FEBS Letters 579 (2005) 100–104 FEBS 29092

Overexpression of a family of RPEL proteins modifies cell shape

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Received 25 October 2004; revised 1 November 2004; accepted 4 November 2004

Available online 30 November 2004

Edited by Veli-Pekka Lehto

Abstract Proteins containing RPEL motifs (e.g., MAL) are important in the regulation of gene expression by the actin cytoskeleton. Screening the ENSEMBL database for RPEL proteins identified four additional proteins that contain RPEL motifs and nuclear localisation sequences, three of which (RPEL-A, RPEL-B and RPEL-C) are expressed in adult mouse tissues with different expression profiles. The mRNAs encoding RPEL-B and RPEL-C were subject to alternative splicing. Expression of these genes in cells indicated that they had a marked effect on cell shape. Furthermore, when expressed with a nuclear localised actin all of the different forms became restricted to the nucleus.

Keywords: Actin; Cytoskeleton; Cell shape; RPEL; Subcellular localization

1. Introduction

In addition to providing structure to cells, the cytoskeleton is an important contributor to cell adhesion, cell movement and cell shape through the formation of lamellipodia and filopodia. The cytoskeleton is also an important regulator of gene expression and the degree of actin polymerisation regulates the expression of genes that encode cytoskeletal components [1,2]. Increased actin polymerisation has been shown to increase the activity of the transcription factor serum response factor (SRF). One mechanism by which this increase in activity has been suggested to occur is through the re-localisation of the SRF co-activator MAL from the cytoplasm to the nucleus in the presence of polymerised actin or the activation of the small monomeric GTPase Rho [3]. MAL has been shown to bind to unpolymerised actin (G-actin) and in the presence of excess G-actin MAL is retained in the cytoplasm. In the absence of G-actin MAL moves into the nucleus. MAL contains three repeats of an RPEL motif (RPXXXEL) that are required for it to bind to actin [3]. These data suggest that other proteins with RPEL repeats may be important actin binding proteins.

We screened the ENSEMBL database and identified a small family of genes that encoded RPEL containing proteins. All of the proteins identified contained nuclear localisation sequences (NLSs), suggesting that they may integrate nuclear and cytoplasmic functions. In this report, we describe the cloning and tissue distribution of these genes. Furthermore, we examine the localisation of the proteins, their ability to interact with actin, effect on cell shape and on promoter activity.

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2. Materials and methods

2.1. Plasmids, RT-PCR and cloning

pSM22α-197-luc was derived from pSM22α-197-CAT by subcloning the pSM22α-197 promoter into pGL3. The actin-NLS vectors were a generous gift from Dr. Richard Treisman. RT-PCR was performed as described previously [4], on RNA isolated from mouse tissues using the RNeasy kit. RPEL cDNA was amplified using the appropriate primers and cloned into pGEM-T easy (Promega). Clones with the correct open reading frame were subcloned into pcDNA3 using *Eco*RV and *Not*1 sites. To make EGFP-fusion proteins, the *Ecor*V–*Not*I fragments were cloned in frame with an N-terminal EGFP gene in pCDNA3.

2.2. Primers

RPELAF	TATGATATGATGCAAACAGCCAACCAGATGCTAAG
RPELAR	TATTGTACAAAATACTGAATGACACTGGGTCACAG
RPELBF	TATGATATCATGGGCATGGACAGTGTGGAAGCAG
RPELBR	TATTGTACATCATGGGCGATGGTAGCGTGTAAAG
RPELCF	TATGATATCATGTATCTGCTAGGGCCGTGGAGCT
RPELCR	TATTGTACACTCAAGAGTAATCCCACTGTTAAG
RPELCLF	TATGATATCATGGCGGCTTCTTCG
RPELCLR	TGTTTTGAAGACAGCAGAGCACTCAAG
RPELDF	TATGATATCATGATACACATCGGAGAGGAAGCTA
RPELDR	TATTGTACACTATCTGTCTTGAATGCGTCTACT

2.3. Cell culture and luciferase assays

C2C12 cells were grown as described previously [5]. Cells were seeded into 24 wells plates at a cell density of 1.8×10^4 cells/well. After 24 h, the cells were washed with serum free DMEM and incubated with a mixture of 400 ng of DNA (made up as indicated in the legends) complexed with 2 μl of lipofectamine (Invitrogen) in OptiMEM (Invitrogen). Cells were incubated with the lipid/DNA mixture for 5 h before the medium was replaced with DMEM supplemented with 10% FCS. Forty-eight hours after transfection, *Firefly* and *Renilla* luciferase activity were measured using the Dual-Luciferase™ reporter assay (Promega).

