Abstract The post-transcriptional control of mRNA levels is a very powerful mechanism which allows cells to quickly change the amount of the som proteins. In this study, we wanted to analyze whether the Brn-3b transcription factor, essential for the proper development of mouse retinal ganglion cells, is subjected to such post-transcriptional regulation. In particular, due to its conservation amongst different species, we wanted to study the role of its 3' untranslated region (3'UTR).

We show that the 3'UTR of the Brn-3b mRNA does indeed contain regulatory sequences that mediate mRNA degradation upon serum starvation-induced differentiation of ND7 neuroblastoma cells. The specific region mediating this effect has been characterized and two different microRNAs that potentially regulate the stability of Brn-3b have been identified. Moreover we show that Dicer, one of the key enzymes in the production of microRNAs, is strongly up-regulated in ND7 cells subjected to differentiation.

Keywords: 3'UTR; Brn-3b; microRNA; Differentiation; Dicer

1. Introduction

Brn-3a and Brn-3b, members of the POU IV class of transcription factors are expressed in different parts of the central and peripheral nervous system [1–5]. While mice lacking Brn-3a die a few days after birth, Brn-3b knock-out mice are viable but exhibit a specific and dramatic loss of up to 70% of retinal ganglion cells (RGC) [6].

Our laboratory has previously shown that when the ND7 neuroblastoma cell line is induced to differentiate via serum removal, Brn-3b levels decrease while Brn-3a is increased [7]. The switch between Brn-3a and Brn-3b is extremely important and allows neuronal cells to activate different subset of genes involved in the cellular routing toward differentiation (Brn-3a) [8–12], or proliferation (Brn-3b) [13–16]. A stringent regulation of Brn-3a and Brn-3b expression is thus essential for cellular fate and development.

The control of gene expression and overall protein synthesis is a key process in the metabolism of a cell. Post-transcriptional regulation of mRNA half-life or the regulation of its translation allows cells to have a very fine and rapid control over protein synthesis. One of the known mechanisms involved in the modulation of mRNA stability resides in their 3'UTR, a region containing motifs, such as the AU-rich regions (AREs) which regulate mRNA half-life [17,18].

In this work, we wanted to understand whether Brn-3b mRNA is subjected to a post-transcriptional regulation in ND7 cells induced to differentiate and whether this process is mediated by its 3'UTR. This region, in fact, exhibits between 99% and 97% nucleotide identity between human and mouse and mouse and rhesus monkey, respectively (our unpublished observation), suggesting a possible important role due to its conservation.

The data presented herein show that the 3'UTR of Brn-3b mediates the degradation of a chimaeric reporter mRNA. This process is induced by differentiation stimuli applied to ND7 cells and is likely to be mediated by two different microRNAs. Furthermore we also present evidence that Dicer, one of the key enzymes in the production of microRNAs, is strongly up-regulated in ND7 cells induced to differentiate.

2. Materials and methods

2.1. Constructs and vectors

The 3'UTR of Brn-3b (NM_138944) was amplified from mouse genomic DNA with the following primers: 3'UTR-SacI: 5'gagcttcgttgcgggaacagaagcgggctggc3' and 3'UTR-BamHI: 5'ggagcctggcagcaatagcag3'. The resulting circa product was sub-cloned in the multiple cloning site of pEGFP-C1, downstream of GFP. The mutants in the microRNA target sequence were obtained via the QuickChange site directed mutagenesis (Stratagene) following manufacturer's instruction with the following primers: mir-23(upstream)-Rw5'-ctggcttggtctgggtgcccgtttaccggaattgtt3', mir-23(downstream)-Fw5'-ttcggctgcttggctgcgtgctc3', mir214-Rw5'-ctggcttggtctgggtgcccgtttaccggaattgtt3', mir-23(downstream)-Fw5'-gttctacgccaagcagtgcaggctg3', mir128-Fw5'-ctggcttggtctgggtgcccgtttaccggaattgtt3', mir214-Rw5'-ctggcttggtctgggtgcccgtttaccggaattgtt3', mir128-Fw5'-ctggcttggtctgggtgcccgtttaccggaattgtt3', mir128-Rw5'-ctggcttggtctgggtgcccgtttaccggaattgtt3'. The sequential deletion of Brn-3b was performed with the Bal-31 nuclease (New England Biolabs). All constructs have been checked by sequencing.

