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APPL1 endocytic adaptor as a fine tuner of Dvl2-induced transcription

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ABSTRACT

APPL1 is a multifunctional endocytic adaptor which acts at different steps of various signaling pathways. Here we report that APPL1 interacts with Dvl2, a protein known to activate the canonical and non-canonical Wnt pathways. APPL1 synergizes with Dvl2 and potentiates transcription driven by AP-1 transcription factors, specifically by c-Jun, in non-canonical Wnt signaling. This function of APPL1 requires its endosomal recruitment. Overproduction of APPL1 increases Dvl2-mediated expression of AP-1 target gene encoding metalloproteinase 1 (*MMP1*) in a JNK-dependent manner. Collectively, we propose a novel role of APPL1 as a positive regulator of Dvl2-dependent transcriptional activity of AP-1.

1. Introduction

It is commonly accepted that endocytic proteins can regulate cellular signaling at different levels, including transcription by modulating the activity of nuclear factors [1,2]. APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif 1) and its less studied homolog APPL2 are examples of endocytic proteins able to regulate intracellular signaling. APPL1 localizes to a subpopulation of early endosomes (termed APPL endosomes) due to interactions with Rab5 and Rab21 GTPases [3,4]. However, this protein resides also in the cytoplasm and shuttles to the nucleus partially due to interactions with the NuRD complex [3,5]. APPL1 binds several transmembrane receptors and kinases involved in signal transduction [6–9]. Recently, we discovered that APPL1 can induce nuclear accumulation of RelA leading to expression of NF-κB target genes [10]. We also showed that APPL1 and APPL2 modulate transcription of β-catenin/TCF target genes in canonical Wnt signaling via interactions with histone deacetylases and reptin [11].

Dishevelled (Dvl) adaptor proteins act at early stages of Wnt signaling thanks to interactions with multiple partners [12]. Both the canonical (β-catenin-dependent) and non-canonical Wnt pathways are activated by overexpression of Dvl. These primarily cytoplasmic proteins can also translocate to the nucleus [13,14]. Upon overexpression they form cytosolic puncta of unclear nature, postulated to represent either protein assemblies [15] or endocytic structures in agreement with the proposed crucial role of Dvl in Frizzled receptor internalization [16,17].

Dvl proteins regulate the canonical Wnt pathway at, at least, two steps: (i) ligand-induced phosphorylation of LRP5/6 receptors and (ii) phosphorylation-dependent degradation of β-catenin. Initially, Dvl-mediated recruitment of Axin-bound GSK3β and/or CKI kinases stimulates phosphorylation of LRP by these kinases at the plasma membrane [18,19]. Consequentially, Dvl-mediated titration of Axin away from the β-catenin destruction complex compromises phosphorylation of β-catenin, thus preventing its subsequent degradation [20].

Planar cell polarity (PCP) signaling, one of the non-canonical Wnt pathways, is also regulated by Dvl proteins. Dvl2 activates a small GTPase Rac which in turn stimulates the c-Jun N-terminal kinase (JNK), a potent regulator of the PCP pathway [21,22]. This mechanism operates during dendrite growth [13] and cell

Abbreviations: AP-1, activating protein 1; APPL, adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif; CKI, casein kinase I; Dvl, Dishevelled; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; LRP, low-density lipoprotein receptor-related protein; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; NuRD, nucleosome remodeling deacetylase; PCP, planar cell polarity; PDZ, post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1); PH, pleckstrin homology; PTB, phosphotyrosine binding; TCF, T-cell factor

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movements in embryogenesis [23]. Moreover, Dvl proteins bind to and activate Daam1 [24] which induces ROCK kinase responsible for cytoskeleton remodeling [25].

The JNK substrate c-Jun belongs to the family of activating protein 1 (AP-1) transcription factors. They act as dimers of basic leucine zipper (bZIP) domain containing mainly proteins which belong to the subfamilies of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra1 and Fra2) (reviewed by [26]). AP-1 homo- and heterodimers bind DNA at the heptamer consensus sequence known as the TPA-responsive element (TRE) [27]. A large number of AP-1 proteins provide broad combinatorial possibilities of dimers with different binding specificities to tightly regulate expression of numerous genes [28]. It was demonstrated that c-Jun exhibits transcriptional activity when phosphorylated at Ser63 and Ser73 by the JNK kinase [29]. Moreover, one of the c-Jun transcriptional targets is c-Jun itself since its promoter region contains a consensus sequence for AP-1 binding [30]. Probably, this positive autoregulation loop constitutes a mechanism for prolonging the signals from extracellular stimuli.

Here, we report that APPL1 interacts and synergizes with Dvl2 to regulate AP-1-dependent transcription in non-canonical Wnt signaling.

2. Materials and methods

2.1. Cell lines and transfections

HEK293T, Hela, Wnt3a-expressing mouse L cells (ATCC; CRL-2647) and parental L cells (CRL-2648) were maintained in DMEM with 10% fetal bovine serum, 2 mM L-glutamine. The conditioned media from L cells were obtained as recommended by the supplier.

siRNA (30 nM) was delivered to HEK293T cells using HiPerFect reagent (Qiagen), plasmids were delivered with Lipofectamine 2000 (Invitrogen) according to manufacturers' instructions. For immunoprecipitation assay HEK293T cells were transfected in 6-well plate with 2 μg of each plasmid and harvested 24 or 48 h after transfection. For RT-PCR experiments HEK293T cells were transfected in 12-well plate with 1.5 μg of each plasmid and harvested 24 h after transfection. For luciferase assay HEK293T cells were transfected for 24 h in 96-well plate with 50 ng of reporter (pAP-1-luc) plus 25 ng of normalization construct (pRL-TK) along with different amounts of APPL1- or Dvl2-encoding plasmids, or with siRNA for 48 h and then with plasmid DNA for the next 24 h. For microscopic analysis Hela cells were transfected in 24-well plates with 0.5 μg of Dvl2-encoding plasmid using FuGene (Roche) reagent and fixed 24 h after transfection.

