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Differential regulation of SC1/PRDM4 and PRMT5 mediated protein arginine methylation by the nerve growth factor and the epidermal growth factor in PC12 cells☆

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HIGHLIGHTS

- SC1/PRMT5 histone methyltransferase activity is down-regulated by NGF, but not EGF.
- NGF reduces the nuclear, but not the cytosolic SC1/PRMT5-mediated HMTase activity.
- SC1 and PRMT5 are found in the nucleus and the cytosol of primary mouse neurons.

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ABSTRACT

During neuronal development, the neuroepithelial stem cells (NSCs) initially undergo proliferative divisions, later switching to neurogenic ones whereby one NSC and a post-mitotic neuron are generated. We recently showed that a member of the PRDM family of transcriptional regulators, PRDM4/SC1, recruits a type II protein arginine methyltransferase, PRMT5, to maintain the "stem-like" cellular state of the embryonic mouse cortical NSCs. However, little is known about the regulation of activity of this complex under proliferation- or differentiation-inducing growth conditions.

In the present work I investigate the regulation of SC1/PRMT5-mediated methylation activity in PC12 cells treated with EGF or NGF. I present evidence that NGF down-regulates SC1/PRMT5 methyltransferase (MTase) activity and that the reduction in SC1/PRMT5 MTase activity occurs mainly in the nucleus. I suggest that high levels of SC1/PRMT5 activity are associated with the proliferative state of the cells.

1. Introduction

During cortical development, the neuroepithelial stem cells initially proliferate to increase their numbers and later differentiate generating neurons and glia [20,23]. In part the decisions of the developing NSCs to continue proliferation or to commit to a differentiation programme are cell-intrinsic [14,15]. Additionally, extracellular growth factors regulate cell fate decisions of the precursor cells during development. Intriguingly, various growth factors bind to their cognate receptors to activate seemingly identical downstream pathway, leading to dramatically different cellular responses. For example, neurotrophins, e.g. the Nerve Growth Factor (NGF), activate receptor tyrosine kinases, but so do growth factors such as the Epidermal Growth Factor (EGF) [4]. However, the biological responses are either differentiation and cell cycle exit or proliferation, respectively [4].

Neurotrophins regulate many aspects of neuronal development via activation of two types of receptors, the low affinity p75 neurotrophin receptor, p75NTR, and a family of receptor tyrosine kinases, the Trk receptors, TrkA, TrkB and TrkC [24]. PC12 cells provide a convenient cellular system to investigate the differences in the signalling mechanisms of the neurotrophins and proliferation-inducing factors, e.g., EGF. PC12 cells express both the p75NTR and TrkA receptors and respond to NGF by differentiating into sympathetic-like neurons and to EGF or serum by proliferating [30]. The duration of ERK kinase activation is one of the critical parameters in distinguishing cellular response to either NGF or EGF in these cells [3,19,21].

We recently demonstrated that a p75NTR interacting protein, PRDM4/SC1 (referred to as SC1 henceforth), recruits a type II protein arginine methyltransferase, PRMT5, to direct histone arginine methylation in the neural precursor cells from the developing

Abbreviations: SC1, Schwann cell factor 1; PRMT, protein arginine methyltransferase; PRDM, positive regulatory domain protein; NGF, nerve growth factor; EGF, epidermal growth factor; NSC, neural stem cells; (H)MTase, (histone) methyltransferase.

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mouse cortex [7]. Moreover, one of the PRMT5-mediated histone modifications, namely histone H4 arginine 3 symmetric dimethylation (H4R3me2s) is a signature of the early proliferating neuroepithelium prior to the onset of neurogenesis [5]. Together these observations suggest that high levels of methyltransferase (MTase) activity of the SC1/PRMT5 complex may maintain the proliferative status of the NSCs. Recent investigations into the mechanisms responsible for growth- or differentiation-promoting activities identified PRMT5 as one of the critical modulators of EGF- or NGF-mediated biological responses in PC12 cells demonstrating that NGF reduces PRMT5 enzymatic activity [2]. I reasoned that SC1/PRMT5-mediated protein/histone arginine methylation may represent a subset of targets for PRMT5 methylation and that the activity of this complex may be differentially regulated by different types of growth factors, e.g., EGF and NGF in PC12 cells.

I demonstrate that NGF down-regulates SC1/PRMT5-mediated MTase activity consistent with the previously published observations that PRMT5 activity in PC12 cells is dampened by NGF [2]. Moreover, NGF-induced reduction in SC1/PRMT5-mediated MTase activity is confined to the nucleus, but not the cytosol of PC12 cells. EGF, however, sustains similar amounts of MTase activity by the complex in both cellular compartments. Finally, I show that both SC1 and PRMT5 are found in the nucleus and the cytosol of the early born primary mouse cortical neurons.

2. Materials and methods

2.1. Cell culture and transfections

PC12 cells were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) horse serum (HS), 5% (v/v) foetal calf serum (FCS) and glutamine. For growth factor treatment the serum content was lowered to 1% HS. EGF (PeproTech) was used at 20 ng/ml, NGF (PeproTech) - at 50 ng/ml. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 48 h post-transfection cells were treated with the indicated growth factors, lysed and processed for the histone methyltransferase activity (HMTase) as described [7].

