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The Sumo protease Senp7 is required for proper neuronal differentiation

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ABSTRACT

Covalent attachment of the Small ubiquitin-like modifier (Sumo) polypeptide to proteins regulates many processes in the eukaryotic cell. In the nervous system, Sumo has been associated with the synapsis and with neurodegenerative diseases. However, its involvement in regulating neuronal differentiation remains largely unknown. Here we show that net Sumo deconjugation is observed during neurogenesis and that Sumo overexpression impairs this process. In an attempt to shed light on the underlying mechanisms, we have analyzed the expression profile of genes coding for components of the sumoylation pathway following induction of neuronal differentiation. Interestingly, we observed strong upregulation of the Senp7 protease at both mRNA and protein levels under differentiation conditions. Sumo proteases, by removing Sumo from targets, are key regulators of sumoylation. Strikingly, loss-of-function analysis demonstrated that Senp7 is required for neuronal differentiation not only in a model cell line, but also in the developing neural tube. Finally, reporter-based analysis of the Senp7 promoter indicated that Senp7 was transiently activated at early stages of neuronal differentiation. Thus, the Sumo protease Senp7 adds to the list of factors involved in vertebrate neurogenesis.

1. Introduction

Accurate neuronal differentiation requires tight regulation during development. Alterations in this process may lead to severe human diseases like cancer or neurodegenerative disorders. In the developing neural tube of vertebrates, neuronal progenitors proliferating close to the lumen (ventricular zone, VZ) progressively exit the cell cycle to migrate into the pial surface (mantle layer, ML) to accomplish neuronal differentiation. It is well known how a variety of transcription factors control this process [1,2]. However, other levels of regulation are less understood. Post-translational regulation through covalent attachment of the Small ubiquitin-like modifier (Sumo) to other proteins appears more and more implicated in controlling important biological processes [3].

Three functional Sumo molecules have been described in vertebrates. Sumo1 is normally conjugated to proteins regulating many biological processes, but exerting a prominent role in transcription repression [4]. On the contrary, most Sumo2 and Sumo3, usually referred as Sumo2/3 due to the high identity they share in the amino acid sequence, are free in the cell and rapidly conjugated to proteins in response to a variety of stress conditions [5]. Covalent attachment of Sumo to proteins involves Sumo maturation by proteolysis of several C-terminal amino acids, activation by the heterodimer Sae1/Uba2 (the E1 enzyme), which requires ATP, transfer to the conjugating enzyme Ubc9 (the E2 enzyme), and conjugation to a target protein, in many cases with the help of a Sumo ligase (the E3 enzyme), which facilitates transfer of Sumo from Ubc9 to the substrate [6]. Deconjugation from targets, as well as maturation, relies on the action of specific proteases of the Senp and Desi families [7–9]. Thus, ligases and proteases are key regulators of sumoylation.

In relation to the nervous system, sumoylation participates in the synapsis and has been associated with neurodegenerative diseases [10,11]. In addition, we have previously reported that sumoylation of the Krox20-associated Nab co-factors is involved in hindbrain development [12]. However, very little is known about the implication of Sumo in the process of neuronal differentiation. To shed light on this, we have investigated how the different components of the sumoylation pathway are regulated following induction of neuronal differentiation. An expression analysis revealed that the Sumo protease Senp7 gene was strongly upregulated following induction of neuronal differentiation. Upregulation was also confirmed at the protein level. These results prompted us to functionally investigate the involvement of Senp7 in neuronal differentiation. Our results indicate that Senp7 is required for proper neuronal differentiation. To our knowledge, this is the first evidence of the involvement of a regulatory component of the sumoylation pathway in the process of neurogenesis in vertebrates.

