ACTIN CYTOSKELETON REMODELING BY THE ALTERNATIVELY SPICED ISOFORM OF PDLIM4/RIL

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Running head: Dominant-negative effect of alternatively-spliced product of PDLIM4/RIL

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RIL (product of PDLIM4 gene) is an actin-associated protein that has previously been shown to stimulate actin bundling by interacting with actin-crosslinking protein α-actinin-1 and increasing its affinity to filamentous actin. Here we report that the alternatively spliced isoform of RIL, denoted here as RILaltCterm, functions as a dominant-negative modulator of RIL-mediated actin reorganization. RILaltCterm is regulated at the level of protein stability and this protein isoform is accumulated particularly in response to oxidative stress. We show that the alternative C-terminal segment of RILaltCterm has a disordered structure that directs the protein to rapid degradation in the core 20S proteasomes. Such degradation is ubiquitin-independent and can be blocked by binding to NAD(P)H quinone oxidoreductase NQO1, a detoxifying enzyme induced by prolonged exposure to oxidative stress. We show that either overexpression of RILaltCterm or its stabilization by stresses counteracts the effects produced by full-length RIL on organization of actin cytoskeleton and cell motility. Taken together the data suggest a mechanism for fine-tuning actin cytoskeleton rearrangement in response to stresses.

RIL (also known as PDLIM4) is a member of ALP/Enigma family of PDZ and LIM domain-containing adapter proteins found in association with actin cytoskeleton (1,2). ALP/Enigma genes/proteins are highly conserved throughout the evolution with a single member in C. elegans (alp-1/eat-1) and D. melanogaster (tungus), and 7 different genes in mammals (3-6). Most of the family members, including the C. elegans prototype, associate with actin cytoskeleton via α-actinins (ALP/PDLIM3 (7-9), CLP-36/PDLIM1 (10-12), Mystique/PDLIM2 (13), RIL/PDLIM4 (12,14), Enigma Homolog/ENH/PDLIM5 (15) and ZASP/Cypher/PDLIM6) (16-18), or Filamin A (PDLIM2) (13) or β-tropomyosin (Enigma/PDLIM7) (19). The EHN/PDLIM5 has also been reported to affect actin structure by binding the Spine-Associated RapGAP (SPAR) (20). Proteins of the PDLIM family mostly function to maintain the structure of Z-discs and are responsible for the integrity of muscle fibers by stabilizing the actin filaments (4,21,22). Thus, ablation of Alp or Cypher/ZASP genes leads to cardiomyopathies and/or skeletal myopathies in various animal models from fly to zebrafish to mouse (23-26), while mutations in hCypher have been shown to cause similar pathologies in humans (22,27-29). In non-muscle cells PDLIM proteins fulfill similar role in stabilizing actin stress fibers. Specifically, binding to RIL increases the affinity of α-actinin-1 to filamentous actin (F-actin), leading to dramatic rearrangement of actin cytoskeleton (1). Alternative splicing adds yet another level of complexity and flexibility to fine-tuning the function of ALP/ENIGMA proteins. With the exception of the CLP-36, the PDLIM genes were reported to express up to 6 alternatively spliced mRNA species that often include LIM-less isoforms. These splice variants can be tissue type-specific (such as cardiac Zasp/Cypher and skeletal muscle Zasp/Cypher in mice (30) or smooth/cardiac...
muscle smAlp and skeletal muscle scAlp (31)) or developmental stage-specific (embryonic Enh isoform vs. adult Enh variants expressed in murine heart (32)) and can be altered in response to stress, e.g. pressure overload in the heart (32). Interestingly, the shorter LIM-less isoforms of Enh have been shown to negatively regulate the longer Enh variant (15,32). Transcripts from the RIL gene are expressed as two alternatively spliced mRNA species (2). In comparison to the full-length RIL the shorter isoform lacks the LIM domain at its C-terminus, and its function remains unknown (2). Physiological processes affected by RIL function are poorly understood although its expression has been reported to be altered in various cancers as well as in inflammatory conditions (33-46). Whether alternative splicing plays any role in modulating RIL function in these pathological states has not been addressed.

In the present study we characterize function of the shorter alternatively spliced isoform of RIL denoted here as RILaltCterm. We report that RILaltCterm is intrinsically unstable due to an unstructured region at its C-terminus. Degradation of this isoform is 20S proteasome dependent, being modulated by binding to NQO1. We show that RILaltCterm is stabilized in response to oxidative stress and/or UV irradiation. Once stabilized the RILaltCterm homodimerizes with full-length RIL to change its subcellular localization leading to rearrangement of actin cytoskeleton and attenuation of cell migration.

**EXPERIMENTAL PROCEDURES**

**Plasmids:** Constructs expressing N-Flag and N-HA-tagged full-length RIL, RILaltCterm (Fig. 1A) and control deletion mutant RILΔCterm (lacking both exon6 and exon7 sequences that code for the LIM domain and/or alternative C-terminal peptide) were generated by subcloning corresponding PCR-amplified cDNA fragments into XbaI and XhoI sites of pLCMV-N-Flag or pLCMV-N-HA, respectively. EGFP-RILaltCterm and Luc-RILaltCterm were constructed by subcloning PCR-amplified RILaltCterm cDNA fragment corresponding to amino acids 217-246 (30 C-terminal residues) in-frame with EGFP into AvrII and BamHI sites of pLCMV-EGFPfus or in-frame with luciferase gene into XhoI and BamHI sites of pLCMV-Luc. For generation of Luc-RIL-Clike (control construct) a 1nt frame shift and an in-frame stop codon were introduced into cDNA sequence corresponding to 30 C-terminal residues of RILaltCterm by PCR and the resulting fragment was ligated in-frame with the luciferase gene into XhoI and BamHI sites of pLCMV-Luc. pLCMV-Luc-Stop refers to the non-fused luciferase expression construct.