2.4. Fluorescence microscopy

For determination of RPEL localisation, C2C12 cells were seeded into 4 well lab-tek chamber slides (Nunc) at a cell density of 1.5×10^4 cells/well. The cells were transfected 24 h later with 470 ng of DNA (as indicated in the figure legends) and 2 μl of lipofectamine as above. Cells were fixed 24 h later with 4% paraformaldehyde in PBS for 15 min and washed prior to imaging. To detect FLAG tagged actins, fixed cells were permeabilised for 10 min in 0.3% Triton X-100 in PBS, incubated for 30 min in PBS containing 1% BSA followed by 1 h with anti-M2 flag antibody (Sigma) and 1 h with anti rabbit-Cy3 antibody (Jackson). Images were captured using an Olympus TX 70 inverted microscope coupled to Ultraview LCI confocal imaging system (Perkin–Elmer) in the epifluorescence mode.

0014-5793/$22.00
doi:10.1016/j.febslet.2004.11.054

3. Results

3.1. Cloning and tissue distribution of the RPEL genes

We screened the ENSEMBL database (www.ensembl.org) for genes that encoded proteins with an RPEL motif. This analysis showed that the mouse genome contained 7 such genes, three of which were known (MAL [3], MRTF-B [6] and myocardin [7]). However, four sequences were from predicted but unidentified genes (ENSMUSG00000027525, ENSMUSG00000028897, ENSMUSG00000038587 and ENSMUSG00000053616). To determine whether these genes were expressed in the adult mouse and to obtain clones for them, primers were designed to amplify each of the coding sequences and used in RT-PCRs. RT-PCR for RPEL-A using primers

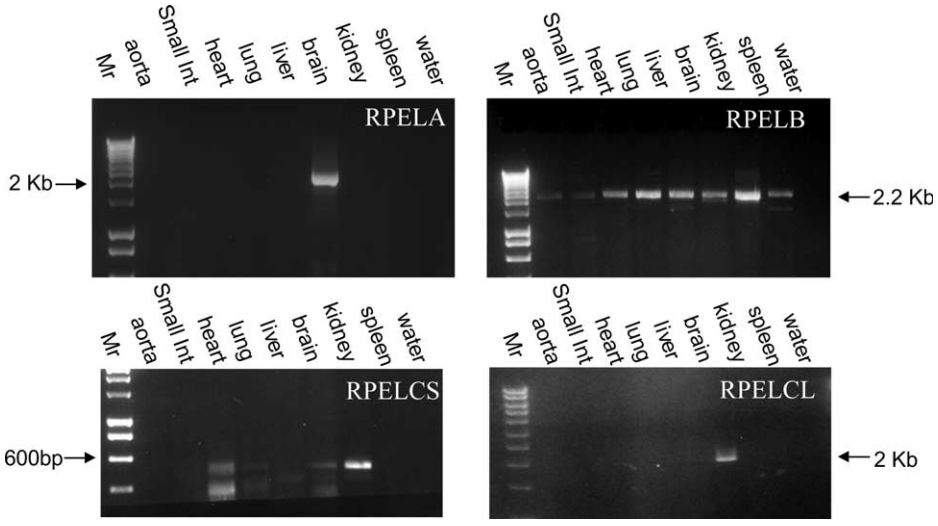

Fig. 1. Tissue distribution of RPEL gene expression. RT-PCR for RPEL-A, -B, -CS and -CL was performed on RNA isolated from the indicated tissues and analysed on a 1% agarose gel. Markers are Hyperladder I (Bioline).

