In addition to regulating cell motility, contractility, and cytokinesis, the actin cytoskeleton plays a critical role in the regulation of transcription and gene expression. We have previously identified a novel muscle-specific actin-binding protein, STARS (striated muscle activator of Rho signaling), which directly binds actin and stimulates serum-response factor (SRF)-dependent transcription. To further dissect the STARS/SRF pathway, we performed a yeast two-hybrid screen of a skeletal muscle cDNA library using STARS as bait, and we identified two novel members of the ABLIM protein family, ABLIM-2 and -3, as STARS-interacting proteins. ABLIM-1, which is expressed in retina, brain, and muscle tissue, has been postulated to function as a tumor suppressor. ABLIM-2 and -3 display distinct tissue-specific expression patterns with the highest expression levels in muscle and neuronal tissue. Moreover, these novel ABLIM proteins strongly bind F-actin, are localized to actin stress fibers, and synergistically enhance SRF-dependent activation of SRF. Conversely, knockdown of endogenous ABLIM expression utilizing small interfering RNA significantly blunted SRF-dependent transcription in C2C12 skeletal muscle cells. These findings suggest that the members of the novel ABLIM protein family may serve as a scaffold for signaling modules of the actin cytoskeleton and thereby modulate transcription.

The actin cytoskeleton controls a wide range of cellular processes, particularly those that require shape changes, such as cell motility, contractility, mitosis and cytokinesis, axon outgrowth, as well as endocytosis and secretion (reviewed in Refs. 1 and 2). Beyond these mechanical functions, actin has also been shown to play a critical role in the regulation of transcription and gene expression, either through its direct association with nuclear chromatin-remodeling proteins (reviewed in Ref. 3) or indirectly through cytoplasmic changes in cytoskeletal actin dynamics (4). The latter effects are tightly controlled by the state of actin polymerization, i.e. the equilibrium between incorporation of monomeric actin (G-actin) at the barbed end of a filament and the dissociation of actin from the pointed end of polymerized actin (F-actin). This process, referred to as “actin treadmilling,” is regulated by several signal transduction cascades that converge on actin-binding proteins such as cofillin/actin-depolymerizing factor, profilin, and β-thymosin (reviewed in Ref. 5).

The Rho family of GTPases, including the best characterized members Rho, Rac, and Cdc42, serve as molecular switches in the regulation of a wide variety of signal transduction pathways (6, 7), in particular actin polymerization and stress fiber formation (8). Rho GTPases alternate between two conformational states, the active state (bound to GTP) and the inactive state (bound to GDP). This balance is, in turn, carefully regulated by numerous activators (guanine nucleotide exchange factors (GEFs)) and inactivators (GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors). Rho effector molecules include the kinase p160 ROCK and the mammalian homologue of diaphanous (mDia), both of which promote stress fiber formation (9). Furthermore, RhoA signaling has been shown to stimulate the transcriptional activity of the serum response factor (SRF) via changes in actin dynamics (4, 10, 11). SRF, a MADS (MCM1, Agamous, Deficiens and SRF) box containing transcription factor, regulates expression of immediate-early genes as well as muscle-specific genes (reviewed in Ref. 12). More recently, the mechanism whereby SRF senses increased levels of polymerized actin has been elucidated (13, 14). The SRF-co-activator MRTF-A/MAL, a member of the myocardin family of SRF-binding transcription factors (15, 16), is sequestered in the cytoplasm of unstimulated cells by association with unpolymerized actin. Upon activation of RhoA, actin becomes polymerized and thus releases MRTF-A/MAL, which translocates to the nucleus to associate with SRF.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ413177, DQ413176, DQ413175, DQ413174.

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5 The abbreviations used are: GEF, guanine nucleotide exchange factor; ABLIM, actin-binding LIM protein; MRTF, myocardin-related transcription factor; SRF, serum-response factor; GST, glutathione S-transferase; siRNA, small interfering RNA; EST, expressed sequence tag; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
Two Novel Members of the ABLIM Protein Family

We previously identified a novel muscle-specific actin-binding protein, STARS (striated muscle activator of Rho signaling), which directly binds actin and co-localizes with actin stress fibers (17). STARS stimulates SRF-dependent transcription through a mechanism that requires actin polymerization and Rho GTPase activation. In muscle cells, STARS regulates the nuclear import of the myocardin-related transcription factors (MRTFs) via depletion of the G-actin pool, thus establishing a mechanism of STARS-dependent SRF activation (18). Interestingly, in cardiomyocytes STARS is localized to the sarcomere, thus providing a potential link between contractile function and signaling. In this regard, several recent reports suggest that the sarcomere indeed serves a critical role in sensing biomechanical stress and activation of downstream signaling pathways (19–22). An important function of Rho-dependent signaling in this context (reviewed in Ref. 23) is further supported by the finding that Rho is not only activated upon pressure overload and biomechanical stress in cardiomyocytes (24) but is also required for the ensuing hypertrophic response (25–28). Finally, a RhoA-specific GEF, p63 Rho GEF, has been identified that is highly expressed in the heart and also localized to the sarcomere (29), further supporting the hypothesis that sarcomeric Rho signaling plays an important role in striated muscle tissue.

