF regulatory domain contains three copies of the RPEL motif (core sequence RPxxxEL; Pfam accession number: PF02755) (Finn et al, 2006), each of which functions as an actin-binding element (Guettler et al, 2008). Mutations at invariant positions within each RPEL motif impair interaction with G-actin and de-repress the activity of the MRTF proteins (Miralles et al, 2003; Vartiainen et al, 2007; Guettler et al, 2008). Similarly, myocardin, the constitutively nuclear and active founding member of the myocardin family to which the MRTFs belong (Wang et al, 2001), has a greatly reduced affinity for actin, reflecting sequence variations in its RPEL motifs (Guettler et al, 2008). These observations have led to the proposal that MRTF relocalisation and activation are regulated directly by actin through RhoA-induced alterations in the availability of G-actin (Miralles et al, 2003; Vartiainen et al, 2007; Guettler et al, 2008). RPEL motifs also mediate G-actin binding by members of the Phactr/Scapinin family of phosphatase-1-binding proteins, but here their functional significance is unknown (Sagara et al, 2003; Allen et al, 2004).

The actin monomer comprises four subdomains: in the actin filament, subdomains 1 and 3 are exposed at the barbed end, whereas subdomains 2 and 4 are exposed at the pointed actin filament end. F-actin assembly is regulated by actin concentration, by ATP hydrolysis and by interactions
between actin and regulatory proteins that control filament nucleation, polymerisation, severing or maintenance of the G-actin pool itself (reviewed by Pollard, 2007). A common feature in many G-actin-binding proteins is an amphipathic helix that engages a hydrophobic cleft separating actin subdomains 1 and 3, an interaction which is also likely to occur between actin protomers in F-actin (Holmes et al, 1990; Dominguez, 2004; Chereau et al, 2005).

The molecular basis for the RPEL<sup>MAL</sup>:G-actin interaction, and how this relates to sequence conservation within the motif, were unknown. MAL:actin interaction interferes with F-actin assembly (Posern et al, 2004). MAL:actin binding is disrupted by profilin, swinholide A, jaspakinolide, cytotochalasin D and tetramethylrhodamine actin modification, but is compatible with LatB and DNase I binding (Posern et al, 2004; SG, unpublished observations). Taken together with previous structural studies of actin interactions (Kabsch et al, 1990; Schutt et al, 1993; Morton et al, 2000; Otterbein et al, 2001; Klencin et al, 2005), these data suggest that interaction between the RPEL motifs and actin is likely to involve the subdomain 1–3 hydrophobic cleft. However, the RPEL motif shares no obvious sequence similarities with other cleft-binding domains such as the WH2/verprolin domains (Dominguez, 2004). Here, we present crystal structures of two RPEL peptides from MAL individually bound to G-actin. Each RPEL peptide presents two consecutive helices that bind actin in a similar manner to two non-contiguous helices in the vitamin-D-binding protein (DBP):G-actin complex. This observation draws attention to four conserved positions shared by most G-actin cleft-binding proteins. Structural and biophysical data combined with cell-based reporter assays show that the sequence conservation that defines the RPEL motif reflects its activity as an actin-binding element crucial to the regulation of MAL in vivo.

**Results**

For our structural analyses, we assembled purified skeletal muscle G-actin bound to latrunculin B (LatB) and ATP with individual 32-residue RPEL peptides from murine MAL. These peptides corresponded to RPEL<sup>1MAL</sup>, RPEL<sup>2MAL</sup> and RPEL<sup>3MAL</sup> and are known to bind actin efficiently in vitro (Guettler et al, 2008). High-resolution structures of the RPEL<sup>1MAL</sup>-LatB–actin:ATP and RPEL<sup>2MAL</sup>-LatB–actin:ATP complexes (hereafter shortened to RPEL<sup>1MAL</sup>:G-actin complexes) were subsequently determined and refined, but we were unable to crystallise the RPEL<sup>3MAL</sup>:G-actin complex (Supplementary Table 1). When bound to actin, RPEL<sup>1MAL</sup> and RPEL<sup>2MAL</sup> each contain two helices (z1 and z2) connected by a short loop and end with a short C-terminal capping (C-cap) region (Ermolenko et al, 2002) (Figure 1A). The observed helical contents of RPEL<sup>1MAL</sup> and RPEL<sup>2MAL</sup> are consistent with secondary structure predictions (56, 65 and 47%, respectively, for each of the three MAL RPEL peptides). However, circular dichroism (CD) experiments revealed that each RPEL peptide is largely unstructured in solution (z-helical content of 5.2, 4 and 4.3%, respectively) (Supplementary Figure S1), indicating that the observed RPEL peptide secondary structure is induced on binding actin.

