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Research article

Sumoylation regulates the transcriptional activity of different human NFAT isoforms in neurons

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HIGHLIGHTS

- Sumoylation represses the transcriptional activity of Ca^{2+}-regulated NFAT proteins.
- Repression of the transcriptional activity of NFAT proteins is cell-type specific.
- In cortical neurons, sumoylation represses the transcriptional activity of NFATc1 and NFATc2 isoforms.
- In hippocampal neurons, sumoylation represses the transcriptional activity of NFATc1, NFATc2, and NFATc3 isoforms.
- Sumoylation represses the transcriptional activity of all NFATs in HEK293-FT cells.

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ABSTRACT

In the nervous system, four calcium/calcineurin-regulated members of the nuclear factor of activated T-cells (NFAT) family of transcription factors, NFATc1-c4, are involved in many developmental and functional processes, such as corticogenesis, synaptogenesis, synaptic plasticity and neurotransmission, that all need precise gene regulation. Therefore it is important to understand molecular events that contribute to the regulation of the transcriptional activity of specific NFAT isoforms. Previously, we have shown that there are a number of alternative splice variants of NFAT genes expressed in the brain and that neuronal activity leads to isoform-specific transactivation capacities of different human NFAT proteins. Here we looked at the effect of sumoylation as a possible regulator of the transcriptional activity of different human NFAT isoforms in rat primary cortical and hippocampal neurons in response to membrane depolarization and compared the results to those obtained from non-neuronal HEK293-FT and BHK-21 cells in response to calcium signaling. Our results show that in primary hippocampal neurons, sumoylation represses the transcriptional activity of NFATc1, NFATc2, and NFATc3 isoforms, whereas in cortical neurons, transactivation capacity of only NFATc1 and NFATc2 is repressed by sumoylation. In non-neuronal cells, however, transcriptional activity of all four NFAT isoforms is repressed by sumoylation in HEK293-FT cells, while only NFATc1 and NFATc2 isoforms are affected by sumoylation in BHK-21 cells. Altogether, our results show that sumoylation represses the transcription activation capacities of NFAT isoforms and that the effect is cell type-specific.

1. Introduction

Gene transcription is largely regulated by DNA sequence-specific transcription factors. Yet, various aspects of transcription factor function can be regulated by post-translational modifications (PTMs). In the case of nuclear factor of activated T-cells (NFAT), four members of this transcription factor family, NFATc1-c4, are highly modified by phosphorylation in their N-terminal regulatory domain, which keeps them inactive in the cytoplasm; dephosphorylation by the calcium/calmodulin-dependent protein phosphatase calcineurin triggers a conformational change exposing nuclear localization signals which allows transcriptionally active forms of NFATs to be translocated to the nucleus [1]. In the nucleus, NFAT proteins bind their DNA consensus sequence together with their nuclear partner proteins via C-terminal Rel homology domain (RHD), thereby regulating their target genes, which in the nervous system are involved in many developmental and functional processes such as corticogenesis, synaptogenesis, synaptic plasticity and neurotransmission [2]. The regulation of

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NFATs' activity is achieved by several kinases that either act in the cytoplasm to maintain their phosphorylated state or in the nucleus by rephosphorylating NFATs and promoting their nuclear export [2]. Additionally, transcriptional activity of NFAT proteins has also been shown to be regulated by sumoylation [3–5].

Sumoylation is a lysine-targeted PTM in which the members of the small ubiquitin-like modifier (SUMO) family are covalently bound to target proteins. Similarly to ubiquitination, SUMO conjugation to targets is achieved by three enzymatic steps catalyzed by activating enzyme E1 (Sae1/Sae2 heterodimer in mammals), conjugating enzyme E2 (a single protein UBC9), and various E3 SUMO ligases, which help to improve SUMO conjugation and substrate selection. Sumoylation is a highly dynamic process, where deconjugation of SUMO is performed by SUMO proteases (nine in mammals), which differ in their subcellular localization and specificity towards SUMO paralogs [6]. Although sumoylation plays an important role in a wide range of cellular processes, it has emerged as an important regulator of neuronal and synaptic function [7]. The components of the sumoylation machinery are temporally and spatially regulated in the developing rat brain [8] and are redistributed upon membrane depolarization at hippocampal synapses [9]. These findings suggest that sumoylation is involved in neuronal differentiation in the developing brain and in synaptic plasticity in the adult – processes implicated also by NFAT proteins.

Previously, we have shown that in response to membrane depolarization, the transcriptional activity of different human NFAT proteins is isoform-specific in neurons [10]. For example, NFATc3 and NFATc4 are the strongest transcriptional activators in neurons, and NFATc1 and NFATc2 display isoform-specific transcription activation capacities [10]. Here, we studied whether sumoylation is involved in the regulation of the transcriptional activity of human NFAT proteins in neurons. For that, we mutated the predicted sumoylation sites of different human NFAT isoforms and studied the transactivation capacities of mutated and wild-type NFAT isoforms in rat primary cortical and hippocampal neurons in response to membrane depolarization. In addition, we analyzed the transactivation capacities of different wild-type and SUMO-mutant NFAT isoforms in non-neuronal HEK293-FT and BHK-21 cells treated with the calcium ionophore ionomycin in combination with the protein kinase C (PKC)-activating phorbol dibutyrate (PdBu).

