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Actin Dynamics Control SRF Activity by Regulation of Its Coactivator MAL

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Summary

Rho GTPases regulate the transcription factor SRF via their ability to induce actin polymerization. SRF activity responds to G actin, but the mechanism of this has remained unclear. We show that Rho-actin signaling regulates the subcellular localization of the myocardin-related SRF coactivator MAL, rearranged in t(1;22)(p13;q13) AML. The MAL-SRF interaction displays the predicted properties of a Rho-regulated SRF cofactor. MAL is predominantly cytoplasmic in serum-starved cells, but accumulates in the nucleus following serum stimulation. Activation of the Rho-actin signaling pathway is necessary and sufficient to promote MAL nuclear accumulation. MAL N-terminal sequences, including two RPEL motifs, are required for the response to signaling, while other regions mediate its nuclear export (or cytoplasmic retention) and nuclear import. MAL associates with unpolymerized actin through its RPEL motifs. Constitutively cytoplasmic MAL derivatives interfere with MAL redistribution and Rho-actin signaling to SRF. MAL associates with several SRF target promoters regulated via the Rho-actin pathway.

Introduction

Serum response factor (SRF) is a MADS-box transcription factor, which controls growth-factor-regulated immediate-early (IE) genes such as c-*fos* and cytoskeletal actin, and numerous muscle-specific genes (for review, Arsenian et al., 1998). SRF forms regulatory complexes with other transcriptional regulators through its DNA binding domain. At the c-*fos* promoter, for example, its interaction with members of the ternary complex factor (TCF) family of Ets-domain proteins allows control of transcription via MAP kinase signaling (for review, Treisman, 1994). Inactivation of SRF impairs IE and muscle-specific gene expression, causes a non-cell-autonomous gastrulation defect, and alters cell adhesive properties (Arsenian et al., 1998; Schratt et al., 2002, 2001; Weinhold et al., 2000).

SRF activity is regulated independently of its interaction with TCF by a novel signaling pathway controlled by Rho-family small GTPases (Hill et al., 1995). Alterations in actin dynamics are both necessary for the activation of SRF by extracellular signals and sufficient for its activation in the absence of signal (Sotiropoulos et al., 1999; Mack et al., 2001). Activation of Rho induces actin polymerization through two effector pathways: the ROCK-LIM kinase-cofilin pathway stabilizes F actin (Geneste et al., 2002; Sotiropoulos et al., 1999), while the mDia1 pathway promotes its assembly (Copeland and Treisman, 2002; Tominaga et al., 2000). Expression of certain inactive mutant forms of LIMK1, mDia1, and another cytoskeletal regulator, VASP, can effectively interfere with serum-induced SRF activation (Copeland and Treisman, 2002; Geneste et al., 2002; Grosse et al., 2003). Moreover, overexpression of either wild-type or nonpolymerizable actin itself also inhibits signaling to SRF (Posern et al., 2002; Sotiropoulos et al., 1999). These data suggest that SRF activity is potentiated by depletion of either the G actin pool or a G actin treadmilling intermediate.

The mechanism by which Rho-actin signaling controls SRF activity has remained obscure. Signaling requires the sequences within the SRF DNA binding domain, which mediate TCF binding (Hill et al., 1993), but the TCFs themselves do not respond to Rho-actin signaling (Gineitis and Treisman, 2001; Hill et al., 1995). Signaling also requires sequences at the N terminus of the SRF DNA binding domain and/or the integrity of the SRF binding site on DNA (Hill et al., 1994). These observations suggest a model in which Rho-actin signaling regulates a SRF cofactor, which interacts with the SRF DNA binding domain (Hill et al., 1994).

Recent studies identified myocardin, a heart-specific SRF coactivator (Wang et al., 2001). Myocardin is closely related to MAL/MKL1, a ubiquitously expressed gene rearranged in t(1;22)(p13;q13) AML (Ma et al., 2001; Mercher et al., 2001) and to MAL16, the site of a lethal insertion mutation in the mouse (Mercher et al., 2001; Skarnes et al., 1992). The mouse homologs were subsequently shown to potentiate SRF activity (MRTF-A and MRTF-B; Wang et al., 2002), and a mouse MAL isoform, BSAC, was isolated in a screen for antiapoptotic factors (Sasazuki et al., 2002). Here, we show that MAL is a G-actin-associated SRF coactivator, which is redistributed from the cytoplasm to the nucleus in response to Rho-induced actin polymerization.

Results

MAL Expression in NIH3T3 Cells

The three MAL family proteins each comprise a conserved N-terminal RPEL domain followed by an ORF of approximately 800 residues containing several conserved motifs (Figure 1A; Mercher et al., 2001; Wang et al., 2001, 2002). We used the EST database and published human MAL/MKL sequences (Ma et al., 2001; Mercher et al., 2001) to identify and isolate mouse MAL homologs. The predominant class of MAL mRNA in NIH3T3 cells is predicted to encode a protein, MAL(met),

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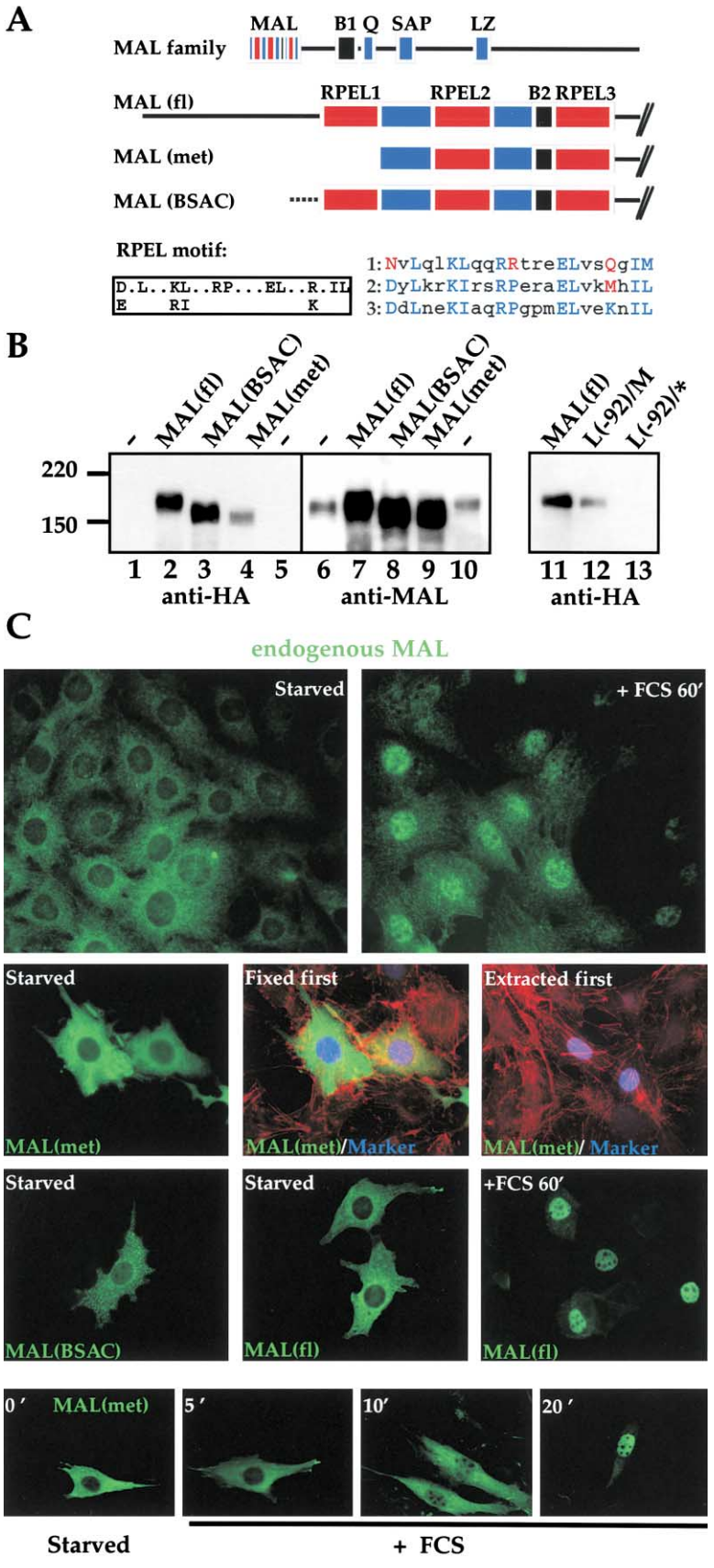

Figure 1. Mouse MAL Proteins Accumulate in the Nucleus upon Serum Stimulation

(A) Conserved sequence motifs in the mouse MAL protein family. RPEL motifs (pfam PF02755) are indicated by red bars, basic boxes are in black, and other conserved elements in blue. Structures of three different MAL N termini are shown below. Bottom, sequences of the three N-terminal RPEL motifs: blue, framework residues; red, divergence from consensus.

(B) NIH3T3 MAL initiates translation N-terminal to the first N-terminal ATG codon. Lanes 1–12, lysates from NIH3T3 cells expressing C-terminally HA-tagged MAL(fl) or derivatives, MAL(BSAC) or MAL(met), or vector alone (200 ng each) were analyzed by immunoblotting with antibodies against HA (lanes 1–5 and 11–13) or MAL (lanes 6–10).

