The p150-Spir protein provides a link between c-Jun N-terminal kinase function and actin reorganization

Ines M. Otto*, Thomas Raabe†, Ulrike E.E. Rennefahrt*, Peer Bork‡, Ulf R. Rapp* and Eugen Kerkhoff*

The Jun N-terminal kinase (JNK) is a downstream effector of Rac and Cdc42 GTPases involved in actin reorganization [1–3]. A role of the *Drosophila* JNK homologue, Basket (DJNK/Bsk), in the regulation of cell shape changes and actin reorganization arises from its function in the process of dorsal closure [4–6]. One potential mechanism for induction of cytoskeletal changes by JNK is via transcriptional activation of the *decapentaplegic* gene (*dpp*, a member of the TGFβ superfamily) [6]. A direct link between JNK signalling and actin organization has not yet been found, however. We have identified a novel DJNK-interacting protein, p150-Spir, that belongs to the Wiscott–Aldrich syndrome protein (WASP) homology domain 2 (WH2) family of proteins involved in actin reorganization [7,8]. It is a multidomain protein with a cluster of four WH2 domains, a modified FYVE zinc-finger motif [9], and a DEJL motif, a docking site for JNK [10], at its carboxy-terminal end. In mouse fibroblasts, p150-Spir colocalized with F-actin and its overexpression induced clustering of filamentous actin around the nucleus. When coexpressed with p150-Spir in NIH 3T3 cells, JNK translocated to and colocalizes with p150-Spir at discrete spots around the nucleus. Carboxy-terminal sequences of p150-Spir were phosphorylated by JNK both *in vitro* and *in vivo.* We conclude that p150-Spir is a downstream target of JNK function and provides a direct link between JNK and actin organization.

Addresses: *Institut für medizinische Strahlenkunde und Zellforschung, Universität Würzburg, Versbacher Strasse 5, 97078 Würzburg, Germany. †Institut für Genetik, Biozentrum am Hubland, Universität Würzburg, 97074 Würzburg, Germany. ‡European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany.

Correspondence: Eugen Kerkhoff
E-mail: Kerkhoff@mail.uni-wuerzburg.de

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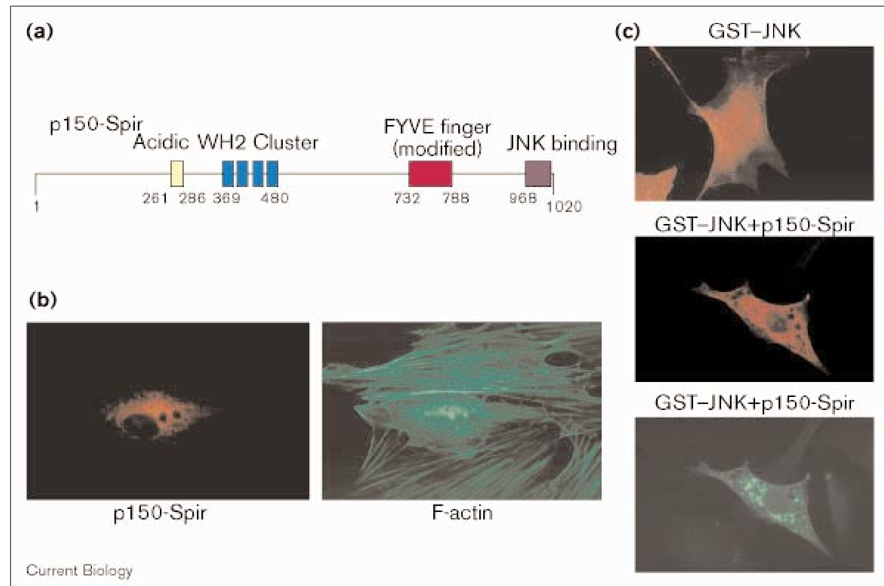
Results and discussion