**Structure of the RPEL<sup>2MAL</sup>:G-actin complex**

We first describe the higher resolution RPEL<sup>2MAL</sup>:G-actin structure as it has a canonical RPEL sequence as defined in the Pfam database (Figure 1A). RPEL<sup>2MAL</sup> wraps around actin, making intimate contacts with the subdomain 1–3 hydrophobic cleft and a ledge on subdomain 3 (Figure 1B). A total surface area of 1070 Å<sup>2</sup> is buried within the interface, accounting for almost 30% of the RPEL<sup>2</sup> surface area and 60% of RPEL<sup>2</sup> residues. Helix z1<sup>RPEL2</sup> (residues 115–123) binds within the actin hydrophobic cleft in a similar manner to WH2-containing proteins that engage this region (Figure 1B; Dominguez, 2004). z1<sup>RPEL2</sup> runs from front to back in the standard view of actin (Figure 1B), making hydrophobic contacts through residues L118, I122 and the aliphatic portion of K121 to the base of the actin subdomain 1–3 hydrophobic cleft (Figure 1C). The Nc of K121 adopts two conformations, one hydrogen bonding with the G146<sup>actin</sup> main chain carboxyl oxygen and the other forming a salt bridge with the side chain of E167<sup>actin</sup>. The invariant arginine, R125 in RPEL<sup>2MAL</sup>, is located within the short loop (defined hereafter as the R-loop) connecting the helices z1<sup>RPEL2</sup> and z2<sup>RPEL2</sup> (Figures 1B and 2A). The R125 side chain, which is critical for actin interaction (Guettler et al, 2008), forms a cation–π interaction with the Y169<sup>actin</sup> side chain through its guanidino group, as well as a side chain hydrogen bond with the main chain carbonyl oxygen of E167<sup>actin</sup> (Figure 1C). Furthermore, R125 makes a salt bridge with the C-terminal carboxylate of F375<sup>actin</sup>, the C-terminal residue of actin. The four residues within the R-loop have an extended conformation. The conserved proline, P126, constrains the R-loop backbone and stabilises the acute angle between z1<sup>RPEL2</sup> and z2<sup>RPEL2</sup>. Its carbonyl oxygen, together with the R128 main chain nitrogen, hydrogen bond with the Y169<sup>actin</sup> hydroxyl moiety (Figure 1C). Y169<sup>actin</sup> therefore has a central and crucial function to RPEL association having its side chain anchored through R-loop hydrogen bonds and pinned between the R125 side chain and those from R128/L131 (see below) (Figure 2B, right panel).

Helix z2<sup>RPEL2</sup> (residues 128–134) and its C-cap residues 135–137 contact a ‘ledge’ on actin subdomain 3 centred on Y166<sup>actin</sup>. The only other actin-binding protein shown to engage this region of actin in a similar manner is the structurally unrelated DBP (Otterbein et al, 2002; Verbven et al, 2003) (see Discussion section). Contacts with the subdomain 3 ledge are predominantly hydrophobic involving RPEL2 side chains L131, I136 and L137 (Figure 1C; Supplementary Figure S2B, right panel). Overall, the RPEL<sup>2MAL</sup>:G-actin structure reveals that the majority of RPEL motif sequence conservation occurs at positions that mediate direct interactions with actin (Figure 2A) or intra-RPEL molecular interactions within the actin complex. The RPEL motif thus reflects the preservation of a functional G-actin-binding element.

