The molecular function of AT-rich DNA binding proteins in the developing brain

PhD Thesis
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I. Introduction

A central question in developmental neuroscience is the regulation of differentiation. Some factors and pathways are already identified that regulate this process. Most factors control multiple steps, and there is also a cross-talk between the different pathways, which makes the picture even more complex. First, neuronal progenitors have to commit to the neuronal fate, then they stop dividing, and finally, the immature neurons undergo fate acquisition and differentiate into mature neurons.

Most of these factors act by binding to their specific cognate sequence, while others recognize a specific secondary DNA structure. AT-rich regions can have such special structure with a narrow minor groove that can be read by proteins. Two distinct groups of AT-rich DNA are present in the mammalian genome: long AT-islands and short AT-tracks. AT-islands are distinct large domains consisting of highly atypical AT-rich sequences. The AT content of these domains ranges from ~80 to 100% AT over their entire length of 200-1000 bp or more. Some AT-islands function as Matrix Attachment Regions (MARs): the association of MARs with the nuclear matrix may serve to structurally define the borders of chromatin domains and thus participate in the regulation of transcription.

In eukaryotic cells the DNA is present in the form of chromatin. Chromatin is a protein-DNA complex, where the major proteins are histones. Covalent modifications of the DNA and histones determine whether the DNA strand is tightly wound in nucleosomal arrays (heterochromatin), and thus poorly accessible to transcriptional proteins, or if the DNA strand resides in a more relaxed transcriptionally active state (euchromatin). Acetylation of histone tails at certain amino acids promotes the “open” structure, while methylation is generally connected with the “closed” state of chromatin. Alteration of the chromatin structure utilizing energy derived from ATP hydrolysis is called “chromatin remodeling” and is one of the known transcriptional regulatory mechanisms. One of these complexes is called Nucleosome Remodeling and histone Deacetylation (NuRD) complex.

In this thesis, we were investigating transcriptional mechanisms that can contribute to the cell fate acquisition in the developing brain. We chose the enkephalin (ENK) gene as a model system. The ENK gene encodes peptide neurotransmitters that mediate important neuropsychiatric functions involving emotion, social behavior, aggression and reward. The expression of the ENK gene in the adult brain is highly cell-specific and spatially restricted. Many of the enkephalinergic neurons are localized in the striatum. Most of the other brain structures, such as cerebral cortex, are devoid of enkephalinergic neurons. We set out to investigate the developmental process that restricts the ENK expression to a subset of neurons. ENK expression starts in rats around embryonic day 16 (E16) in the caudal–ventral part of the striatum; around E20, positive cells appear in the entire striatum, and shortly after birth the expression reaches the adult levels, so we concentrated on the developmental stage from E16 to P4. Using a variety of techniques, several far upstream regions of the ENK gene were identified that may play a role in its developmental expression regulation.

One of these regions was the AT-rich region that can be found approximatelly -2500bp upstream of the ENK gene in rats, mice as well as humans. Two proteins were identified that bind to this sequence: one was AUF1, a protein that is known for regulating mRNA stability, and SATB2, a protein similar to the MAR-binding SATB1.

II. Methods

1. Semi-quantitative reverse transcription PCR (RT-PCR) was used to compare the SATB2 mRNA levels between different ages and organs.

2. Western blotting and single, as well as double, immunhistochemistry was used to detect proteins.

3. Sections were viewed in a Leica DM RXA microscope or the Zeiss Pascal Laser Scanning Confocal Microscope (LSCM). The appropriate fluorescent channels and images were merged using TIFFany3 (Caffeine Software, Inc.) to create the final pictures. The quantification of the SATB2 and MTA2 or HDAC1 double and single positive areas was done by analysing the scanned images of immunhistochemically stained hemispheres with a Mathematica 5.2 script (Wolfram Reaserch Inc., Urbana, IL).

4. DNA Affinity Preincubation Specifity Test of Recognition (DAPSTER) assay was used to assess the specifity of DNA-protein interaction *in vitro*.

