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ARHGAP30 is a Wrch-1-interacting protein involved in actin dynamics and cell adhesion

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ABSTRACT

The atypical Rho GTPase Wrch-1 has been proposed roles in cell migration, focal adhesion dissolution, stress fibre break down and tight junction heterogeneity. A screen for Wrch-1 binding-partners identified the novel RhoGAP protein, ARHGAP30, as a Wrch-1 interactor. ARHGAP30 is related to the Cdc42- and Rac1-specific RhoGAP CdGAP, which was likewise found to bind Wrch-1. In contrast to CdGAP, ARHGAP30 serves as a Rac1- and RhoA-specific RhoGAP. Ectopic expression of ARHGAP30 results in membrane blebbing and dissolution of stress-fibres and focal adhesions. Our data suggest roles for ARHGAP30 and CdGAP in regulation of cell adhesion downstream of Wrch-1.

1. Introduction

Rho GTPases are pivotal regulatory units in signal transduction pathways that regulate cell morphogenesis, cell adhesion, cell proliferation and cell migration [1]. The atypical Rho GTPase Wrch-1 (also known as RhoU) was originally identified in a screen for genes upregulated in response to Wnt-1 in mouse mammary cells, hence the name Wnt responsive Cdc42 homologue (Wrch-1) [2]. It was proposed that Wrch-1 link Wnt-1 to downstream responses, such as cell transformation and gene transcription [2]. Wrch-1 is functionally and structurally distinct from other members of the Rho GTPases since it contains a proline-rich N-terminal extension, which has been found to bind SH3 domain containing proteins such as Nck and Grb2 [3,4]. Although it harbours a measurable GTP-hydrolysis activity, the high intrinsic nucleotide exchange activity overrules this enzymatic process and result in a constitutively active, GTP-bound, status of Wrch-1 [3–5]. This property implicates that the activity of the protein must be regulated by other factors than the canonical Rho GEFs and RhoGAPs; however how this regulation is achieved is currently not known. Moreover, Wrch-1 and the closely related Chp (RhoV) do not harbour any functional CAAX boxes. Instead, membrane targeting of the proteins is conferred by palmitoylation [6,7].

Ectopic expression of Wrch-1 in fibroblasts result in formation of filopodia associated with loss of focal adhesion and stress fibres [2–4,8]. The latter phenotype can be seen as a rounding up of the cells, a phenomenon which, in this case, is not associated with increased apoptosis [3]. The formation of Wrch-1 filopodia occurs in a Cdc42- and Rif-independent manner and requires the direct interaction to the non-receptor tyrosine kinase Pyk but, importantly, the Wrch-1:Pyk2 interaction is regulated by Src [10]. Wrch-1 is also involved in the regulation of cell:cell contacts in a PAR6-dependent manner [9]. Moreover, the protein is working as transforming agent in common *in vitro* assays for cell transformation, such as growth in soft agar [4,7]. We sought to study the biological functions of Wrch-1 by identifying novel binding partners for the protein. Employing a yeast two hybrid cDNA library from EBV-transformed human B-cells, we identified ARHGAP30, a previously uncharacterized RhoGAP domain-containing protein, as a candidate Wrch-1-binding protein. ARHGAP30 is closely related to the Cdc42-specific RhoGAP CdGAP [11], and together they form a subgroup of the RhoGAP proteins. The two proteins differ in tissue expression and they also differ in their affinity for the different members of the Rho GTPases. We show that Wrch-1 binds ARHGAP30 and CdGAP in co-immunoprecipitation assays. Furthermore, we show that the proteins have roles downstream of Wrch-1 in cytoskeletal regulation and dissolution of focal adhesions.