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RPELCS  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
RPELCL  ~MAASSEDDI DRRPIRRVRS KSDTPYLAEA RISFNLGAAE EVERLAAMRS DSLVPGTHTP PIRRRSKFAN LGRIFKPWKW RKKK.SEKFK
RPELA   MQTANQMLSL NFRRMKSGTA AVRTRARHRP PGSGPSRCGD EMDQTPPARS EPLVSGIRTP PVRRNSKLAT LGRIFKPWKW RKKK.NEKLK
RPELB   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MGM DSVEAGDTTP PTKRKSKFSA LGKIFKPWKW RKKKSSDKFK

RPELCS  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
RPELCL  HTSAALERKI SMRQSREELI KRGVLKEIYD KDGELS.... .......... .ISNEDDS.. . LEN..... .....GQSLS SSQLSLPALS
RPELA   QTTSALEKKM AGRQGREELI KQGLLEMMEQ DSENKA.... .......... .CSPKEGSQP VQSE..... .....PPAGE QETLTSEGAQ
RPELB   ETSEVLERKI SMRKPREELV KRGVLLEDPE QDGEDSGKLS HAALKNGHTT PIGSARSSSP VLVEEEPERS LRNLTPEEES KKRLGSTGSQ

RPELCS  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
RPELCL  EMEPVPMPRD PCSYEVLQAS DI.MDGPV.. .SEESPSASE SGVLLSQDPS A.KPVLFLPP K.....KSAA FPGDHEETPV KQLSLHKQPP
RPELA   PGSPSASGTD QVSQDELLSS DAHLDDTA.. .NIPSASTAE EADAGSLLPT TDEPSQALAG S.....DSLD SPPRSLERSV SQLPSPPLLP
RPELB   PNSEAEPGPE HAPKQPLLPP KRPLSSSCEA KEVPAGSTAR SVSSTSGSTT VTSAATTAAT DMTKTVKTLP AAPASANTAA TTTAPAKQPP

RPELCS  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
RPELCL  ALPPKPTARI ANHLTDP.GA PVKLPCLPVK LSPPLPPKKV L..ICMPVGG PELTLASYAA QKSSQQAVAQ HHHT...VLP SQMQHQLQYG
RPELA   TPPPKASSKA TKNVTGQ.AA LFQGP....S MKNNEPALRG Q..LATPTGS PHVTTV.... .......... .HRP...LPP SRVMEELHRA
RPELB   IPPPKPAQRN SNPIIAELSQ AMNSGTVLSK PSPPLPPKRG IPSTSIPSLE PAASFTTKTA NDQREKTVSL CLEPPLIIPP SSPSPPLPTH

RPELCS  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
RPELCL  SHGQHLPSST GTLPMHPSGC RMIDELNKTL AMTMQRLESS EQRVPCSTSY HSSGLH.... .......... .......... ..........
RPELA   LATKHRQDSF .......QGR ECRGSPKKRM DVRLSRTSSM ERGKERDEAW SFDGAS.... .......... .......... ..........
RPELB   IPPEPPRSPL VPAKTFQIVP EVEFSSSSDL FQDISQQEDQ KTEVPKKIQD QSFGESHIPS RLPPLPLHIR IQQALTSPLP VTPPLEGTHR

RPELCS  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
RPELCL  ........SS DGITKAGPMG LPEIRQVPTV VIECDDNKEN VPHEPDYED. .......SPC LYGREEEEE. .......... ..EEDEDDDA
RPELA   ........EN KW........ ........TA TKDSEENKEN LMLSSELKD. .......DML LY........ .......... ...QDEEALN
RPELB   AHSLLFENSD SFSEDTGTLG RTRSLPITIE MLKVPDDEEE EQTCPFVEDV TSTSATPSLP LCLREEEKES DSDSEGPIKY RDEEDDDDD

RPELCS  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~MYLLGPWS SPAPGGYDMV QKLFLDFFRR RLSQRPTAEE LEQRNILKPR NEQEEQEEKR
RPELCL  SLYTSSLAMK VCRKDSLAIK LSNRPSKREL EEKNIL.PRQ TDEE.RLELR QQIGTK.LTR RLSQRPTAEE LEQRNILKPR NEQEEQEEKR
RPELA   DSIISGTLPR KCKKELLAVK LRNRPSKQEL EDRNIF.PRR TDEE.RQEIR QQIEMK.LSK RLSQRPAVEE LERRNILKQR NDQTEQEERR
RPELB   ESHQSALANR VKRKDTLAMK LSSRPSEPET NLNS..WPRK SKEEWN.EIR HQIG.NTLIR RLSQRPTAEE LEQRNILQPK NEADRQAEKR