5. To show the in vitro protein-protein interactions, we used the co-immunoprecipitation assay (Co-IP).

6. We used chromatin immunoprecipitation (ChIP) assay, optimalized to the developing brain, to prove the in vivo interaction between the AT-rich region of the ENK gene and the chosen proteins.

7. To prove the repressor activity of SATB2, we transiently transfected C6 glioma cells with reporter constructs and SATB2 expressing plasmids. Trichostatin A (TSA) treatment, and HDAC inhibitor was used to show that the SATB2 mediated repression is dependent on HDAC1 activity.

III. Aims

1. Our first goal was to determine the developmental expression pattern of AUF1 and SATB2. Both proteins were known to be expressed in the brain, however, there was no information about their developmental expression pattern during the critical phase of cortical development, which is when the enkephalinergic "decision-making" takes place.

2. The second goal was to show that the interaction between the AT-binding proteins and the AT-rich DNA element of the ENK gene (AT^{ENK}) is present *in vitro* as well as *in vivo.* Even though both AUF1 and SATB2 were isolated based upon their binding to the AT^{ENK}, there was no direct proof if the binding is specific, or if it is present in the developing brain, *in vivo,* as well.

3. The third aim was to identify potential partner protein(s) which may interact with SATB2. Is SATB2 independently acting as a regulator of gene expression, or as a part of a complex? SATB2 is a close homologue to SATB1, the hematopoietic master regulator, which is known to interact with members of multiple chromatin remodeler complexes. As the two SATB proteins share a high homology at the aminoacid level, especially at DNA- and protein binding domains, it was reasonable to assume that SATB1 and SATB2 might have similar partner proteins.

4. Next, we wanted to determine the spatio-temporal distribution of these partner proteins during the critical phase of cortical development.

5. Since such interactions would likely influence the mechanism of gene regulation by SATB2, it was an important goal to find out the role of the interactory proteins.

6. The final goal was to determine how SATB2 regulates gene expression *in vivo*, and if it requires any co-factors for its regulation.

IV. Results

1. The developmental expression pattern of AUF1

We characterized the distribution of AUF1 proteins in the developing brain. At embryonic day 16 (E16) practically all cells populating the ventricular zone (VZ) were immunoreactive for AUF1. In addition, many cells in the pallidal subventricular zone and in the telencephalic wall were positive for AUF1, suggesting its involvement in the proliferation and/or differentiation of neuronal progenitors. At E18, practically all cells in the developing cortex were AUF1 immunoreactive; however, in the striatum only a subset of cells was immunoreactive. The proportion of AUF1+ cells in the striatum seemed proportional to the population of neurons that are expressing other neurotransmitter genes, but not ENK in the adult striatum. At postnatal day 2 (P2), the majority of AUF1 immunoreactive cells were found in regions that contain very few or no enkephalinergic neurons in the adult, including the cerebral cortex, septum, thalamus, etc. Using higher power imaging, we found that AUF1 immunoreactivity in the developing rat brain is exclusively nuclear. Within the nucleus, AUF1 immunoreactivity was not distributed evenly: it appeared by sub nuclear speckle-like "hot-spots" while most of the nucleoplasm lacked immunoreactivity. *(A. Dobi et al., 2006, JBC and 2005. Neuroscience poster).*

2. AUF1 specifically binds to the AT-rich region of the ENK *in vitro* and *in vivo*

DNA Affinity Preincubation Specifity Test of Recognition (DAPSTER) assay was used to show that AUF1 specifically binds to the double stranded AT-rich DNA of the rat ENK gene (rAT^{ENK}) *in vitro*. The binding is specific, since it could be competed with specific double stranded wild type DNA, but not with mutant competitor.