In an attempt to further dissect the Rho/STARS/SRF pathway in muscle tissue, we performed a yeast two-hybrid screen of a skeletal muscle cDNA library using STARS as bait. From this screen we identified two novel members of the ABLIM protein family, ABLIM-2 and -3, as STARS-interacting molecules. ABLIM-1 was originally found in human retina as well as in the sarcomeres of murine cardiac tissue and was postulated to regulate actin-dependent signaling (30). Likewise, ABLIM-2 and -3 display distinct tissue-specific expression patterns with the highest expression levels in muscle and neuronal tissue. Moreover, these novel ABLIM proteins are localized to actin stress fibers and show a sarcomeric localization in striated muscle. We show that ABLIM-2 and -3 both strongly bind F-actin and can augment STARS-dependent SRF activation, suggesting that this new protein family serves as a scaffold for signaling modules of the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—An amino-terminally truncated mouse STARS cDNA (encoding amino acids 234–375) was fused to the GAL4 DNA binding domain (plasmid Pas1; Clontech) and was used as bait in a yeast two-hybrid screen of ~1 × 10⁶ clones of a human skeletal muscle cDNA library (Clontech), as described (31). Briefly, clones displaying differential growth on selective plates, lacking histidine, leucine, and tryptophan, were picked and replated for overnight. Clones displaying differential growth were picked and replated for on selective plates, lacking histidine, leucine, and tryptophan, as described (31). Human ABLIM-3 was fused with a GST domain (Pgex2T vector) as described (32) to allow for GST pulldown experiments.

Northern Blot Analysis and Radioactive in Situ Hybridization—Multiple tissue Northern blots (Clontech) containing mouse and human poly(A) RNA were hybridized overnight at 65 °C with [32P]dCTP-labeled (Rediprime II random prime labeling system; Amersham Biosciences) cDNA probes corresponding to the open reading frame of mouse and human ABLIM-2 and -3, respectively. Serial washes were conducted with 2× SSC, 0.1% SDS, and 0.2× SSC, 0.1% SDS at 65 °C. Autoradiography was performed at −80 °C for 24–48 h with an intensifying screen.

For radioactive in situ hybridization, RNA probes corresponding to sense and antisense strands of ABLIM-2 and ABLIM-3 cDNAs were prepared, using T7 and T3 RNA polymerase (Roche Applied Science) and [35S]-labeled UTP. Sections of mouse embryos at various time points were subjected to in situ hybridization, as described (33). Sense probes were used as negative controls.

Generation of an ABLIM-2-specific Antiserum and Western Blot Analysis—A peptide consisting of 15 amino-terminal amino acids (NH₂-SQPQAHAPLEKPAS-OH) of mouse ABLIM-2 was synthesized (Biosynthesis) and used to generate antisera in rabbits. The amino terminus was chosen to generate an isoform-specific antiserum, because this part of the protein does not display significant homology to the other two ABLIM family members (supplemental Fig. 1). Similarly, 14 amino-terminal amino acids of mouse ABLIM-3 (NH₂-PYQDSPRPGGSN-OH) were utilized to generate an ABLIM-3 antiserum. IgG was purified from rabbit serum using protein A-Sepharose beads (Amersham Biosciences) and subsequently used for Western blotting as well as immunostaining of mouse skeletal muscle cryosections.

Immunostaining—The subcellular localization of ABLIM-2 was determined in cryosections of mouse hindlimb skeletal muscle tissue using indirect immunofluorescence. Cryosections were air-dried and fixed in 4% paraformaldehyde for 5 min, followed by three washes with PBS, permeabilization with 0.3% Triton X-100 (Sigma), and blocking in 3% horse serum for 1 h. Primary antibodies were incubated for 1 h at the following dilutions: polyclonal anti-ABLIM-2 1:100, polyclonal anti-STARS 1:50, and monoclonal anti-sarcomeric actin (Sigma) 1:200. Secondary antibodies conjugated to either fluorescein or TRITC (Vector Laboratories) were also incubated for 1 h at a
dilution of 1:250. Transfected C2C12 cells were rinsed with PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized, and blocked with 0.1% Triton X-100 and 2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Cells were then incubated with primary antibodies (anti-FLAG monoclonal antibody (Sigma), 1:200; anti-ABLIM-2 polyclonal antibody, 1:75) in 2% BSA in PBS for 1 h. Secondary antibodies conjugated to fluorescein or TRITC (Vector Laboratories) were used at 1:200. Vectashield medium with 4’,6’-diamidino-2-phenylindole (Vector Laboratories) was used for mounting.

Tissue Culture, Immunoprecipitations, and Reporter Gene Assays—293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM l-glutamine, and penicillin/streptomycin. 2

Platinum PCR was performed in an ABI 7700 thermocycler applying the SYBR Green PCR SuperMix-UDG (Invitrogen). Resulting data were normalized to 18 S rRNA.