**Distinct differences in RPEL<sup>1MAL</sup> and RPEL<sup>2MAL</sup> R-loop actin contacts**

The RPEL<sup>1MAL</sup>:G-actin structure shares many of the interactions seen in the RPEL<sup>2MAL</sup>:G-actin complex. These are made by structurally equivalent hydrophobic residues in helix z1 (residues 72–79), helix z2 (residues 84–92) and the C-cap (Supplementary Figure S2A and B). The RPEL<sup>1MAL</sup> interac-
tion surface with actin is slightly smaller than for RPEL2MAL (811 Å²) despite the higher affinity (see below). Core contacts from the R-loop R81 side chain and R84 main chain, which stack either side of Y169, are preserved (Figure 2B). However, there are significant differences in the way R-loop RPEL1 contacts actin, mainly reflecting its non-canonical REL core sequence (Figure 1A). The R-loop RPEL1 follows a trajectory distinct from R-loop RPEL2 with an r.m.s. difference of 2.9 Å over 12 C-alpha atoms (calculated by superposing only their respective actin partners), indicating a degree of structural plasticity (Figure 2C). This difference most likely reflects the substitution of the canonical RPEL proline by R82, which makes RPEL1-specific actin contacts, specifically a salt bridge with E167actin and a hydrogen bond with the phenolic oxygen of Y166actin (Figure 2C; Supplementary Figure S2A). These contacts draw the RPEL1 MAL peptide away from Y169actin such that the C-alpha atom of invariant R81 RPEL1 is 3.0 Å from the equivalent R125 of RPEL2 (Figure 2B and C). R-loop main chain hydrogen bond distances to the Y169actin side chain are therefore much longer in RPEL1MAL. Strikingly, R81 is unable...
Figure 2  The RPEL1MAL and RPEL2MAL R-loops make different contacts with G-actin. (A) Summary of RPEL1/RPEL2 interactions with G-actin mapped onto a HMM (Hidden Markov Model) representation for the RPEL motif (Schuster-Bockler et al., 2004). Helices 1a and 2a are highlighted in pink and conserved residues from the RPEL HMM are highlighted in yellow. Boxes with solid lines indicate RPELMAL side chain-mediated interactions with actin, whereas those with dashed lines describe RPELMAL main chain-mediated interactions. Interactions that are conserved between RPEL peptides 1 and 2 are shown in black text and RPEL1- and RPEL2-specific ones in blue and green, respectively. (B) Close-up view of RPEL1MAL and RPEL2MAL interactions close to Y169actin. Left panel, RPEL1MAL:G-actin; right panel, RPEL2MAL:G-actin. A 2mFo-DFc electron density map calculated around each RPEL is shown in blue contoured at 1σ. Note that F375 is disordered in the RPEL1:actin complex. (C) Comparison of RPEL1MAL (cyan) and RPEL2MAL (green) motifs following superposition of their respective actin subunits. Important RPEL and G-actin residues described in the text are highlighted. Selected actin residues are shown in dark blue (contacting RPEL1) and dark green (contacting RPEL2), respectively. (D) Loss of F375 of β-actin affects binding of MAL RPEL motifs differentially. NIH3T3 fibroblasts transiently expressing either wild-type FLAG–β-actin (W) or FLAG–β-actin-AF375 (A) lacking the C-terminal residue were lysed and extracts were probed with bacterially produced GST or GST–RPEL peptide fusions as indicated. NIH3T3 cell lysates (input) and bound material were subjected to SDS–PAGE and western blotting for detection of the FLAG tag (WB: anti-FLAG) or endogenous β-actin (WB: anti-β-actin). Ponceau stain of the membrane indicates the levels of GST fusion proteins.
to reach and form a salt bridge with the C-terminal carboxylate of F375<sup>actin</sup>, which is instead disordered (Figure 2B; Supplementary Figure S2A). To the best of our knowledge, only the twinfilin C-terminal domain has been shown earlier to make contact with the carboxylate of F375<sup>actin</sup> (Paavilainen et al., 2008).

To validate these apparent differences in how RPEL<sub>1</sub> MAL and RPEL<sub>2</sub> MAL engage actin, we tested whether complex formation by RPEL<sub>1</sub> MAL and RPEL<sub>2</sub> MAL is differentially sensitive to deletion of F375<sup>actin</sup> using GST–RPEL pull-down assays (Guetttler et al., 2008). RPEL<sub>1</sub> MAL and RPEL<sub>2</sub> MAL recovered exogenous wild-type β-actin and endogenous β-actin efficiently from total cell lysates, but only the RPEL<sub>3</sub> MAL G-actin interaction was sensitive to deletion of F375<sup>actin</sup>, in agreement with our structural data (Figure 2D). RPEL<sub>3</sub> MAL was also sensitive to the F375<sup>actin</sup> deletion, despite its much lower apparent affinity for G-actin (Figure 2D), and would thus be predicted to bind in a similar manner to RPEL<sub>2</sub> MAL.

Fluorescence anisotropy validation of MAL RPEL:G-actin interaction

To confirm the structural features of each RPEL<sub>1</sub> MAL–G-actin interaction, we performed fluorescence anisotropy assays using N-terminally FITC-conjugated RPEL peptides analogous to those used for the structural studies. Peptides were incubated with LatB-bound G-actin (peptide 0.5 μM; LatB-actin 0–59 μM) and the actin-binding affinity was calculated from anisotropy by nonlinear regression. The results are shown in Figure 3A. We also included an analysis of MAL RPEL3, for which no structural data are available. The wild-type peptides corresponding to RPEL1 and 2 bound relatively tightly, with apparent dissociation constants (K<sub>d</sub>) of 1.0 ± 0.3 and 1.9 ± 0.1 μM, respectively, whereas the RPEL3 peptide bound weakly (K<sub>d</sub> of 28.9 ± 1.1 μM). These affinities differ slightly from those determined earlier as discussed in the Materials and methods section, but are generally comparable (Guetttler et al., 2008).