Chromatin immunoprecipitation (ChIP) was performed to prove that AUF1 binds to rAT^{ENK} as well as the TATA region of the ENK gene *in vivo*. *(A. Dobi et al., 2006, JBC and 2005. Neuroscience poster).*

3. Developmental expression of SATB2 mRNA and protein in the rat brain

We analyzed the developmental pattern of SATB2 mRNA expression by semi-quantitative RT-PCR. We detected low levels of SATB2 mRNA in the rat telencephalon at E14, then abundance gradually increases and reaches the highest abundance at P2, then it is gradually decreasing and only very low level was detected in the adult cortex. We detected no SATB2 mRNA in the neonatal thymus, a rich source of SATB1 mRNA at this developmental stage.

Similarly, when using immunoblot, we found that the highest amount of SATB2 protein is between E18 and P2; we could not detect any at E14 and in the adult.

Using immunhistochemistry, we have found that SATB2 positive cells were restricted to the developing cortex. Immunoreactive cells were first detected at E16 in the superficial layers of the cortex. At E18, the number of SATB2 positive cells increased substantially. The SATB2 stained area further increased at P2 and then sharply declined by P4. When labeling dividing cells by BrdU staining, we found no double-labelled cells, and there was a clear boundary between BrdU and SATB2 immunoreactive cells at both ages investigated. These findings demonstrated that SATB2 is expressed in a subpopulation of postmitotic neurons. *(M. Szemes and A. Gyorgy et al., 2006, Neurochem. Res.)*

4. SATB2 interacts with two members of the NuRD complex in vitro

SATB2 and the interacting proteins were co-immunoprecipitated from nuclear extracts isolated from the late embryonic (E18) cortex. MTA2 and HDAC1 were both co-precipitated with SATB2.

We used the the DAPSTER assay to show the specificity of binding of the protein complex to the rAT^{ENK} regulatory sequence. We detected both SATB2 and SATB1 along with MTA2 and HDAC1 in the bound fraction when nuclear extract was pre incubated with no oligo or with mutant, but not wild-type oligo prior to affinity chromatography. *(A. Gyorgy, 2006, submitted)*

5. SATB2 interacts with chromatin remodeling factors *in vivo*.

Using chromatin immunoprecipitation the AT-rich region of the ENK gene was successfully amplified from samples immunoprecipitated with SATB2, HDAC1 or MTA2 antibodies, as well as from the CHRAC17 -precipitated sample, indicating in vivo interaction of these proteins with the AT^{ENK} region. These experiments demonstrated that SATB2 binds to the AT-rich region of the ENK gene *in vivo* in the developing brain, along with three other proteins that are part of chromatin remodeling complexes. *(A. Gyorgy, 2006, submitted)*

6. Cellular co-localization of SATB2, MTA2 and HDAC1

We have mapped the spatial and temporal distribution of SATB2/MTA2 or SATB2/HDAC1 double positive cells by immunohistochemistry using E18 and P2 brain sections. SATB2 and HDAC1 or MTA2, respectively, were showing overlapping localization in the developing cortex in both ages. At E18 the SATB2 (+) regions are mostly restricted to the neocortex. MTA2 and HDAC1 expression is more widely spread, but not generally abundant as it was expected. HDAC1 as well as MTA2 expression are both limited to the neocortex at the age of P2. Confocal microscopy shows nuclear co-localization of HDAC1/SATB2 and MTA2/SATB2. The double positive areas substantially increase from E18 to P2. *(A. Gyorgy, 2006, submitted)*

7. SATB2 represses reporter gene expression in an AT-rich dsDNA and HDAC1 dependent manner

Transiently transfecting C6 glioma cells with plasmids containing an AT-rich or mutant binding sites, we found that the reporter gene activity substantially decreased in the presence of SATB2 in an AT-rich DNA dependent manner. When adding trichostatin A (TSA), an HDAC1 inhibitor, the repression was actually reversed to a slight transactivation, showing that SATB2 in itself is not enough to exert a repression on the reporter gene: it requires HDAC activity as well. *(A. Gyorgy, 2006, submitted)*

V. Discussion

Our goal was to identify nuclear proteins that are expressed in the developing brain and contribute to the developmental regulation of the ENK gene. Two proteins that fit in this category, AUF1 and SATB2 have been identified. Interestingly, AUF1 and SATB2 are two structurally unrelated proteins, however, both bind specifically to the AT-rich DNA of the ENK gene. In the developing brain the expression of both proteins is spatially and temporally restricted, and both have nuclear localization.