2. Materials and methods

2.1. Plasmid constructs

All transfection constructs, except reporter constructs and NeuroD2 and E12 expression constructs, were derived from vector pAdRSV-Sp

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[13]. As reporter plasmids we used pEGFP-N1 and pmCherry-N1 (Clontech, Mountain View, CA, USA). To generate a reporter GFP construct under the control of the promoter region of the mouse and chicken *Senp7* genes, a 1500-bp DNA fragment upstream of the transcription initiation point of the *Senp7* genes was PCR-amplified and cloned into the pEGFP-C2 plasmid (Clontech) in substitution of the CMV promoter. Expression constructs for NeuroD2, E12, NeuroM, Neurogenin2, Sumo1 and Sumo2 have been previously described [12,14,15]. Expression vectors for small hairpin RNA molecules (shRNA) were based on vector pSuper (OligoEngine, Seattle, WA, USA). Target sequences in shRNA constructs for *Senp7* were as follows, mouse: 5′-TTACAGCCTCCTCATG AGA-3′ and 5′-TCAGACTCATTGCCTTCGA-3′ (we chose this latter for the results presented), chicken: 5′-GGATTCTGTTGCTCAGACA-3′ and 5′-GAAGAAAGCTGGAGAAGAA-3′ (we chose this latter for the results presented). Control shRNA for P19 and chick embryos was described in [16].

2.2. Cell culture and transfections

P19 cells (originally obtained from Regine Losson laboratory, Illkirch, France), authenticated and tested for contamination, were cultured in α-modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 7.5% calf (HyClone) and 2.5% fetal bovine sera (Sigma, St Louis, MO, USA). Transfections were performed with Lipofectamine 2000 (Invitrogen, Life Technologies, Paisley, UK) 72 h before harvesting cells. For transfection monitoring, the green fluorescent protein expression vector pEGFP-N1 (Clontech) was used. For neuronal differentiation, all *trans* retinoic acid (RA) (Sigma) was used at 0.5 μM in α-modified Eagle's medium supplemented with 5% fetal bovine serum for 2 or 4 days in non-adherent dishes.

2.3. Cell extracts and western blot

Preparation of SDS-based denaturing and NP40-based non-denaturing cell extracts was according to [17]. Except when indicated, free Sumo and sumoylated proteins were analyzed using SDS-based denaturing extracts. Western blot was conducted with PVDF membranes (Bio-Rad) using the chemiluminescent ECL system (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. A ChemiDocXRS apparatus (Bio-Rad, Hercules, CA, USA) was used for measuring chemiluminescence. Antibodies: mouse anti-Sumo1 (1:1000 from concentrates, Developmental Studies Hybridoma Bank, Iowa, IA, USA, 21C7), mouse anti-Sumo2/3 (1:1000 from concentrates, Developmental Studies Hybridoma Bank, 8A2), rabbit anti-Senp7 (1:1000, abcam, Cambridge, UK, ab187126), goat anti-Senp3 (P-18) (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-46640), mouse anti-α-tubulin (1:5000, Sigma-Aldrich, T9026), rabbit anti-Oct3/4 (H-134) (1:1000, Santa Cruz Biotechnology, sc-9081), rabbit anti-neuron specific βIII-tubulin (1:2000, abcam, ab18207), goat anti-rabbit and anti-mouse HRP-conjugated antibodies (1:10000, Sigma-Aldrich, A6154, A4416), donkey anti-goat HRP-conjugated antibody (1:10000, Bethyl, Montgomery, TX, USA, A50-201P).

2.4. Quantitative PCR

Quantitative PCR was used for analysis of gene expression levels. Total RNA was isolated with the RNAsy kit (QIAGEN, Hilden, Germany). Retrotranscription of RNA was performed with the Superscript III enzyme (Invitrogen). Real-Time PCR reactions were performed with the SensiMix SYBR Low-ROX kit (BIOLINE, London, UK) in the Applied Biosystems 7500 FAST Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Endogenous *Rn18S* was used for normalization. Algorithms for calculation of relative units and normalization of values according to primer efficiency have been previously described [18]. Sequence of primers for expression analysis of *Sumo1*, *Sumo2*, *Sumo3*, *Sae1*, *Uba2*, *Ubc9* (*LOC102641751*), *Pias1*, *Pias2*, *Pias3*, *Pias4*, *Senp1*, *Senp2*, *Senp3*, *Senp5*, *Senp6*, *Senp7*, *Desi1*, *Oct4* (*Pou5f1*), *Tubb3*, and *Rn18S*, are described in Table S1.