Loss-of-contact alanine substitutions of both helix z1 hydrophobic residues (z1<sup>AA</sup> mutations), which contact the hydrophobic cleft of actin, virtually abolished detectable interaction with actin for all three RPEL peptides (Figure 3A). A similar result was observed when loss-of-contact alanine substitutions were introduced at hydrophobic residues within helix z2 and its C-cap sequence (z2<sup>AAA</sup> mutations). This region makes hydrophobic contacts with the subdomain 3 ledge. z2<sup>AAA</sup> mutations within RPEL<sub>1</sub> MAL greatly reduced binding but nonetheless binding was still detectable (K<sub>d</sub> = 24.0 ± 1.5 μM) (Figure 3A). Combination of both z1<sup>AA</sup> and z2<sup>AAA</sup> mutations eliminated measurable actin binding of all three RPEL peptides (data not shown). Mutations at the conserved RPEL arginine and proline residues had context-specific effects, consistent with the distinct molecular interactions revealed in the structures. Mutation of the invariant RPEL arginine residue (RPEL1, R81; RPEL2, R125; RPEL3, R169) abolished measurable actin binding for RPEL2 and 3, but only reduced RPEL1 binding (K<sub>d</sub> = 17.7 ± 2.4 μM). The relatively small effect of the RPEL1 R81A mutation may reflect its failure to engage the F375<sup>actin</sup> carboxylate, whereas the RPEL1<sup>AAA</sup>-specific ionic interaction between R82 and E167<sup>actin</sup> provides a compensatory effect. Consistent with this, mutation of both R81 and R82 of RPEL<sub>1</sub> MAL to alanine effectively reduced the RPEL1-actin affinity (K<sub>d</sub> = 44.5 ± 3.5 μM), whereas the charge-reversal mutation RR81/82DD rendered binding undetectable.

The effect of alanine substitution of the conserved RPEL proline residue was also context-dependent. The RPEL<sub>2</sub> MAL P126A mutant reduced affinity by 16-fold (K<sub>d</sub> = 30.8 ± 2.3 μM), consistent with an important role for the proline in maintaining the R-loop conformational integrity. In contrast, the analogous alanine substitution in RPEL<sub>1</sub> MAL, which contains an arginine at this position, reduced affinity only 3.5-fold (K<sub>d</sub> = 3.7 ± 0.5 μM), whereas conversely, replacement of RPEL<sub>2</sub> MAL P126 with arginine (analogous to RPEL<sub>1</sub> MAL), also reduced binding affinity (K<sub>d</sub> = 19.5 ± 3.1 μM). The different contacts seen in the two structures are thus reflected in contrasting roles for the conserved RPEL R and P residues in RPEL<sub>1</sub> MAL and RPEL<sub>2</sub> MAL, respectively (see Discussion). Residues I122 and P126 of RPEL<sub>2</sub> MAL are substituted by G and S, respectively, in the RPEL2 motif of myocardin (Figure 1A). These substitutions eliminate crucial G-actin contacts and are likely to account for the weak actin affinity exhibited by myocardin (Guetttler et al., 2008). Alanine substitution at the conserved RPEL glutamate, which does not make direct contact with actin, had only a small effect on RPEL<sub>1</sub> MAL–G-actin binding affinity (E86A, K<sub>d</sub> = 2.7 ± 0.4 μM), and did not affect RPEL<sub>2</sub> MAL–G-actin interaction (E130A, K<sub>d</sub> = 1.9 ± 0.2 μM). The conservation of this residue among the family of RPEL motifs may reflect an additional conserved role unrelated to actin binding (see Discussion).

RPEL<sub>3</sub> MAL retains all the equivalent residues to RPEL<sub>2</sub> MAL that make direct interaction with actin, yet its affinity for actin is an order of magnitude lower than that of either RPEL<sub>1</sub> MAL or RPEL<sub>2</sub> MAL (Guetttler et al., 2008). We hypothesised that non-consensus residues might be impairing RPEL3 binding to actin. Mapping the MAL RPEL3 sequence onto the RPEL1/RPEL2 structures identified that G171 immediately before helix z2<sup>RPEL</sup> could introduce considerable flexibility into the R-loop. More importantly, a proline residue at position 172 has no main chain amide available to hydrogen bond to the Y169<sup>actin</sup> hydroxyl group. To test this hypothesis, we generated an ‘RPEL2-like’ RPEL<sub>3</sub> MAL peptide by replacing RPEL3 G171/P172 by the corresponding residues from RPEL<sub>2</sub> MAL, E and R. This substitution improved actin-binding affinity almost six-fold, to 4.8 ± 0.1 μM, compared with the wild-type RPEL<sub>3</sub> MAL peptide, with actin binding remaining dependent on hydrophobic contacts between z2<sup>RPEL</sup> and the subdomain 3 ledge contact (Figure 3A).