(C) Serum induces MAL nuclear accumulation. Top row, endogenous NIH3T3 MAL. Second row, cells expressing MAL(met)-HA and NlexA transfection marker (Posern et al., 2002) (50 ng each) stained for MAL-HA, F actin, and marker either directly, or following extraction with 0.5% Triton X-100 (right image). Third row, cells expressing MAL(fl)-HA or MAL(BSAC)-HA (50 ng each) were treated as indicated and stained directly for HA. Bottom row, time course of MAL(met)-HA nuclear accumulation. MAL(met)-HA was predominantly cytoplasmic in 82 ± 3% of serum-starved cells, and predominantly nuclear in 83 ± 2% of serum-stimulated cells.

with its presumptive ATG codon N-terminal to RPEL motif 2. However, in this mRNA, the ORF remains open N-terminal to this ATG codon and is conserved between human and mouse at 104 of 105 preceding codons, including RPEL motif 1 (see Experimental Procedures). We were unable to detect a second mRNA isoform, MAL(BSAC), which contains all three RPEL motifs (Sasazuki et al., 2002) in our NIH3T3 cells.

The intact MAL cDNA, including its 5′ UTR, and the individual MAL(met) and MAL(BSAC) ORFs were inserted into appropriate expression plasmids and transiently expressed in NIH3T3 cells. The C-terminally HA-tagged MAL(met) reading frame produced a protein of Mr ~150 kDa (Figure 1B, lane 4); in contrast, expression of the full-length cDNA produced a polypeptide of Mr ~180 kDa, which we term MAL(fl) (Figure 1B, lane 2). MAL(BSAC) migrated with Mr ~160 kDa (Figure 1B, lane 3). MAL(fl) comigrated with endogenous NIH3T3 cell MAL, detected using an antiserum directed against MAL(met) codons 1–170 (Figure 1B, lanes 6 and 7; Supplemental Figure S1A available at http://www.cell.com/cgi/content/full/113/3/329/DC1). To investigate MAL(fl) initiation further, we mutagenized Leu-92, the first leucine codon in the conserved MAL ORF: a TAG codon abolished MAL(fl) synthesis, while an ATG codon reduced but did not abolish it (Figure 1B, lanes 11–13). Thus, NIH3T3 MAL mRNAs mostly initiate translation at codon Leu-92 or shortly N-terminal to it.

MAL Relocalizes to the Nucleus upon Serum Stimulation

We used the MAL antiserum to investigate the cellular localization of endogenous MAL in NIH3T3 cells. In serum-starved cells, endogenous MAL exhibited diffuse cytoplasmic staining, but became predominantly nuclear following stimulation with serum or LPA (Figure 1C). Transiently expressed MAL(met) and MAL(fl) also exhibited diffuse cytoplasmic staining in serum-starved cells, while MAL(BSAC) exhibited a punctate cytoplasmic localization, suggesting that its variant N-terminal sequences are involved in targeting within the cytoplasm (Figure 1C). The proteins did not colocalize with the F actin cytoskeleton and were completely extracted from the cells by mild detergent treatment (Figure 1C; Supplemental Figure S2A available at above website). Upon serum stimulation, all three MAL isoforms rapidly accumulated in the nucleus; accumulation was readily apparent after 10 min and virtually complete by 20 min, persisting for up to three hours (Figure 1C; data not shown). Serum stimulation did not affect MAL protein levels (Supplemental Figure S1C available at above website). Regulated nuclear accumulation of MAL proteins in response to extracellular signals suggests that it might play a role in Rho-actin signaling to SRF.

DNA Binding Properties of the MAL-SRF Complex

We next investigated the interaction between MAL and SRF using gel mobility-shift assays. Extracts from cells expressing MAL(fl), MAL(BSAC), or MAL(met) proteins generated small amounts of a low mobility complex (Figure 2A, lanes 2–4). This complex was not formed on a probe containing point mutations which prevent SRF binding and was supershifted by both anti-MAL and anti-SRF antibodies (data not shown; see Supplemental Figures S3A and S3B available at http://www.cell.com/cgi/content/full/113/3/329/DC1). Substantially greater amounts of MAL-SRF complex were generated upon removal of the entire MAL N-terminal sequence, including all the RPEL motifs, indicating that these sequences inhibit MAL-SRF complex formation (Figure 2A, lane 5). All the MAL derivatives also formed complexes with the SRF DNA binding domain (Figure 2, lane 7; Supplemental Figure S3B available at above website). MAL was detectable, albeit weakly, in SRF immunoprecipitates from serum-stimulated cells (Supplemental Figure S3C available at above website).

Previous studies indicate that the altered-specificity SRF derivative SRF-M2 is unable to respond to signaling through the Rho pathway when bound to its cognate binding site SRE.LM (Hill et al., 1993, 1994). MALΔN did not interact with the SRF-M2:SRE.LM complex, consistent with a potential role in Rho signaling (Figure 2B). Functional studies also indicate that any Rho-actin pathway cofactor should compete with TCF for a common surface on SRF (Hill et al., 1994; Murai and Treisman, 2002). We therefore tested whether the Elk-1 B box sequence, which directly interacts with SRF (Hassler and Richmond, 2001; Ling et al., 1997), could compete with MAL for interaction with SRF. Wild-type B box peptide, but not a peptide containing the mutation Y159A, which cannot bind SRF (Ling et al., 1997), inhibited complex formation in a dose-dependent manner (Figure 2C). The MAL-SRF interaction thus has two critical properties expected of the putative Rho-actin coactivator.

We examined the domains of MAL required for interaction with SRF. Deletion of basic region B1 completely abolished MAL-SRF complex formation, while removal of Q-rich region significantly reduced it; deletion of the SAP domain had no effect (Figure 2A, lanes 8, 9, and 11). Deletion of the LZ motif significantly reduced MAL-SRF complex formation, but substantially increased its mobility (Figure 2A, lane 10). Deletion of the LZ also abolished the ability of MAL to self-associate in a coimmunoprecipitation assay (Figure 2D). These data suggest that MAL preferentially binds SRF as a dimer, although monomeric MAL remains competent for weak interaction. Taken together, these data show that MAL interacts with the SRF DNA binding domain, that it binds preferentially as a dimer, and that MAL and TCF proteins interact with SRF in a mutually exclusive manner.

MAL Potentiates the Activity of the Rho Pathway

We used reporter gene assays to assess transcriptional activation by various MAL derivatives (Figure 3A). In serum-starved cells, reporter activity was strongly potentiated by increasing amounts of MAL(met) and was not further stimulated by coexpression of wild-type SRF, which alone inhibited reporter activity slightly (Figure 3B, left; Hill et al., 1993). Potentiation of SRF activity occurred at MAL(met) expression levels at which nuclear MAL was detectable even in serum-starved cells (data not shown). In contrast, MAL(met) expression did not activate a reporter controlled by the mutant SRF binding

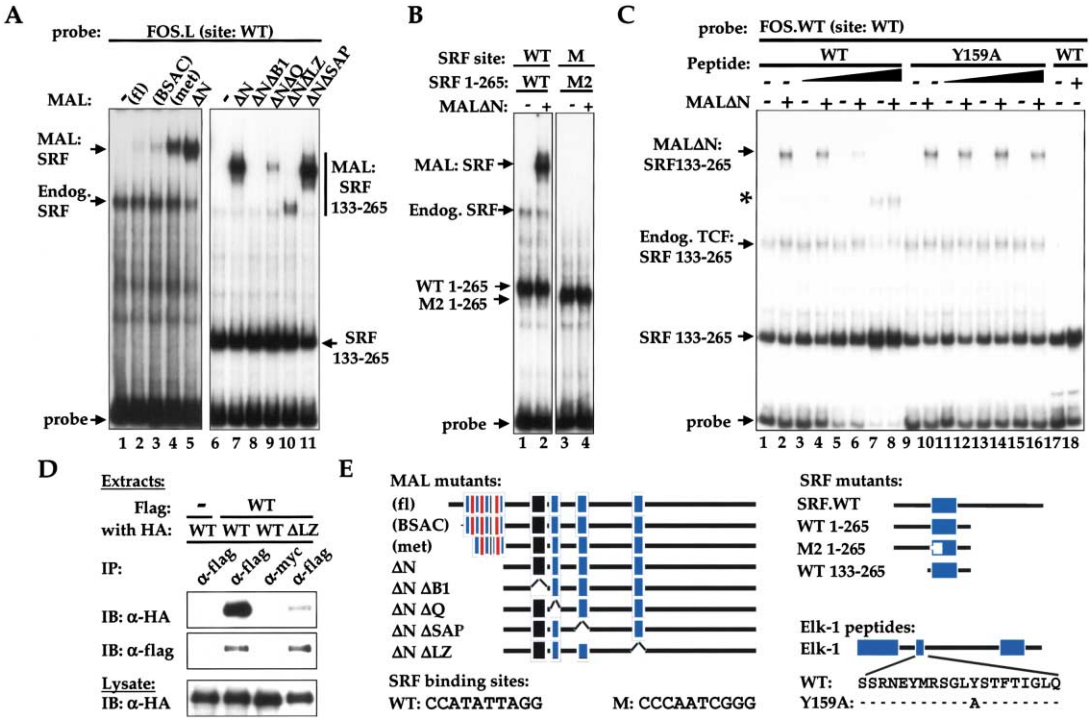

Figure 2. The MAL-SRF-DNA Complex

(A) Complex formation between MAL and SRF. Gel mobility-shift assays were performed using whole-cell extracts from cells expressing MAL(fl), MAL(BSAC), MAL(met), or MAL deletion mutants; extracts were assayed either alone (lanes 1–5) or with added recombinant SRF DNA binding domain (SRF 133-265; lanes 6–11).

(B) MAL does not interact with altered-specificity SRF-M2 at its cognate site. Extracts from cells expressing MALΔN with either wild-type SRF or SRF-M2 were analyzed using the indicated probes.