2.5. In ovo electroporation and immunofluorescence

Electroporation of fertilized eggs (*Gallus gallus*), preparation of embryos and immunofluorescence on neural tube sections or P19 cells were conducted as described previously [14,15]. No ethical considerations apply for egg and chick embryo manipulation. For monitoring electroporation, the green fluorescent protein expression vector pEGFP-N1 (Clontech) was used at a concentration of 0.3 μg/μl. Antibodies: rabbit anti-neuron specific ßIII-tubulin (1:500, abcam, ab18207), mouse anti-neurofilaments (1:200 from concentrates, Developmental Studies Hybridoma Bank, Iowa, IA, USA, 3A10), donkey anti-rabbit DyLight-549 (1:800, Jackson Immunoresearch, Suffolk, UK, 711-505-152), donkey anti-mouse Cy3 (1:800, Jackson Immunoresearch, 715-165-150). Cell nuclei were exposed by DAPI (Sigma) staining. Fluorescent images of cells and embryo sections were acquired on Leica Microsystems GmbH (Wetzlar, Germany) epifluorescence DM6000 and confocal TCS SP5 microscopes, respectively. Single focal plane images were obtained in both cases, and processed with Leica LAS AF and Adobe Photoshop softwares.

2.6. Statistical analysis

Data were analyzed using the Student's *t*-test. Statistically significant *P*-values were as follows: * < 0.05, ** < 0.01, *** < 0.001.

3. Results

3.1. Retinoic acid-mediated neuronal differentiation results in free Sumo pool changes

As a first attempt to investigate the involvement of sumoylation in neuronal differentiation we focused on studying whether the process involves changes in the levels of Sumo conjugation. Thus, we evaluated Sumo conjugation under proliferation and neuronal differentiation conditions. We took advantage of the pluripotent P19 cell line, a mouse teratocarcinoma cell line easy to differentiate into neurons by treatment with retinoic acid (RA) [19]. After four days of treatment, although cells have not achieved terminal differentiation, they have reached an interesting state of differentiation, as the pluripotency marker Oct4 is completely downregulated while the neuronal marker ßIII-tubulin starts to be abundantly expressed (Fig. 1A). In immunoblots, together with unconjugated (free) Sumo, anti-Sumo1 and anti-Sumo2/3 antibodies detect a smear of bands of high molecular mass (>70 kDa) corresponding to sumoylated proteins (Fig. 1A). In addition, in immunoblots with anti-Sumo1 antibodies, sumoylated RanGAP1 is clearly identified as a band of about 80 kDa. To estimate Sumo conjugation we decided to compare the amount of unconjugated Sumo in cell extracts prepared under denaturing conditions with unconjugated Sumo in cell extracts prepared under non denaturing conditions as described in [17]. Denaturing conditions preserve conjugated Sumo from the action of the endogenous Sumo specific proteases. Thus, measured free Sumo corresponds to the naturally existing free Sumo pool. On the other hand, non-denaturing conditions enable Sumo deconjugation from targets; so measured free Sumo corresponds to total Sumo in the cell. Interestingly, RA-treated cells exhibited a 2-fold increase in both free Sumo1 and Sumo2/3 pools as detected under denaturing conditions, while no changes were observed in total Sumo as measured under non-denaturing conditions (Fig. 1). Increase in free Sumo1 mostly correlated with reduced levels of sumoylated RanGAP1. By contrast, increase in free Sumo2/3 correlated with a decrease in Sumo2/3 high molecular mass bands.