Integrity of RPEL:G-actin contacts is required for MAL regulation

We observed earlier that mutations of the conserved core arginines, which lower RPEL<sub>1</sub> MAL–G-actin affinity, result in partial or complete nuclear accumulation of MAL protein in serum-starved cells, potentiate its transcriptional activity and uncouple its activation from Rho signalling (Guetttler et al., 2008). We therefore tested whether the structure-based mutations that disrupt actin binding have a similar effect on MAL function in vivo. The loss-of-hydrophobic-contact mutations in helix z1, helix z2 and the flanking C-cap (z1<sup>AA</sup> and z2<sup>AAA</sup>) were introduced into full-length MAL (Figure 3B), and the mutant proteins were expressed by transient transfection in NIH3T3 cells. Activity was monitored by assessment of the
proteins’ subcellular localisation (Figure 3D) and by their ability to activate a co-transfected reporter gene for their transcription factor target SRF (Figure 3C and D).

Alanine substitutions at each core RPEL arginine substantially increased MAL nuclear localisation and SRF reporter activity, with mutations in RPEL1MAL having a lesser effect than those in the other motifs, as reported previously (Guettler et al. 2008). Similar results were obtained on in vivo down-regulation of myocardin family SRF coactivators.

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Figure 3 In vitro and in vivo validation of the RPEL1MAL and RPEL2MAL structures. (A) Fluorescence anisotropy assay for characterisation of the RPEL1MAL-G-actin interaction. Anisotropies of FITC-conjugated 32 amino-acid RPEL peptides at a concentration of 0.5 μM were measured over a range of LatB-actin concentrations. Anisotropy values were normalised by subtracting the anisotropy obtained in the absence of LatB-actin from all anisotropies for each peptide and multiplied by 1000. Graphs correspond to one of three experiments done in duplicate. Dissociation constants (Kd) for RPEL1MAL-G-actin interactions were calculated by nonlinear regression from each duplicate after normalisation using GraFit software (see Materials and methods). Kd values shown are means from three independent experiments with s.e.m. (B) Schematic representation of N-terminal MAL mutated peptides used for luciferase reporter assays and immunofluorescence. The mutated region is shown in red. (C) SRF reporter activation by structure-derived MAL point mutants. The indicated MAL derivatives were expressed with and without C3 transferase coexpression in serum-starved NIH3T3 cells. Reporter activation was normalised to reporter activation conferred by SRF-VP16 or SRF-VP16 plus C3 transferase. x23, 1x3, 12x and xxx refer to MAL derivatives described earlier (Guettler et al. 2008): x23, R81A; 1x3, R25A; 12x, R169A; xxx, R81A R25A R169A. Data from three independent experiments are shown. Error bars, s.e.m. (D) Subcellular localisation of structure-derived MAL point mutants. The localisation of the indicated constructs was scored as predominantly nuclear (nuc), comparable to SRF-VP16 plus C3 transferase coexpression in serum-starved NIH3T3 cells. Reporter activation was normalised to reporter activation conferred by SRF-VP16 or SRF-VP16 plus C3 transferase. x23, 1x3, 12x and xxx refer to MAL derivatives described earlier (Guettler et al. 2008): x23, R81A; 1x3, R25A; 12x, R169A; xxx, R81A R25A R169A. Data from three independent experiments are shown. Error bars, s.e.m.

Discussion

Implications for RPEL1MAL:G-actin interactions and regulation of myocardin family SRF coactivators

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Alanine substitutions at each core RPEL arginine substantially increased MAL nuclear localisation and SRF reporter activity, with mutations in RPEL1MAL having a lesser effect than those in the other motifs, as reported previously (Guettler et al. 2008). Similar results were obtained on in vivo down-regulation of myocardin family SRF coactivators.
RPEL motifs in the N-terminal regulatory domain be competent to bind G-actin. Together with our previous demonstration that signalling induces changes in MAL-actin interaction in vivo (Vartiainen et al., 2007), our data are consistent with a model in which alterations to actin loading onto the regulatory domain control MAL nuclear accumulation.

Actin binding is required for Crm1-dependent MAL nuclear export (Vartiainen et al., 2007) and is also likely to inhibit activity of a putative nuclear import signal within the RPEL2–RPEL3 linker (Vartiainen et al., 2007; Guettler et al., 2008). It is thus likely that different actin-bound states of the regulatory domain will exhibit different interactions with import and export factors. We identified earlier a stable 3:1 actin–MAL complex in gel filtration experiments (Vartiainen et al., 2007). This complex can effectively sequester actin from polymerisation, so the arrangement of the actin molecules in it must differ from that occurring within the actin filament (Posern et al., 2004). The relevance of this complex to MAL regulation, in terms of its competence to bind import factors or to recruit Crm1, remains unclear. Although the existence of the 3:1 actin–MAL complex is consistent with each RPEL engaging one actin molecule in the manner described in this study, this awaits direct confirmation. Our current work is focused on elucidation of the structure of the 3:1 actin–MAL complex.