(C) MAL and Elk-1 compete for the same binding site on SRF. Complex formation between MALΔN and SRF(133–265) was challenged with increasing amounts of wild-type or mutant Elk-1 B box peptides. Note specific SRF-peptide interaction, revealed by increased binding of full-length SRF (present in the extract; asterisk) and of SRF 133–265 (lanes 7, 8, 15, and 16).

(D) MAL dimerizes through the LZ motif. Extracts from cells expressing MAL(met) derivatives were analyzed by immunoprecipitation and immunoblotting.

(E) Proteins, peptides, and SRF binding sites. Proteins are shown with conserved domains in blue the SRF-M2 mutation in white. Sequences of the DNA binding sites for SRF and SRF-M2 are shown below.

site SRE.LM, which cannot bind wild-type SRF (Figure 3B, right; Hill et al., 1994). Coexpression of the inactive altered-specificity SRF derivative SRF-M2, which can bind SRE.LM but cannot respond to Rho-actin signaling, did not allow efficient activation by MAL, consistent with the failure of SRF-M2 to interact with MAL in vitro (Figure 3B, right).

To assess the role of MAL in Rho-actin signaling to SRF more directly, we examined its effect on serum-induced transcription. At low expression levels, MAL (met) substantially potentiated serum-induced SRF reporter activity, and at high expression level also strongly stimulated reporter activity in serum-starved cells (Figure 3C). Expression of C3 transferase, which ADP-ribosylates and inactivates Rho, strongly inhibited reporter activation by MAL(met) in both serum-starved and -stimulated cells (Figure 3D). In contrast, C3 transferase expression did not affect reporter activation by MALΔN, suggesting that the N-terminal RPEL domain may control sensitivity to Rho-actin signaling (Figure 3D; see below). Expression of MAL(met)C471, which lacks C-terminal sequences, did not activate the reporter in serum-starved cells, and indeed strongly inhibited its response to serum stimulation (Figure 3E). MAL(met)C471 expression also inhibited reporter activation by activated forms of RhoA and mDia1, both of which activate Rho-actin signaling intracellularly (Figure 3E). This observation suggests that MAL contains a C-terminal activation domain, as does myocardin, and that MAL derivatives which lack this domain can bind SRF but not activate transcription. Consistent with this idea, LexA fusion proteins containing the MAL C-terminal sequences strongly activated a LexA reporter gene; however, serum stimulation did not alter transcriptional activation by these fusions (Figure 3F).

Nuclear Accumulation of MAL Requires Rho-Actin Signaling

We next tested the ability of known inhibitors of Rho-actin signaling and other pathways to inhibit serum-induced nuclear accumulation of MAL. Serum-induced nuclear accumulation of MAL(met), MAL(fl) and MAL (BSAC) was prevented upon inactivation of Rho with C3 transferase or toxin B (Figure 4A; data not shown). Treatment of cells with latrunculin B, which sequesters actin monomer and prevents SRF activation via the Rho-

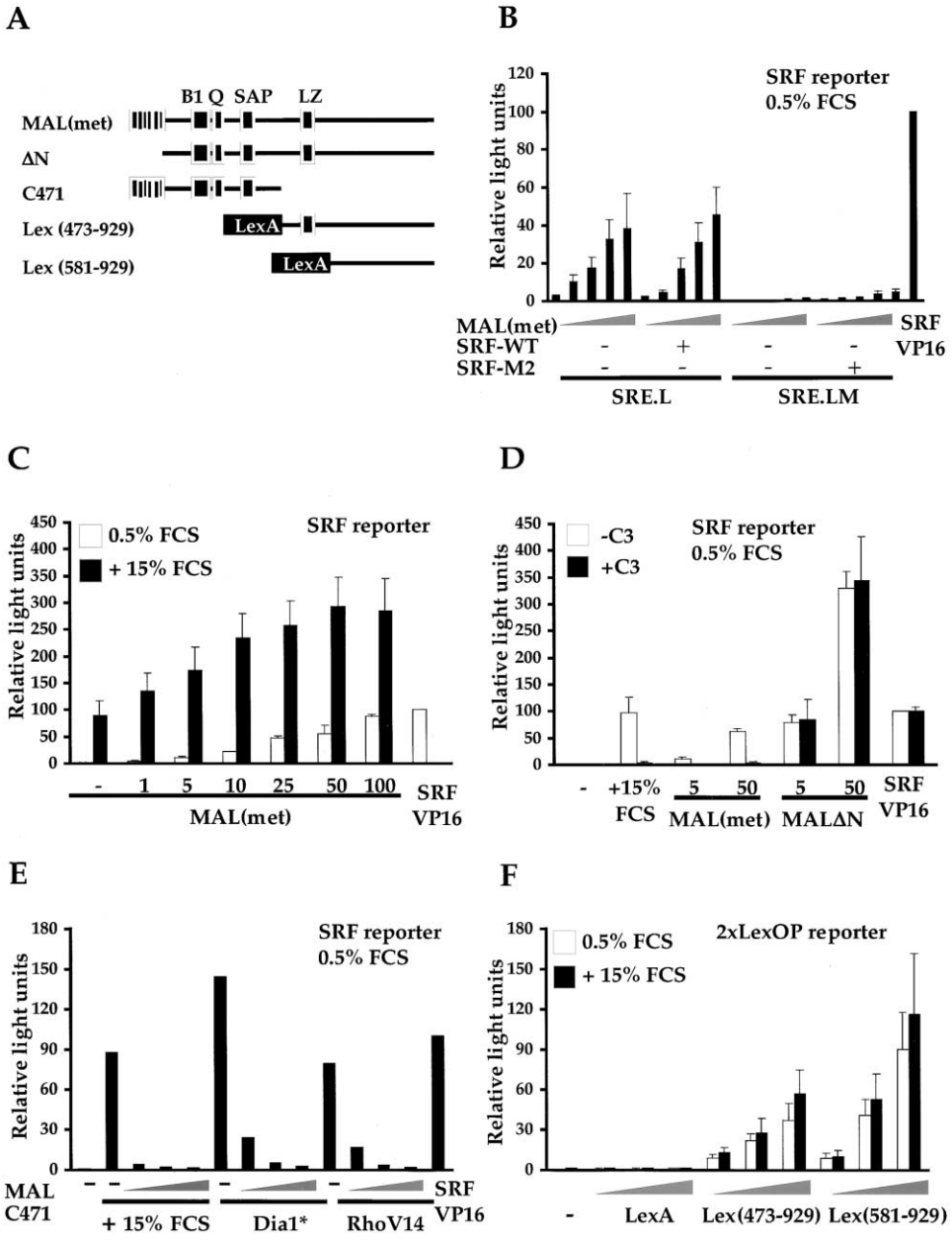

Figure 3. Reporter Analysis

Transfected cells were maintained in 0.5% serum and serum-stimulated where indicated.

(A) MAL derivatives used.

(B) MAL activates wild-type but not altered-specificity SRF. NIH3T3 cells were transfected with SRE.L2.luc or SRE.LM2.luc reporter plasmids and 0, 5, 50, 100, or 250 ng MAL(met) expression plasmid, with MLV.SRF or MLV.SRF-M2 (50 ng) as indicated.

(C) MAL potentiates the serum-induced activity of SRF. NIH3T3 cells were transfected with SRF reporter 3D.ALuc and the indicated amounts (ng) of MAL(met) expression plasmid.

(D) Rho-dependent activation of SRF by MAL(met) requires MAL N-terminal sequences. NIH3T3 cells were transfected with the indicated plasmids with or without C3 transferase (50 ng).

(E) MAL(met)C471 inhibits signaling to SRF. NIH3T3 cells were transfected with 3D.ALuc and increasing amounts (0.1, 0.5 and 1.0 μg) of MAL(met)C471 expression plasmid, and stimulated with 15% FCS or by coexpression of activated RhoA.V14 or activated mDia1 (F1F2; Copeland and Treisman, 2002) (100 ng each) as indicated.

(F) The MAL C terminus activates transcription. NIH3T3 cells were transfected with LexOP2.luc reporter and 5, 50, and 250 ng expression plasmid encoding the indicated LexA derivatives.

actin pathway (Sotiropoulos et al., 1999) completely blocked serum-induced nuclear accumulation of transiently expressed MAL(met) (Figure 4A). No inhibition of serum-induced MAL(met) translocation was observed upon inhibition of MEK-ERK signaling by U0126, of $G\alpha_i$ and ERK signaling by pertussis toxin, of the Rho effector