We identified p150-Spir in a yeast two-hybrid screen to search for novel DJNK/Bsk interaction partners. The cDNA encoding p150-Spir contains an open reading frame of 1,020 amino acids (Figure 1a). Transient transfection experiments in mouse fibroblasts revealed that the cDNA directs expression of a protein that migrates with an apparent molecular weight of approximately 150 kDa on an SDS–polyacrylamide gel (data not shown). The *Spire* (*spir*) gene was independently cloned by transposon tagging [11] and exhibits significant homology to the maternal gene *pem-5* [12] isolated from the ascidian *Ciona savignyi* (Figure 1b). *Spire* is a maternal effect gene that affects both the dorsal–ventral and anterior–posterior axes of the *Drosophila* egg and embryo [11,13]. It is required for localization of determinants within the developing oocyte to the posterior pole and to the dorsal anterior corner [11]. Disruptions found in *spir* mutants can be mimicked by treatment with cytochalasin D [11,14], which inhibits actin polymerization, indicating a role for the *spir* gene product in the regulation of the actin cytoskeleton. Comparison of *Drosophila* p150-Spir sequences to human EST clones reveals a high conservation between the p150-Spir sequences of *Drosophila* and human.

The p150-Spir protein contains an acidic domain, a cluster of four WH2 domains [7,8], a modified FYVE zinc finger domain [9] and a carboxy-terminal DEJL motif (docking site for Erk and JNK containing an LXL motif; see later [10]; Figure 1a). WH2 domains bind monomeric actin and WH2 family proteins such as WASP and WAVE are involved in actin reorganization [8,15–17]. Sequences encompassing the Spir WH2 domains interact directly with monomeric actin [11]. As was shown for WASP [15] and WAVE [16], transient expression of p150-Spir in adherent mammalian cells induced clusters of filamentous actin around the nucleus, which colocalized with p150-Spir in all transfected cells (Figure 1b). A modified FYVE zink-finger motif is located in the central region of the protein (Figure 1a) [9]. The modified structure lacks a pocket of basic amino acids between cysteines 3 and 4 of the FYVE finger structures that is necessary for the binding of phosphatidylinositol 3-phosphate.

We have identified p150-Spir in a yeast two-hybrid screen as a DJNK-interacting protein. The smallest fragment that still binds DJNK consists of the carboxy-terminal 53 amino acids. A DEJL motif, characterized by a cluster of basic amino acids amino-terminal to an L/I–X–L/I motif (in the single-letter amino acid code), is located in this carboxy-terminal sequence (amino acids 997–1014 of p150-Spir, KQKRSSARNRTIQNLTLD) [10]. DEJL domains have been shown to mediate the docking of the Erk and JNK

Figure 1



The p150-Spir protein has multiple domains and is involved in actin reorganization. **(a)** A unique array of structural motifs in p150-Spir: an acidic region, four WH2 domains, which bind monomeric actin, a modified FYVE zink-finger structure and a JNK-binding domain at the carboxyl terminus containing a DEJL motif. (**b**) The p150-Spir protein is involved in actin reorganization. Transient expression of a Myc-epitope-tagged version of p150-Spir (plasmid BJ4-Myc-p150-Spir) in NIH 3T3 mouse fibroblasts by lipofection induces the formation of F-actin clusters around the nucleus, which colocalize with the p150-Spir protein. Myc–p150-Spir was visualized by staining with the anti-Myc 9E10 antibodies and a tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Filamentous actin was visualized by costaining with fluorescein isothiocyanate (FITC)-conjugated phalloidin (green). Cells were fixed in a 3.7% paraformaldehyde solution and permeabilized by treatment with 0.2% Triton X-100 in PBS. (**c**) The p150-Spir and JNK proteins colocalize when coexpressed in NIH 3T3 cells. Cells were transfected by lipofection with pEBG-GST-JNK (GST–JNK) or cotransfected with pEBG-GST-JNK and pcDNA3-Myc-p150-Spir (GST–JNK + p150-Spir) expression vectors. At 36 h post transfection, the cells were fixed and permeabilized as described above and immunostained with a mouse anti-GST antibody and a TRITC-conjugated secondary antibody (red) and a FITC-conjugated anti-Myc 9E10 antibody (green).