**Cells and transient transfection:** 293T, HeLa, A549, H1299, MDA-MB-435S, A431, U2OS and RAW264.7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and appropriate antibiotics. Transient transfection was performed using LipofectAMINE and Plus reagent (Invitrogen Inc.).

**Lentiviral transduction:** Lentiviral particles were packaged as described (47) using pGag1 and pRev2 helper plasmid set and pseudotyped with the VSV-G protein. Supernatants containing viral particles were collected every 12 hours, pooled and concentrated by PEG-8000 precipitation. For infection of target cells viral preparations were diluted in complete growth media supplemented with 4 μg/ml polybrene. The expression was assayed 48 h post infection.

**Reagents and antibodies:** Translation inhibitor cycloheximide (at 1-5 μg/ml) was from ICN Biomedicals; proteasome inhibitor MG-132 (N-Carbobenzoxyl-L-leucinyl-L-norleucinal, at 20 μM), inhibitors of serine proteases AEBSF (4-(2-Aminoethyl) benzenesulfonylfluoride hydrochloride at 0.4 mM), calpain inhibitor Calpeptin (at 0.1 mM), inhibitors of serine and cysteine proteases AEBSF (4-(2-Aminoethyl) benzenesulfonylfluoride at 0.4 mM), calpain inhibitor Calpeptin (at 0.1 mM) were from Calbiochem; NQO1 inhibitor dicoumarol (at 400 μM) was from Acros Organics; cysteine protease inhibitors E-64 (trans-epoxysuccinyl-L-leucylamido-4-guanidinobutane at 5 μM), aspartic protease inhibitor Pepstatin A (at 5 μg/ml), serine and cysteine protease inhibitor Leupeptin (at 50 μM), hypoxia mimetic DFO (desferroxamine mesylate, at 300μM), H2O2 (at 0.5 mM), antioxidants NAC (N-acetyl-L-cysteine, at 0.5 mM) and Ebselen (organo-
selenium compound, at 0.02 mM), β-Gal substrate ONPG (o-nitrophenyl β-D-galactopyranoside) were from Sigma. LPS from E. coli was purchased from Sigma. FLAG mAb M2 and anti-Flag rabbit polyclonal IgGs were from Sigma, GAPDH mAb from Meridian Life Science, and HA mAb clone 12CA5 from rat was from Roche. Antibodies against GFP (Ab-2) were from NeoMarkers, mAbs against ubiquitin (P4D1), anti-NQO1 (C-19) rabbit polyclonal IgGs, anti-PDLIM4/RIL (D-8) mAbs predicted to recognize both isoforms of RIL and anti-actin (C-11) goat polyclonal IgGs from Santa-Cruz, RIL-specific goat polyclonal antibodies were acquired from Abcam and anti-α-actinin-1 mAbs were from Millipore.

RT-PCR: Total RNA from cells was extracted using TRizol reagent (Invitrogen) according to manufacturer’s protocol. First cDNA strand was synthesized from oligo(dT)12-18 primer using SuperScript First-strand Synthesis System (Invitrogen). For PCR amplification the following primer pairs were used: RIL (RIL4ex sense 5'-CTCGCATTCCAGTCCCTCACATT-3’ and RIL5ex antisense 5’-TCTAGCATGCCCTGCA-AGTAGC-3’), β-actin (beta-actin sense 5'-GCTTGCCATCCAACCACTCAGTCTTG-3' and beta-actin antisense 5'-GCGTCTCCTTGGCTGTTTGGACAG-3'), EGFP (EGFP sense 5'-TGACCCTGAAGTTCATCTGCACCA-3' and EGFP antisense 5’-TGTGGCGGATCTTGAAGTTCACCT-3’).

Polyribosome isolation and analysis: 10^7 HeLa cells transfected with either full-length RIL, RILaltCterm of RILΔCterm control truncation mutant were treated with 50 μg/ml cycloheximide for 5 min, harvested by scraping and washed with ice-cold PBS with 50 μg/ml cycloheximide. Cell pellet was resuspended in 20 volumes of polysome lysis buffer (0.14 M NaCl, 25 mM MgCl₂, 10 mM Tris-HCl pH 8.0, 0.5% NP-40, 1 mM DTT, 1000 U/ml RNAsin (Promega) and 50 μg/ml cycloheximide), spun down at 2000g and supernatant applied onto 30% sucrose/lysis buffer cushion and centrifuged at 250,000g for 4 hours at 4°C. RNA from supernatant and pellet was isolated with TRizol reagent (Invitrogen), reverse transcribed and analyzed by PCR.

Reporter assays: H1299 cells were transfected with Luc-stop, Luc-RILaltCterm or Luc-RIL-Clike and a plasmid constitutively expressing β-galactosidase for transfection efficiency normalization. Cells were briefly washed in PBS and lysed in either Reporter Lysis Buffer (Promega) for subsequent assessment of luciferase luminescence with Luciferase Assay Substrate or in β-gal staining buffer (1 mM MgCl₂, 250 mM Tris-HCl, pH 7.4, 0.02% NP-40, 2 mg/ml ONPG in PBS), incubated at 37°C and assessed by photometry at 405 nm.

