Cellular Signalling 20 (2008) 1471–1477

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Sprouty 2 disturbs FGFR3 degradation in thanatophoric dysplasia type II: A severe form of human achondroplasia

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ARTICLE INFO

Article history:
Received 3 August 2007
Received in revised form 20 March 2008
Accepted 1 April 2008
Available online 10 April 2008

Keywords:
Thanatophoric dysplasia
Achondroplasia
FGFR3
Sprouty
RTK
c-Cbl
Receptor degradation

ABSTRACT

Thanatophoric dysplasia is a member of the achondroplasia family of human skeletal dysplasias, which result from FGFR3 mutations that exaggerate this receptor's inhibitory influence on chondrocyte proliferation and differentiation in the skeletal growth plate. We have previously reported that defective lysosomal degradation of activated receptor contributes to the gain-of-function of the mutant FGFR3. We now provide evidence that this disturbance is mediated by the receptor's kinase activity and involves constitutive induction and activation of Spry2. Our findings suggest that activated Spry2 may interfere with c-Cbl-mediated ubiquitination of FGFR3 by sequestering c-Cbl. They provide novel insight into the pathogenesis of this group of human skeletal dysplasias and identify a mechanism that potentially could be targeted therapeutically.

1. Introduction

Achondroplasia (ACH) is the prototype of a group of human chondrodysplasias that range in severity from lethal thanatophoric dysplasia types I and II (TDI and TDII) to relatively mild hypochondroplasia [1,2]. These disorders result from dominantly inherited mutations of fibroblast growth factor receptor 3 (FGFR3), a transmembrane receptor tyrosine kinase (RTK) that serves as a major negative regulator of linear bone growth. The mutations are gain-of-function, i.e., activating mutations associated with increased tyrosine kinase activity and augmentation of FGFR3 signals [3–5]. These inhibitory signals are propagated through STAT, MAP kinase and other downstream pathways to diminish proliferation and terminal differentiation of growth plate chondrocytes [6–9].

Several mutation-specific mechanisms have been proposed to explain how different disease-related mutations lead to gain of receptor function. They include stabilization and induction of FGFR3 dimerization, which is necessary for receptor activation, by the transmembrane and cysteine substitution mutations of ACH and TDI, respectively, and kinase domain mutations of TDII that constitutively activate the receptor's intrinsic kinase activity [4,10,11]. We have recently uncovered a mechanism that may be common to ACH, TD and other activating FGFR3 mutations. Our findings suggest that mutant receptors are degraded more slowly than their wild type (WT) counterparts allowing for accumulation of actively signaling receptors and an increase in overall FGFR3 signal strength [12]. Our results point to a disturbance in c-Cbl-mediated ubiquitination of the mutant receptors as the underlying defect. Normally, receptor ubiquitination serves as a targeting signal for trafficking activated RTKs to lysosomes for degradation; consequently, defective ubiquitination slows turnover [13]. Similar disturbances have been reported in tumors due to accumulation of mitogenic RTKs [14–16].

The RTK trafficking defects in some cancers reflect somatic, loss of function mutations of c-Cbl or mutations that disrupt binding of c-Cbl to its RTK substrates, both of which impair receptor ubiquitination [14–16]. Neither of these mechanisms seems likely in the ACH group of disorders in which mutations in different regions of FGFR3 lead to

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doi:10.1016/j.cellsig.2008.04.001

the ubiquitination disturbance. More plausible is that the ubiquitination defect shared by the different FGFR3 mutations reflects the increased tyrosine kinase activity that they share [1,3]. Indeed, constitutive kinase activation has been proposed to influence maturation and trafficking of RTKs, including FGFR3 [17,18]. Also consistent with this notion is that the disturbance we previously observed in FGFR3 ubiquitination correlates with the extent of the increase in kinase activity associated with the specific mutation, i.e., moderate increase in both kinase activity and ubiquitination defect in ACH and marked increase in both kinase activity and ubiquitination defect in TD [3,12].

In this paper, we examine the possibility that accumulation of mutant FGFR3 is mediated by excessive kinase activity acting through induction and constitutive activation of Sprouty 2 (Spry2). Originally discovered in *Drosophila*, four Sprys have been identified in mammals [19,20]. They are classically induced by and antagonize MAP kinase signaling in response to several growth factors including FGF, EGF (epidermal growth factor), VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor) and SCF (stem cell factor/kit ligand) [20–24]. Spry2 has been implicated as an FGF antagonist during lung morphogenesis and limb development and outgrowth [25–27]. Its postnatal function is less well defined, but it expressed in many adult tissues [27].