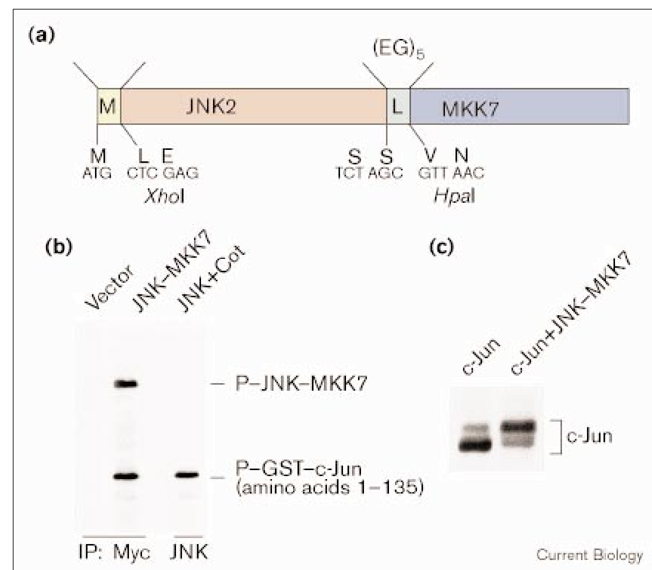
mitogen-activated protein (MAP) kinases to either activating kinases or substrate proteins [10]. To further characterize the interaction between p150-Spir and JNK, we determined the localization of the two proteins when transiently expressed in NIH 3T3 cells. As shown in Figure 1b, p150-Spir accumulated in punctate spots located around the nucleus. Expression of a fusion protein between glutathione-S-transferase and JNK (GST–JNK) revealed both a cytoplasmic and nuclear distribution of the kinase (Figure 1c). In the presence of p150-Spir, GST–JNK was translocated from these areas and accumulated at places where p150-Spir was located (Figure 1c). The colocalization of the proteins was detected in all cells expressing both proteins but was not found in cells coexpressing GST alone with p150-Spir (data not shown).

Erk and JNK MAP kinases are recruited to substrate proteins via docking sites, enabling the kinases to phosphorylate serine or threonine residues adjacent to prolines (S/TP motifs) [10]. As p150-Spir contains a JNK docking site (the DEJL motif) and several potential S/TP phosphorylation motifs, we sought to determine whether p150-Spir is a phosphorylation target of JNK. In a recent report, a highly specific, constitutively active Erk was generated by the fusion of Erk2 to its upstream activator Mek1 (Erk2–Mek1-LA) [18]. Analogously, we fused JNK2 (rat) to its upstream activator MKK7 (mouse) via a linker region (Figure 2a). The fusion protein, JNK–MKK7, is a constitutively active Jun N-terminal kinase. JNK–MKK7 phosphorylated amino-terminal c-Jun sequences *in vitro* (Figure 2b) and induced an electrophoretic mobility shift of the c-Jun protein when coexpressed in NIH 3T3 cells, indicating an *in vivo* c-Jun phosphorylation (Figure 2c). MKK7 activates JNK by phosphorylating a TPY motif in the central region of JNK. JNK–MKK7 exhibited autophosphorylation (Figure 2b) and interacted with a phospho-specific antibody recognizing activated JNK protein (data not shown). A similar construct (JNKK2–JNK1) was recently shown to be a specific, constitutively active c-Jun kinase [19].

We first investigated whether p150-Spir sequences are phosphorylated by JNK *in vitro*. Indeed, we found that arsenite-activated JNK1, immunoprecipitated from NIH 3T3 cell lysates, phosphorylated a carboxy-terminal fragment of the p150-Spir protein in an *in vitro* immune-complex kinase assay (Figure 3a). Erk and p38 MAP kinases precipitated from the same lysates exhibited a basal phosphorylation activity which could not be increased by arsenite stimulation of the cells (data not shown). Coexpression of p150-Spir with MLK3-activated JNK2 (data not shown; mixed lineage kinase (MLK3) is an upstream activator of JNKs [2]) or JNK–MKK7 (Figure 3b) in mouse fibroblasts induced several slower migrating forms of the p150-Spir protein during SDS–PAGE.