Co-immunoprecipitation and Western blotting: For straight immunoblotting cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Na deoxycholate, 1% NP-40, 0.1% SDS with protease inhibitor cocktail (Roche)) and lysate pre-cleared by centrifugation (10,000×g, 4°C for 10 min). For co-immunoprecipitation studies cells expressing the necessary constructs were washed with ice-cold PBS, harvested by scraping and lysed on ice for 10 min in NET buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 1% NP-40 supplemented with protease inhibitors). Cell lysates were pre-cleared by centrifugation as described above and 500 μg aliquots were subjected to immunoprecipitation with anti-Flag M2 agarose (Sigma). Detergent-soluble fractions were obtained by harvesting the cells on ice by scraping and subsequent lysis in ice-cold CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100) supplemented with protease inhibitors for 10 min. Detergent-insoluble material was spun down at 10,000g and pellets were resuspended in 2% SDS, 50 mM Tris, pH 7.5. For Western blotting protein lysates and prestained molecular mass standards were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in PBS with 0.1% Tween-20 (PBS-T), incubated with primary antibodies, extensively washed in PBS-T, and stained with horseradish peroxidase-conjugated secondary species-specific IgG (1:10,000) (Santa-Cruz). Immunoreactivity was visualized by enhanced chemiluminescence (ECL) kit (GE Healthcare).
**Immunocytochemistry:** U2OS cells expressing Flag- or HA-tagged RIL, or HA- or Flag-tagged RILΔCterm, or both were grown on glass coverslips, fixed in 3.7% formaldehyde for 15 min, permeabilized in 0.5% Triton X-100 in PBS for 15 min, blocked in 3% BSA/PBS for 1h, then incubated with primary rabbit anti-Flag (1:500) and rat anti-HA (1:500) antibodies in blocking solution, washed in PBS, incubated with secondary AlexaFluor 350 or AlexaFluor 594 conjugated antibodies (1:1000, Invitrogen) and counterstained with FITC-labeled phalloidin in blocking buffer. Slides were extensively washed in PBS and mounted in Fluoromount G (Southern Biotech). Fluorescent pictures were taken by a Qimaging camera using Leica 1.25NA 40× objective. Images were processed and assembled using Photoshop (Adobe).

**F-actin content measurement:** U2OS cell transduced with RILaltCterm or empty vector were grown in triplicate wells of 24-well plates, subjected to 0.5 mM H2O2 treatment for 4 hours where indicated and fixed by 4% formaldehyde (methanol-free) in cytoskeleton buffer (10 mM MES, pH 6.1, 138 mM KCl, 3 mM MgCl2, 2 mM EGTA and 0.32 M sucrose) to prevent actin depolymerization. After permeabilization by 0.1% Triton X-100 in PBS cells were blocked in 3% BSA and stained with FITC-conjugated phalloidin. F-actin content was quantified using spectrofluorimeter (Wallac Victor3). Data were normalized by counterstaining with DAPI to quantify DNA content.

**Migration assays:** For the chemotactic migration assay RAW264.7 macrophage-like cells expressing Flag-tagged RILaltCterm or empty vector were starved overnight in 1% FBS, treated with 0.5 mM H2O2 for 3 hours where indicated, detached and 5×10⁴ cells were seeded in triplicates into the upper chamber of transwell system (8 µm membrane pore size). 1 µg/ml LPS added to the lower chamber was used as chemotactant. Cells were allowed to migrate for 6 hours, when non-migrated cells were removed from the upper chamber with a cotton swab, membranes were fixed in methanol and the cells were stained with DAPI. Five random fields were photographed per well and nuclei of migrated cells were automatically counted using NIH ImageJ software. For the haptotactic migration analysis U2OS cells were transfected with RILaltCterm and RILΔCterm constructs or empty vector control and 2×10⁵ cells were plated per well of a 6-well plate. Cell were left to attach overnight and spontaneous motility was followed by time-lapse microscopy for 5 hours using Leica DMI 6000B microscope equipped with 5× NA 0.12 Leica objective and heated motorized stage with humidified CO2 chamber operated by Leica LAS AF software. Images were taken every 5 minutes and average migration velocity calculated using NIH ImageJ Manual Tracking plugin. Approximately 20 cells per experimental group were tracked.

**RESULTS**

Alternatively spliced isoform of RIL contains unstructured C-terminal segment. Unlike major full-length isoform of RIL transcript, minor alternatively spliced isoform originally described by Bashirova et al. (2) and denoted here as RILaltCterm lacks the sequences of exon 6. The exon skipping results in a frameshift in exon 7, leading to deletion of a C-terminal segment (106 amino acids) containing LIM domain and its substitution with a short (22 amino acids) peptide (Figure 1A). Analysis of the amino-acid composition in the alternative product of RIL reveal high content of proline (P), glutamate (E), serine (S) and threonine (T) residues at its C-terminus. Such regions tend to be unstructured that is characteristic to protein-destabilizing PEST sequences (48). Indeed, analysis by globular domain/disorder predicting machines DisEMBL (49), GlobPlot (50) and PONDR (51) also suggests that the C-terminus of RILaltCterm forms a non-folded disordered structure (Figure 1B).

It has been previously suggested that PEST sequences are present in proteins with high turnover rate and can signal to degradation (52,53). In order to test if RILaltCterm is instable we expressed by lentiviral transduction into A549 cells Flag-tagged proteins corresponding to full-length RIL, RILaltCterm...
and RILΔCterm truncation mutant lacking the frame shifted segment. Although either of the constructs was equally expressed at the RNA level, and on Westerns there were strong bands corresponding to the full-length and RILΔCterm proteins, the level of FlagRILaltCterm protein was undetectable (Figure 1C) suggesting that the unfolded protein segment might be responsible for the effect.