RPELCS  EIKRRLTRKL SQRPTVEELR ERKILIRFSD YVEVADAQDY DRRADKPWTR LTAADKAAIR KELNEFKSTE MEVHELSRHL TRFHRP
RPELCL  EIKRRLTRKL SQRPTVEELR ERKILIRFSD YVEVADAQDY DRRADKPWTR LTAADKAAIR KELNEFKSTE MEVHELSRHL TRFHRP
RPELA   EIKQRLTRKL NQRPTVDELR DRKILIRFSD YVEVARAQDY DRRADKPWTR LSAADKAAIR KELNEYKSNE MEVHASSKHL TRFHRP
RPELB   EIKRRLTRKL SQRPTVAELL ARKIL.RFNE YVEVTDAHDY DRRADKPWTK LTPADKAAIR KELNEFKSSE MEVHVDSKHF TRYHRP
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Fig. 2. Alignment of the protein sequences for the RPEL proteins. Amino acids identical in two or more of the sequences are shaded. The RPEL motifs (solid lines) and the NLS (dotted lines) are underlined.

RPELAF and RPELAR amplified a PCR product of approximately 2 kb from brain but not from any other tissue (Fig. 1). Cloning and sequencing of this product showed that it encoded a protein identical to that encoded by ENSMUSG00000027525. RT-PCR for RPEL-B using primers RPELBF and RPELBR produced a 2.2-kb PCR product from all the samples tested (Fig. 1). Cloning and sequencing of the product identified it as the transcript from the gene ENSMUSG00000028897. Amongst the clones sequenced for RPEL-B was a product 164 bp shorter than the majority of the clones, suggesting that RPEL-B is alternatively spliced (see below). RT-PCR using primers RPELCF and RPELCR produced a 500-bp product from the kidney, with weak PCR products also present in the heart, lung and brain (Fig. 1). Cloning and sequencing confirmed that the product was the transcript from ENSMUSG000000385870. Comparison of this sequence with the NR database identified an additional larger transcript with the Accession No. NM_198419. We therefore termed the initial

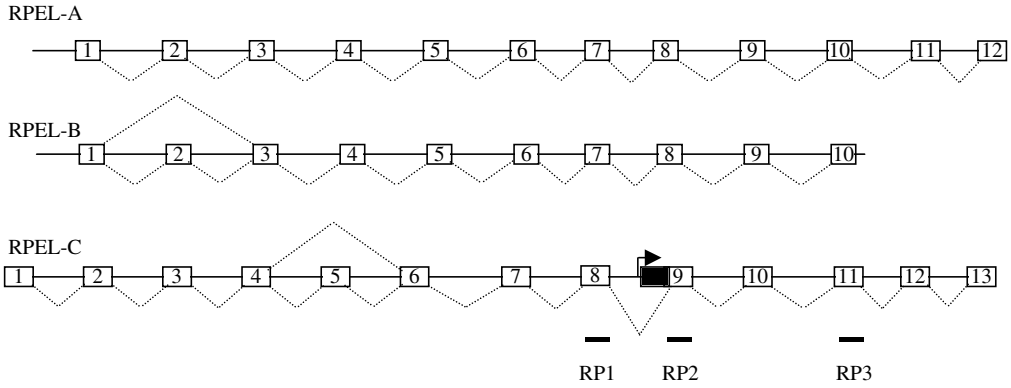

Fig. 3. Organisation of the RPEL genes. Intron–exon organisation of the RPEL genes. Introns (lines) and Exons (numbered boxes) are not drawn to scale. The splicing pattern for the mRNAs identified is shown as dotted lines and the extra sequence present in RPEL-CS is shown as a filled in box with the putative additional promoter shown as an arrow. The diagram has been drawn to align the exons coding for each RPEL motif. RPELs are indicated by the lines (RP = RPEL).

