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Research Article

STI1 antagonizes cytoskeleton collapse mediated by small GTPase Rnd1 and regulates neurite growth

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ABSTRACT

Rnd proteins comprise a branch of the Rho family of small GTP-binding proteins, which have been implicated in rearrangements of the actin cytoskeleton and microtubule dynamics. Particularly in the nervous system, Rnd family proteins regulate neurite formation, dendrite development and axonal branching. A secreted form of the co-chaperone Stress-Inducible Protein 1 (STI1) has been described as a prion protein partner that is involved in several processes of the nervous system, such as neurite outgrowth, neuroprotection, astrocyte development, and the self-renewal of neural progenitor cells. We show that cytoplasmic STI1 directly interacts with the GTPase Rnd1. This interaction is specific for the Rnd1 member of the Rnd family. In the COS collapse assay, overexpression of STI1 prevents Rnd1–plexin-A1-mediated cytoskeleton retraction. In PC-12 cells, overexpression of STI1 enhances neurite outgrowth in cellular processes initially established by Rnd1. Therefore, we propose that STI1 participates in Rnd1-induced signal transduction pathways that are involved in the dynamics of the actin cytoskeleton.

Introduction

The small GTPase family of proteins comprises a group of molecules that switch between an active GTP-bound form and an inactive GDP-bound state to regulate their activity. Among the GTPases described, Cdc42, Rac1 and RhoA are the best characterized proteins, with roles in the regulation of cytoskeletal plasticity [13,8].

The Rnd family (Rnd1, Rnd2 and Rnd3) was first characterized as a different branch of the Rho GTPase family [29]. These proteins

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have low intrinsic GTPase activity and are constitutively active. The Rnd proteins are expressed in different tissues: Rnd1 was found in the liver, brain and human myometria during pregnancy, and Rnd2 expression is highest in the testis [8]. Rnd3 has a ubiquitously low expression level, which changes with diverse stimuli and conditions [36]. Rnd1 and Rnd3 proteins are associated with lipid rafts, and their interaction with the protein Syx prevents degradation [15,34]. The Rnd family has been implicated in rearrangements of the actin cytoskeleton and microtubule dynamics, leading to cell adhesion, migration, and invasion. During neural development, these GTPases affect neurite outgrowth and branching. Rnd1 has been implicated in process extension in PC-12 cells [1], the extension of axons [22], and in the elongation of dendritic spines and dendrite development in rat hippocampal neurons [18,19].

Morphological changes induced by Rnd1 are mediated by p190 RhoGAP through an antagonistic effect on RhoA [47]. Moreover, the microtubule regulator SCG10 binds Rnd1 and acts as a downstream effector on axonal extension [22]. Rnd1 is also involved in axonal guidance and cytoskeleton collapse by interacting with the cytoplasmic domains of semaphorin receptors [49,32,33,48]. Rnd1 interaction with plexin-B1 regulates RhoA activity by interacting with PDZ-RhoGEF, a Rho-specific-GEF in the Sema-4D pathway [14,32]. Furthermore, it was recently reported that Rnd1 regulates the collapse of COS-7 cells after plexin-B1, -B2 or -B3 stimulation by soluble Sema-4A [48].

Stress-Inducible Protein-1 (STI1) is a co-chaperone that associates directly with Hsp70/Hsp90 heat shock proteins and modulates their chaperone activities [30]. We have demonstrated that a secreted form of STI1 [23,16] binds to prion protein [50] and that this complex modulates processes such as neurite outgrowth [25,3], neuroprotection [9], astrocyte development [2], neuronal protein synthesis [37] and neural progenitor cells self-renewal [39]. Therefore, we propose that the secreted form of STI1 has neurotrophin-like activity [26].

STI1 is abundantly expressed in the cytoplasm and has been recently found to be associated with the actin cytoskeleton [46]; however, the function of this form of the protein in neuronal processes is unknown. Because Rnd proteins regulate cytoskeletal rearrangement, we decided to investigate a possible interaction between STI1 and the Rnd1 GTPase as an alternative way to regulate Rnd1 activity.