The siRNAs used were as follows: ABLIM-1, sense 5’-CAGAGCAUUCCACAAUA-3’ and antisense 5’-UGGAUGUGGAAAUUCUGGG-3’ (Eurogentec, Belgium). The siRNAs for ABLIM-2 and -3 were obtained from Dharmacon (ON-TARGET plus; catalogue numbers 058308 (ABLIM-2) and 041985 (ABLIM-3)). Negative control siRNA duplexes (OR-0030-NEG05) were purchased from Eurogentec. Primers for real time PCR analyses were designed using the primer3 soft-

RESULTS

Yeast Two-hybrid Screen with STARS and Molecular Cloning of ABLIM-2 and ABLIM-3—To identify novel STARS-interacting proteins in striated muscle tissue, we performed a yeast two-hybrid screen of a human skeletal muscle cDNA library using the evolutionarily conserved carboxyl terminus of STARS (amino acids 234–375) as bait. This region of STARS has been shown to be necessary and sufficient for actin binding and for stimulation of SRF-dependent transcription (17). From this screen, we identified three β-galactosidase-positive clones encoding for skeletal muscle actin (ACTA1), confirming our previous findings that STARS is an actin-binding protein (17). Two additional independent clones encoded a novel open reading frame. Data bank comparisons revealed partial identity of these clones with a previously uncharacterized protein fragment, KIAA1808 (GenBank™ accession number BAB47437). To obtain a full-length clone, we designed primers, using the longest human EST sequences available in the database, and subsequently cloned the entire open reading frame (supplementary Fig. 1), encoding 649 amino acids (GenBank™ accession number DQ413176). We also identified a shorter splice variant encoding 559 amino acids (Fig. 1C and see supplemental Fig. 1) (GenBank™ accession number DQ413177), as well as the mouse homologue (GenBank™ accession number DQ413175), which displays high homology to the human pro-

Two Novel Members of the ABLIM Protein Family
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A

Four LIM Domains

B

Human and Mouse ABLIM-1, -2, and -3

Drosophila UNC115

C. elegans UNC115

C

human ABLIM1V1

human ABLIM2V2

human ABLIM1V2

human ABLIM2V2
tein (Fig. 1A). These novel proteins contain four LIM domains as well as a carboxyl-terminal villin domain (Fig. 1A). Further querying of the data base revealed high homology with the actin-binding LIM protein (ABLIM), which had originally been found in human retina (30). Moreover, the data base contained a sequence for another previously uncharacterized putative protein fragment with homology to both ABLIM and KIAA1808 and KIAA0843 (GenBank™ accession number BAA74866). Utilizing the EST data base, we designed primers for the human homologue of this putative novel protein and subcloned the open reading frame from human heart cDNA (Fig. 1A and supplemental Fig. 1) (GenBank™ accession number DQ413174). The identical domain architecture (Fig. 1B), as well as the high homology between these three proteins, suggests that they constitute a novel protein family, and we thus designated the newly identified members ABLIM-2 and ABLIM-3 (Fig. 1). Interestingly, ABLIM proteins are evolutionary conserved with (single) homologues in Caenorhabditis elegans (UNC-115) and Drosophila (D-UNC-115) (Fig. 1, A and B).

ABLIM-2 and -3 Are Expressed in a Tissue-specific Pattern—Because STARS is expressed in a highly striated muscle-specific fashion, we examined if ABLIM-2 and -3 might display a similar expression pattern. An adult human multiple tissue Northern blot revealed that ABLIM-2 is highly expressed in skeletal muscle (Fig. 2A, left panel) and at lower levels in brain, spleen, and kidney. In heart and skeletal muscle, at least two distinct bands could be observed, consistent with the existence of splice variants. No significant expression was detected in the heart. Because we identified two possible splice variants of human ABLIM-2 (Fig. 1C), we examined the tissue distribution of the longer splice variant (hABLIMv1), using a probe specific for the 270 nucleotides unique to this isoform. However, we observed the same expression pattern as shown in Fig. 2A (data not shown). In contrast to ABLIM-2, ABLIM-3 is predominantly expressed in human heart and brain, whereas the murine ABLIM-3 homologue displays a somewhat broader tissue distribution that also includes lung and liver.

Temporospatial Expression Patterns of ABLIM-2/-3—To analyze the temporospatial expression patterns of ABLIM-2 and ABLIM-3, we performed radioactive in situ hybridization experiments (Fig. 2B). At embryonic day (E) 15.5 of mouse development, ABLIM-2 is predominantly expressed in skeletal muscle tissue, including the diaphragm, and to a lesser extent, in the central nervous system. At the same time point, ABLIM-3 also displays strong expression in skeletal muscle. These findings demonstrate a developmental regulation of ABLIM gene expression, especially of the expression of ABLIM-3, which is highly expressed during embryonic development (Fig. 2B) but down-regulated in adult skeletal muscle (Fig. 2A).