Several considerations suggest that in the context of the MAL N-terminal regulatory domain the RPEL motifs do not function independently in a ‘beads-on-a-string’ manner. First, the high apparent affinity of the intact regulatory domain for actin compared with individual RPEL peptides suggests that cooperative actin–actin interactions may facilitate complex formation. Second, the non-canonical RPEL1 motif, and its distinct mode of actin binding, has been selected throughout metazoan evolution, as have the sequences responsible for the low affinity of the RPEL3 motif, suggesting the motifs have distinct functional roles. Third, comparative studies of MAL and its constitutively nuclear relative myocardin suggest that actin-regulated nuclear accumulation appears determined by the RPEL1–RPEL2 unit, RPEL3 being interchangeable (Vartiainen et al., 2007; Guettler et al., 2008). It will be interesting to examine whether RPEL3 loads actin last in an ordered assembly of multiple actin molecules onto the triple RPEL repeat region of MAL, and the potential functional significance of this for MAL cytoplasmic–nuclear shuttling.

In addition to controlling nuclear accumulation of MAL, actin binding also appears to repress the ability of nuclear MAL to activate transcription through SRF (Vartiainen et al., 2007; Guettler et al., 2008). At this level, MAL-bound actin may modulate the formation of ternary complexes of MAL, SRF and DNA, recruit transcriptional repressors or interfere with the formation of active transcription complexes.

The RPEL motif binds G-actin similarly to DBP

To understand how MAL is able to compete with other actin-binding proteins, including the highly abundant G-actin-buffering proteins profilin and thymosin β4 (Pollard and Borisy, 2003), we compared our RPEL peptide:G-actin structures with other actin-binding protein structures. This analysis showed that ‘cleft-and-ledge’ contacts from RPEL1 and 2 are strikingly similar to those made by vitamin D-binding protein (DBP), a large multi-domain actin-sequestering protein quite unrelated to the RPEL motif (Otterbein et al., 2002; Verbven et al., 2003) (Figure 4A). DBP uses two helices, structurally equivalent to those of RPEL1/2, to engage both the actin subdomain 1–3 hydrophobic cleft and the subdomain 3 ledge of actin (Figure 4A). This region of DBP and RPEL2MAL superposes with an r.m.s. difference of 1.5 Å over 17 C-alpha atoms. The DBP helices are non-contiguous, however, being separated by over 100 amino acids in the primary sequence, and DBP therefore has no equivalent of the RPEL R-loop (Figure 4A). At least five structurally equivalent residues are shared by the RPEL motif and DBP. These include L184DBP and L188DBP (from helix x1); K191DBP, which hydrogen bonds to E167actin main chain (equivalent to R81/R125 of RPEL1/2); V294DBP and F298DBP, which contact the subdomain 3 ledge (Figure 4A, DBP residue numbers are taken from 1MA9 coordinates). The latter residue resides within a lengthened helix, which replaces the C-cap attached to the α2RPEL, but has an analogous function to α2RPEL C-cap residues I92/I136, which contact the subdomain 3 ledge. The unexpected similarity of actin contacts between RPEL and DBP raises the possibility that other cleft-and-ledge actin-binding proteins may yet be found.

An extended family of G-actin cleft-binding proteins

Similar to DBP and gelsolin, RPEL1 and RPEL2 of the MRTFs contain a helix that binds in the forward direction of the hydrophobic cleft in actin, a frequently used site for actin-binding proteins (McLaughlin et al., 1993; Robinson et al., 1999; Otterbein et al., 2002; Verbven et al., 2003; Burtnick et al., 2004; Paavilainen et al., 2008) (Figure 4B and C). ‘Forward’ is defined as the peptide ligand (N–C) running front-to-back in the conventional actin view (Dominguez, 2004) (Figure 1B). The unexpected similarity between RPEL and DBP actin contacts close to the subdomain 3 ledge (Figure 4A) prompted us to perform structure-based sequence alignments with other actin cleft-binding proteins to examine common structural features. Previous analysis identified three hydrophobic residues (designated A, B and C herein) that are present in most actin cleft-binding proteins (Dominguez, 2004) (Figures 4C and 5). Inclusion of RPEL and DBP contacts with actin identified an additional, highly conserved interaction involving a basic residue (designated D; see Figure 5), which was not explicitly described earlier as being conserved in both forward and reverse orientations. Residues A and D are present independently of the orientation of the cleft-binding helix, and they superpose well between the different structures (Figure 4C). There is more variability in the interactions made by residues B and C; for example, the hydrophobic residue C in the RPELMAL x1 and DBP helices is oriented towards the cleft floor rather than the side of the cleft. This positions residue B, which is in some instances a lysine residue, outside the cleft on the subdomain 3 surface but still able to contribute hydrophobic contacts via the aliphatic portion of its side chain (Figure 4C; see Supplementary Figure S2A).