The electrophoretic mobility shift of p150-Spir strongly suggests that it was phosphorylated by JNK on multiple sites. The slower migrating forms were abolished by

Figure 2

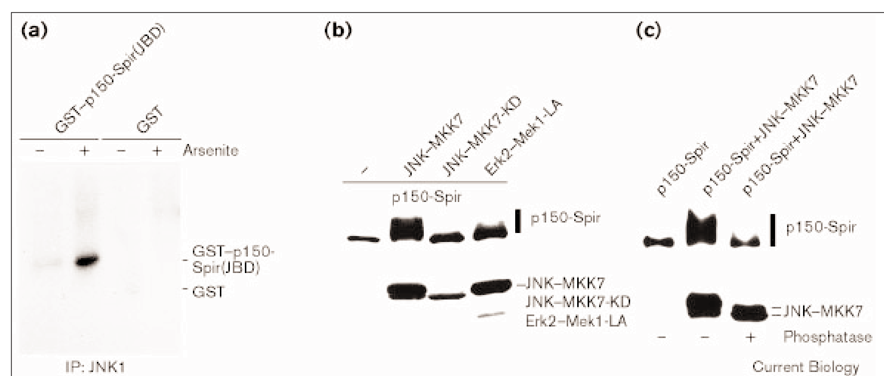


Fusion of the JNK protein with its upstream activator MKK7 generates a constitutively active c-Jun N-terminal protein kinase. **(a)** Structure of the JNK–MKK7 fusion protein. Amino acids 2–426 of rat JNK2 (GenBank accession number L27128) were fused via an $(EG)_5$ linker peptide (L) to amino acids 2–346 of mouse MKK7 (GenBank accession number AF026216). In addition, a Myc epitope tag (amino acids 410–419 of c-Myc; M) was fused to the amino terminus of the protein. **(b)** JNK–MKK7 phosphorylates c-Jun *in vitro*. NIH 3T3 cells were transfected by lipofection with pcDNA3 (Vector), pcDNA3-Myc-JNK-MKK7 (JNK–MKK7), or the pcDNA3-JNK and pCMV5-CotΔC expression plasmids (Jnk+Cot; the Cot (Tpl-2) kinase is an upstream activator of JNK proteins [2]) . The cells were lysed and proteins were subsequently immunoprecipitated (IP) with anti-Myc or anti-JNK1 antibodies. The kinase activity was analysed using *in vitro* complex kinase assays employing GST–c-Jun (amino acids 1–135) as a substrate. Autoradiographs of phosphorylated (P) proteins from kinase reactions separated by SDS–PAGE and blotted onto nitrocellulose membranes are shown. **(c)** JNK–MKK7 activity induces an electrophoretic mobility shift of c-Jun *in vivo*. NIH 3T3 cells were transfected with a c-Jun expression vector (pMT-35) alone or cotransfected (lipofection) with a JNK–MKK7 expression vector. Total cell lysates were separated using SDS–PAGE and analysed by immunoblotting with the anti-c-Jun antibody.

phosphatase treatment, demonstrating that the mobility shifts were due to phosphorylation of p150-Spir protein (Figure 3c). Supporting these data, a kinase-inactive mutant of JNK–MKK7 (containing the JNK mutations K55A and K56A and the MKK7 mutation K76E, called JNK–MKK7-KD) did not induce an electrophoretic mobility shift of p150-Spir (Figure 3b). To analyse the specificity of JNK phosphorylation of p150-Spir *in vivo*, we coexpressed p150-Spir with the constitutively active Erk2–Mek1-LA fusion protein [18]. Although expressed to the same levels as JNK–MKK7, Erk2–Mek1-LA induced only a very weak electrophoretic mobility shift of p150-Spir, indicating a preferential phosphorylation of p150-Spir by JNK (Figure 3b). All experiments were repeated at least three times with identical results.