To check possible difference in efficiency of polyribosome entrance we checked the mRNA content of different RIL isoforms bound by polyribosomes in transfected HeLa cells. As shown in Figure 1D, there was no significant variation in polyribosome entrance of different species of RIL mRNAs and mRNA of GFP used as loading/transfection control.

Fusion with the alternative C-terminus of RIL destabilizes reporter proteins. To check the ability of alternatively spliced C-terminus of RIL to destabilize heterologous proteins we appended the last 30 amino acids of RILaltCterm to C-termini of either EGFP or luciferase to generate EGFP-RILaltCterm or Luc-RILaltCterm, respectively. Western analysis indicates that the abundance of EGFP-RILaltCterm fusion protein was significantly lower compared to the unmodified EGFP, while corresponding mRNA levels were not significantly different (Figure 2A). Similarly, A549 cells transduced with the EGFP-RILaltCterm construct demonstrated a dramatically decreased fluorescence, compared with the A549 cells transduced with similar construct carrying control EGFP (Figure 2B). The luciferase fusion construct was tested by expression in H1299 cells, in parallel with the non-fused luciferase construct and an additional control construct Luc-RILaltCterm. The latter construct has similar to the Luc-RILaltCterm structure, except for a one nucleotide insertion leading to an unrelated frame shifted C-terminal peptide. Fusion of the alternative C-terminal segment of RIL to luciferase resulted in a dramatic (almost 100-fold) decrease in enzymatic activity, while the RIL-Clike unrelated peptide had no significant effect on luminescent signal (Figure 2C).

Next we decided to test whether fusion with the RILaltCterm peptide changes stability of luciferase protein. Cells expressing Luc-RILaltCterm along with two control constructs were treated with protein synthesis inhibitor cycloheximide and protein decay was monitored by measuring luciferase activity at different time points. Indeed, activity of Luc-RILaltCterm decayed steeply with a half-life of ~1 hour while the unmodified protein along with the Luc-RIL-Clike control demonstrated a much slower decline (Figure 2D). The results suggest that RIL alternative C-terminus contains a PEST motif that can destabilize heterologous target proteins.

RILaltCterm is degraded by the core 20S proteasome. It has been previously reported that PEST sequences may be recognized by ubiquitin E3 ligases and target the protein to 26S proteasome-dependent degradation. To check possible ubiquitination of RILaltCterm lysates from 293T cells transiently expressing FlagRILaltCterm and treated with proteasome inhibitor MG-132 were immunoprecipitated with anti-Flag agarose, separated on gels and Western blots were probed with anti-ubiquitin antibody. Here Flag-p53 was used as positive control as it is well established that p53 is ubiquitinated by HDM2 (54,55). High-molecular weight smears characteristic of poly-ubiquitination could only be detected in case of p53 but not RILaltCterm (Figure 3A). These results indicate that RILaltCterm is not ubiquitinated and hence its degradation could be ubiquitin-independent.

Rapid degradation of RILaltCterm could be mediated either by non-proteasomal degradation, or by 20S proteasomes. There is a number of studies showing that disordered/unfolded protein regions can be exposed and thus targeted to lysosomes (56) or easily accessible to other proteases (57,58). Specifically, it has been previously reported that PEST sequences can be recognized and cleaved by calpain (59). Also, compelling evidence has recently emerged that proteins harboring unstructured regions can be degraded “by default” by core 20S proteasome (60). To probe these protein degradation pathways we screened a panel of known protease inhibitors with defined specificities in 293T cells to assess their effect on the
abundance of transiently expressed RILaltCterm protein. Regarding the 20S proteasome degradation pathway testing, we used its activator dicoumarol. As shown in Figure 3B, inhibition of neither calpain nor cysteine, serine or aspartic proteases had any significant effect on the RILaltCterm protein level (Figure 3B, lanes 8-11). Proteasomal inhibitor MG-132, on the other hand, stabilized RILaltCterm to a high extent (compare lanes 3 and 5), yielding as much protein as both full-length RIL and RILΔCterm used as stable positive controls (lanes 2 and 4). This result implies the involvement of proteasomes in RILaltCterm degradation. At the same time dicoumarol treatment significantly enhanced the degradation making RILaltCterm protein undetectable (lane 6). Taken together with the observation that no ubiquitination of RILaltCterm could be detected, this latter result provides strong evidence that the core 20S proteasome pathway is involved in the degradation process. The protein levels of both full-length RIL (not shown) and RILΔterm truncation mutant (Figure 3C) were not significantly affected by inhibition (by MG-132) or activation (by dicoumarol) of 20S proteasomal degradation, which provides further evidence for the important role of RIL alternative C-terminus in regulation of protein stability. NQO1 is known to modulate degradation in the 20S proteasome by binding to its targets. Indeed, the RILaltCterm protein can interact with endogenous NQO1, as shown by co-immunoprecipitation in HeLa cells (Figure 3D). Moreover, the alternatively spliced RIL isoform is significantly accumulated upon over-expression of recombinant NQO1 in a dose-dependent manner (Figure 3E) and the protection is abolished by the NAD(P)H antagonist dicoumarol. The results establish that the stability of RILaltCterm isoform is controlled by the core 20S proteasome degradation machinery.