In contrast to its better known role as an inducible RTK inhibitor, Spry2 has been shown to promote RTK signaling in certain contexts through its ability to bind and sequester c-Cbl [20,28–35]. Our observations based on comparison of WT to constitutively active TDII mutant FGFR3 in cultured cells, suggest that this alternate mechanism involving Spry2 sequestration of c-Cbl may be relevant to the pathogenesis of TDII and potentially other members of the ACH group of disorders.

2. Materials and methods

2.1. Cell lines and tissues

Cell culture experiments were carried out in both COS-7 and TRex-293 (Invitrogen) cells maintained in DMEM supplemented with 10% FCS in a humidified incubator with 5% CO_2 at 37 °C. COS-7 cells stably expressing WT or TDII FGFR3-GFP were prepared by retroviral transduction under conditions designed to yield one copy of vector per infected cell as reported previously [12]. COS-7 cells stably expressing WT kinase dead (kd) or TDII kd FGFR3-GFP were prepared by lentiviral transduction using the pLenti-6 system (Invitrogen) and under conditions designed to yield 1-copy of vector per infected cell, per manufacturer's recommendation. Flp-In™ TRex-293 cell line (Invitrogen) expressing the Tet repressor and containing a single integrated Flp Recombination Target (FRT) site was used to generate tetracycline-inducible cell lines expressing GFP-tagged WT, WT kd, TDII, and TDII kd FGFR3 as follows: GFP-tagged receptors in the pcDNA5/FRT/TO cassette were co-transfected with pOG44, containing the Flp-recombinase at a 1:9 molar ratio using Lipofectamine 2000 (Invitrogen). Recombined cells were selected for hygromycin resistance and confirmed by western blot analysis.

Proximal tibial growth plate tissues were prepared from 1 week-old mice homozygous for a K644E knock-in allele corresponding to K650E TDII mutation in humans [36]. The mouse protocol was approved by the Institutional Animal Care and Use Committee. Growth plate tissues were isolated from a 17 week gestation human fetus with clinical and radiographic findings typical of TDII and a normal control human fetus 18 weeks gestation. DNA sequencing confirmed that the affected fetus had characteristic heterozygous K650E FGFR3 mutation. The human fetal tissues were obtained through the International Skeletal Dysplasia Registry under a protocol approved by Cedars-Sinai Research Institute Institutional Review Board (DK).

2.2. Reagents and antibodies

FGF1 (gift from M. Mohammadi) and FGF2 (Calbiochem) were used at final concentrations of 10 ng/ml and 25 ng/ml respectively in the presence of heparin (10 U/ml). We observed comparable responses to FGF1 and FGF2 at these doses as assayed by Erk phosphorylation. Bafilomycin (LC Laboratories, Woburn, MA) was used at 1 μM and MG132 (Calbiochem) was used at 30 μM. Anti-HA and anti-c-Cbl (C15) antibodies were obtained from Santa Cruz Biotechnology. 4G10 anti-phosphotyrosine antibody was a gift from Brian Druker, Oregon Health & Science University. Antibodies to Erk1/2 and pErk1/2 were purchased from Cell Signaling Technology, anti-GFP (Immunocytochemistry) from Abcam, anti-GFP (Western blotting) and anti-V5 from Invitrogen and anti-actin and anti-α tubulin from Sigma. Antibody to mouse Spry2 was a gift from Gerhard Christofori, University of Basel [21]; it was also purchased from Sigma (S-1444). Alexa dye conjugated secondary antibodies were purchased from Molecular Probes and HRP conjugates from Amersham.