ABLIM-2 and -3 Are Expressed in Distinct Regions of the Brain—Given that ABLIM-2 and -3 are both expressed in brain, we sought to further define their expression profiles in central nervous tissue and thus conducted analyses of adult mouse brain sections. Interestingly, these experiments revealed very distinct and nonoverlapping expression patterns for ABLIM-2 and -3. Although human ABLIM-2 is predominantly expressed in skeletal muscle tissue and brain (left panel), human and mouse ABLIM-3 are expressed in the heart and to a lesser extent in brain (middle and right panel). ABLIM-2 and -3 transcripts were detected by radioactive in situ hybridization of mouse embryo sagittal sections at day 15.5 of embryonic development using an antisense probe. A sense probe served as negative control. ABLIM-2 is mainly expressed in skeletal muscle tissue, i.e. the diaphragm, and, to a lesser extent, in the central nervous system (top panel). Similarly, ABLIM-3 displays strong expression in skeletal muscle (lower panel).

FIGURE 1. Protein sequence alignment for ABLIM proteins. A, alignment of the human ABLIM (hABLIM-1 and -2 (variant V1 and V2), and -3), mouse ABLIM (mABLIM-1 and -2), Drosophila melanogaster (D-UNC-115), and C. elegans (UNC-115) protein sequence reveals high homology, particularly within the LIM domains (top) and the carboxyl-terminal villin domain (bottom). (D. melanogaster (D-UNC-115 amino acids 134–172 are not shown). B, the modular architecture of ABLIM proteins is characterized by the presence of four LIM domains and a carboxyl-terminal villin domain (VHD). C, for the human ABLIM-2, two splice variants were identified, encoding a longer (649 amino acids) and shorter isoform (559 amino acids), respectively.

FIGURE 2. Expression patterns of human and mouse ABLIM-2 and -3. A, Northern blot analysis of poly(A) multiple tissue RNA of human and mouse origin reveals a tissue-specific expression pattern of ABLIM-2 and -3. Although human ABLIM-2 is predominantly expressed in skeletal muscle tissue and brain (left panel), human and mouse ABLIM-3 are expressed in the heart and to a lesser extent in brain (middle and right panel). B, ABLIM-2 and -3 transcripts were detected by radioactive in situ hybridization of mouse embryo sagittal sections at day 15.5 of embryonic development using an antisense probe. A sense probe served as negative control. ABLIM-2 is mainly expressed in skeletal muscle tissue, i.e. the diaphragm, and, to a lesser extent, in the central nervous system (top panel). Similarly, ABLIM-3 displays strong expression in skeletal muscle (lower panel).
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Protein Expression and Subcellular Localization of ABLIM-2—To analyze the expression pattern of the ABLIM-2 protein, we generated antisera against a synthetic peptide specific for mouse ABLIM-2. Western blots (Fig. 4A) confirmed the expression of ABLIM-2 in skeletal muscle and brain. Interestingly, at least two distinct bands could be detected, suggesting either different splice products of the ablim-2 gene or tissue-specific differential post-translational modifications of the protein. We also generated an antisera against ABLIM-3 that detects the protein when overexpressed in COS cells (data not shown). However, this antisera displays a high background when used in tissue preparations and was not useful for further analyses.

Staining of mouse skeletal muscle hindlimb cryosections with the anti-ABLIM-2 antibody (Fig. 4B, upper panels) revealed a striated pattern, consistent with a sarcomeric or costameric localization of the protein. Similarly, staining of skeletal muscle cryosections with a polyclonal anti-STARS antibody (17) revealed the same pattern (Fig. 4B, lower panels), suggesting that ABLIM proteins and STARS co-localize in vivo.

ABLIM-2 and -3 Bind F-actin—Both ABLIM-2 and -3 contain a "villin domain" (also called villin headpiece domain) composed of 74 amino acids at their carboxy termini. This domain is highly conserved among the three different ABLIM isoforms (supplemental Fig. 1), as well as in ABLIM proteins from other species, including C. elegans (UNC-115) and Drosophila (D-UN-115), and has been shown to mediate actin binding (34, 35). We thus directly tested the actin-binding properties of full-length ABLIM-2 and -3, respectively, by conducting an actin co-sedimentation assay using in vitro-translated protein. Without F-actin, ABLIM-2 and -3 were almost exclusively detected in the supernatant (Fig. 5A, lanes 1 and 2). Upon addition of F-actin, ABLIM-2 and -3 co-sedimented quantitatively with F-actin and were nearly completely cleared from the supernatant (Fig. 5A, lanes 3 and 4). As a positive control, we used α-actinin known for its strong actin binding capability. As expected, α-actinin co-sedimented entirely with the F-actin pellet (Fig. 5A, lanes 5 and 6). In contrast, BSA, included as a negative control, did not co-sediment with actin (Fig. 5A, lanes 7 and 8). These results confirm the direct association of ABLIM-2 and -3 with actin. Constructs that encoded for the isolated villin domain of ABLIM-2 and -3 failed to produce sufficient quantities of stable protein, thus it remains unresolved if this domain is sufficient for actin binding of ABLIM proteins.

In addition, we performed immunofluorescence experiments in C2C12 cells to determine whether ABLIM proteins co-localize with actin. Using phalloidin as a marker for the actin cytoskeleton, we found that cells overexpressing ABLIM-2 display a substantially overlapping staining pattern (Fig. 5B), further supporting the notion that ABLIM proteins bind actin in vivo.