2.2. Cell culture

The neuroblastoma ND7 and the breast cancer MCF-7 cell line were routinely cultured in DMEM supplemented with 10% Foetal Calf Serum (FCS) at 37°C, 95% humidity and 5% CO2. Transfection was performed with GeneJuice (Novagen) according to the manufacturer’s instruction. SH-SY5Y cells were cultured in 50% MEM, 50% Hams F12 supplemented with 15% FCS. For all the serum starvation experiments, cells were transfected with the indicated constructs and the following day (day 0) incubated in full medium with or without FCS in order to induce differentiation for the indicated days.
2.3. RNA extraction, real-time RT-PCR, Northern blot

For mRNA turnover experiment ND7 cells were transfected and treated as described above. Post-transfection cells (48 h) were treated with actinomycin D (7.5 μg/ml) for 0, 4 and 8 h [19]. Total RNA, extracted with Trizol (Invitrogen), has been subjected to real-time RT-PCR reaction using SYBR I Green technology on the DNA Engine Opticon System (MJ Research) as previously described [20] with primers specific for GFP and for neomycin for normalization purposes.

For Northern blot analysis of small RNA, 30 μg of total RNA were loaded and run on a 15% polyacrilamide, 7 M UREA gel. The pre-hybridized membrane was incubated overnight with the appropriate probes at 42°C [176]. Probes: U6-gcaggggccatgctaatcttctctgtatcg; mir-214-ctgcctgtctgtgcctgctgt; mir-128-aaaagagaccggttcacgtga; mir-23-gaaaatccctggcaatgtgat, were end labeled with T4 Kinase in the presence of γ32P-ATP. Washes were done at room temperature in 2·SSC, 0.1% SDS. Membranes were then exposed to a phosphor-imager screen.

2.4. Western blot

Transfected cells were lysed with RIPA buffer (10 mM TRIS pH 7.5, 150 mM NaCl, 0.2% NP-40) supplemented with protease inhibitors (mini-cocktail, Roche). Membranes were probed with an anti-GFP antibody (Sigma G-1544), stripped and reprobed with anti-neomycin antibody (Upstate) for normalization purposes. For Dicer, an anti-Dicer antibody (St. Cruz) was used and data normalized with anti-p85 (Upstate). Western blots were scanned and bands analyzed via the QuantityOne software (Biorad). All experiments were repeated at least in triplicate. Error bars indicate S.D.

3. Results

3.1. The 3’ UTR of Brn-3b mediates the down-regulation of a chimaeric reporter construct

The 3’ UTR of Brn-3b was sub-cloned downstream of GFP in the peGFP expression vector. The peGFP::3bUTR and the empty peGFP control constructs were transfected into neuroblastoma ND7 cells which were then induced to differentiate via serum removal and harvested at different time points. After 1, 3 or 5 days, GFP levels were assessed by Western blot and normalized with neomycin, which is expressed from the same vector. As shown in Fig. 1A serum removal induces a progressive and strong reduction of GFP expression in the construct containing the 3’UTR of Brn-3b compared to the ‘empty’ vector.

In order to confirm the result, we repeated the experiment in the SH-SY5Y human neuroblastoma and in the MCF-7 human breast cancer cell line. As shown in Fig. 1B, C, the levels of the chimaeric mRNA are reduced in the SH-SY5Y but not in the MCF-7 cell line following serum removal thus suggesting that the chimaeric mRNA is not intrinsically unstable and that its down-regulation seems to be specific for neuronal cell lines and to occur in both human and mouse cells.
We next addressed whether the reduction of GFP levels in cells transfected with the GFP::3b-3’UTR is due to increased degradation of its mRNA or to the inhibition of its translation. Total RNA extracted from ND7 cells transfected as described above, serum starved and treated with Actinomycin D, was subjected to real-time RT-PCR. As shown in Fig. 1D, the levels of the mRNA containing the 3’UTR of Brn-3b are strongly and rapidly reduced compared to the control. This suggests that the observed reduction of GFP at the protein level is due to the degradation of its mRNA and caused by sequences contained within the 3’UTR of Brn-3b. Since both constructs are under the control of the CMV constitutive promoter, in fact, no differential transcriptional regulation can occur, and post-transcriptional regulation is therefore responsible for reduced GFP levels.

3.2. Deletion analysis of the 3’UTR of Brn-3b

In order to map the region of the 3’UTR of Brn-3b responsible for this effect, a series of deletion constructs were made with the Bal-31 nuclease and assessed for their sensitivity to differentiation-induced degradation using the assay described above (Fig. 2A). As shown in Fig. 2B, while the Bal1 deletion mutant still responds to serum removal like the wild-type, the Bal4 and Bal7 mutants have lost this ability, suggesting that they no longer possess the responsive in cis sequence.

