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The Ras suppressor Rsu-1 binds to the LIM 5 domain of the adaptor protein PINCH1 and participates in adhesion-related functions

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Abstract

Rsu-1 is a highly conserved leucine rich repeat (LRR) protein that is expressed ubiquitously in mammalian cells. Rsu-1 was identified based on its ability to inhibit transformation by Ras, and previous studies demonstrated that ectopic expression of Rsu-1 inhibited anchorage-independent growth of Ras-transformed cells and human tumor cell lines. Using GAL4-based yeast two-hybrid screening, the LIM domain protein, PINCH1, was identified as the binding partner of Rsu-1. PINCH1 is an adaptor protein that localizes to focal adhesions and it has been implicated in the regulation of adhesion functions. Subdomain mapping in yeast revealed that Rsu-1 binds to the LIM 5 domain of PINCH1, a region not previously identified as a specific binding domain for any other protein. Additional testing demonstrated that PINCH2, which is highly homologous to PINCH1, except in the LIM 5 domain, does not interact with Rsu-1. Glutathione transferase fusion protein binding studies determined that the LRR region of Rsu-1 interacts with PINCH1. Transient expression studies using epitope-tagged Rsu-1 and PINCH1 revealed that Rsu-1 co-immunoprecipitated with PINCH1 and colocalized with vinculin at sites of focal adhesions in mammalian cells. In addition, endogenous P33 Rsu-1 from 293T cells co-immunoprecipitated with transiently expressed myc-tagged PINCH1. Furthermore, RNAi-induced reduction in Rsu-1 RNA and protein inhibited cell attachment, and while previous studies demonstrated that ectopic expression of Rsu-1 inhibited Jun kinase activation, the depletion of Rsu-1 resulted in activation of Jun and p38 stress kinases. These studies demonstrate that Rsu-1 interacts with PINCH1 in mammalian cells and functions, in part, by altering cell adhesion.
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Keywords: Rsu-1; Leucine rich repeat; PINCH; LIM domain; Ras; Jun kinase

Introduction

Rsu-1 is a highly conserved, ubiquitously expressed single copy gene that encodes a leucine rich repeat (LRR) protein [15,40]. LRR motifs have been identified in a wide range of proteins and are implicated in protein–protein association [26,27]. Rsu-1 was isolated in an expression cloning assay based on its ability to suppress transformation by the Ras oncogene [15]. Ectopic expression of Rsu-1 cDNA prevented transformation by the Ras oncogene, but not Raf or Src, and inhibited anchorage-independent growth of human tumor cell lines [15,41,46]. Our previous studies demonstrated that Rsu-1 expression blocked the activation of Jun kinase but not Erk kinase, inhibited Rho (ROCK) kinase activity, and altered the actin cytoskeleton organization [32,46].

The human Rsu-1 locus maps to 10p13, a region that is deleted in high-grade gliomas, and an alternatively spliced Rsu-1 mRNA that encodes a truncated and unstable protein product occurs in 30% of high-grade gliomas [38]. The introduction of an Rsu-1 vector into a human glioma cell line, which contained a single copy of chromosome 10 and did not express endogenous Rsu-1, blocked tumor formation in a nude mouse xenograft model [41].

To understand the mechanism by which Rsu-1 blocked growth of tumor cells, we screened for Rsu-1 binding proteins using the well-established yeast dual-hybrid system for the detection of in vivo binding of proteins [2,11,18,49]. All of the Rsu-1 binding proteins that were identified in our screen contained the LIM domain protein PINCH1.

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PINCH1 is an adaptor protein consisting of five LIM domains (LIM 1–5) [36] that localizes to focal adhesions [29,42]. The LIM 1 domain of PINCH1 binds to the amino terminal ankyrin repeat domain of the integrin-linked kinase (ILK) and can modulate ILK activity [43]. The LIM 4 domain of PINCH1 binds to the third SH3 domain of the SH2–SH3 protein Nck2 [45,47].

Binding of integrins to their ligands results in a number of events including the activation of focal adhesion kinase (FAK) and syk, the activation of molecules involved in organization of actin cytoskeleton (filamin, talin, and paxillin), and the generation of lipid second messengers [37]. ILK is a component of focal adhesions and fibrillar adhesions and it plays a role in cell matrix-adhesion functions [16]. The carboxyl terminal region of ILK binds to the cytoplasmic domain of the beta 1, 2, or 3 subunits of the integrins [20] and ILK is activated as a result of integrin engagement [14,16]. Recent studies demonstrated that in addition to binding PINCH1 [44], the amino terminal ankyrin repeat region of ILK also interacted with the LIM domain proteins PINCH2 [42] and paxillin [34].