**RILaltCterm is involved in reorganization of actin cytoskeleton.** Reorganization of actin cytoskeleton is a tightly regulated process. As an actin-associated protein the full-length RIL is known to alter F-actin turnover (1). The alternatively spliced isoform could act as a modulator for fine-tuning the processes. The PDZ domain of RIL is known to associate with the LIM-domain of RIL in a yeast two-hybrid system (61). No evidence for such interaction in mammalian cells has been reported so far. By co-immunoprecipitation we found that both short isoforms of RIL (RILaltCterm and RILΔCterm) are able to bind endogenous full-length RIL in U2OS cells (Figure 4A). FlagRILaltCterm precipitated significantly lower amounts of endogenous RIL because of its very high turnover rate and low abundance. On the other hand, the deletion mutant RILΔCterm that lacks the degradation signal was strongly enriched in the precipitate and was able to pull down large amounts of endogenous RIL. We used RILΔCterm as a stable variant of RILaltCterm in the subsequent experiments.

Next we asked if interaction between the full-length and the shorter RIL isoforms would affect its intracellular distribution and the ability to induce formation of actin stress fibers. U2OS cells were cotransfected with constructs expressing Flag- or HA-tagged full-length RIL and RILaltCterm and subjected to fractionation based on detergent solubility. Overexpression of RILaltCterm isoform shifted distribution of the full-length RIL from Triton X-100 insoluble (cytoskeleton and nuclei) to detergent-soluble (cytosol) fraction (Figure 4B). Similarly, we analyzed distribution of α-actinin-1, an actin cross-linking protein and a marker for fibrillar actin. Upon introduction of RILaltCterm, the full-length RIL failed to increase the content of cross-linked actin fibers as evidenced by α-actinin distribution between cytoskeleton and cytosol. Same results were obtained using RILΔCterm (data not shown) confirming that both short isoforms of RIL are functionally similar.

Change in full-length RIL localization in the presence of RILΔCterm was confirmed by immunofluorescent staining (Figure 4C). When expressed alone, the full-length RIL displayed a fibrillar pattern with characteristic thick fibers and occasional clusters (Figure 4C, upper left panel), consistent with previous reports (1), while the RILΔCterm isoform stained more diffusely in the cytoplasm with thin fibers forming a dense mesh-like pattern (Figure 4C, lower left panel). Although occasional fibrillar
structures could still be observed, the filaments were short, thin and disorganized. On the other hand, when both isoforms were coexpressed in the same cell the full-length RIL no longer formed thick fibrillar structures and its staining has changed to a mesh-work of thin fibers similar to that of the RILΔCterm isoform (Figure 4C, right panel). The RILaltCterm isoform had similar effect on the staining pattern of full-length RIL although fewer RILaltCterm-positive cells could be detected due to the high turnover rate (data not shown). We quantified the proportion of RIL-expressing cells with thin, thick fibers or mixed RIL staining pattern in the presence or absence of the short isoform of RIL. Indeed, expression of RILΔCterm decreased substantially the number of cells with thick RIL fibers from 49.5±2.6% to 29.2±2.8% while increasing the number of cells with thin fibers from 18.8±6.1% to 34.9±7.5% (Figure 4D).

As a final step we analyzed the effect of RIL isoforms on F-actin morphology (Figure 4E). When compared to non-transfected control (asterisk), cells expressing the full-length RIL exhibited increased formation of actin cables (Figure 4E, arrows) and the protein colocalized markedly with the stress fibers. Coexpression with the short isoform changed dramatically distribution of the full-length RIL to the mesh-work of thin fibers and the content of F-actin was significantly reduced (arrowheads). Collectively, the results provide compelling evidence that RILaltCterm can participate in actin cytoskeleton reorganization by modifying distribution on the full-length isoform of RIL.

RILaltCterm is stabilized in response to oxidative stress. Binding of NQO1 to some of its targets is increased following γ-irradiation leading to protein stabilization (62-64). Ionizing radiation promotes formation of reactive oxygen and nitrogen species (ROS and RNS, respectively) that induce oxidative stress response. To find physiological conditions favoring accumulation of the RILaltCterm isoform we checked whether oxidative stress induced by hydrogen peroxide treatment affects the 20S proteasomal pathway. Consistent with previous reports UV irradiation and to a lesser extent H2O2 treatment induced accumulation of the RILaltCterm isoform (Figure 5A), while ROS and RNS scavengers NAC and ebselen as well as the hypoxia-mimetic DFO decreased slightly the abundance of RILaltCterm. In agreement with our previous results (Figure 3E), stabilization of RILaltCterm after oxidative stress coincided with the increase in expression of NQO1 (Figure 5B). Immunoblot analysis on non-transfected cells treated with proteasome inhibitor MG-132 or increasing concentrations of H2O2 (using antibodies predicted to recognize both isoforms of RIL) detected an additional band that was identified as RILaltCterm by mass-spec analysis of immunoprecipitated material (Figure 5C). The signal intensity increased dose-dependently with ROS challenge and was comparable to RILfull-length in abundance.

Stabilization of RILaltCterm in response to oxidative stress induces reorganization of actin cytoskeleton. As we found that the short isoform of RIL can modify intracellular localization of full-length RIL we checked if stabilization of RILaltCterm in response to oxidative stress would produce similar effect. Indeed, we found that treatment with hydrogen peroxide of U2OS cells expressing RILaltCterm shifted full-length RIL from detergent insoluble (or cytoskeleton-associated) to detergent soluble (cytosol) fraction (Figure 5D). Moreover, accumulation of RILaltCterm after prolonged (4 hr) exposure to H2O2 significantly decreased the content of polymerized actin compared to vector-treated cells (Figure 5F). In keeping with these results, marked redistribution of endogenous RIL from cytoskeleton-bound state to cytosol could be readily detected in non-transfected MDA-MB-435S and A431 cells (Figure 5E).