Interaction of ABLIM Proteins with STARS—To confirm that the interaction of ABLIM-2 with STARS identified in the yeast two-hybrid screen also occurred in mammalian cells, we performed co-immunoprecipitation experiments in transfected COS cells (Fig. 6A, left panel). Both ABLIM-1 and ABLIM-2 co-precipitated with STARS (Fig. 6A, lanes 2 and 3), whereas no
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signal was observed with vector alone (lane 1), confirming the data obtained in yeast. Regarding ABLIM-3, a weak interaction with vector alone was observed; thus we also performed GST pulldown experiments to confirm the interaction between STARS and ABLIM-3 (Fig. 6A, right panel). In vitro translated ABLIM-3 was incubated with GST-STARS and GST alone, respectively, which resulted in a selective pulldown only in the presence of GST-STARS. Taken together, these data suggest that STARS is able to interact with all three members of the ABLIM protein family. Immunofluorescence experiments with C2C12 cells co-transfected with FLAG-tagged STARS and ABLIM-2 also revealed co-localization of both proteins (Fig. 6B).

ABLIM-2 and -3 Stimulate STARS Activity—The actin cytoskeleton has been shown not only to regulate cell motility, contractility, and cytokinesis but also to play a critical role in the regulation of transcription and gene expression. Specifically, the state of actin polymerization controls the activity of the transcription factor SRF (4). Consistent with this notion, we have previously shown that STARS activates SRF-dependent transcription via stabilization of the actin cytoskeleton (18). Therefore, we sought to determine whether either ABLIM-2 or ABLIM-3 could modulate STARS-dependent activation of a luciferase reporter construct controlled by the SM22 promoter, which contains two essential SRF-binding sites and is highly sensitive to STARS activity. When transfected alone, neither ABLIM-2 nor ABLIM-3 was able to activate the SM22-luciferase reporter (Fig. 6C). However, in combination with STARS, both ABLIM proteins enhanced STARS activity more than 2-fold in a dose-dependent fashion (Fig. 6C). To exclude the possibility that co-transfected ABLIM proteins stabilize STARS and thereby indirectly enhance its transcriptional effects, we tested STARS expression levels in the presence and absence of ABLIMs. Neither ABLIM-2 nor ABLIM-3 overexpression altered STARS-levels (supplemental Fig. 2). We also tested if ABLIMs could enhance STARS activity in C2C12 cells. When transfected with ABLIM proteins alone, we observed modestly enhanced activity of the SM22-luciferase reporter in these cells (data not shown). However, when ABLIMs were co-transfected with STARS, no synergy was observed, suggesting either a saturation effect because of endogenous STARS in C2C12 cells (17) or a cell type-specific effect of ABLIM proteins on SRF-dependent transcription.

Reduction in SRF-dependent Transcription by ABLIM siRNA—To test whether ABLIMs are not only sufficient but also required for the activation of the SM22 promoter, we performed knockdown experiments of the endogenous ABLIM1–3 in C2C12 skeletal muscle cells, utilizing small interference RNAs against ABLIM-1, -2, and -3, as well as unspecific control siRNA. ABLIM-1–3 mRNA knockdown was confirmed by real time PCR, revealing a reduction of mRNA abundance of ABLIM-1 by 62%, of ABLIM-2 by 90%, and of ABLIM-3 by 92%, respectively.

Knockdown of each individual ABLIM isoform already led to a significant decrease in the activation of the SRF-dependent SM22-luciferase reporter (Fig. 6D). Moreover, combining siRNAs against ABLIM-2 and ABLIM-3 further reduced transcriptional activity. Finally, ablation of all three ABLIMs resulted in an even more pronounced attenuation of SRF-dependent luciferase reporter gene activity.

To test whether ablation of ABLIMs also attenuates STARS-mediated activation of the reporter, we repeated the combined ABLIM knockdown in the presence of overexpressed STARS (Fig. 6D). Again, a significant attenuation was observed.

DISCUSSION

STARS is a striated muscle-specific actin-binding protein that stimulates SRF-dependent transcription in a Rho-dependent fashion (17). In an effort to identify additional components
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FIGURE 6. ABLIM proteins interact with STARS and stimulate its activity. A, to confirm the interaction of ABLIM-2 with STARS identified in the yeast two-hybrid screen, co-immunoprecipitation experiments with transfected COS cells were performed (left panel). Both ABLIM-1 and ABLIM-2 co-precipitated with STARS (lanes 2 and 3), whereas no signal was observed with vector alone (lane 1). GST pulldown assay using GST-STARS and in vitro translated ABLIM-3 confirmed an interaction between these proteins (right panel). IP, immunoprecipitation. B, immunofluorescence experiments utilizing C2C12 cells co-transfected with cDNAs encoding Myc-tagged STARS and ABLIM-2 revealed co-localization of both proteins by immunostaining with anti-Myc monoclonal antibody detected with anti-rabbit IgG conjugated to Texas Red and anti-ABLIM-2 polyclonal antibody detected with anti-rabbit IgG conjugated to fluorescein-4. C, to determine whether ABLIM proteins modulate STARS-dependent activation of the SM22 promoter, which contains two essential SRF-binding sites and is highly sensitive to STARS activity, luciferase reporter assays were performed. When transfected alone, neither ABLIM-2 nor ABLIM-3 was able to activate the SM22-luciferase reporter in COS cells. However, in combination with STARS both ABLIM proteins dose-dependently enhanced STARS activity. D, to test whether endogenous ABLIMs are required for SRF-dependent transcription, C2C12 skeletal muscle cells were transfected with siRNAs directed against ABLIM-1, ABLIM-2, or ABLIM-3, all individually leading to a significant decrease in activation of the SM22-luciferase reporter. When co-transfecting siRNAs for ABLIM-2 and -3 or ABLIM-1, -2, and -3, luciferase activity was further inhibited. In combination with overexpression of STARS, the knockdown of all three ABLIM proteins significantly still attenuated activation of the SRF reporter gene. *, p < 0.001 versus control; #, p < 0.01 versus ABLIM-1, -2, or -3 alone; †, p < 0.05 versus ABLIM-2 and -3 combined.