SATB2 shares a high amino acid homology with another AT-rich dsDNA binding protein, SATB1, which is known to interact with members of NuRD and CHRAC/ACF chromatin remodeling complexes. It also binds to MARs in the genome, just as SATB2.

We have shown that SATB2 also interacts with two members of the NuRD complex binding to the AT-rich region of the ENK gene by performing DAPSTER, co-immunoprecipitation and chromatin immunoprecipitation assays. The double immunohistochemical stainings also confirmed this finding showing that the majority of SATB2 expressing cells also express MTA2 and HDAC1. Our finding that these two members of NuRD are not ubiquitously expressed in the developing rat brain points to the possibility that these proteins and the complexes they are participating in are much more specific than it was originally thought.

These observations pointed us to the conclusion that SATB2, just as SATB1 suppresses gene expression by functioning as a “guide” for chromatin remodeling complexes. It is also tempting to hypothesize that given the relative abundance of AT-rich sequences in the genome, SATB2 might regulate the phenotypical differentiation of cortical neurons through repressing multiple genes at the same time, which is a crucial pre-requisite of fate acquisition.

VI. Publications

1. Szemes M, Gyorgy A, Paweletz C, Dobi A, Agoston DV *The first two authors contributed equally to this work*
Isolation and Characterization of SATB2, a Novel AT-rich DNA Binding Protein Expressed in Development- and Cell-Specific Manner in the Rat Brain.
Neurochem Res. 2006 Apr 4. PMID: 16604441
2. Albert Dobi, Marianna Szemes, Cheol Lee, Miklos Palkovits, Francis Lim, Andrea Gyorgy, Mark A. Mahan, Denes V. Agoston
AUF1 is expressed in the developing brain, binds to AT-rich dsDNA and regulates enkephalin gene expression
Accepted with modicications: Journal of Biological Chemistry (JBC), 2006.
3. Andrea Gyorgy, Marianna Szemes, Denes V. Agoston
The special AT-rich DNA binding protein SATB2 is co-expressed with chromatin remodellers in a subset of developing neocortical neurons and regulates gene expression through altered histone acetylation
Submitted J. Neurochemistry, 2006.

Conference posters
A) D.V. Agoston, C. Lee, A. Dobi, M. Palkovits, A. Gyorgy, M. Szemes.
The hematopoietic master-regulator SATB1 is expressed in the developing brain and regulates the development of the enkephalin phenotype through chromatin modifications.
Program No. 35.7. 2004. Society for Neuroscience.
B) A. Gyorgy, C. Lee, M. Szemes, A. Dobi, D.V. Agoston. Molecular and functional multitasking: the regulator of mRNA stability AUF1/hnRNP D controls gene expression upon binding to AT-rich dsDNA, participates in chromatin remodeling and is involved in regulating adult de novo neurogenesis.
Program No. 478.8. *2005* Society for Neuroscience

Other publications
4. Rutkai E, Gyorgy A, Dorgai L, Weisberg RA
Role of secondary attachment sites in changing the specificity of site-specific recombination. J Bacteriol. 2006 May;188(9):3409-11. PMID: 16621836
5. Kovacs L, Marczinovits I, Gyorgy A, Toth GK, Dorgai L, Pal J, Molnar J, Pokorny G.
Clinical associations of autoantibodies to human muscarinic acetylcholine receptor 3(213-228) in primary Sjogren's syndrome.
Rheumatology (Oxford). 2005 Aug;44(8):1021-5. Epub 2005 May 11. PMID: 15888503
6. Marczinovits I, Kovacs L, Gyorgy A, Toth GK, Dorgai L, Molnar J, Pokorny G.
A peptide of human muscarinic acetylcholine receptor 3 is antigenic in primary Sjogren's syndrome.
J Autoimmun. 2005 Feb;24(1):47-54. Epub 2005 Jan 20. PMID: 15725576

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