Actin polymerization/disassembly balance mirrored by F-actin content is intimately involved in regulation of cell migration. Actin polymerization at the leading edge is the driving force behind lamellipodia protrusion while actin cables within the cell body function as tracks for myosin during tail retraction (39,65). Hence we hypothesized that decreased F-actin content would lead to attenuated cell motility. To address this question experimentally we subjected RAW 264.7 monocytic cells to transwell chemotaxis assay. Monocytes
extravasate and migrate along the gradient of chemoattractants while being exposed to oxidative stress associated with inflammation (65). Cells transduced with RILaltCterm or empty vector were preconditioned by 0.5 mM H$_2$O$_2$ for 4 hours and then loaded into upper chamber of a transwell system. Cells were induced to migrate towards LPS added to lower chamber as chemoattractant. Consistent with previous data, under oxidative stress conditions RILaltCterm markedly inhibited cell migration compared to empty vector. On the other hand, the alternatively spliced isoform of RIL failed to elicit any changes in motility in untreated monocytes (Figure 5G). Accumulation of the short RIL isoform (mimicked by the stable deletion mutant RILΔCterm) decreased mean migration velocity in a model of spontaneous haptotactic cell motility by almost 40% in U2OS cells (Figure 5H) corroborating previous results. These data allowed us to conclude that actin cytoskeleton rearrangement mediated by stabilization of RILaltCterm in response to oxidative stress might attenuate cell migration thus contributing to cellular stress response mechanisms.

DISCUSSION

Results of the present study suggest a function for an alternatively spliced isoform of RIL (PDLIM4) gene product. The isoform originates from skipping exon 6 sequences during splicing, which results in a 84 amino acids shorter protein, compared to major full-length RIL (2). The alternative splicing joining exon 5 and exon 7 removes the LIM domain and produces frame shift leading to a totally unrelated stretch of 22 amino-acids at the C-terminus. Functional characterization of RIL gene has been carried out in different laboratories with cDNA constructs corresponding to the major full-length protein. RIL was implicated in regulation of actin stress fiber turnover. RIL protein binds to α-actinin, enhances its ability to interact with actin filaments and promotes formation of actin stress fibers, although details of this mechanism are not clearly understood (1).

No information regarding functional properties of the alternative isoform of RIL have been reported to date, although numerous examples for other genes suggest that alternative splicing can substantially modify functions of the genes (66-68). RILaltCterm is extremely unstable and the protein can be hardly indentified by Western analysis even when overexpressed from recombinant constructs. To reveal basis for its instability we analyzed protein structure of the C-terminal segment that is translated from an alternative frame of exon 7 and found a PEST like disordered protein structure (69) commonly associated with low protein stability (52,53,59,60,70,71) due to its preferred targeting by E3 ubiquitin ligases, calpain or by degradation in the core 20S proteasomes. Placing the C-terminal segment of RILaltCterm to GFP or luciferase was capable to reduce dramatically the protein half life (from 48 and 12h, respectively, to less than 1h), suggesting that this 30 amino acid sequence could be used as a model protein-destabilizing instrument.

In eukaryotes the vast majority of short-lived proteins are degraded by 26S proteasome complex following the process of poly-ubiquitination that marks the proteins for destruction. Regulatory 19S subunit of the proteasome recognizes the poly-ubiquitin chain conjugated to the target protein, unfolds the protein and passes it on to the catalytic core 20S subunit where it is hydrolyzed to short peptides or single amino acids (72). We found no evidences of poly-ubiquitinylation of RILaltCterm in transfected cells, while transfected control p53 produced characteristic high molecular weight smears on Western blots developed with anti-ubiquitin antibody.

In bacteria and archaea the proteasome lacks both the regulatory subunit and ubiquitin-conjugating machinery (73), and a similar ubiquitin-independent 20S proteasome pathway has been found in eukaryotes (74,75). It is generally accepted that the 20S proteasome can degrade unfolded proteins (76) that may be partially denatured as a result of stresses or protein “ageing” (57,77). On the other hand, a large subclass of proteins feature intrinsically disordered regions that can either be folded upon binding to their targets or function as flexible linkers and thus remain unstructured (71). The intrinsically disordered proteins tend to be inherently unstable, being rescued from
degradation through masking unstructured parts by certain binding partners (78). Degradation in the 20S proteasomes can be regulated by NAD(P)H quinone oxidoreductase-1 (NQO1). The ubiquitously expressed NQO1 associates with the 20S proteasome (63) and acts as its gate-keeper (60) by preventing degradation of proteins as diverse as ornithine decarboxylase (ODC) (79) and p53 (64). We found that dicoumarol, an inhibitor of NQO1, enhances substantially degradation of RILaltCterm. In addition, by co-immunoprecipitation we found that RILaltCterm interacts with endogenous NQO1. While overexpression of recombinant NQO1 results in accumulation of the alternative isoform, treatment with dicoumarol abolishes the protection. Collectively, the results indicate that fast degradation of RILaltCterm is achieved by the 20S proteasome machinery.