2.2. siRNA, plasmids

ON-TARGETplus SMARTpool siRNA targeting human APPL1 (Dharmacon, cat # L-005138-00-0005) and non-targeting control SMARTpool (cat# D-001810-10-20) were employed at 30 nM concentration.

The following plasmids were used: pCMV-Dvl2 (kind gift from Prof. Jacek Otlewski, University of Wroclaw); pcDNA3-based constructs of untagged and N-terminally myc-tagged APPL1 and APPL2, pEYFP-C3-APPL1ΔC4, pcDNA3-MycHisB-APPL1ΔC4, pcDNA3-MycHisB-APPL1 K280E/Y283C/G319R, pcDNA3-MycHisB-APPL1-A318D, pcDNA3-MycHisB-APPL1-G320A [3,4]; pRL-TK (kind gift from Dr. Vladimir Korinek, Institute of Molecular Genetics, Prague), pAP-1-luc (Stratagene), A-Fos construct was provided by Dr. Charles Vinson (Addgene plasmid #33353) [31]. pGEX-6P-3/APPL1-N (amino acids 1–428), pGEX-6P-3/APPL1-C (amino acids 429–709), pGEX-6P-3/APPL2-N (amino acids 1–377), pGEX-6P-3/APPL2-C (amino acids 378–664) were previously described [3,11].

2.3. Antibodies

Rabbit polyclonal anti-APPL2 (Ab5002) and anti-APPL2 (Ab4567) antibodies were previously described [11]. Anti-Dvl2 (sc-8026), anti-phospho-c-Jun Ser63/73 (sc-16312), anti-c-Jun (sc-45), anti-GAPDH (sc-25778), anti-lamin A/C (sc-7292) and anti-β-catenin (sc7199) were from Santa Cruz Biotechnology; anti-Myc tag (05-419) and anti-Axin1 (2087) from Cell Signaling; anti-β-catenin (610154) from BD Transduction Laboratories and anti-GSK3β (44-610) from Invitrogen.

The following secondary antibodies were used: horseradish peroxidase-conjugated (Jackson ImmunoResearch), fluorophore-conjugated (IRDye 680 and IRDye 800CW; LI-COR Biosciences), AlexaFluor®488-conjugated anti-mouse and AlexaFluor®568-conjugated anti-rabbit antibodies (Invitrogen).

2.4. Western blotting

HEK293T cells were lysed in RIPA buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, protease inhibitor cocktail (6 μg/ml chymostatin, 0.5 μg/ml leupeptin, 10 μg/ml antipain, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin A and 10 μg/ml 4-amidinophenylmethanesulfonyl fluoride hydrochloride; Sigma–Aldrich) and phosphatase inhibitor cocktails (Sigma–Aldrich, cat# P0044, P5726). Protein concentration was measured with BCA Protein Assay Kit (Thermo Scientific). Samples of 10–50 μg total protein were subjected to SDS–PAGE. Resolved proteins were transferred to nitrocellulose membrane (Whatman), probed with specific antibodies, and detected with enhanced chemiluminescence or Odyssey infrared imaging system (LI-COR Biosciences). Quantitative analysis of bands detected on LI-COR Odyssey platform was performed with Image Studio Software (LI-COR Biotechnology).

2.5. Immunoprecipitation and GST pull-down

Immunoprecipitation assay was performed as described previously [5].

GST, GST–APPL1-N (comprising 428 amino acids from the N-terminus) and GST–APPL1-C (comprising amino acids 429–709), GST–APPL2-N (comprising 377 amino acids from the N-terminus) and GST–APPL2-C (comprising amino acids 378–664) fusion proteins used in pull-down assays as baits were expressed and purified according to the manufacturer's instructions (GE Healthcare). Isopropyl β-D-thiogalactoside (Sigma–Aldrich) at a concentration of 0.5 mM was used to induce the expression. The lysates of Dvl2 overexpressing cells were incubated overnight at 4 °C with constant rotation with equal amounts of glutathione–Sepharose beads (GE Healthcare) complexed with GST, GST–APPL1-N, GST–APPL1-C, GST–APPL2-N or GST–APPL2-C fusion proteins. Beads were washed 5 times with the wash buffer used for immunoprecipitation. GST-fusion proteins together with bound proteins were eluted with 10 mM glutathione in 50 mM Tris/HCl, pH 8.0, for 15 min at room temperature with shaking. Eluates were resuspended in Laemmli buffer, subjected to 10% SDS–PAGE and immunoblotted for the proteins of interest.

2.6. Luciferase assays

HEK293T cells at 50–60% confluence were transiently transfected with the appropriate plasmids and/or siRNA, and lysed with passive lysis buffer (Promega). The following amounts of transfected plasmids were used in Fig. 2A and B: Dvl2 – 12.5 ng/well, APPL1 – 12.5 ng/well, A-Fos – 25 ng/well; in Fig. 2C: Dvl2 – 6 ng/well, APPL1 – 10 ng/well, APPL1mut1 – 37.5 ng/well, APPL1mut2 – 37.5 ng/well, APPL1mut3 – 75 ng/well (to obtain comparable