2.2. Immunoprecipitation and methylation assays

PC12 cells were harvested in immunoprecipitation (IP) buffer (50mM Tris–HCl, pH7.4, 0.5% (v/v) Nonidet P-40, 300 mM NaCl) supplemented with the protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails 1 and 2 (Sigma), processed as previously described and collected on protein A/G beads (Santa Cruz) [7]. After washing, beads with immunoprecipitated (IPed) proteins were processed for a radioactive in vitro HMTase assay as previously described using a mixture of purified calf thymus histones (Roche Applied Science) [7]. Products of HMTase reactions were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) and visualized using fluorography.

2.3. Subcellular fractionation of the cells and histone extraction

PC12 cells treated as specified were harvested and processed for cytoplasmic and nuclear fractionation. Cells were rinsed in cold PBS on ice, scraped off the plates, resuspended in Hypotonic Buffer A (HBA) (10 mM HEPES-K^+ pH7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 DTT) supplemented with protease (Sigma) and phosphatase inhibitors (Sigma) and pelleted by centrifugation at 1000 rpm for 5 min at 4 °C. HBA was supplemented with NP40 to a final concentration of 0.5% and added to the pellet to lyse the cells for 10 minutes are 4 °C. Nuclei were pelleted at 3000 rpm for 2 min at 4 °C and the supernatants collected as the cytosolic fraction, aliquoted and stored at −80 °C. Pellets containing nuclei were washed with HBA without NP40, followed by resuspension in buffer C (20 mM HEPES-K^+ pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 DTT, 25% Glycerol) supplemented with protease and phosphatase inhibitors. Nuclear proteins were extracted for 30 min on ice with regular vortexing. The suspension was centrifuged at 4 °C for 10 min and the supernatant containing nuclear proteins collected, aliquoted and stored at −80 °C or used for HMTase assays immediately.

Histones were extracted from PC12 cells as follows. Cells were washed twice in cold PBS, lysed in Triton extraction buffer (TEB: PBS, 0.5% Triton X 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (v/v) NaN_3) for 10 min on ice, centrifuged at 2000 rpm for 10 min at 4 °C, the supernatants discarded, pellets washed again in half the volume of TEB and centrifuged as before. Pellets were resuspended in 0.2 N HCl and extracted overnight at 4 °C. Extracts were centrifuged at 2000 rpm for 10 min at 4 °C and the supernatants used for SDS-PAGE.

2.4. Western blotting, immunocytochemistry and antibodies used

Western blotting was performed in TBST buffer (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20). For IP's and Western blotting the following antibodies were used: anti-myc (Upstate, 1:1000 for Western blotting), anti-H3 (Abcam, 1:1000), anti-H4 (Abcam, 1:500), anti-H4R3me2s (Abcam, 1:1000), anti-H4R3me2a (asymmetric dimethyl, Active Motif, 1:1000), anti-TBP (Abcam 1:2000), anti-α-tubulin (Abcam, 1:5000). The following antibodies were used for immunocytochemistry: anti-TuJ1 (Sigma, 1:500), anti-PRMT5 (Upstate Biotech, 1:100), anti-SC1/PRDM4 (a gift from Pilar Perez and Moses Chao (1:40) and our own (1:100) [6,7]). For immunocytochemical detection of antigens, primary neural stem cells were processed as previously described [7]. Fluorescent images were collected using the Leica Microsystems SPE confocal microscope. The following secondary antibodies were used: goat anti-rabbit Alexa 488, goat anti-mouse Alexa 568 (Invitrogen).

2.5. Primary neural stem cell cultures

Primary cortical neural stem cells were isolated from time mated mouse E10.5 embryos according to a published protocol [7] and plated at a density of 2.5×10^5 cells/13 mm on glass cover slips. NSCs were cultured in DMEM supplemented with 40 ng/ml Neurotrophin-3 (NT-3) (Pepro Tech), 0.25% FCS, B27 supplement, sodium pyruvate and glutamine (all from Invitrogen). Animal experiments were approved by the University College London local ethical committee and conformed to the UK Animals (Scientific Procedures) Act 1986. Project license number PPL 70/6697.

3. Results

3.1. NGF down-regulates SC1/PRMT5-mediated HMTase activity

We recently demonstrated that SC1 recruits the protein arginine methyltransferase, PRMT5, to mediate symmetric dimethylation of histone H4 on arginine 3 (H4R3me2s) [7] and showed that H4R3me2s modification mediated by PRMT5 is a "signature" of proliferating neuroepithelium in the developing mouse cortex [5]. These observations suggest that the SC1/PRMT5-mediated MTase activity may be subject to regulation by extracellular signals which instruct the NSCs to either proliferate or differentiate. To investigate whether SC1/PRMT5 MTase activity is regulated by differentiation or proliferation-inducing factors, I used PC12 cells which respond to NGF and EGF by differentiating into sympathetic-like neurons or by proliferating, respectively [30]. To determine if SC1 interacts with PRMT5 in PC12 cells, I transiently transfected PC12 cells with either Myc-tagged SC1- (mycSC1) or