of this pathway, we performed a yeast two-hybrid screen with STARS and discovered a new member of the ABLIM family, ABLIM-2, which is closely related to ABLIM/Limatin, originally found in human retina (30) and postulated to function as a tumor suppressor (36). Homology searches identified an additional member of this protein family, ABLIM-3. The interaction of STARS with ABLIM-2 and -3 was confirmed by co-immunoprecipitation and was further supported by the co-localization of STARS and ABLIM-2 as detected by immunofluorescence. Although ABLIM-2 is strongly expressed in skeletal muscle tissue (but not in heart), as well as in non-muscle tissues, such as brain and spleen (Fig. 2A), ABLIM-3 is predominantly expressed in heart as well as in brain, liver, and lung. The complementary expression patterns of ABLIM-2 and -3 in striated muscle imply that STARS interacts in vivo with ABLIM-2 in skeletal muscle and ABLIM-3 in cardiac muscle, respectively. Moreover, the observed ABLIM expression in other tissues suggests that this protein family also confers STARS-independent effects.

Actin Binding of ABLIM Proteins and Their Potential Role in Transcriptional Regulation—Similar to ABLIM, ABLIM-2 and -3 contain four LIM domains and a carboxy-terminal villin headpiece domain, which mediates actin binding in several proteins, such as villin and dematin (34). Consistent with this prediction, both ABLIM-2 and -3 strongly bind F-actin (Fig. 5A) and co-localize with actin stress fibers (Fig. 5B). Interestingly, in addition to the ABLIM-2 clones, our yeast two-hybrid screen also revealed two other villin domain-containing proteins, advillin and the muscle-specific splice variant of supervillin archvillin (data not shown), suggesting that STARS may bind to many if not all members of this protein family. It remains to be seen whether the villin domain of ABLIM proteins alone is sufficient for actin binding and/or if additional actin-binding domains exist in this novel protein family. Of note, several of the “villin headpiece” domain-containing proteins have been implicated in signal transduction and transcriptional regulation (37–39).

Potential Roles of ABLIM Proteins in Striated Muscle—Given that ABLIM-2 and -3 were identified as binding partners for the muscle-specific protein STARS, what could
tion to biomechanical stress. Moreover, the small GTPase Rho, which acts downstream of STARS, has been shown to be required for cardiomyocyte hypertrophy in response to different stimuli, including biomechanical stress (26–28, 41). Thus, it is tempting to speculate that ABLIM proteins might participate in sarcomeric stress signaling. This notion is further supported by the fact that LIM domain proteins in general function as key transducers of signals from the cytoskeleton toward the nucleus (reviewed in Ref. 42). Muscle-enriched LIM proteins include ALP, MLP/CRP3, cypher/ZASP/oracle, and FHL-2, all of which are Z-disk proteins (43). Interestingly, FHL-2 has recently been shown to physically interact with SRF and to inhibit SRF-dependent gene expression in response to RhoA by competing with MAL/MRTF-A (44).

Potential Roles of ABLIM Proteins in the Brain—ABLIM-1 was originally identified in retina and brain (30). We now show that both ABLIM-2 and -3 are expressed in the central nervous system as well, whereas the ABLIM-binding protein STARS is a muscle-specific protein, suggesting that ABLIM proteins have additional functions beyond modulating STARS activity. Interestingly, ABLIM-2 and -3 are expressed in distinct and only partially overlapping regions of the brain. For example, in the cerebellum ABLIM-2 is expressed exclusively in the Purkinje cell layer, whereas ABLIM-3 is confined to internal granular cells. Conversely, the olfactory bulb stains strongly positive for ABLIM-3, whereas ABLIM-2 is only moderately expressed. Both genes are expressed in the hippocampus, whereas ABLIM-2 is detected in the CA1, CA2, and CA3 fields, and ABLIM-3 is found selectively in CA2 and CA3. Taken together, these data imply specific and distinct functions of the individual ABLIM family members in the brain.