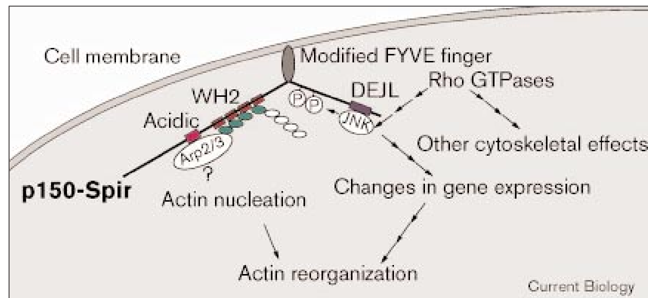
In conclusion, the JNKs are downstream effectors of the Rac and Cdc42 GTPases involved in actin reorganization [1,5]. Microinjection of activated Rac and Cdc42 proteins into cells induces changes in actin structures within minutes, before *de novo* protein synthesis [20,21]. Here we report the identification of a WH2 family protein p150-Spir, involved in actin reorganization, as a phosphorylation target of JNK activity. Strikingly, others have shown that amino-terminal sequences of Spir interact with Rho family GTPases in yeast two-hybrid experiments [11]. One possible mechanism of actin regulation by Rho

Figure 3



The p150-Spir protein is a phosphorylation target of JNK activity. **(a)** Activated JNK phosphorylates p150-Spir *in vitro*. Carboxy-terminal sequences of p150-Spir (amino acids 902–1020) containing the JNK binding domain (JBD) were fused to GST. GST and GST–p150-Spir(JBD) were purified from *E. coli* and analysed as phosphorylation substrates for JNK proteins that had been immunoprecipitated from NIH 3T3 cell extracts untreated or treated with arsenite. Autoradiographs of proteins from kinase reactions separated by SDS–PAGE and blotted onto nitrocellulose membranes are shown. **(b)** The p150-Spir protein is phosphorylated by JNK activity *in vivo*. NIH 3T3 cells were transfected by lipofection with either DNA vectors directing the expression of either the Myc-epitope-tagged p150-Spir alone or in combination with either JNK–MKK7, a kinase inactive mutant of JNK–MKK7 (JNK–MKK7-KD), or the constitutively active Erk2–Mek1-LA. Total protein lysates were analysed by immunoblot using anti-Myc antibodies. **(c)** The JNK–MKK7-induced electrophoretic mobility shift of p150-Spir is due to protein phosphorylation. Phosphatase treatment (+) of p150-Spir proteins immunoprecipitated from NIH 3T3 cell extracts cotransfected with plasmids encoding Myc–p150-Spir and Myc–JNK–MKK7 abolishes the electrophoretic mobility shift of p150-Spir induced by JNK–MKK7. An immunoblot developed with anti-Myc antibodies is shown.

Figure 4



A model of p150-Spir regulation and function. JNK binds to and phosphorylates p150-Spir. This may regulate the function of p150-Spir in actin reorganization. The modified FYVE zinc-finger structure may be involved in membrane localization of the protein. To initiate actin polymerization, an actin trimer must be formed. Therefore, the four WH2 domains by themselves could be sufficient to nucleate actin polymerization by bringing four actin monomers (small ovals) in contact to each other. Alternatively, the Arp2/3 protein complex, which induces actin nucleation and binds acidic regions adjacent to the WH2 domains of the WASP family proteins [8,23], may bind the acidic region of p150-Spir. In addition, JNK-induced target gene expression has also been shown to be required for cytoskeletal reorganization processes [6].

GTPases could therefore be the activation of a MAP kinase, which subsequently phosphorylates an actin regulator such as WAVE [22] or p150-Spir, thus regulating a role in either actin nucleation or the localization of actin regulating proteins (Figure 4). Future experiments will illuminate the cellular functions of p150-Spir and its regulation by JNK phosphorylation.

Supplementary material

Supplementary material including additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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