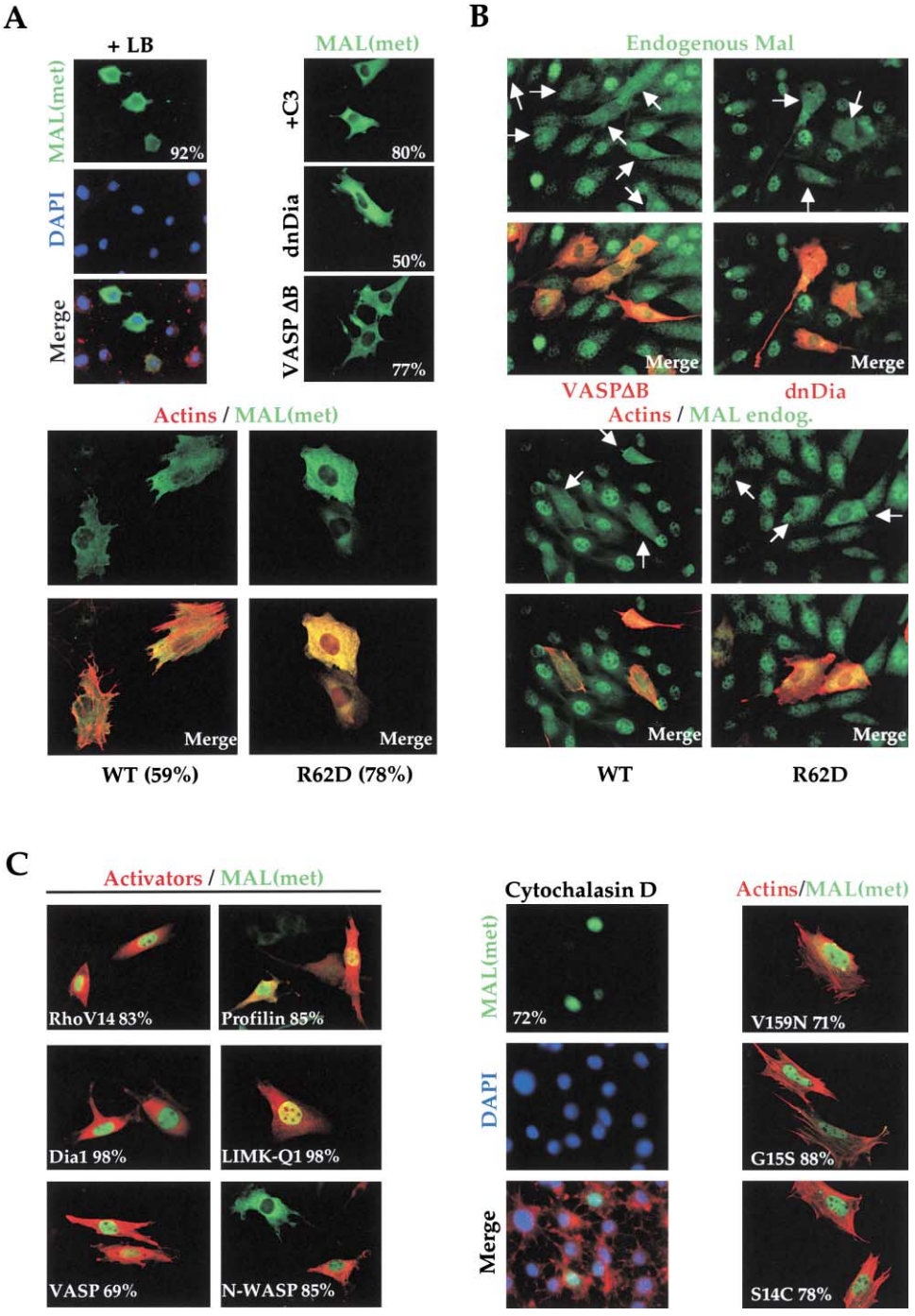

Figure 4. Nuclear Translocation of MAL Involves RhoA-Actin Signaling

Transfected NIH3T3 cells were processed for immunofluorescence as indicated after serum stimulation (A and B) or serum starvation (C).

(A) Transiently expressed MAL(met) requires Rho-actin signaling for nuclear accumulation. Cells expressed flag- or HA-MAL(met) (50 ng) with C3 transferase (50 ng), interfering myc-Dia1 (F1F2Δ1; Copeland and Treisman, 2002) (200 ng) or flag-VASP (ΔB; Grosse et al., 2003) (200 ng), or different flag-actins (wild-type, 1 μg; R62D, 200 ng). Where indicated cells were pretreated with latrunculin B and additionally stained for DNA (DAPI) and F actin (phalloidin). Figures give proportion of cells with predominantly cytoplasmic MAL.

(B) Nuclear accumulation of endogenous MAL requires Rho-actin signaling. Cells expressed interfering myc-Dia1, flag-VASP, or actin derivatives as in (A). Arrows indicate transfected cells.

(C) Activation of RhoA-Actin signaling induces nuclear accumulation of MAL(met). Cells expressing HA- or flag-tagged MAL(met) (50 ng) with indicated activators (50 ng), actins (200 ng) or vector. Where indicated, cells were treated with cytochalasin D, and additionally stained for DNA and F actin. Figures give proportion of cells with predominantly nuclear MAL.

kinase ROCK by Y27632, or of PI-3 kinase signaling by LY294002 (data not shown).

We previously demonstrated that inactive deletion derivatives of mDia1 (F1F2Δ1) and VASP (VASPΔB) can specifically interfere with operation of the Rho-dependent signaling pathway to SRF (Copeland and Treisman, 2002; Grosse et al., 2003). Expression of these mutants effectively blocked serum-induced nuclear accumulation of both transiently expressed MAL(met) (Figure 4A) and endogenous MAL (Figure 4B). Overexpression of either wild-type β actin or its nonpolymerizable mutant derivatives β actin G13R and R62D is also sufficient to inhibit Rho-actin signaling to SRF (Posern et al., 2002; Sotiropoulos et al., 1999). Actin overexpression inhibited serum-induced nuclear accumulation of transiently expressed MAL(met) or endogenous MAL (Figures 4A and 4B, lower images). Taken together, these results show that operation of the Rho-actin pathway is required for MAL nuclear translocation in response to serum.

Alterations in Actin Dynamics Induce MAL Nuclear Accumulation

Many proteins involved in Rho GTPase signaling to actin dynamics can promote actin polymerization and SRF activation upon overexpression either as wild-type or activated forms. These include RhoA, Cdc42, and Rac (Hill et al., 1995); LIM kinase (Geneste et al., 2002; Sotiropoulos et al., 1999); profilin (Sotiropoulos et al., 1999); mDia1 and mDia2 (Copeland and Treisman, 2002; Sotiropoulos et al., 1999; Tominaga et al., 2000); VASP (Grosse et al., submitted; Sotiropoulos et al., 1999); and WASP and N-WASP (Geneste et al., 2002; Sotiropoulos et al., 1999). We therefore tested the effect of expressing active forms of these proteins on MAL subcellular localization. In each case, expression was sufficient to induce nuclear translocation of MAL(met) in serum-starved cells (Figure 4C; data not shown).

SRF can also be activated in the absence of extracellular signals by certain actin binding drugs, which interfere directly with the actin treadmilling cycle (Sotiropoulos et al., 1999). Cytochalasin D and swinholide A, which do not promote actin polymerization but nevertheless activate SRF, induced nuclear accumulation of transiently expressed MAL (Figure 4C; data not shown). Jasplakinolide, which stabilizes F actin and activates SRF (Sotiropoulos et al., 1999), also efficiently induced MAL nuclear accumulation (data not shown). Expression of β actin mutants S14C and V159N, which apparently stabilize F actin and strongly activate SRF (Posern et al., 2002) also induced efficient MAL(met) nuclear accumulation, as did expression of a third mutant of this type, actin G15S (F.M. and G.P. unpublished data; Figure 4C). These data establish an intimate correlation between activation or inhibition of Rho-actin signaling and accumulation of MAL in the nucleus or cytoplasm, respectively.

MAL Nuclear Accumulation Involves MAL N- and C-Terminal Sequences

To define sequences required for regulated nuclear accumulation of MAL, we analyzed relocalization of mutant MAL derivatives following serum stimulation of transfected NIH3T3 cells. Data are summarized in Figure 5A. MAL(fl) and MAL(BSAC) exhibited regulated translocation upon serum stimulation, as did MAL(met) (Figure 5A), indicating that sequences N-terminal to the MAL (met) ATG, including RPEL motif 1, are not absolutely required for regulation. In contrast, MALΔN, which lacks all the RPEL motifs, was constitutively nuclear-localized in serum-starved cells (Figures 5A and 5B). We tested the functional significance of RPEL motifs 2 and 3 by introducing point mutations into their conserved core. MAL(met) derivatives containing point mutations in both RPEL motifs (RR33/77DD or PP34/78AA) were also nuclear in serum-starved cells (Figures 5A and 5B). Removal of greater extents of N-terminal sequence generated proteins, which were distributed diffusely throughout the cell regardless of stimulation (Figures 5A and 5B). We used C3 transferase coexpression to test whether nuclear accumulation of MALΔN and the RPEL mutants required functional Rho. In the presence of C3, both MALΔN and the MAL(met) RPEL mutants remained nuclear (Figure 5B), and their ability to activate transcription was unaffected (Figure 3D; data not shown). These data establish that the integrity of the N-terminal domain, particularly RPEL motifs 2 and 3, is required for linkage of MAL nuclear accumulation to the Rho-actin signal pathway. The function of the RPEL motifs will be analyzed further below.

We also examined the effect of deleting other conserved elements of MAL. Deletion of MAL(fl) sequences C-terminal to residue 471 resulted in predominantly nuclear localization of the protein even in serum-starved cells, as did truncation of MAL(met) to residue 471 or 256 (Figure 5A). Appropriate regulation could be restored to the MAL(met)C471 truncations by addition of MAL C-terminal sequences (Figure 5A). In contrast to the MALΔN and the RPEL mutants, however, both MAL(fl)C471 and MAL(met)C471 required functional Rho for nuclear accumulation, remaining cytoplasmic upon inactivation of Rho by C3 transferase (Figure 5B). A deletion derivative of MAL(met) lacking the Q-rich domain (MAL(met)ΔQ) behaved in a similar manner (Figure 5B). Deletion of the SAP or LZ motifs had no effect on regulation (Figure 5A). These results show that the C-terminal and Q box sequences are not required for the response to signal per se, suggesting that they may mediate cytoplasmic retention or nuclear export.