What could be the function of ABLIM proteins in neuronal tissue in vivo? It has been demonstrated that the C. elegans homologue of ABLIM proteins, UNC-115, plays a role in axon guidance, a process dependent on actin dynamics (45). In UNC-115 mutant worms, axon outgrowth is markedly impaired (46). Moreover, it has been shown that UNC-115 mediates axon pathfinding signals downstream of the Netrin receptor UNC-40 and the small GTPase Rac (47, 48), confirming a critical role of this ABLIM homologue in delivering signals toward the actin cytoskeleton. It is reasonable to anticipate that ABLIM proteins may play a similar role in vertebrates. This notion is supported by data from Erkman et al. (49) who reported that expression of a dominant-negative ABLIM-1 (lacking the actin-binding villin domain) results in axon pathfinding defects in the chicken optic pathway. Recently, Lu et al. (50) reported the first mouse knock-out of an ABLIM protein, ABLIM-1. This work focused on the role of ABLIM-1 in photoreceptors and inner retinal neurons and found no obvious morphological or functional defects, including axonal growth and pathfinding. However, these investigators chose to selectively ablate the longest of three different splice variants of the mouse abl-1 gene, because this transcript was found to be enriched in retinal tissue. Thus, the possibility remains that either one of the other two abl-1 transcripts and/or abl-2 and -3 could compensate for the lack of this particular abl-1 splice variant.
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dependent transcription. Moreover, siRNA-mediated knockdown of ABLIMs inhibited STARS-mediated activation of this reporter, further supporting the notion that this novel protein family modulates STARS-dependent regulation of SRF.

In summary, ABLIM-2 and -3 both strongly bind F-actin and modulate actin-dependent SRF activity via binding of STARS. Given that ABLIM proteins display only moderate direct effects on the activity of the Rho/STARS/actin/SRF pathway, we favor the hypothesis that this novel protein family rather serves as a scaffold for the integration of cytoskeletal signaling pathways (Fig. 7). Because UNC-115/ABLIM was shown to be a Rac effector in C. elegans, an intriguing possibility would be that ABLIM integrates signals from the small GTPases Rac and RhoA (via STARS) toward the actin cytoskeleton. Moreover, the presence of four LIM domains, which mediate protein-protein interactions, suggests that additional ABLIM-interacting proteins should exist. Their identification will allow further dissection of the roles of ABLIMs in actin-dependent signaling. Finally, it will be interesting to see if ABLIMs also play a role in muscle or neurological diseases as implied by the functional deficits in UNC-115/ABLIM-deficient nematodes.

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Addendum—While this manuscript was in preparation, the sequence of ABLIM-2 was independently submitted to the data base by Xu et al. (GenBank™ accession number AAP23233) and Klimov et al. (52) (GenBank™ accession number CAG38375). In reverse transcription-PCR experiments, the latter group observed predominant expression of rat ABLIM-2 in the brain (52). The human ABLIM-2 clones we identified contain additional sequences, likely due to the presence of splice variants.