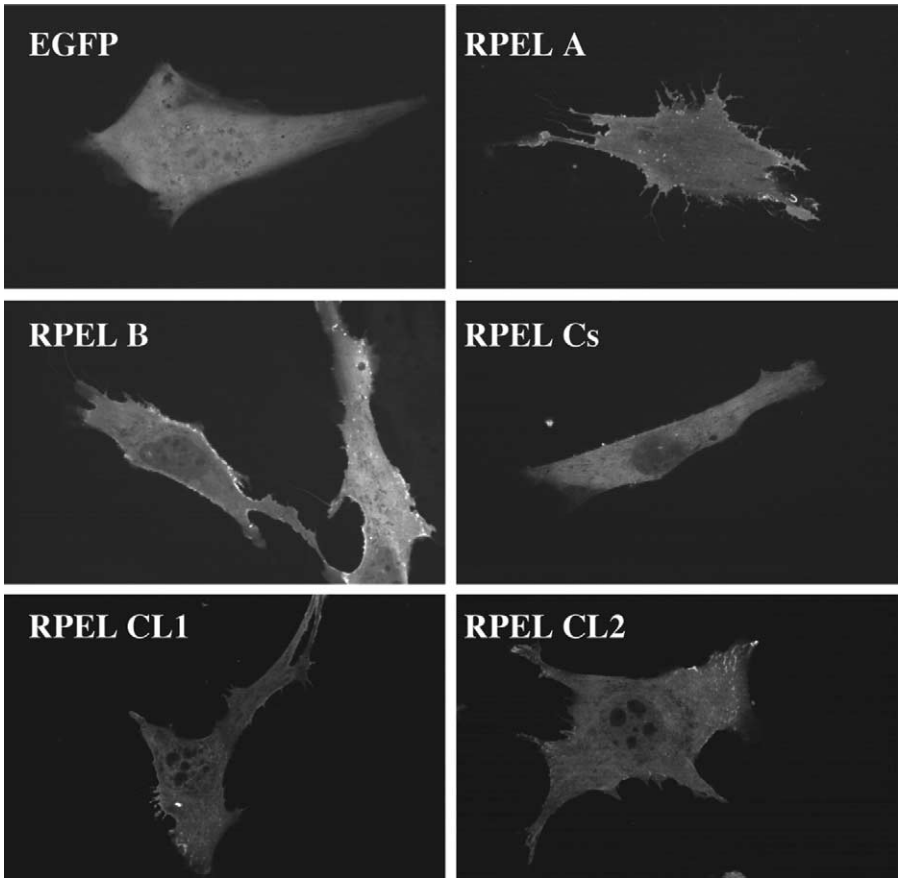

Fig. 4. Localisation of EGFP–RPEL proteins. C2C12 cells were transfected with 470 ng of EGFP–RPEL expression vector. The cells were fixed 24 h after transfection and analysed by epifluorescence microscopy.

product RPEL-CS. To clone the larger transcript, new primers (RPELCLF and RPELCLR) were designed. These reactions produced a product of approximately 2 kb from the kidney alone (Fig. 1). Cloning and sequencing of this product identified two additional isoforms of RPEL-C, which differed by approximately 200 bp and were therefore named RPEL-CL1 and RPEL-CL2. No product was obtained from primers for RPEL-D from any of the samples used in the PCRs.

3.2. Protein sequence comparison

Comparison of the coding sequences for the proteins indicated that the C-termini of the proteins were approximately 90% similar and that all but RPEL-CS contained three RPEL motifs and a NLS (Fig. 2). RPEL-CS contained the last two RPEL motifs and the NLS. The N-terminal domains of all of the proteins were very different apart from a conserved bipartite NLS in RPEL-A, RPEL-B and RPEL-CL (Fig. 2). If RPEL-D is made as a protein, it is the most divergent of the four members of this family. Whilst RPEL-D has all three RPEL motifs and a NLS in the C-terminus it lacks the N-terminal bipartite NLS and has a C-terminal extension not present in the other proteins.

3.3. Gene structure

Comparison of the cDNA sequences with the mouse genome showed that the coding sequence of all of the genes was split into at least 10 exons (12 exons for RPEL-A on chromosome 2, 10 exons for RPEL-B on chromosome 4 and 13 exons for RPEL-C on chromosome 13, Fig. 3). The sequence lacking in the shorter form of RPEL-B contained exon 2 of the coding sequence (Fig. 3). Comparison of the different isoforms of RPEL-C indicated that the difference between RPEL-CL1 and RPEL-CL2 was the absence of exon 5 sequence in RPEL-CL2 mRNA. Analysis of the RPEL-CS sequence showed that RPEL-CS consisted of exons 9–13 of RPEL-CL1 with an additional sequence at the 5′-end of the RPEL-CS cDNA that was not present in RPEL-CL1 or RPEL-CL2. This additional sequence was found to be in the intron upstream of RPEL-CL exon 9 (Fig. 3), suggesting that the RPEL-C gene has two promoters, one driving expression of RPEL-CL and one for RPEL-CS.