To investigate sequences required for nuclear import in response to signal, we focused on two regions rich in basic residues: B1, a basic region homologous to one first identified in myocardin (Wang et al., 2001), and B2, a related sequence between RPEL motifs 2 and 3 (see Figure 1A). Removal of the MAL(met) B1 region, which abolishes complex formation with SRF, reduced the efficiency of serum-induced nuclear accumulation such that many cells exhibited only partial MAL accumulation in the nucleus (Figure 5A). This suggests that B1 is involved in nuclear import, and consistent with this view, removal of B1 from the constitutively nuclear mutant MALΔN resulted in its relocation to the cytoplasm (Figure 5A). While removal of B2 alone had no effect on MAL(met) regulation, removal of both B1 and B2 prevented its serum-induced nuclear accumulation (Figure 5A; see below).

The results presented in this section thus show that:

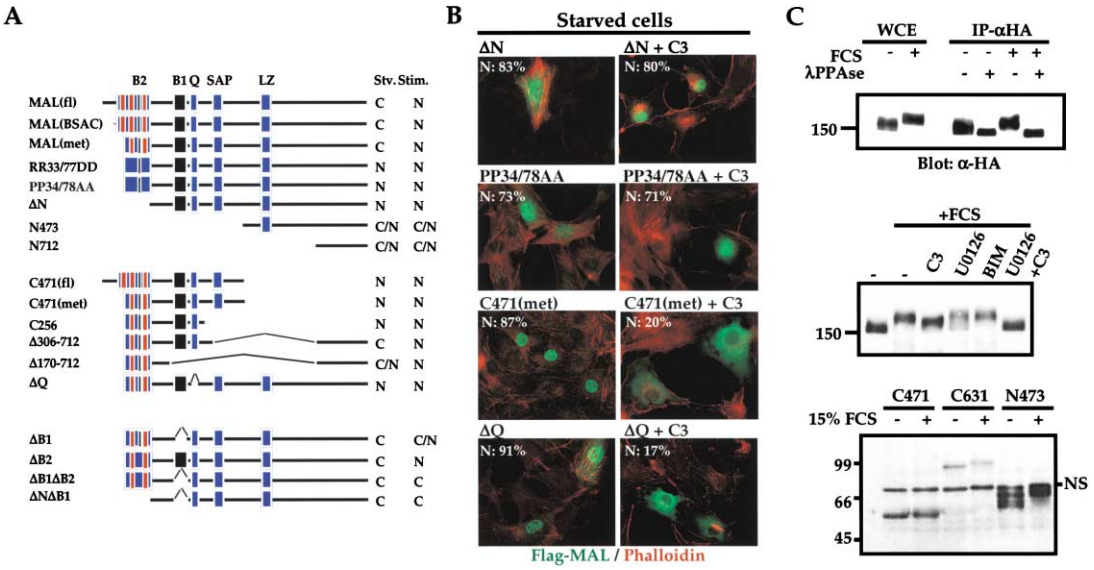

Figure 5. MAL N- and C-Terminal Sequences Have Regulatory Functions

(A) MAL domains required for serum-induced MAL redistribution. Cells expressing MAL derivatives (50 ng) were serum-starved or -stimulated as indicated before staining for the MAL epitope tag. N and C, predominantly nuclear or cytoplasmic localization in >80% of the cells, respectively; N/C, localized throughout the cell.

(B) Dependence on Rho distinguishes two types of nuclear MAL mutant. Cells expressed MAL derivatives (50 ng) with or without C3 transferase (50 ng). Merged images for MAL and F actin are shown. Proportions of cells exhibiting predominantly nuclear MAL localization are shown.

(C) Serum-induced MAL(met) phosphorylation. Cells expressing MAL(met) or derivatives (200 ng) were left unstimulated or serum-stimulated for 1 hr and analyzed by immunoblotting. Top, extracts were analyzed directly or following immunoprecipitation and treatment with λ phosphatase. Center, MAL in cells treated with U0126 (10 μM) or bisindolylmaleimide (BIM; 10 μM) or with coexpressed C3. Bottom, cells expressing different MAL truncations. NS, non-specific product.

(1) signal serum-regulated nuclear accumulation of MAL(met) requires RPEL motifs 2 and 3; (2) the MAL Q box and C-terminal sequences are required for nuclear export or cytoplasmic retention; and (3) the basic motifs B1 and B2 are required for nuclear accumulation in response to Rho-actin signaling.

MAL C-Terminal Sequences Are Subject to Serum-Induced Phosphorylation

Serum stimulation results in the activation of multiple kinase-mediated signal pathways downstream of both Rho and Ras. We therefore tested whether MAL nuclear accumulation correlated with its phosphorylation. On SDS-PAGE, endogenous MAL and transfected MAL (met) exhibited an apparent increase in M_r upon serum stimulation (Figure 5C, top; Supplemental Figure S1C available at http://www.cell.com/cgi/content/full/113/3/329/DC1). This reduction in mobility was sensitive to treatment with λ phosphatase, indicating that it results from phosphorylation; this may occur at serine or threonine residues, since MAL did not react with anti-phosphotyrosine antibodies (Figure 5C, top). To assess the relevance of Rho and Ras signaling for MAL phosphorylation, we used C3 transferase coexpression and U0126 treatment to inactivate Rho-actin and MEK-ERK signaling, respectively. Inactivation of Rho substantially reduced but did not prevent the serum-induced increase in MAL M_r, while U0126 had a lesser effect; treatment of C3-expressing cells with U0126, however, abolished it (Figure 5C, center). A PKC inhibitor, bisindolylmaleimide, had no effect (Figure 5C, center). Both Rho and ERK signaling therefore contribute to phosphorylation of MAL.

We used different truncation derivatives of MAL to gain insight into the location of the phosphorylations. MAL(met) lacking sequences C-terminal to position 471 exhibited a minimal change in M_r following serum stimulation (Figure 5C, bottom). In contrast MALN473, which comprises residues 437–929 alone, was phosphorylated upon serum stimulation, as was MAL(met)C631, which lacks sequences C-terminal to residue 631 (Figure 5C, bottom). These data indicate that the C-terminal sequences of the protein are likely targets for phosphorylation. We note, however, that the C-terminally truncated MAL mutant MAL(met)C471 remains dependent on signaling for nuclear import. Phosphorylation is thus not a prerequisite for the control of MAL nuclear accumulation by its N-terminal domain via Rho-actin signaling.

MAL Associates with Actin via the RPEL Motifs

The results presented above show that inhibition of Rho-actin signaling or overexpression of actin itself can inhibit nuclear translocation of MAL through a mechanism dependent on the integrity of the N-terminal RPEL motifs. We therefore investigated whether this reflects the association of MAL with actin in vivo. First, mutant actins designed to localize to novel cellular locations were constructed and their ability to alter the localization of coexpressed MAL(met) tested by immunofluorescence assay. Wild-type actin or nonpolymerizable actin R62D tagged with the SV40 NLS efficiently accumulated in the nucleus, and in the presence of these actins coex-

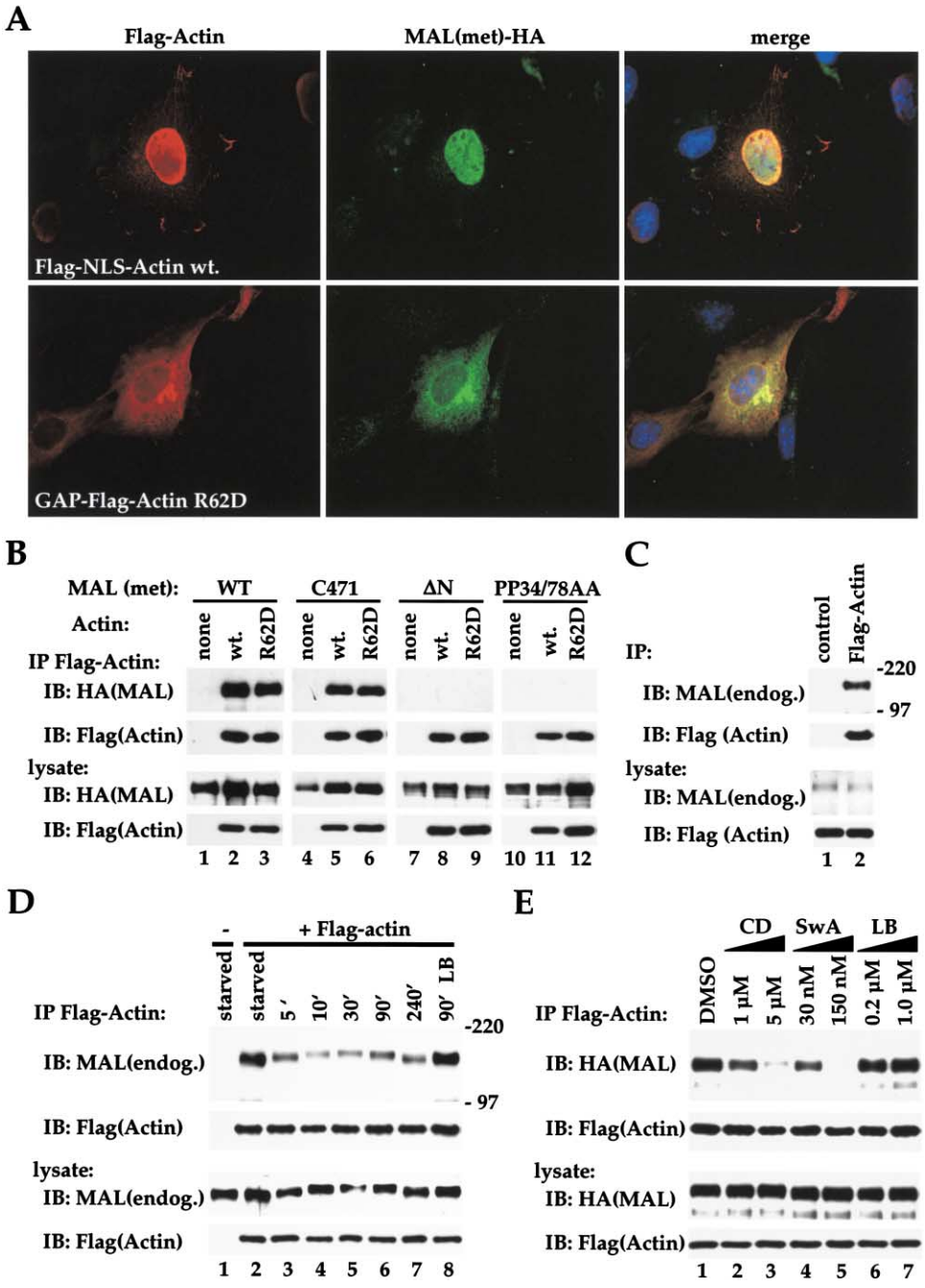

Figure 6. Actin Interacts with the MAL RPEL Domain

(A) MAL colocalizes with actin. Serum-starved transfected cells expressed the indicated actins (500 ng) together with HA-tagged MAL(met) (100 ng). In this experiment, 48% of cells expressing GAP-R62D exhibited perinuclear localization of both actin and MAL(met). NLS-actin was nuclear in 78% of transfected cells, and of these 96% also displayed nuclear MAL(met). Wild-type actin did not affect MAL(met) localization in unstimulated cells (data not shown).