All the Bal-31 mutants have been sequenced and analysed using bioinformatics tools for known sequences that might mediate mRNA stability at the post-transcriptional level [21] but no AU-rich or other known sequences were found. The region deleted in the Bal4 and the Bal7 mutants, though, is putatively targeted by three different microRNAs: mir-23, mir-128, and mir-214 (http://cbio.mskcc.org/mirnaviewer/), one of which, mir-23, has two target sites on the 3’UTR of Brn-3b (Fig. 2A). These microRNAs putatively bind to highly conserved (90–100%) sites on the 3’UTRs of mouse and human Brn-3b (Fig. 3 right column), suggesting a possible role due to their evolutionary conservation. Furthermore none of these putative microRNAs have any matching binding sites on the 3’UTR of the closely related Brn-3a transcription factor suggesting specificity for Brn-3b (data not shown).

3.3. Mutation of the microRNA sites on the 3’UTR of Brn-3b

The target sequences of the three candidate microRNAs on the 3’UTR of Brn-3b were individually mutagenised by changing three out of the seven nucleotides, known as the “seed”, essential for the binding of the microRNA to its target sequence (Fig. 3 right-hand column) and the resulting constructs have been assessed by Western blot. As shown in Fig. 3 (left column), the mutagenesis of the sites recognized by mir-23 (Fig. 3A, B), mir-214 (Fig. 3C) but not of mir-128 (Fig. 3D) abolishes the down-regulation of GFP levels. This result is entirely consistent with our observation that the Bal1 mutant, which lacks the mir-128 binding site, still responds to serum removal, while Bal4 and Bal7 mutants, lacking the mir-23 and mir-214 binding sites no longer have this response. The downstream mir-23 target site seems more important than the upstream one. This is also reflected by the nucleotide similarity between mouse and human and the overall match of the mir-23 microRNA on its target sites with mir-23/downstream showing an overall similarity on its target site which is higher than the one of mir-23/upstream. Moreover, mir-128, which in our assay does not seem to have any role in the regulation of the chimaeric construct, has a very low consensus on its target site on the Brn-3b 3’UTR, especially at the critical 5’ “seed” end of the microRNA.

Intriguingly, mutations of the microRNA sites are associated with reduced GFP levels. This is particularly evident in the mir23/mir23 double mutant (Fig. 3E), in which both mir-23 sites have been mutagenised and in the mir23/mir23/mir214 (Fig. 4F) triple mutant in which the mir-214 site has been also mutagenised.

Fig. 2. Deletion analysis of the 3’UTR of Brn-3b. (A) Schematic representation of the microRNA binding sites on the wild-type 3’UTR of Brn-3b and on the Bal-31 deletion mutants used in this study. The effect of these deletions on the production of GFP is graphically represented in (B) and is the average of at least five independent experiments.
Fig. 3. Effect of the mutation on the microRNA binding sites. The alignment of the microRNA binding sites on the 3’UTR of mouse and human Brn-3b is represented in the right-hand column. The 5’ end of the microRNAs (seed) is type faced in gray and the mutations sites introduced in this study are in ‘bold’ black. The effect of these mutations on GFP levels is displayed on the graph in the left column. The effect of the mir23/mir-23 double mutation and the mir-23/mir-23/mir214 triple mutation on the stability of the reporter construct is displayed in panels E and F, respectively. All experiments have been performed by serum-starving cells for two days after transfection with the indicated constructs.
3.4. Effect of the GFP chimaeric constructs on endogenous Brn-3a and Brn-3b levels

We then analysed the effect of the transfection of the pEGFP::3bUTR on the levels of the endogenous Brn-3a and Brn-3b mRNA in cells induced to differentiate. As mentioned in Section 1 in fact, while Brn-3a levels increase, Brn-3b levels are sharply reduced in ND7 cells induced to differentiate via serum removal. Furthermore, although almost identical at the protein level, Brn-3a and Brn-3b share a very little similarity in their 3'UTR (data not shown). In ND7 cells transfected with the pEGFP::3bUTR, but not the ‘empty’ GFP vector, the endogenous Brn-3b mRNA levels are not reduced following serum removal (Fig. 4a). In the same conditions the levels of the endogenous Brn-3a transcription factor mRNA increase during serum removal as expected (Fig. 4b).