REFERENCES

1. Chen, H., Bernstein, B. W., and Bamburg, J. R. (2000) Trends Biochem. Sci. 25, 19–23
2. Carlier, M. F., Le Clainche, C., Wiesen, S., and Pantaloni, D. (2003) Bioessays 25, 336–345
3. Randò, O. J., Zhao, K., and Crabtree, G. R. (2000) Trends Cell Biol. 10, 92–97
4. Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999) Cell 98, 159–169
5. Wear, M. A., Schafer, D. A., and Cooper, J. A. (2000) Curr. Biol. 10, 891–895
6. Sorokin, E. M., and Chernoff, J. (2005) J. Cell. Biochem. 94, 225–231
7. Etiene-Manneville, S., and Hall, A. (2002) Nature 420, 629–635
8. Ridley, A. J., and Hall, A. (1992) Cell 68–75
9. Wulfkuhle, J. D., Donina, I. E., Stark, N. H., Pope, R. K., Pestonjamasp, K. N., Niswonger, M. L., and Luna, E. J. (1999) J. Biol. Chem. 274, 341–347
10. Hill, C. S., Wynne, J., and Treisman, R. (1995) Cell 81, 1159–1170
11. Miano, J. M. (2003) J. Mol. Cell. Cardiol. 35, 577–593
12. Miralles, F., Posern, G., Zaroymidou, A. L., and Treisman, R. (2003) Cell 113, 329–342
13. Posern, G., Miralles, F., Guettler, S., and Treisman, R. (2004) EMBO J. 23, 3973–3983
14. Wang, D. Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J. A., Nordheim, A., and Olson, E. N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14855–14860
15. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) Cell 105, 851–862
16. Arau, A., Spencer, J. A., and Olson, E. N. (2002) J. Biol. Chem. 277, 24453–24459
17. Kuwahara, K., Barrientos, T., Pipes, G. C., Li, S., and Olson, E. N. (2005) Mol. Cell. Biol. 25, 3173–3181
18. Knoll, R., Hoshijima, M., Hoffman, H. M., Person, V., Lorenzen-Schmidt, I., Bang, M. L., Hayashi, T., Shiga, N., Yasukawa, H., Schaper, W., McKenna, W., Yokoyama, M., Schorn, N. J., Omens, J. H., McCulloch, A. D., Kimura, A., Gregorio, C. C., Poller, W., Schaper, J., Schultheiss, H. P., and Chien, K. R. (2002) Cell 111, 943–955
19. Geiger, B., and Bershadsky, A. (2002) Cell 110, 139–142
20. Torsoni, A. S., Fonseca, P. M., Crosera-Alberto, D. P., and Franchini, K. G. (2003) Ann. I. Physiol. 284, C1411–C1419
21. Hoshijima, M., Sah, V. P., Wang, Y., Chien, K. R., and Brown, J. H. (1998) J. Biol. Chem. 273, 7725–7730
22. Sah, V. P., Hoshijima, M., Chien, K. R., and Brown, J. H. (1996) J. Biol. Chem. 271, 31185–31190
23. Pan, J., Singh, U. S., Takahashi, T., Oka, Y., Palm-Leis, A., Herbelin, B. S., and Baker, K. M. (2005) J. Cell. Physiol. 202, 536–553
24. Souchet, M., Portales-Casamar, E., Mazuara, D., Schmidt, S., Leger, J. L., Robert, P., Berrebi-Brerand, L., Bril, A., Gout, B., Debant, A., and Calmes, T. P. (2002) J. Cell Sci. 115, 629–640
25. Roof, D. J., Hayes, A., Adamian, M., Chishti, A. H., and Li, T. (1997) J. Cell Biol. 138, 575–588
26. Frey, N., and Olson, E. N. (2002) J. Biol. Chem. 277, 13998–14004
27. Frey, N., Richardson, J. A., and Olson, E. N. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14632–14637
28. Pan, J., Singh, U. S., Takahashi, T., Oka, Y., Palm-Leis, A., Herbelin, B. S., and Baker, K. M. (2005) J. Cell. Physiol. 202, 536–553
29. Souchet, M., Portales-Casamar, E., Mazuara, D., Schmidt, S., Leger, J. L., Robert, P., Berrebi-Brerand, L., Bril, A., Gout, B., Debant, A., and Calmes, T. P. (2002) J. Cell Sci. 115, 629–640
30. Roof, D. J., Hayes, A., Adamian, M., Chishti, A. H., and Li, T. (1997) J. Cell Biol. 138, 575–588
31. Kim, A. C., Peters, L. L., Knoll, J. H., Van Huffel, C., Ciccioretti, S. L., Kley, P. W., and Chishti, A. H. (1997) Genomics 46, 291–293
32. Vermeulen, W., Vanhaesebroeck, P., Van Troys, M., Verschuren, M., Fant, F., Goethals, M., Ampe, C., Martins, J. C., and Borremans, F. A. (2004) Protein Sci. 13, 1276–1287
33. Krieg, P. A., and Olson, E. N. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 661–666
34. Mahadeva, H., Brooks, G., Lodwick, D., Chong, N. W., and Samani, N. J. (2002) FEBS Lett. 521, 100–104
35. Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Zhu, W., Kadowaki, T., and Yazaki, Y. (1999) Circ. Res. 84, 458–466
36. Kadmars, J. L., and Beckerle, M. C. (2004) Nat. Rev. Mol. Cell. Biol. 5, 920–931
37. Frank, D., Kuhn, C., Katus, H. A., and Frey, N. (2006) J. Mol. Med. 84, 446–468
38. Philippar, U., Schratt, G., Dieterich, C., Muller, J. M., Galgoczy, P., Engel, F., Keating, M. T., Gertler, F., Schule, R., Vinogron, M., and Nordheim, A. (2004) Mol. Cell 16, 867–880
39. Lin, C. H., Thompson, C. A., and Forscher, P. (1994) Curr. Opin. Neu-
Two Novel Members of the ABLIM Protein Family

46. Lundquist, E. A., Herman, R. K., Shaw, J. E., and Bargmann, C. I. (1998) *Neuron* **21**, 385–392
47. Gitai, Z., Yu, T. W., Lundquist, E. A., Tessier-Lavigne, M., and Bargmann, C. I. (2003) *Neuron* **37**, 53–65
48. Struckhoff, E. C., and Lundquist, E. A. (2003) *Development* **130**, 693–704
49. Erkman, L., Yates, P. A., McLaughlin, T., McEvilly, R. J., Whisenhunt, T., O’Connell, S. M., Krönes, A. I., Kirby, M. A., Rapaport, D. H., Berman, J. R., O’Leary, D. D., and Rosenfeld, M. G. (2000) *Neuron* **28**, 779–792
50. Lu, C., Huang, X., Ma, H. F., Gooley, J. J., Aparacio, J., Roof, D. J., Chen, C., Chen, D. F., and Li, T. (2003) *Neuroscience* **120**, 121–131
51. Wei, L., Roberts, W., Wang, L., Yamada, M., Zhang, S., Zhao, Z., Rivkees, S. A., Schwartz, R. J., and Imanaka-Yoshida, K. (2001) *Development (Camb.)* **128**, 2953–2962
52. Klimov, E., Rud’ko, O., Rakhmanaliev, E., and Sulimova, G. (2005) *Biochim. Biophys. Acta* **1730**, 1–9