3.4. Localisation of EGFP–RPEL proteins and effect on cell shape

As all of the proteins had at least one NLS as well as sequences previously shown to bind to actin, we generated N-terminal EGFP–RPEL fusion proteins expression vectors to determine whether the expressed proteins became localised to one specific compartment or were present throughout the cell. Transfection of these vectors into C2C12 cells showed that all of the proteins with the bipartite NLS (i.e., all except RPEL-CS) were present throughout the cells (Fig. 4). Conversely, approximately 50% of the cells expressing EGFP–RPEL-CS showed nuclear exclusion of this protein (Fig. 4), raising the possibility that the N-terminal NLS is important in nuclear localisation. The cytoplasmic EGFP fluorescence in cells transfected with RPEL-A and RPEL-B was often distributed inhomogeneously with stronger fluorescence at the edge of the cells and punctate fluorescence in the cytoplasm (Fig. 4). However, this fluorescence did not co-localise with staining for focal adhesion kinase (data not shown).

The long forms of the EGFP–RPEL fusion proteins had a significant effect on cell shape (Fig. 4). Cells expressing these proteins had a rough appearance rather than the smooth edges of cells transfected with EGFP alone. All of the cells transfected with the fusion proteins for RPEL-A, B, C1 and C2 had hair-like cytoplasmic extensions of varying lengths that were not observed on the control cells (Fig. 4).

3.5. Association of the EGFP–RPEL proteins with actin

To determine the effect of actin on RPEL localisation, cells were transfected with the EGFP–RPEL fusion protein in the presence of FLAG tagged wild-type actin or a mutant FLAG tagged actin that contained a NLS [3]. Cotransfection of wild-type actin had no effect on the localisation of the EGFP–RPEL proteins (Fig. 5). However, in the presence of the mutant actin containing the NLS all of the EGFP–RPEL isoforms became localised to the nucleus, indicating that the RPEL proteins interact with actin (Fig. 5).

3.6. Effect of the RPEL proteins on transcription

To determine whether these RPEL proteins modified gene expression, their effect on the activity of a fragment of the SM22α promoter [8] (which is activated by MAL) was determined. In these experiments, the RPEL proteins caused a weak inhibition of promoter activity (approximately 20%, Table 1).

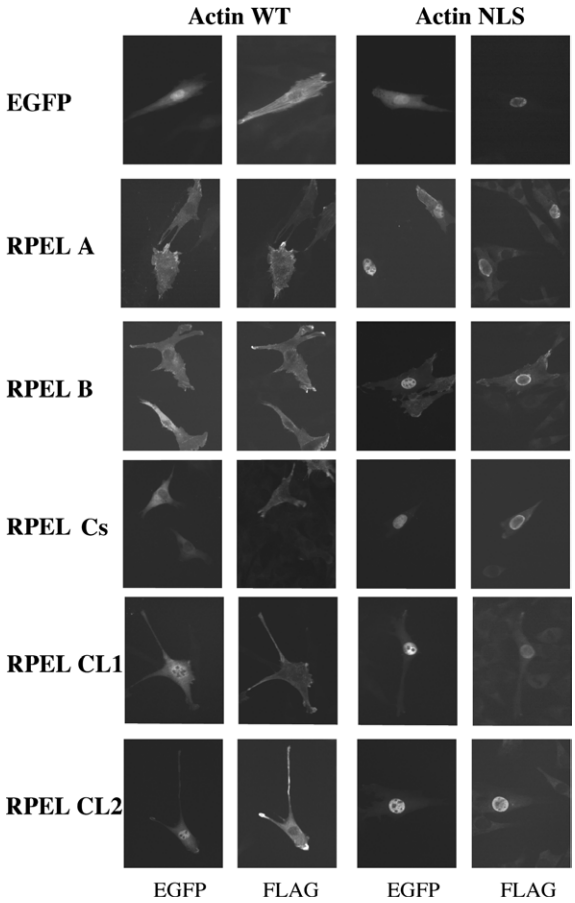

Fig. 5. EGFP–RPEL proteins co-localise with nuclear targeted actin. C2C12 cells were transfected with 350 ng of EGFP–RPEL expression vector and 120 ng of pEF-FLAG actin expression vector. The cells were fixed 24 h after transfection, stained for FLAG as described in Section 2 and analysed by epifluorescence microscopy.