(B–E) MAL coimmunoprecipitates with actin. Serum-starved cells expressing flag-tagged actin with or without HA-tagged MAL(met) (1 μg each) were stimulated with serum or actin binding drugs as indicated. Extracts were analyzed directly (lysate panels) or following immunoprecipitation with anti-flag antibody (IP panels). LB, latrunculin B; CD, cytochalasin D; SwA, swinholide A.

(B) Actin association requires the N-terminal RPEL motifs.

(C) Endogenous MAL coimmunoprecipitates with actin.

(D) Serum stimulation reduces association of actin with endogenous MAL. Cells were serum-stimulated for the indicated times. A subinhibitory level of actin (500 ng) was expressed for this experiment.

(E) Actin binding drugs which activate SRF disrupt actin-MAL association.

pressed MAL(met) was also nuclear, even in the absence of signal (Figure 6A, top; data not shown). Similarly, actin R62D tagged with a GAP43 membrane targeting sequence (McCabe and Berthiaume, 1999) exhibited strong perinuclear staining in about half the transfected cells, presumably at the Golgi, as did coexpressed MAL (met) (Figure 6A, bottom). These results suggest that actin physically associates with MAL in vivo, and that this is not dependent on actin's ability to polymerize.

Actin-MAL interactions were investigated more directly using a coimmunoprecipitation assay. Extracts from cells expressing HA-tagged MAL(met) with or without different flag-actins were immunoprecipitated with flag antibodies and tested for the presence of HA-tagged MAL proteins. In these experiments, flag-actin is expressed at around the same level as endogenous actin, as assessed by quantitative DNAse I and phalloidin staining for cellular G- and F actin, respectively (Posern et al., 2002). MAL(met), MAL(fl), and MAL(BSAC) were all readily detectable in immunoprecipitates of wild-type flag-actin (Figure 6B, lanes 1 and 2; data not shown), as was endogenous NIH3T3 MAL (Figure 6C). MAL did not coimmunoprecipitate with flag-hSmad4 or with anti-myc beads (data not shown). Interaction of MAL(met) with actin did not require sequences C-terminal to residue 471, but was absolutely dependent on the N-terminal domain, in particular the integrity of RPEL motifs 2 and 3 (Figure 6B, compare lanes 2, 5, 8, and 11). Association was not affected by the deletion of basic motif B2, which lies between RPEL2 and RPEL3 (data not shown). The nonpolymerizable actin mutant R62D interacted with MAL(met) in a similar way to wild-type actin (Figure 6B), consistent with the notion that it is unpolymerized actin which controls MAL activity (Posern et al., 2002; Sotiropoulos et al., 1999).

We used the coimmunoprecipitation assay to test whether activation of Rho-actin signaling alters actin-MAL association. Serum stimulation caused a transient decrease in the amount of endogenous MAL coimmunoprecipitated with flag-actin, reaching a minimum at 10 min following stimulation and recovering slowly thereafter (Figure 6D, lanes 2–7). Latrunculin B treatment, which inhibits SRF activation, effectively blocked serum-induced dissociation of the actin-MAL complex (Figure 6D, lane 8). Similar results were obtained with cotransfected MAL(met) (data not shown). Unlike latrunculin B, cytochalasin D and swinholide strongly activate SRF (Sotiropoulos et al., 1999), and treatment of cells with these drugs also substantially reduced actin association with both contransfected MAL(met) (Figure 6E) and endogenous MAL (data not shown). This observation is consistent with our previous proposal that cytochalasin D and swinholide, but not latrunculin B, disrupt the regulatory function of G actin (Sotiropoulos et al., 1999). Together, these results show that actin associates with MAL via the RPEL motifs and that Rho-actin signaling leads to dissociation of this complex.

Functional MAL Is Required for SRF Activation

To establish the relevance of MAL function for SRF activation, we first studied dominant interfering mutants of MAL. Three basic box mutants which remain cytoplasmic upon serum stimulation were described above: MALΔNΔB1, MAL(met)ΔB1B2, and MAL(met)ΔB1 (see Figure 5A). We reasoned that these mutants might sequester endogenous MAL proteins in the cytoplasm, since they retain the LZ sequence required for MAL dimerization (see Figures 2A and 2D).

We used immunofluorescence assays to test whether cytoplasmic MAL mutants can prevent nuclear accumulation of intact MAL. Derivatives of the proteins lacking the LZ sequence served as controls. The basic box mutants MAL(met)ΔB1B2 and MAL(met)ΔB1 strongly inhibited nuclear accumulation of coexpressed MAL(met) upon serum stimulation, provided that they contained the LZ (Figure 7A, top; data not shown). Both mutants substantially reduced serum-stimulated activity of a cotransfected SRF reporter in a dose-dependent manner, and this required the LZ (Figure 7B). Expression of MAL (met)ΔB1B2 also strongly inhibited serum induction of a chromosomal SRF reporter gene, but did not prevent activation of the cellular c-*fos* and egr-1 genes, which are regulated independently of Rho-actin signaling (Figure 7C; Sotiropoulos et al., 1999). Expression of MAL (met)ΔB1B2 and MAL(met)ΔB1 did not affect serum-induced activity of an Elk-1 reporter gene system (data not shown). The MAL(met)ΔB1B2 and MAL(met)ΔB1 mutants thus represent specific inhibitors of Rho-actin signaling, which act at the level of MAL rather than SRF. These data establish that functional MAL is required for SRF activation by extracellular signals.

MAL Associates with SRF Targets Regulated by Rho-Actin Signaling

Finally, we used chromatin immunoprecipitation (ChIP) assays to test whether MAL associates specifically with genes activated by Rho-actin signaling. We used the actin binding drug swinholide to specifically activate actin-responsive SRF target genes, which include vinculin, SRF, and cyr61, but not c-*fos* and egr-1 (K. Murai and R.T., unpublished data; Gineitis and Treisman, 2001; Sotiropoulos et al., 1999). SRF was specifically associated in the ChIP assay with all the above-mentioned promoters in both untreated and swinholide-treated cells (Figure 7D, lanes 3 and 4). Following swinholide treatment, MAL became associated with the promoters of the SRF, vinculin and cyr61 genes but not those of c-*fos* or egr-1 (Figure 7D, lanes 7 and 8). In contrast SAP-1, a member of the TCF family of SRF cofactors, was associated only with the egr-1 promoter in both untreated and swinholide-treated cells. The SRF cofactors MAL and SAP-1 therefore associate differentially with SRF target genes.

Discussion

In this paper, we have identified the MAL protein as a signal-regulated SRF coactivator. MAL activity is controlled by a mechanism in which activation of actin polymerization by Rho induces its redistribution from the cytoplasm to the nucleus. The effect of activated or inhibitory derivatives of components of the Rho-controlled actin polymerization pathway on the subcellular distribution of MAL correlates completely with their effect on SRF activity. Both wild-type cytoskeletal actin and nonpolymerizable actin mutants associate with