In the present study, we show that STI1 specifically binds Rnd1 and blocks morphological collapse induced by the interaction of Rnd1 and plexin-A1 in COS-7 cells. Moreover, STI1 and Rnd1 co-expression in PC-12 cells inhibits the formation of short new processes and enhances the establishment of longer neurites.

Experimental procedures

Production of polyclonal antibodies

The expression vector pQE-30 containing the coding sequence of human Rnd1 [22] was employed to produce His_6-Rnd1 protein in *Escherichia coli* M15 cells. Heterologous protein was purified on Ni-NTA beads under denaturing conditions, following the manufacturer's protocol (Qiagen). A specific polyclonal anti-Rnd1 antibody was raised by immunizing mice with recombinant Rnd1 (Fig. S1A and B). Mouse His_6-STI1 and GST proteins were purified from bacterial lysates, and polyclonal anti-STI1 antibody was raised in rabbit as previously described (Fig. S1C) [50]. All experiments involving animals were monitored by the Committee for Animal Experimentation in the Setor de Ciências Biológicas – Universidade Federal do Paraná.

GST-pull-down assay

GST pull-down assays were performed as previously described [38], with some modifications. Briefly, the glutathione S-transferase-tagged human Rnd1, murine Rnd2, RhoD, RhoG [38,49] or the pGEX4T2 empty vector were expressed in the BL21 (DE3) pLysS bacterial strain, and cells were suspended in ice-cold PBS, supplemented with 2 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 10% glycerol and complete protease inhibitor cocktail (Roche), and lysed in a French press. Bacterial extracts were cleared by centrifugation and stored at −80 °C until use. Whole adult mice brains were homogenized in TLB buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 1.5 mM $MgCl_2$, 4 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4) for 30 min on ice. Cleared extracts were incubated for 2 h at 4 °C with glutathione-Sepharose 4B beads. After 5–7 washes with TWB (0.1% Triton X-100 in TLB buffer), bound proteins were eluted from the beads by boiling in Laemmli buffer for 5 min at 100 °C. The pulled-down proteins were analyzed by immunoblotting with anti-STI1 and anti-GST antibodies.

Immunoprecipitation

Whole mice brain extracts were used for Rnd1 co-immunoprecipitation assays with anti-STI1. Brains were homogenized on cold lysis buffer (50 mM Tris–HCl pH 7.4, 0.2% sodium deoxycholate, 0.5% Triton X-100, plus complete protease inhibitor cocktail) for 30 min on ice and centrifuged for 15 min at 10,000*g* at 4 °C. Supernatants (500 μg) were pre-cleared with protein-A Sepharose (GE Healthcare) cross-linked to rabbit non-immune IgG for 1 h at room temperature. After incubation, cleared lysates were incubated with rabbit IgG anti-STI1 and rabbit non-immune IgG cross-linked to protein-A Sepharose for 2–3 h at 4 °C. Sepharose beads were washed with lysis buffer, and bound proteins were analyzed as described above. Antibodies were cross-linked to Sepharose beads employing disuccinimidyl suberate-chemistry, as recommended by the manufacturer (Pierce).

Binding assays

Binding assays were performed as previously described [10]. Briefly, 1 μg of recombinant GST–Rnd1, GST and BSA were immobilized on 96-well polystyrene plates (Maxisorb, Nunc) at 4 °C for 16 h. Non-specific binding sites were blocked with 1% BSA for 2 h at room temperature. Increased amounts of His_6-STI1 (0.1 μg, 0.2 μg, 0.5 μg and 1 μg) were added. The reactions proceeded for 16 h at 4 °C followed by extensive washes with PBS. Bound His_6-STI1 was detected with anti-STI1 (1:3000) and anti-rabbit HRP conjugated antibody (BD Bioscience), followed by reaction with o-phenylenediamine/H_2O_2 substrate. Absorbance was measured at 490 nm.