3.5. Expression of microRNAs and Dicer in ND7 cells

We next wanted to measure the expression levels of candidate microRNAs. Total RNA extracted from ND7 cells grown in full serum or serum starved, was subjected to Northern blot analysis. All microRNAs were detected after several days of exposure and the expression of mir-23, mir-214 but not mir-128, were increased in ND7 cells subjected to serum removal (Fig. 5a). We next evaluated whether Dicer, one of the key enzymes involved in the production of mature microRNAs, is expressed and differentially regulated in ND7 cells subjected to serum removal. As shown in Fig. 5b, not only Dicer is expressed in ND7 cells but it is actually strongly up-regulated following serum removal (Fig. 5c).

4. Discussion

In the past years, a new class of molecules named microRNAs (or miRNAs), have been shown to be involved in the direct regulation of mRNA translation by binding to their 3'UTR. These 22–23 nucleotide long single stranded RNA molecules are produced in a wide variety of organisms from long precursor transcripts [22–24] which, initially folded into double-stranded RNA molecules, are subjected to a controlled maturation and shortening process by two proteins with RNaseIII activity, Drosha in the nucleus and Dicer in the cytoplasm. One of the two strands of the resulting 22–23 nucleotide long double-stranded RNA, is then incorporated into the RISC multiprotein (RNA Induced Silencing Complex). Via the specificity provided by the incorporated microRNA, the complex is able to bind the target mRNA and directly modulate its degradation or inhibition of translation [25–27].

The Brn-3b transcription factor, a POU IV-class protein, is modulated in retinal ganglion cells (RGCs) during development. By using different approaches we have established that its 3'UTR is involved in the modulation of a reporter mRNA via the targeting of microRNAs. The individual mutagenesis of these microRNA binding sites allowed us to determine that mir-23 and mir-214 but not mir-128 are likely to be involved in the down-regulation of Brn-3b upon serum removal in ND7 cells. Furthermore the downstream mir-23 binding site is part of a consensus sequence termed ‘K-box’ (eUGUAU). This was originally identified in the 3'UTR of Notch pathway target genes such as basic helix–loop–helix repressors and members of the Bearded family proteins [28]. It is thus possible that Brn-3b is a member of the proteins targeted by the Notch pathway during RGCs development.
Although the mutagenesis of the microRNA binding sites on the 3′UTR of Brn-3b seems sufficient to alleviate serum starvation-induced down-regulation of GFP::3bUTR, these mutants display overall lower GFP levels compared to the ‘wild-type’ GFP vector. Our data suggests that, besides a role in the down-regulation of the GFP::3bUTR chimaeric construct exerted by microRNAs as a response to a specific stimulus, the mutagenesis of the 3′UTR of Brn-3b seems to be causing per se its increased degradation. This is especially evident in the mir-23/mir-23 double mutant (Fig. 3E) or in the mir-23/mir-23/mir-214 triple mutant (Fig. 3F) in which the overall levels of GFP, instead of increasing due to the elimination of progressively more microRNA binding sites, are instead lower than their wild-type counterpart. These data suggest that the down-regulation of the different mutant GFP::3bUTR constructs is a process which possibly involves two separate mechanisms. One, mediated by microRNAs, seems to be involved in the specific down-regulation of the GFP::3bUTR mRNA as triggered by serum removal; the other instead seems to be causing a microRNA- and differentiation stimuli-independent degradation of the chimaeric mRNA. Although at the present time the mechanism involved in the latter phenomenon is not clear, we might speculate that, as described previously [29,30], the chimaeric mRNA harbouring a mutated 3′UTR has an altered secondary structure and is therefore degraded.

The role of the 3′UTR of Brn-3b in the degradation of the chimaeric GFP mRNA has also been indirectly confirmed by evaluating its effect on the modulation of the endogenous Brn-3b transcription factor. In comparison to cells transfected with the GFP empty control vector, the endogenous Brn-3b mRNA is not down-regulated in cells transfected with the pGFP::3bUTR reporter construct. The 3′UTR of the exogenous construct, in fact, is likely to be titrating the microRNAs involved in the down-regulation of Brn-3b. This is also confirmed by the lack of any effect on the closely related Brn-3a transcription factor which is known to be up-regulated in differentiating ND7 cells. Since the 3′UTR of Brn-3a and Brn-3b do not have any similarity, these data confirm the specificity of the observed effect as mediated by the 3′UTR of Brn-3b. This effect could be also experimentally used to specifically block the down-regulation of Brn-3b or any other mRNA which are modulated via their 3′UTR and control their levels in vitro at a post-transcriptional level.

The analysis of microRNA expression levels has allowed us to show that mir-23, mir-214 and mir-128 are expressed in our in vitro at a post-transcriptional level. The analysis of microRNA expression levels has allowed us to show that mir-23, mir-214 and mir-128 are expressed in our in vitro at a post-transcriptional level.

References