Genetic studies in *C. elegans* demonstrated that PINCH, ILK, and integrins function together in muscle cell attachment assembly [22] with mutations in PINCH or ILK resulting in similar phenotypes. The *C. elegans* and *Drosophila* homologs of ILK do not have associated kinase activity but appear to function exclusively in integrin attachment [13,31]. In mammalian cells, both kinase activity and attachment functions have been ascribed to ILK. Inhibition of PINCH–ILK interaction in mammalian cells decreased cell spreading and reduced motility, suggesting that PINCH is required for ILK function in these activities [52]. A more recent study has demonstrated that both members of the PINCH1–ILK complex are required for the localization of the complex to focal adhesions [42]. In addition, the ILK binding protein CH-ILKBP/actopaxin has been reported to participate in linking ILK to paxillin, thus providing another potential mechanism to localize ILK to focal adhesions [33].

The results presented in this paper demonstrate that Rsu-1 also binds to PINCH1. Our data show that the LIM 5 domain of PINCH1 binds to Rsu-1 and that Rsu-1 colocalizes with PINCH1 in focal adhesions. Because of the potent negative effects of Rsu-1 on anchorage-independent growth, this finding links Rsu-1 and the PINCH1–ILK complex to the regulation of cell survival signaling in the absence of cell attachment.

Experimental procedures

Two-hybrid screening

Bait plasmids encoding Rsu-1 GAL4 binding domain (BD) proteins were constructed in vectors from Clontech (BD Biosciences Clontech, PaloAlto, CA) as follows. Plasmid 3v120 contained the entire mouse Rsu-1 ORF (amino acids 1–277) inserted in frame with the GAL4 BD in pAS2-1, and plasmid 3v125 contained the entire mouse Rsu-1 ORF inserted in frame with the GAL4 BD in pGBKT7. Rsu-1 fragments were amplified with specific primers encoding restriction enzymes, digested and inserted into the vectors. All constructs were verified by DNA sequencing.

The Rsu-1 plasmid 3v125 was used to screen a mouse embryo cDNA library inserted in the GAL4 activating domain (AD) vector pAD-GAL4-2.1 (Stratagene, La Jolla, CA). The screening was performed in yeast strain AH109 (BD Biosciences Clontech, Palo Alto, CA) by PEG-lithium acetate co-transformation of the bait with library plasmids released from phage. Co-transfection was performed using a commercially available kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's directions. Transformed AH109 cells were divided into two pools. Half were plated on SD $His^-/Leu^-/Trp^-$/X-α-Gal with 5 mM 3-AT (TDO) and half were plated on SD $Ade^-/His^-/Leu^-/Trp^-$/X-a-Gal with 1 mM 3-AT (QDO). The plates were incubated at 30° for 14 days and blue colonies were picked beginning at 5 days. Colonies were retested on the above media and those that grew were inoculated into SD liquid culture, grown overnight, and used for recovery of plasmid DNA. Plasmid DNA was recovered from yeast DNA obtained by lyticase lysis protocol and column purification. The yeast plasmid DNA preparation was used as a template for amplification of insert DNA using vector-derived primers. The yeast DNA was also used for the recovery of library plasmids by electroporation into DH10B cells and selection on LB plates containing Ampicillin. The recovered plasmids were retested for ability to interact with the Rsu-1 bait plasmid 3v125 by co-transfection into AH109 and plating on the SD Leu^-/Trp^-, TDO, and QDO media. Plasmids positive for interaction were sequenced using vector-derived primers and the sequence information was used to perform Blast searches.

Subdomain mapping of the PINCH1 binding domain

To perform subdomain mapping of the PINCH1 interaction sites, deletion constructs of PINCH1 were prepared as GAL4 AD fusion proteins in pGADT7 (BD Biosciences Clontech, Palo Alto, CA). These constructs were tested for interaction with full-length Rsu-1 Gal 4 BD fusion proteins. The PINCH1 subdomain plasmids were constructed by amplification of PCR products using forward primers containing a restriction site (*Bam*HI or *Eco*RI) that allowed in-frame insertion of the fragment into pGADT7. The reverse primer for all constructs that encompassed LIM 5 contained a *Xho*I site. The subdomain constructs encoded the following regions of PINCH1: PINCH1 LIM 2–5, aa 74–331; PINCH1 LIM 3–5, aa 145–331; PINCH1 LIM 4–5, aa 202–331;