2. Materials and methods

2.1. Isolation and cloning of ARHGAP30

The yeast two-hybrid screen employed to identify ARHGAP30 as a binding partner for the constitutively active mutant of Wrch-1 (Wrch-1/Q107L) has been described before [10]. EST clones

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encoding the human ARHGAP30 were obtained from Geneservice Ltd., Cambridge, UK. Two clones were used for further characterisation: BI906570, which encoded the full-length S-ARHGAP30 and BM464735, which encoded the C-terminal portion of L-ARHGAP30, including the glutamic acid-rich segment. In order to obtain a full-length L-ARHGAP30, the N-terminal fragment of S-ARHGAP30 was ligated to the C-terminal fragment of L-ARHGAP30. The cDNAs were subsequently subcloned into the mammalian expression vectors pRK5Myc and pRK5FLAG. pRK5Myc–CdGAP was a generous gift from N. Lamarche-Vane, Montreal, Canada. The vectors expressing the constitutively active Rho GTPases have been described before [10]. Nucleotide sequencing was done on an ABI Prism 310 Genetic Analyzer.

2.2. Reagents and antibodies

Mouse monoclonal anti-Myc (9E10) antibodies were from Covance; rabbit polyclonal anti-Myc and mouse monoclonal anti-phospho-tyrosine (PY99) antibodies were from Santa Cruz Biotechnology; mouse monoclonal anti-FLAG (M2) and mouse monoclonal anti-α-tubulin antibodies were from Sigma–Aldrich; tetramethyl rhodamine isothyocyanate (TRITC)- and aminomethylcoumarin acetate (AMCA)-conjugated antibodies were from Jackson ImmunoResearch; AlexaFluor488-conjugated antibodies and AlexaFluor488- and AlexaFluor350-coupled phalloidin were from Invitrogen (Molecular Probes); TRITC-labelled phalloidin was from Sigma–Aldrich. Rabbit polyclonal anti-CdGAP was a generous gift from N. Lamarche-Vane, Montreal, Canada.

2.3. Cell cultivation, transfection and immunoprecipitation procedures

Maintenance of HEK293T cells, normal mouse embryonic fibroblasts (MEFs) and porcine aortic endothelial cells stably expressing the human platelet-derived growth factor receptor β (PAE/PDGFRβ cells) was carried out as described before [10]. The transfection and staining procedures has been described before [10]. Microscopy analysis was performed on a Zeiss Axiovert 40 CFL equipped with an AxioCam MRm digital camera employing the AxioVision software.

2.4. Purification of recombinant proteins and GAP assay

A fragment encoding the GAP domain (amino-acid residues 1–215) of ARHGAP30 was subcloned in the pGEX2T vector. Recombinant GST-fusion proteins were produced in *Escherichia coli* and purified as described before [12]. The GAP assay followed the procedure by Self and Hall [13]; briefly, recombinant Rho GTPases were preloaded with [γ^{32}P]-GTP in the presence of equimolar amounts of GST-ARHGAP30-GAPdomain. Hydrolysis activity was determined by taking aliquots from the incubation mixtures intervals as described in Fig. 3. Samples were collected on nitrocellulose filters and washed and the filters were subjected to scintillation counting.

3. Results and discussion

We performed a yeast two hybrid screen to identify binding partners for the constitutively active mutant of Wrch1 (Wrch1/Q107L). Yeast, *Saccharomyces cerevisiae*, cells expressing Wrch1/Q107L fused to the GAL4 DNA binding-domain was transformed with a cDNA library derived from EBV transformed human B-cells fused to the GAL4 activation domain [10]. Clones expressing candidate Wrch1/Q107L-interacting proteins were recovered and subjected to sequence analysis. One set of the clones encoded a non-characterised RhoGAP domain-containing protein, named ARHGAP30. The gene appears to encode two major splice-variants which we called L-ARHGAP30 and S-ARHGAP30 for the long and short splice form, respectively (Fig. 1A). The only difference between the two is the absence of a glutamic acid-rich stretch of amino acid residues in S-ARHGAP30. ARHGAP30 is a paralogue to the ubiquitously expressed RhoGAP domain-containing protein CdGAP [11]. *In silico* analysis of the UniGene data-bank indicate that ARHGAP30 has a relative restricted expression pattern. It is expressed in different cells of haematopoietic origin but also in endocrine cells, connective tissue, muscle, and lung cells.