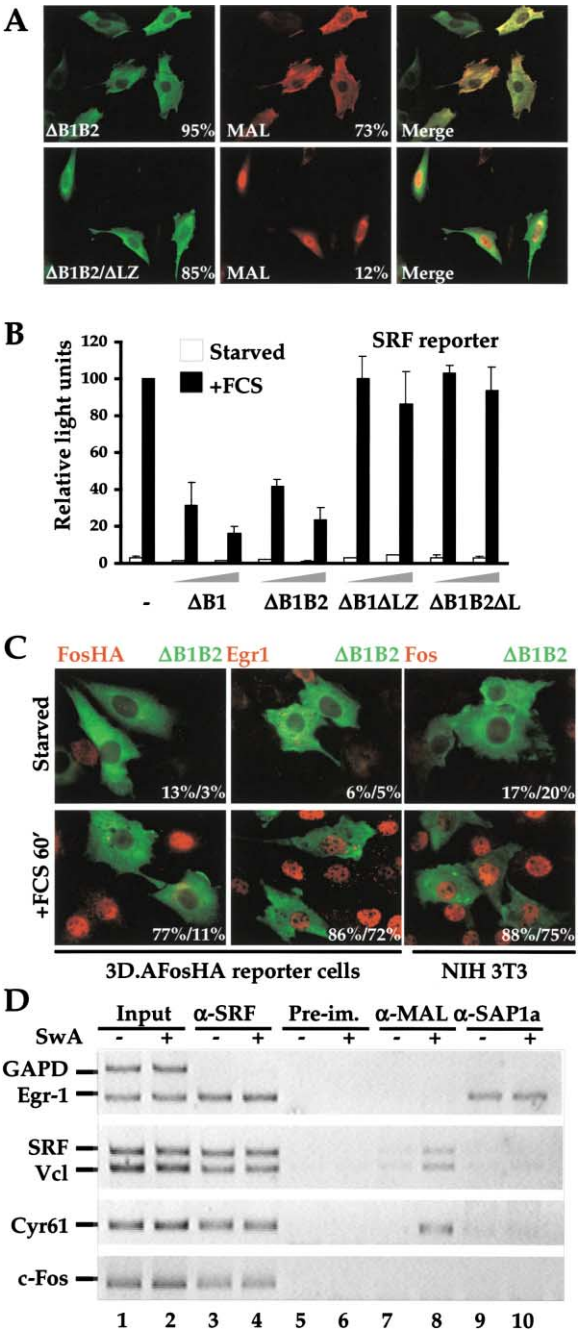

Figure 7. MAL Regulates SRF Target Promoters In Vivo

(A) Cytoplasmic MAL(met) deletion mutants retain wild-type MAL (met) in the cytoplasm. Cells expressing cytoplasmic flag-MAL(met) derivatives ΔB1B2 or ΔB1B2ΔLZ (250 ng) with intact HA-tagged MAL(met) (50 ng) were serum-stimulated before staining as indicated. Proportion of cells with predominantly cytoplasmic MAL(met) is indicated.

(B) Cytoplasmically restricted MAL derivatives inhibit SRF activation. SRF reporter 3D.ALuc activity was assessed in cells expressing MAL derivatives as indicated.

(C) Interfering MAL mutants specifically inhibit Rho-actin signaling to SRF. Serum-starved or -stimulated 3AD.AfosHA reporter or NIH3T3 cells expressing MALΔB1B2 (250 ng) and stained for inhibitory MAL proteins, SRF reporter product, Egr-1, or Fos. Marker-positive cells in untransfected/MALΔB1B2-expressing cells are indicated.

(D) MAL associates with SRF targets regulated by Rho-actin signaling. Serum-starved cells were treated with 1 μM swinholide A for 30 min. Chromatin immunoprecipitates were prepared and analyzed by PCR as indicated.

MAL N-terminal RPEL motifs, which are also required for the response to Rho signaling. Nuclear MAL is required for SRF activation via the Rho-actin pathway, and chromatin immunoprecipitation shows that MAL associates specifically with SRF target genes regulated via the pathway. MAL regulation thus provides a direct link between actin cytoskeletal dynamics in the cytoplasm and transcriptional activation in the nucleus. It is likely that aberrant regulation of a subset of SRF target genes forms the basis for transformation in t(1;22)(p13;q13) AML, in which MAL is fused to the RBM/OTT gene (Ma et al., 2001; Mercher et al., 2001). The functional elements of MAL and the structure of the Rho-actin-MAL signal pathway are summarized in Figure 8.

Translation of the predominant MAL RNA present in NIH3T3 cells is initiated upstream of the first ATG codon in the open reading frame, most likely at a leucine codon at position 92. The resulting protein, MAL(fl), which contains all three RPEL motifs, has an Mr similar to that of endogenous NIH3T3 cell MAL. A second MAL isoform, MAL(BSAC), in which the RPEL motifs are preceded by distinct N-terminal sequences, was not detectable in our cells. Both proteins are cytoplasmic in serum-starved cells but accumulate in the nucleus upon serum stimulation. MAL(BSAC) is localized in a punctate pattern rather than diffusely, however, suggesting a targeting role for its N-terminal sequences. Heightened activity of the Rho pathway may account for the nuclear localization of BSAC in 293 cells (Sasazuki et al., 2002). MAL does not colocalize with F actin.

Mutational analysis of MAL revealed a complex set of regulatory sequences (Figure 8A). Two classes of MAL mutant are nuclear in serum-starved cells, but can be distinguished according to whether nuclear accumulation requires functional Rho. MAL mutants which lack the N-terminal RPEL domain, or which contain point mutations in RPEL motifs 2 and 3, do not require basal Rho activity for nuclear accumulation, indicating that the RPEL motifs are required for Rho-regulated nuclear accumulation. In contrast, MAL mutants which lack the Q-box or C-terminal sequences still require functional Rho for their accumulation in the nucleus. This indicates that these sequences are not required for the response to Rho signaling per se. Instead, they may mediate either export of MAL from the nucleus or its retention in the cytoplasm. Two basic motifs, B1 and B2, are required for effective nuclear accumulation of MAL in response to Rho-actin signaling (Figure 8A). Preliminary experiments indicate that the basic motifs do not appear to function as autonomous NLS elements, since they do not promote nuclear localization when linked to pyruvate kinase, a cytoplasmic protein (F.M., unpublished data). Mutants lacking the basic motifs remain cytoplasmic upon serum stimulation and retain wild-type MAL in the cytoplasm: they thus act as Rho-actin pathway-specific SRF inhibitors.

Our studies show that MAL associates with actin in vivo. Although the data are consistent with the view that MAL directly binds actin, we have as yet been unable to

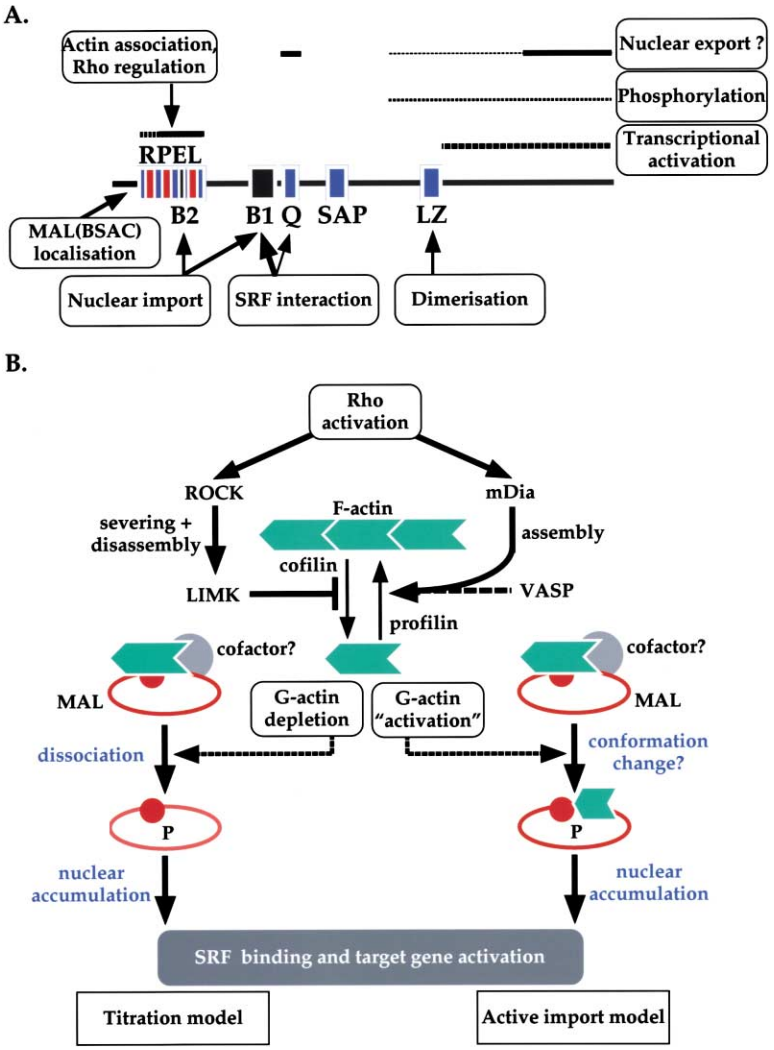

Figure 8. MAL and Its Relationship to Rho Signaling and Actin Dynamics

(A) Functional elements of the MAL protein. Functional elements are shown.

(B) The Rho-actin signal pathway and models for MAL nuclear accumulation. Top, control of actin treadmilling at the levels of F actin assembly and severing/disassembly via the Rho effectors ROCK-LIMK and mDia. Titration model for regulated MAL nuclear accumulation is shown bottom left. Note that the data do not exclude the operation of an additional active import mechanism, shown at the right. Red circle represents either MAL nuclear import signal occluded by actin association or export signal activated by actin association. For discussion see text.

detect direct interaction between the purified proteins, suggesting that additional cofactor(s) may be involved. The RPEL motifs are critical for actin-MAL association, which is abolished by point mutations at the conserved core of motifs 2 and 3. Intriguingly, in myocardin RPEL motif 2 mismatches the consensus (RS—SL), suggesting that it might exhibit a reduced affinity for actin. Indeed, transiently expressed myocardin is nuclear, regardless of Rho signaling, and this is due to its N-terminal RPEL region (F.M., unpublished data). In contrast, the third MAL family protein, MAL16/MRTF-B (Mercher et al., 2001; Wang et al., 2002), contains RPEL motifs similar to those of MAL, suggesting that it may be similarly regulated. In all three MAL family members and the single *Drosophila* MAL ortholog (GenBank NM167965), however, RPEL motif 1 has the variant core sequence RR—EL (Figure 1A), but the functional significance of this is unclear. One other family of RPEL proteins exists, each with a single N-terminal "variant" motif and a C-terminal cluster of three motifs (e.g., GenBank BC012871). It will be interesting to test whether these proteins also associate with G actin.