Table 1
RPEL proteins are weak inhibitors of the SM22α promoter

Sample	Control	MAL	RPEL-A	RPEL-B	RPEL-CS	RPEL-CL1	RPEL-CL2
Fold activation	1	23 ± 1	0.69 ± 0.03	0.76 ± 0.05	0.72 ± 0.05	0.82 ± 0.02	0.82 ± 0.04

C2C12 cells were transfected with 200 ng of pSM22α-197-luc, 100 ng of pRL-TK and 50 ng each of RPEL or MAL in pCDNA3 and pCDNA3. Cells were harvested 48 h after transfection for luciferase assays. The *Firefly* luciferase activity was normalised to *Renilla* luciferase activity. Numbers are fold activation of luciferase activity ± S.E.M. Transfections were set up in triplicate and repeated 3–6 times.

The most effective of the proteins were RPEL-A and RPEL-CS, which inhibited the SM22α promoter by approximately 30%.

4. Discussion

We have identified and cloned a family of proteins that contain RPEL motifs and NLSs. The NLSs are located both within the RPEL motif domain and in the N-terminal region of the larger protein isoforms. Expression of RPEL-B was found in all of the tissues examined, suggesting a general role in cell biology. However, two of the others were expressed in a tissue-restricted fashion with RPEL-A restricted to the brain and RPEL-CS and RPEL-CL expression found mainly in the kidney. This implies that there are more specialised roles for these members of the family either in terms of function or the context within which they function. Two of the genes produced more than one isoform by alternative splicing. However, there was no obvious similarity between the deleted regions of RPEL-B and RPEL-CL and the role of the alternative isoforms is not clear.

To determine the subcellular localisation of these proteins requires the generation of specific antibodies for each of the isoforms and these do not yet exist. In this study, we used the expression of EGFP fusion proteins as a surrogate measurement and therefore determined the localisation of exogenous proteins. The subcellular localisation of the transfected EGFP–RPEL proteins is consistent with the presence of NLSs in the long isoforms and with their ability to interact with actin. During the course of this work, a study identifying scapanin (the human form of RPEL-A) was published [9]. Consistent with the findings presented here, they found that the protein was present in both the nucleus and the cytoplasm of transfected HeLa cells using antibodies and EGFP-fusion proteins. The localisation of these proteins is also consistent with the localisation of MAL in the same cells under serum stimulated conditions (LF unpublished observations).

RPEL containing proteins are found in a wide range of metazoan organisms and at least one protein from each of *Caenorhabditis elegans*, *Drosophila melanogaster* and *Danio Rerio* have a R-X-X-P-W-K-W-(R/K)-X_{0-2}-(K/R)-(K/R)-(K/R) motif, similar to part of the bipartite NLS found in the long forms of the RPEL proteins. The conservation of this sequence across a diverse range of species and a protein family suggests that it is important in the function of the proteins and indicates that the proteins may be involved in nuclear processes.

Consistent with an ability to interact with actin, the EGFP–RPEL proteins caused significant modification in cell shape. It is of interest that expression of one of the proteins, RPEL-A, is restricted to the brain, suggesting that it may be involved in the regulation of cell shape in neural cells. Alternatively, the proteins may be involved in cell movement as the transfected cells had a tendency to detach from the substrate (data not shown). However, the effect of these proteins on cell shape is not just a result of the interaction between actin and the RPEL motifs because other RPEL containing proteins (e.g., MAL) do not have the same effect.

Whether this subfamily of RPEL proteins is involved in the regulation of gene expression remains to be determined. The proteins have a limited effect on the activity of an SM22α reporter gene, suggesting that they do not affect SRF activity, consistent with the lack of an SRF interacting SAP domain. However, the interaction of the proteins with actin suggests that they may compete for actin binding with MAL. Such a competition for G-actin should have led to nuclear localisation of MAL, activation of SRF and thereby increased promoter activity rather than the inhibition of promoter activity seen here. Therefore, the data imply a more complex method of regulation of MAL.

In conclusion, we have analysed a family of proteins that contain multiple RPEL motifs. The members of the family show different tissue specific expression patterns and are able to modify cell shape.

Acknowledgments: This work was supported by the British Heart Foundation. P.R.K. is a British Heart Foundation Basic Sciences Lecturer.

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