Previous observations in yeast two-hybrid system have revealed binding of C-terminal LIM domain of RIL with the PDZ domain located close to the N-terminus (61). Here we have shown by direct immunoprecipitation that the two isoforms of RIL (the full length and RILaltCterm) can heterooligomerize (Figure 4A). The interaction suggested possible mutual functional modulation of the two protein isoforms, such as a dominant-negative effect produced by the alternative isoform. In agreement with previous report (1) we observed localization of full-length RIL along actin stress fibers where it interacts with an actin-bundling protein α-actinin-1 and assists in the formation of thick actin fibers and actin aggregates. We found that the alternatively spliced isoform of RIL displaces full-length RIL from the actin fibers leading to reorganization of the cytoskeleton. Ectopic expression of RILaltCterm isoform induced the release of full-length RIL from actin cytoskeleton is accompanied by a reduction in F-actin content in the cell and reorganization of actin cytoskeleton. As a consequence of its stabilization the RILaltCterm isoform reduced cell migration rate in the chemotactic transwell assay.

NQO1 belong to the phase II detoxifying enzymes that are induced to protect against electrophilic insults, oxidative stress and ionizing radiation (70). Deficiency in NQO1 is observed in various cancers (80). Recently it has been found that NQO1 plays a role in suppressing inflammatory response and in attenuation of macrophage migration induced by LPS (65), although the mechanism of the effect is poorly understood. We hypothesize that the stabilization of RILaltCterm by NQO1 in response to prolonged exposure to oxidative stress and subsequent cytoskeleton reorganization might contribute to gradual curbing of inflammatory reaction. Following exposure to ROS generated by phagocyte respiratory burst direct oxidation of actin results in rapid assembly of filaments (81). Additional cytoskeleton rearrangement is induced through redox activation of signaling pathways affecting Src and Rho (82), while induction of myosin light chain phosphorylation leads to increased contractility and monocytic migration (83). Prolonged exposure to oxidants induces NQO1, which might stabilize RILaltCterm thereby assisting in gradual recovery of the changes in actin cytoskeleton and in cell motility.

Taken together our results indicate that the alternatively spliced isoform of RIL (PDLIM4) can be activated by protein stabilization in response to certain stresses. The induction can modify the ability of full length RIL to modulate organization of actin cytoskeleton and to affect cell motility.
ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. Alternatively spliced isoform of RIL has low stability. A, genomic organization and splice isoforms of the RIL gene. Full-length RIL mRNA includes all 7 exons, the corresponding protein features PDZ and LIM domains and an interdomain linker region. Alternatively spliced isoform of RIL lacks the 6th exon leading a frameshift and a substitution of a short peptide instead of LIM domain. B, identification of a disordered region within the C-terminal region of RILaltCterm by GlobPlot 2.3 (D denotes disorder) and its sequence. Disorder promoting residues are in bold, order promoting in italic, amino acids characteristic for PEST sequences are underlined. C, Flag-tagged full-length RIL, RILaltCterm or RIL truncation mutant were lentivirally expressed in A549 cells. RILaltCterm protein was barely detectable (Western), while there was no significant difference in mRNA levels as detected by RT-PCR. D, full-length RIL, RILaltCterm and RIL truncation mutant mRNAs are equally bound by polyribosomes. HeLa cells were transfected by indicated RIL constructs along with GFP-expressing plasmid as internal control. RNA extracted from polysomal fraction was analyzed by RT-PCR.

Figure 2. Alternative C-terminal peptide of RIL destabilizes heterologous proteins. RIL alternative C-terminal peptide was fused in-frame with GFP and lentivirally expressed in A549 cells (A and B). A, abundance of modified GFP protein was significantly decreased (by Western blot) while mRNA levels remain comparable (RT-PCR). B, decreased fluorescence of cells expressing GFP fused to RIL alternative C-terminus compared to non-modified GFP, as examined by fluorescence microscopy. C and D – Luciferase was fused with RIL alternative C-terminus in-frame (Luc-RILaltCterm) or with a frameshift (Luc-RIL-Clike) as a control and introduced into H1299 cells by transfection. C, Luciferase activity was severely impaired compared to controls (Luc-stop and Luc-RIL-Clike). Transfection efficiency was normalized for by β-galactosidase. D, cells were treated with 1μg/ml cycloheximide for 0, 1, 2, 4 or 8 hours and stability of modified luciferase was assessed based on its enzymatic activity. Luc-RILaltCterm has a lower half-life time relative to controls.

Figure 3. RILaltCterm is degraded by the core 20S proteasome. A, RILaltCterm is not ubiquitinated. HeLa cells expressing Flag-tagged RILaltCterm or p53 were treated with 20μM MG-132 for 6 hours. Protein lysates were subjected to immunoprecipitation with anti-Flag agarose beads and analyzed by Western blot with antibodies specific to ubiquitin and Flag-epitope. B, RILaltCterm is degraded by the core 20S proteasome. Western blot analysis of 293T cells transfected by constructs expressing HA-tagged full-length RIL, RILaltCterm or RILΔCterm and treated with indicated compounds for 5 hours. C, Alternative C-terminus of RIL targets the protein to the 20S proteasome. HeLa cells were transfected by constructs expressing HA-tagged RILaltCterm or RILΔCterm and treated as indicated. Protein lysates were analyzed by Western blotting with anti-HA and anti-tubulin antibodies. D, RILaltCterm interacts with NQO1. HeLa cells were transfected with the indicated Flag-tagged RIL constructs and protein lysates were subjected to immunoprecipitation with anti-Flag agarose. Immune complexes were probed for the presence of NQO1. E, NQO1 protects RILaltCterm from degradation by the 20S proteasome. HeLa cells were transfected with varying amounts of HA-RILaltCterm and FlagNQO1. NQO1 inhibitor dicoumarol was added where indicated. Levels of RILaltCterm, NQO1 and GAPDH proteins were analyzed by immunoblotting with anti-HA, Flag and GAPDH antibodies, respectively.