Considered together, our data suggest a simple scheme for regulation of MAL subcellular localization by Rho-actin signaling (Figure 8B). MAL localization is regulated in response to the level of either G actin itself or a G actin subpopulation defined by interaction with actin binding proteins or nucleotide-loading status. In the simplest model, G actin, perhaps acting with a cofactor, would titrate MAL and thereby occlude nuclear import signals or create a nuclear export signal; in either case, depletion of the G actin pool by Rho-induced actin polymerization would result in MAL nuclear accumulation (Figure 8B, left). MAL nuclear accumulation can also be induced by actin binding drugs, which directly interfere with actin-MAL association, consistent with our previous proposal (Sotiropoulos et al., 1999). We cannot exclude the possibility that an additional positively acting G actin subpopulation exists which binds MAL in such a way as to promote its nuclear accumulation (Figure 8B, right). Our data do not address the mechanism of MAL downregulation: it is likely that additional regulatory mechanisms must operate, since MAL remains nuclear even after the shutdown of target gene expression (see Gineitis and Treisman, 2001).

Serum stimulation also induces MAL C-terminal phosphorylation via both Rho- and MEK-ERK dependent signal pathways. The finding that nuclear accumulation of MAL mutants lacking the C-terminal sequences is still dependent on Rho signaling, however, suggests that

phosphorylation is not a prerequisite for serum-induced MAL nuclear accumulation and SRF activation. Consistent with this idea, we have observed that expression of activating actin mutants (Posern et al., 2002) can induce nuclear accumulation of MAL without its concomitant phosphorylation (F.M. and G.P. unpublished data). Nevertheless, we cannot exclude the possibility that regulated phosphorylation of MAL may control its nuclear accumulation under circumstances in which signaling does not regulate actin dynamics. It also remains possible that phosphorylation regulates nuclear export, cytoplasmic retention, or even transcriptional activation.

The MAL N-terminal sequences act to inhibit MAL-SRF complex formation. We are currently testing whether this reflects association of MAL with actin present in the extracts used for the assays. Inhibition of MAL-SRF complex formation by actin would set a threshold level of signal for SRF activation and would explain the inhibition of SRF activity by expression of NLS-actin (Posern et al., 2002). The properties of the MAL-SRF interaction are consistent with our previous studies of Rho-actin signaling to SRF. MAL does not form complexes with the altered-specificity SRF derivative SRF-M2, which cannot mediate serum-induced transcription activation (Hill et al., 1993, 1994). The peptide competition experiments indicate that the SRF surfaces contacted by MAL overlap with those contacted by TCF, consistent with our previous observations that TCF binding inhibits Rho-actin signaling to SRF (Hill et al., 1994; Murai and Treisman, 2002). In MAL, basic region B1 is essential for interaction with SRF, while in contrast to myocardin (Wang et al., 2001) the Q box is not absolutely required. The lesser contribution of the MAL Q box to SRF interaction may reflect the dimeric nature of MAL compared with myocardin, which appears to bind SRF as a monomer (A-I.Z., unpublished data).

The chromatin immunoprecipitation experiments, DNA binding studies, and analysis of interfering MAL mutants indicate that MAL and the SAP-1 TCF associate with distinct subsets of SRF target genes, consistent with previous studies of signaling to SRF target genes (Gineitis and Treisman, 2001; Murai and Treisman, 2002; Sotiropoulos et al., 1999). At least some muscle-specific genes are responsive to Rho-actin signaling (Mack et al., 2001), and it will be interesting to elucidate the role of MAL at those promoters. Several SRF target genes dependent on Rho-actin signaling are involved in either cytoskeletal maintenance or cell adhesion (Gineitis and Treisman, 2001; Sotiropoulos et al., 1999); moreover, deletion of the mouse SRF gene disrupts cytoskeletal functions and prevents gastrulation (Arsenian et al., 1998; Schratt et al., 2002, 2001; Weinhold et al., 2000). There is increasing evidence that both Rho signaling and SRF target gene expression are involved in the epithelial-mesenchymal transition thought important for tumor progression (Bhowmick et al., 2001; Oft et al., 2002; Psichari et al., 2002). We speculate that MAL signaling controls a specific set of SRF target genes involved in cell morphology, adhesion, and movement.

Experimental Procedures

MAL cDNA Sequences and Mutants

Examination of the mouse and human EST databases reveals three different classes of MAL cDNA (Figure 1A). (1) ESTs such as GenBank BB642124 represent MAL RNAs containing an ORF with first in-frame ATG at RPEL motif 2, followed by 929 codons (MAL(met), Figure 1A; recently published as MRTF-A, AF532597; Wang et al., 2002). This ORF is conserved between human and mouse ESTs at 104 out of the 105 potential codons N-terminal to the first ATG, including RPEL motif 1. (2) ESTs such as BB842845 also contain the MAL(met) open reading frame, preceded by RPEL motif 1, and 11 distinct N-terminal codons bounded by an in-frame stop codon. (3) ESTs such as GenBank BB850403 represent a MAL mRNA isoform encoding the BSAC protein, which contains RPEL motifs 1–3 preceded by a third distinct 14 residue N-terminal sequence following an in-frame ATG codon (see XM128296; Sasazuki et al., 2002). MAL mutants are as follows, numbering for MAL(met): ΔN,1-80; ΔB1, 223-248; ΔB2, 57-65; ΔQ, 264-285; ΔSAP, 349-386; ΔLZ, 520-560; other mutants are described in the Figures.

Expression and Reporter Plasmids

Full details are available on request. MAL(met) cDNA was isolated by PCR from an NIH3T3 cDNA library (Clontech) using EST-derived oligonucleotides. The MAL(met) mRNA 5′UTR was isolated from NIH3T3 cells by RACE-PCR; oligonucleotides were used to create the MAL(BSAC) N terminus. MAL derivatives were expressed with N- or C-terminal flag or HA-tags in EFplink (Sotiropoulos et al., 1999), or for intact MAL(fl), pcDNA3. Expression plasmids for SRF derivatives, Rho proteins and effectors, actins, VASP, and C3 transferase were as described (Copeland and Treisman, 2002; Grosse et al., 2003; Hill et al., 1993, 1994; Posern et al., 2002; Sotiropoulos et al., 1999). GAP43-actin contains an N-terminal MLCCMRRTKQV peptide (McCabe and Berthiaume, 1999). Reporter plasmids were 3DA.Luc and MLV-lacZ (Geneste et al., 2002); SRE.L2.luc, SRE.LM2.luc, and 2LexOP.luc are derivatives of CAT reporters (Sotiropoulos et al., 1999).

Cell Lines and Transfections

NIH3T3 or SRE.FosHA cells in 6-well dishes were transfected using lipofectamine (Invitrogen). DNA inputs (total 1 μg) were 40 ng 3DA.Luc (50ng for SRE.L2.luc, SRE.LM2.luc or 2xLexOPluc), 150 ng MLV-LacZ reference, with expression plasmids as in figure legends. After maintenance in 0.5% FCS for 18 hr, serum stimulation was for 7 hr. Normalized data were expressed relative to reporter activation by the constitutively active SRF derivative SRF-VP16 (50 ng), performed in parallel in every set of transfections, and presented as mean ± SEM of 3 independent experiments (Copeland and Treisman, 2002). For immunofluorescence, transfected cells were kept in DME/1 mg/ml BSA before serum stimulation or drug treatments (Sotiropoulos et al., 1999) for one hour (or as indicated) before processing.

Antibodies and Immunofluorescence

Immunofluorescence and imaging was as described (Sotiropoulos et al., 1999; Posern et al., 2002). Primary antibodies were rabbit anti-flag (Sigma), mouse anti-flag M2 (Sigma), mouse anti-HA 12CA5 (Cancer Research UK and Roche), and mouse anti-myc 9E10 (Cancer Research UK), used at 1/100 to 1/300 dilution. MAL antisera were produced in rabbits immunized with GST-MAL(met) residues 1–170, affinity-purified on protein A, and depleted of anti-GST activity, and used at 1/100 dilution. Secondary antibodies were FITC- and TRITC-anti-mouse and anti-rabbit (DAKO and Jackson Laboratories) used as directed. TRITC-labeled phalloidin (Molecular Probes) was used at 33–66 nM. For quantitation, MAL localization was scored as predominantly nuclear, predominantly cytoplasmic, or evenly distributed in 80–120 cells.

Immunoblotting, Immunoprecipitations, and ChIP

Immunoblotting was by standard techniques. For immunoprecipitations, 2 μg DNA was transfected per 9 cm plate. For actin experiments, cell lysates in RIPA were immunoprecipitated in 1% TX (Posern et al., 2000), using M2 anti-flag or anti-HA agarose beads (Sigma). Actin binding drugs were present throughout processing where appropriate. For dephosphorylation, immunoprecipitates were incubated in λ phosphatase buffer with or without enzyme (NEB) for 3 hr at 30°C. For MAL dimerization assays, extracts were immunoprecipitated with M2 anti-flag or control anti-myc agarose beads. ChIP assays were as described (Alberts et al., 1998) using anti-SRF and anti-SAP1a antibodies (4 μg/ml Santa Cruz, sc-335

and sc-13030, respectively) at 20°C for 1 hr. Primer details are available on request.

Gel Mobility Shift Assays

Gel shift assays used DNA binding probes from c-*fos* promoter mutants ΔTCF (SRE.L) and ΔTCFΔSRF (SRE.LM), with purified SRF(133–265) and MAL derivatives produced by transient transfection (Murai and Treisman, 2002). Peptide competition assays contained 0, 0.8, 4, or 20 μM of Elk-1 B-box peptides (Figure 2C; Ling et al., 1997).

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