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Research Report

Phosphorylation of the Brn-3a transcription factor is modulated during differentiation and regulates its functional activity

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Abstract

Brn-3a is a transcription factor expressed in a subset of neurons of the peripheral nervous system. Its role encompasses the activation of genes involved in neuronal differentiation and survival. While a lot of data have been produced on Brn-3a target promoters, very little is known about the upstream regulatory signals that mediate its activation in response to differentiation. In this work, we describe for the first time that Brn-3a is phosphorylated in IMR-32 neuroblastoma cells in response to differentiation induced by retinoic acid treatment and that its post-translational modification is potentially mediated by the activation of the MAPK/ERK pathway. Furthermore, we show that the mutation of a putative phosphorylated amino acid strongly reduces the ability of Brn-3a to mediate the differentiation of IMR-32 cells.

Theme: Cellular and molecular biology
Topic: Gene structure and function: general

Keywords: Brn-3a; Transcription factor; Retinoic acid; Differentiation; Phosphorylation

1. Introduction

Brn-3a, along with Brn-3b and Brn-3c, is a transcription factor belonging to the class IV of the POU family of transcription factors [3,11,14,29]. Although these three factors are expressed in different subset of neurons, they are all involved in different aspects of neuronal survival and differentiation. Brn-3a in particular is mainly expressed in sensory neurons of dorsal root ganglia and in the trigeminal ganglion [10,11,13,34]. Lack of Brn-3a in knock-out mice is incompatible with survival due to extensive loss of these neuronal cells. It has been shown that Brn-3a can induce neuronal differentiation by regulating the expression of genes such as those encoding SNAP-25 [13], alpha-internexin [2], the neurofilaments [22], and can also inhibit neuronal apoptosis in vitro via the activation of Bcl-2 and Bcl-x [23–25], although some experimental findings suggest that Bcl-2 might have a more complex regulation in vivo [9].

While data have been published in regard to the promoters that are targeted and activated by Brn-3a as well as in regard to the promoter that regulates the synthesis of Brn-3a [12,31], very little is known about the upstream regulatory signals involved in the activation of Brn-3a and whether any post-translational modification such as phosphorylation could be a means to regulate its activity. Phosphorylation is a well-known mechanism that regulates the activity of proteins, by influencing, for example, their tertiary structure, the binding to other proteins or DNA, or by their relocation to different subcellular compartments. The phosphorylation of Oct-2, for example, a member of the class II of the POU family of transcription factors, leads to a down-regulation of its DNA binding ability on target promoters [18] while the phosphorylation of Pit-1, a class-1 POU member, regulates its binding to the ETS-1 transcription factor [1].

In this work, we wanted to understand whether Brn-3a is phosphorylated as a response to differentiation stimuli applied to cells and dissect the pathway that might be involved in its activation. As a differentiation stimulus, we

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decided to use all-trans retinoic acid. This compound has been found in the CNS [15], it induces neurite outgrowth (although not differentiation) in neurons of adult mouse dorsal root ganglia [7], it has been shown to induce neuronal differentiation during embryonic development [8], and it has been previously used as a differentiating agent in the IMR-32 neuroblastoma cell line [6].

In our experiments, we have shown that Brn-3a is phosphorylated in IMR-32 cells when differentiation is induced with retinoic acid, via the activation of the MAPK/ERK pathway. Furthermore, we have identified a specific amino acid within Brn-3a which is a target for phosphorylation and whose mutation is sufficient to drastically reduce the ability of Brn-3a to enhance the differentiation of IMR-32 cells.

2. Materials and methods

2.1. Vectors and constructs

Brn-3a wild type and 121/122 were isolated from a pcDNA vector with *Eco*RI and *Bam*HI and subcloned in frame with the FLAG-tag in the PsG5-FLAG vector digested with *Eco*RI and *BgL*LII. The inserts were sequenced to check for orientation and lack of point mutations. A construct Brn3alLTRpoly containing the Brn-3a long cDNA under the control of the Moloney murine leukemia virus LTR promoter (used previously in our laboratory) was modified such that TCG codons Serine 121 or Serine 122, or both were replaced with GCG (Alanine) codons. The Brn3alLTRpoly plasmid was digested with *Ngo*MIV and *Bst*EII restriction enzymes (Promega) to excise a fragment of approximately 45 bp. Two oligonucleotides were then hybridized to form a replacement fragment, which was ligated into the gel-purified, linearized plasmid. These oligonucleotide pairs had the altered codons required. Primer pairs:

121Fw 5′GTGACCTGCTGGACCACATCGCGTCGC-CGTCGCTCGCGCTCATGG3′
121Rw 5′CCGGCCATGAGCGCGAGCGACGGCGA-CGCGATGTGGTCCAGCAG3′
122Fw 5′GTGACCTGCTGGACCACATCTCGGCGC-CGTCGCTCGCGCTCATGG3′
122Rw 5′CCGGCCATGAGCGCGAGCGACGGCGC-CGAGATGTGGTCCAGCAG3′
121/2F 5′GTGACCTGCTGGACCACATCGCGGCGC-CGTCGCTCGCGCTCATGG3′
121/2Rw 5′CCGGCCATGAGCGCGAGCGACGGCG-CCGCGATGTGGTCCAGCAG3′

Plasmids were ligated using T4 DNA ligase (Promega) and transformed. Resulting constructs were sequenced to determine the presence of the insert and the correctly mutated codons.

2.2. In vitro phosphorylation assay

Depending on the experiments, cells were either pretreated with the indicated kinase inhibitors: PD-98059 (25 μM), LY-294002 (50 μM), or diluent (DMSO) for 45 min before retinoic acid was added to a 25-μM final concentration and incubated for different times, washed twice with ice-cold PBS and lysed on ice with RIPA buffer (10 mM Tris–Cl pH 7.5, 150 mM NaCl, 0.2% NP-40) supplemented with protease inhibitors (mini-cocktail, Roche) and sodium-orthovanadate (1 mM). Cell lysate was spun to collect the membrane debris and an aliquot of the lysate was added to an equivalent volume of GST fusion proteins resuspended in kinase buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$) and incubated with occasional mixing for 30 min at room temperature in the presence of γ^{32}P-ATP (depending on the experiments). The beads were then washed twice in RIPA buffer, resuspended in an appropriate volume of protein sample buffer and run on a 10% PAGE.

2.3. Cell culture and transfection

IMR-32 neuroblastoma cells were routinely grown in DMEM plus 10% fetal calf serum (FCS) at 37 °C, 5% CO_2 and 95% humidity. When needed, cells were either pretreated or not with kinase inhibitors and differentiation induced for an hour with retinoic acid as described previously. For luciferase assays, cells were transfected with the indicated constructs with Fugene® (Roche) according to the manufacturer's instruction. Renilla, under the control of the constitutive thymidine kinase promoter, was co-transfected for normalization purposes. All gene reporter assays have been repeated at least three times in triplicate.

For in vitro labeling, cells were transfected with different FLAG-tagged Brn-3a constructs as described above. After 2 days, cells were incubated for 5 h in the presence of 500 μCi of ^{32}P-orthophosphate in a phosphate-free medium. Retinoic

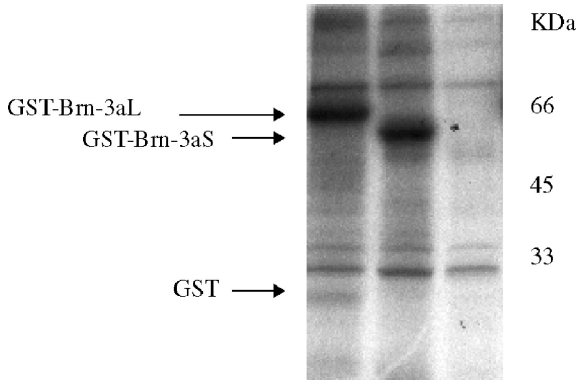

Fig. 1. GST-Brn3aL, GST-Brn3aS, and GST alone were incubated with reticulocyte lysate in the presence of γ^{32}P-ATP and kinase buffer. Proteins were run on a gel that was then dried and exposed to phosphoimager. Both the long and the short form of Brn-3a (3aL and 3aS) can be phosphorylated by kinases present in the reticulocyte lysate, while GST is not.