In summary, our structural analysis provides the first detailed picture of how an RPEL peptide binds to G-actin and suggests functionally important differences between each MAL RPEL motif. To understand how MAL is regulated by higher order RPEL:G-actin assemblies, future experiments will concentrate on G-actin complexes with an intact triple RPEL domain from MAL.
Materials and methods

Plasmids
Sequences encoding mouse MAL RPEL peptides (see GST pull-down assays) were inserted into a vector derived from pET-41a(+) (Novagen; described in Vartiainen et al., 2007) for bacterial expression of GST–(His)_{6}–S-tag fusions (Guettler et al., 2008). Mammalian expression constructs for wild-type mouse MAL(fl)-HA_{2} and human FLAG-β-actin and their mutant derivatives were based on pEF (Sotiropoulos et al., 1999; Miralles et al., 2003). SRF-VP16, C3 transferase and luciferase reporter plasmids were described earlier (Sotiropoulos et al., 1999; Geneste et al., 2002).

Proteins and peptides
Actin was prepared from rabbit skeletal muscle as described earlier (Feuer et al., 1948; Spudich and Watt, 1971). Peptides (both
unlabelled and N-terminally FITC-conjugated) corresponding to the three RPEL motifs of MAL were synthesised and HPLC-purified by the Cancer Research UK Protein and Peptide Chemistry Laboratory (RPEL1Mal: MAL 67–98; RPEL2Mal: MAL 111–142 and RPEL3Mal: MAL 155–186). During the course of these studies, we discovered that the RPEL peptides exhibited varying degrees of methionine oxidation on storage and therefore we subjected all peptides to reduction and re-purification before absorption analysis. Absorption of unlabelled peptide was measured at 215 nm (peptide bond) in an Agilent 8453 UV/Vis spectrophotometer and concentrations were calculated using \( \varepsilon_{215} = 1000 \text{ M}^{-1} \text{ cm}^{-1} \) per peptide bond. Absorption of FITC-conjugated peptides was measured at 492 nm (FITC) in a NanoDrop spectrophotometer (NanoTechnologies) using \( \varepsilon_{492} = 83000 \text{ M}^{-1} \text{ cm}^{-1} \).

**Preparation of LatB-actin**

We used LatB to block actin polymerisation, as successfully used in earlier crystallographic studies of actin (Morton et al., 2000; Hertzog et al., 2004). Briefly, rabbit skeletal muscle actin was dialysed into Mg\(^{2+}\)–G-buffer (2 mM Tris–HCl pH 8.0, 0.3 mM MgCl\(_2\), 0.2 mM EGTA, 0.2 mM ATP and 0.5 mM DTT) and co-incubated overnight at 4°C with a 10-fold molar excess of LatB (Calbiochem), added from a 50 mM stock in DMSO. Un-complexed actin was polymerised for 1 h with Mg\(^{2+}\)–F-buffer (2 mM Tris–HCl pH 8.0, 1 mM MgCl\(_2\), 0.2 mM EGTA, 0.7 mM ATP and 2 mM DTT). FITC-conjugated peptides were used at 0.5 \( \mu \text{M} \), whereas LatB-actin was added from 1 \( \mu \text{M} \) up to 59 \( \mu \text{M} \). Plates were read in a Safire\(_2\) microplate reader (Tecan) after 2 h co-incubation at room temperature to achieve binding equilibrium. The Safire\(_2\) was used in fluorescence polarisation mode (excitation, 470 ± 20 nm; emission, 525 ± 20 nm; 10 reads; integration time, 40 ms) with the manufacturer’s ‘Magellan’ software (version 5.03). Anisotropy (A) was calculated using the formula \( A = \frac{I_{\text{parallel}} - I_{\text{perpendicular}}}{I_{\text{parallel}} + 2I_{\text{perpendicular}}} \), where \( I_{\text{parallel}} \) and \( I_{\text{perpendicular}} \) denote the fluorescence intensities parallel and perpendicular to the excitation

**Figure 5** Structure-based sequence alignment of G-actin-binding proteins engaging the subdomain 1–3 hydrophobic cleft. The alignment is subdivided according to forward and reverse orientations of the actin-binding \( \alpha \)-helix. Red boxes indicate experimentally observed helical regions. Cleft-binding residues A, B, C and D are highlighted as well as the three actin ledge-binding residues. Residue numbering for gelsolin and DBP are taken from the PDB coordinate files indicated and can be interconverted to full length sequence numbering by addition of 51 or 16 residues respectively.