We first confirmed the yeast two hybrid results by coimmunoprecipitation analysis. To this end, FLAG-tagged S-ARHGAP30 was cotransfected with mutants of Wrch-1 in HEK293T cells. The cells were lysed and the presence of S-ARHGAP30 in the Wrch-1 precipitates was determined by immunoblotting. S-ARHGAP30 bound the wild-type and constitutively GTP-bound mutant Wrch-1 (Wrch-1/Q107L) with equal affinity. The Wrch-1/T63N mutant, corresponding to the GDP-bound, inactive conformation of Wrch-1, also bound S-ARHGAP30, indicating that the interaction is not strictly GTP-dependent (Fig. 1B). In contrast, a mutant Wrch-1 lacking the N-terminal extension was unable to bind S-ARHGAP30 indicating the binding requires an intact GTP-bound Wrch-1 protein. We also tested the binding-ability between ARHGAP30 and a panel of Wrch-1 constructs harbouring point mutations in its effector loop. We previously noticed significant differences in the binding specificity between these Wrch-1 mutants and a collection of Wrch-1-binding proteins. PAK1 binds only to Wrch-1/F83G and not to Wrch-1/P80G and Wrch-1/F86C [10]. Nck binds to all three Wrch-1 mutants and PYK2 does not bind to any of them [10]. Interestingly, ARHGAP had an opposite binding pattern compared to PAK1 since it bound to P80G and the F86C mutant of Wrch-1, but not to F83G mutant (Fig. 1B). These observations suggest that these binding partners serve different roles downstream of Wrch-1.

In order to identify the Wrch-1-binding domain on ARHGAP30, deletion mutants of ARHGAP30 were cotransfected with Wrch-1. We found that Wrch-1 bound to the C-terminal part of ARHGAP30 but not to the GAP domain, indicating that ARHGAP30 is unlikely to be a GAP for Wrch-1 (Fig. 1C). Wrch-1 has been shown to localise to vesicular structures in mouse fibroblasts, which was also found in PAE/PDGFRβ cells (Fig. 1D) [3]. We transfected FLAG-tagged S-ARHGAP30 together with Myc-tagged Wrch-1 into PAE/PDGFRβ cells and observed that ARHGAP30 localised in a vesicular pattern in the cytoplasm and the vesicles often coincided with Wrch-1 (Fig. 1E). In contrast, the Wrch-1/F83G-positive vesicles rarely coincided with ARHGAP30 (Fig. 1E).

We have previously found that ectopic expression of Wrch-1 in fibroblasts is associated with filopodia formation, stress fibre breakdown and loss of cell adhesion [3,5,10]. We reasoned that, provided ARHGAP30 serves as a downstream effector of Wrch-1, it should be able to induce a Wrch-1-like cellular response [3,10]. To test this hypothesis, ARHGAP30 was ectopically expressed in PAE/PDGFRβ cells. The subcellular localisation of ARHGAP30 was determined with an antibody against the Myc epitope and the effect on the actin organisation was assayed by staining the cells for filamentous actin with fluorescently labelled phalloidin. S-ARHGAP30 expression resulted in stress fibre dissolution and a loss of cell attachment. These effects were also associated by a vigorous membrane blebbing (Fig. 2A, quantified in Fig. 2B). L-ARHGAP induced a similar phenotype in PAE/PDGFRβ cells (Fig. 2B). The deletion mutants of ARHGAP30 lacking the GAP domain or the C-terminal domains did not induce any significant membrane blebbing or up-rounded phenotype indicating that the full-length protein is required for this phenotype (Fig. 2B). We also analysed the effect of ectopic expression of ARHGAP30 on focal adhesion dissolution and noticed a more or less complete penetrance of this