2.3. Plasmids and transfections

The mouse FGFR3 vectors used for transient transfection were generated by excising the receptor coding region from the corresponding EGFP vectors [12] with HindIII and BamH1 and subcloning into pcDNA6/V5-His (Invitrogen). The K502A mutation that abolishes the receptor's tyrosine kinase activity was introduced using QuickChange site-directed mutagenesis (Stratagene) to generate WT kinase dead (kd) FGFR3-V5-His and TDII kd FGFR3-V5-His [37]. Expression vectors for HA hSpry2 HA hSpry2 Y55F and c-Cbl and 70z-Cbl were gifts from Dafna Bar-Sagi and Brian Druker respectively, and have been previously described [12,33]. Mouse c-Cbl was amplified from pcDNA3.1(−) and cloned into dsRed2-N1 using KpnI and AgeI restriction sites following the manufacturer's recommendation (Clontech). HA-ubiquitin (HA-Ub) was a gift from Dirk Bohmann, University of Rochester [38]. To generate the inducible TRex-293 cell lines, GFP-tagged receptors from the EGFP vector [2] were excised using HindIII and Not1 and directly cloned into pcDNA5/FRT/TO. Transient transfection of COS-7 cells stably expressing FGFR3-GFP was performed using Lipofectamine 2000 (Invitrogen) or Fugene (Roche), following the manufacturer's recommended protocols. Assays were performed 30–48 h later, with or without serum starvation and following FGF1 or FGF2 stimulation. For inhibitor studies cells were serum starved 4 h prior to addition of inhibitor; inhibitor was added and 1 h later cells were stimulated with growth factor. Lysates were harvested 6 h later and receptor levels were evaluated by western blot analysis. For Spry2 induction, TRex-293 cells containing WT and TDII FGFR3-GFP were induced overnight using 2 μg/ml tetracycline. Cells were then serum starved before stimulating with 10 ng/ml FGF1 and 10 U/ml heparin for the indicated times.

2.4. Quantitative RT-PCR

TRIzol reagent for total RNA purification and the SuperScript Choice System (Invitrogen) for cDNA synthesis were used according to the manufacturers' instructions. The expression of Spry2 relative to GAPDH was determined by qRT-PCR using the ABI PRISM 7700 system (Applied Biosystems) with the following primers: sense primer for Spry2 (GGCC-TACTGTCGTCCCAAGAC); antisense primer for Spry2 (AGTGCTGGAGCC-TAGGAGGC); sense primer for GAPDH (CATGTTCGTCATGGGTGTGAAC); and antisense primer for GAPDH: (GTTGTCATGGATGACCTTGGC). Reactions utilized SYBR Green PCR Master Mix reagent (Applied Biosystems) according to the manufacturer's protocol. The Comparative Ct method was applied following the manufacturer's instruction to measure fold-change differences of Spry2 under each experimental condition.

2.5. Western blot and immunoprecipitation

Cells were washed twice with cold PBS and solubilized in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1 mM Na orthovanadate, and protease inhibitor cocktail (Roche). Human growth plate tissues were extracted on ice using a glass-teflon homogenizer. Lysis buffer extracts were clarified by centrifugation (4 °C, 14,000 rpm, 5 min) and protein measured using the Dc Protein Assay (BioRad). Samples were fractionated by SDS-PAGE, transferred to PVDF membranes and subjected to HRP-based Western blot analysis. Chemiluminesence was detected using the SuperSignal West Pico substrate following the manufacturer's instructions (Pierce). For immunoprecipitation, equivalent cell lysates were pre-cleared with 1 μg of rabbit IgG and incubated with antibodies to GFP (FGFR3-GFP), HA (HA-hSpry2, HA-Ub) or c-Cbl. Immunoprecipitates were collected with Protein G PLUS Agarose (Santa Cruz Biotechnology) and solubilized by boiling in gel sample buffer prior to Western blot analysis.

2.6. Half-life

COS-7 cells (1×10^5 cells per well, 12-well dish) were transiently transfected with Fugene following manufacturer's instructions. Forty hours post-transfection cells were washed twice in Cys/Met-free DMEM and incubated in Cys/Met-free DMEM containing 10% dialyzed serum for 30 min. Cells were then pulsed for 30 min in 150 uCi/ml Tran ^{35}S-Label (MP Biomedicals) in Cys/Met-free DMEM containing 10% dialyzed serum, rinsed three times and chased for indicated times in DMEM containing 10% serum, 100 μg/ml cold methionine and 500 μg/ml cold cysteine. Cells were rinsed in PBS, lysed in ice cold lysis buffer for 20 min at 4 °C, transferred to a 1.5 ml tube and cleared by centrifugation. Cleared lysates were immunoprecipitated overnight with 0.56 μg of anti-V5 antibody (Invitrogen) and 25 μl Protein G PLUS Agarose (Santa Cruz). Samples were run out on a 4–12% Novex Gel (Invitrogen), gel dehydrated, and ^{35}S signals were quantified using a STORM 860 Phosphoimager (GE Healthcare). Background was subtracted and signals normalized to total protein. Numbers were plotted for 0.5 h to 7 h time point as percent total receptor versus time, and the best-fit exponential line ($R^2\geq96$) was plotted using EXCEL. Half-life corresponds to the time when each line crossed 50% receptor levels.

2.7. Microscopy

Partially dissected knee joints from 1 week-old WT mice and mice homozygous for the K644E FGFR3 knock-in mutation were fixed in 4% paraformaldehyde and embedded