**CD measurements and spectra deconvolution**

CD spectra were recorded using an Aviv 202SF spectrophotometer in a 0.2 nm path length cell at 20°C. Data were recorded every 0.2 nm with a data acquisition time of 3 s in the range of 188–260 nm. Each peptide was dissolved in 10 mM Tris pH 8, 10 mM NaCl to a final concentration of 250 \( \mu \text{M} \). Each spectrum was the average of three repeated scans. The composition of the secondary structure of each peptide was analysed from CD spectra using the DICHROWEB server (Whitmore and Wallace, 2004) and the algorithm CONTIN (van Stokkum et al., 1990).

**Crystallisation, data collection and structure determination**

RPEL:LatB-G-actin:ATP complexes were prepared at a molar ratio of 3:1 of RPEL:LatB-actin and at a final actin concentration of 12 mg/ml.
 Approximately 10^7 NIH3T3 fibroblast cells on a 150-mm dish were GST pull-down assays in three independent experiments with s.e.m. Sigma). The blot was stained with Ponceau S to reveal bait input. 4–12% SDS–PAGE and western blotting with detection of the FLAG times in binding buffer without protease inhibitors and subjected to was for 2 h in binding buffer at 4°C. Insoluble generated by lysis in binding buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 3 mM MgCl₂, 0.2 mM EGTA, 0.2 mM ATP, 1 mM DTT and protease inhibitors) through syringing and removal of insoluble material by centrifugation. An equivalent of a confluent 150-mm dish of NIH3T3 cells was used for four binding reactions. Binding was for 2 h in binding buffer at 4°C. The resin was washed three times in binding buffer without protease inhibitors and subjected to 4–12% SDS–PAGE and western blotting with detection of the FLAG epitope tag (M2–FLAG–HRP–Sigma) and total β-actin (AC-15; Sigma). The blot was stained with Ponceau S to reveal bait input.

**GST pull-down assays**

Approximately 10^7 NIH3T3 fibroblast cells on a 150-mm dish were transfected with 6 μg of pEF-FLAG-β-actin or its Δβ375 derivative using Lipofectamine reagent (Invitrogen). Cells were maintained in media containing 10% FCS for 1 day and serum-starved in media using Lipofectamine reagent (Invitrogen). Cells were maintained in a medium containing 0.5% FCS for 22 h. Firefly luciferase activity was measured and normalised to Renilla luciferase activity (Dual-Luciferase Reporter Assay System; Promega).

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Heyduk and Lee, 1990: \[K_d = \frac{(R t \cdot C_0)}{(R t \cdot C_{16} + R t \cdot C_{17})} \]

where \( A \) is the measured value of anisotropy; \( A_t \) and \( A_r \) are the anisotropy values corresponding to free and bound peptide, respectively; \( R_t \) and \( L_t \) are the total peptide (‘receptor’) and total LatB-actin (‘ligand’) concentrations, respectively; \( K_d \) is the dissociation constant. \( K_d \) values were derived from samples in three independent experiments with s.e.m. Bubb MR, Govindasamy L, Yarmola EG, Vorobiev SM, Almo SC, F375 derivative

**Structure of the RPEL motif G-actin-binding module**

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**Immunofluorescence microscopy**

Immunofluorescence microscopy was performed as described earlier (Vartiainen et al, 2007; Guettler et al, 2008). NIH3T3 cells (150 000 cells per well in a six-well dish) were transfected with MAL-HA derivative. After transfection, cells were maintained in a medium containing 0.5% FCS for 20 h. Primary antibody was anti-HA (12CA5; Roche). The localisation of each MAL derivative was scored as predominantly nuclear, pancellular or predominantly cytoplasmic in 100 cells.

**Luciferase reporter assay**

Luciferase reporter assays were performed as described earlier (Vartiainen et al, 2007; Guettler et al, 2008). NIH3T3 cells (30 000 cells per well in a 24-well dish) were transfected with SRF reporter p3DA.luc (8 ng), reference reporter ptk-RL (20 ng) plus SRF–VP16 (40 ng) or MAL (10 ng) or MAL derivative (10 ng). Where indicated, C3 transferase was coexpressed (2 ng). After transfection, cells were maintained in a medium containing 0.5% FCS for 22 h. Firefly luciferase activity was measured and normalised to Renilla luciferase activity (Dual-Luciferase Reporter Assay System; Promega).
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