2. Material and methods

2.1. Plasmid constructs

The generation of plasmid constructs encoding wild-type NFAT isoforms has been described elsewhere [10]. For mutating lysine residues to arginine of all predicted sumoylation sites on NFAT isoforms, we designed primers for site-directed mutagenesis (Supplementary Table 1). PCR reactions were performed using Expand High-Fidelity PCR Enzyme Mix (Roche Diagnostics, Risch-Rotkreuz, Switzerland) according to manufacturer's instructions. All constructs were confirmed by sequencing.

2.2. Cell culture

Human Embryonic Kidney 293-FT (HEK293-FT) cells (Invitrogen, Carlsbad, CA, USA) and Baby Hamster Kidney 21 (BHK-21) cells (ATCC, Manassas, VA, USA) were grown as described before [10,11]. Rat primary hippocampal and cortical neurons were cultured as before [10]. All cells were grown at 37 °C in a 5% CO_2 atmosphere.

2.3. Protein electrophoresis and Western blotting

HEK293-FT cells grown on a 6-well plate were transfected with 2 μg of DNA and 4 μg of polyethylenimine reagent (Sigma-Aldrich, St. Louis, MO, USA) per well at ~60–70% confluence. 36 h after transfection, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot were performed as described in [10].

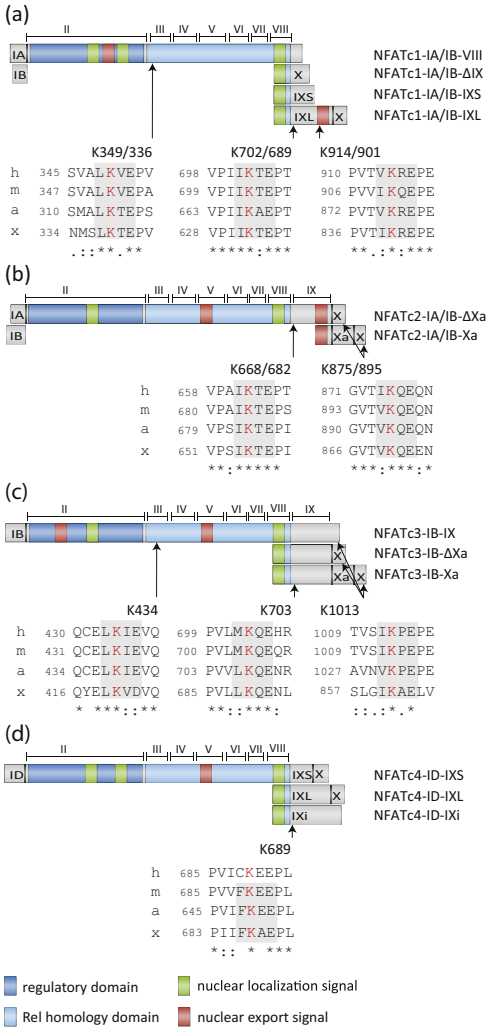

Fig. 1. Mapping sumoylation sites of human NFATc1-c4. Schematic representation of putative sumoylation sites of human NFATc1 (a), NFATc2 (b), NFATc3 (c), and NFATc4 (d) proteins together with multiple protein sequence alignments of sumoylation motifs of *Homo sapiens* (h), *Mus musculus* (m), *Anolis carolinensis* (a), and *Xenopus tropicalis* (x). Potential sumoylated lysines of NFATc1-c4 were predicted using GPS-SUMO algorithm [13] and are indicated with the arrow or highlighted in red in the sequence. Sumoylation motifs with high confidence scores are highlighted in grey. Sequence alignments were generated using Clustal Omega [15]. The names of the isoforms are according to [16]. Exons are numbered with Roman numbers.

2.4. Luciferase assay

For transfection of HEK293-FT and BHK-21 cells, 0.5 μg of DNA and 1 μg of polyethylenimine reagent (Sigma-Aldrich) were used per well of a 48-well plate at ~60–70% confluence. Neuronal cultures were transfected at 6 DIV using 0.5 μg of DNA and 1 μl of Lipofectamine 2000 reagent (Invitrogen) per well of a 48-well plate. Cells were cotransfected with equal amounts of human NFAT wild-type or SUMO mutant construct or empty EGFP vector, NFAT luciferase reporter plasmid pGL4.30[luc2P/NFAT-RE/Hygro] (Promega, Madison, WI, USA), and *Renilla* luciferase reporter vector pRL-TK (Promega) for normalization. Two days post-transfection, cultured neurons were stimulated with 25 mM KCl, and HEK293-FT and BHK-21 cells were stimulated with 1 μM ionomycin (AppliChem, Darmstadt, Germany) plus 1 μM PdBu