Figure 4. RILaltCterm affects actin organization via interaction with full-length RIL. A, RILaltCterm binds to full-length RIL. Lysates from U2OS cells expressing FlagRILaltCterm were immunoprecipitated using anti-Flag agarose and probed with anti-RIL antibody. B, RILaltCterm changes distribution of full-length RIL between cytoskeleton-bound and cytosolic fractions and abrogates the effect of RIL on α-actinin-1. 293T cells were transfected as indicated, fractionated by Triton X-100 solubility and protein distribution analyzed by immunoblotting. C, RILΔCterm affects staining pattern of full-length RIL. U2OS cells were transfected with the indicated RIL constructs, fixed in 4% formaldehyde for 15 min, stained for Flag and HA tags with suitable primary and fluorescently labeled secondary antibodies and analyzed by wide-field fluorescence microscopy. Panel C’ shows enlarged detail of cell regions outlined in C. D, samples were processed as in C and cells exhibiting thick fibers, meshwork of thin fibers or...
mixed staining pattern of full-length RIL in the presence or absence of RILaltCterm were counted. Bars represent an average of 4 independent experiments ± SEM, \( p \)-values calculated by Student’s \( t \)-test.\( E \), U2OS cells expressing Flag-tagged full-length RIL and/or HA-tagged short isoform of RIL were grown on glass coverslips, fixed in 4% formaldehyde and stained with rabbit anti-Flag and rat anti-HA antibodies and then secondary AlexaFluor594 anti-rabbit and AlexaFluor350 anti-rat antibodies. F-actin was visualized by FITC-conjugated phalloidin. Asterisk indicates non-transfected cell, arrows – thick actin/RIL fibers, arrowheads – meshwork of thin actin fibers. Panels \( E^* \), \( E^\downarrow \) and \( E^\uparrow \) represent enlarged detail of cells in \( E \) marked with asterisk, arrows and arrowheads, respectively.

**Figure 5.** Stabilization of RILaltCterm in response to oxidative stress attenuates cell migration. \( A \), RILaltCterm is stabilized in response to oxidative stress. HeLa cells were transfected with construct expressing HA-RILaltCterm and treated as indicated. Stabilization of RILaltCterm was assessed by immunoblotting with anti-HA antibodies. \( B \), RILaltCterm is stabilized in response to oxidative stress concomitantly with induction of NQO1. U2OS cells expressing FlagRILaltCterm were treated with 0.5 mM \( \mathrm{H}_2\mathrm{O}_2 \) for 4 hours, then lysed and probed for Flag, NQO1 or tubulin by Western blotting. \( C \), Oxidative stress induces accumulation of endogenous RILaltCterm. Non-transfected MDA-MB-435S and A431 cells were treated as indicated and protein lysates immunoblotted with anti-RIL antibodies capable of recognizing all isoforms. \( D \), RILaltCterm redistributes full-length RIL from cytoskeleton to cytosol in response to oxidative stress. U2OS cells were treated as in \( B \) and fractionated based on Triton X-100 solubility. Distribution of FlagRILaltCterm and endogenous full-length RIL were analyzed by Western blotting. \( E \), oxidative stress induces redistribution of endogenous RIL from cytoskeleton to cytosol. Non-transfected MDA-MB-435S and A431 were treated with 0.2 mM \( \mathrm{H}_2\mathrm{O}_2 \) where indicated and fractionated based on Triton X-100 solubility. Distribution of endogenous RIL and \( \alpha \)-actinin-1 was analysed by immunoblotting. \( F \), stabilization of RILaltCterm in response to oxidative stress decreases F-actin content. Cells from \( B \) were fixed with formaldehyde in cytoskeleton buffer, permeabilized by 0.1% Triton X-100 and F-actin was stained by FITC-labeled phalloidin. Relative F-actin content was assessed by fluorescence intensity and normalized by DNA content (DAPI staining). Bars represent mean ± SEM, relative to empty vector controls. \( p \)-Values calculated by Student’s \( t \)-test; NS – not significant. \( G \), RAW 264.7 monocytic cells were lentivirally transduced to express RILaltCterm and subjected to transwell chemotaxis assay with or without \( \mathrm{H}_2\mathrm{O}_2 \) treatment in triplicates. Migrated cells were fixed, stained and automatically counted after 6 hours of migration using ImageJ software. Bars represent mean ± SEM, relative to empty vector controls. \( p \)-Values determined by Student’s \( t \)-test, NS – not significant. \( H \), U2OS cells were transfected with RILaltCterm or RILΔCterm constructs or empty vector and spontaneous non-directional haptotactic motility was analysed by time-lapse microscopy for 5 hours. 20 cells in each sample were tracked using ImageJ Manual Tracking plugin. Bars respresent mean velocity (\( \mu \text{m/min} \)) ± SEM. \( p \)-Values calculated by one-way ANOVA, NS – not significant. Panels on the right show representative cell trajectories for each experimental group.
Guryanova et al., Figure 3.
Guryanova et al., Figure 5.

**A**

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**F**

- **GAPDH**
- **Flag**
- **RIL Cterm**
- **RIL full-length**

**G**

- **untreated H$_2$O$_2$**
- **H$_2$O$_2$**

**H**

- **migration velocity, μm/min**
- **number of migrated cells relative to empty vector control**

- **p<0.001**
- **NS**