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Comparative distribution of protein components of the A20 ubiquitin-editing complex in normal human brain

Elaine L. Pranski[a,b,*], Carson D. Van Sanford[a,b], Nirjari V. Dalal[a,b], Adam L. Orr[a,b], Dipan Karmali[a,b], Deborah S. Cooper[b,c], Nichole Costa[b,c], Craig J. Heilman[a,b], Marla Gearing[b,c], James J. Lah[a,b], Allan I. Levey[a,b], Ranjita S. Betarbet[a,b]

[a] Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, United States
[b] Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA 30322, United States
[c] Department of Pathology and Lab Medicine, Emory University School of Medicine, Atlanta, GA 30322, United States

HIGHLIGHTS

- Components of the A20 ubiquitin-editing complex are present in the human brain.
- A20 complex components are mainly expressed in neurons.
- A20 complex components are differentially expressed in various regions of the human brain.

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ABSTRACT

Activation of innate and adaptive immune responses is tightly regulated, as insufficient activation could result in defective clearance of pathogens, while excessive activation might lead to lethal systemic inflammation or autoimmunity. A20 functions as a negative regulator of innate and adaptive immunity by inhibiting NF-κB activation. A20 mediates its inhibitory function in a complex with other proteins including RNF11 and Itch, both E3 ubiquitin ligases and TAX1BP1, an adaptor protein. Since NF-κB has been strongly implicated in various neuronal functions, we predict that its inhibitor, the A20 complex, is also present in the nervous system. In efforts to better understand the role of A20 complex and NF-κB signaling pathway, we determined regional distribution of A20 mRNA as well as protein expression levels and distribution of RNF11, TAX1BP1 and Itch, in different brain regions. The distribution of TRAF6 was also investigated since TRAF6, also an E3 ligase, has an important role in NF-κB signaling pathway. Our investigations, for the first time, describe and demonstrate that the essential components of the A20 ubiquitin-editing complex are present and mainly expressed in neurons. The A20 complex components are also differentially expressed throughout the human brain. This study provides useful information about region specific expression of the A20 complex components that will be invaluable while determining the role of NF-κB signaling pathway in neuronal development and degeneration.

1. Introduction

The NF-κB signaling pathway is well known for its ubiquitous roles in inflammation, immune responses, and control of cell division and apoptosis [21]. These roles of NF-κB signaling are apparent in the central nervous system (CNS) where they can range from neuronal development, synaptic signaling that underlies learning and memory and coordination of immune responses to toxic stimuli [11,14]. Activation of NF-κB is normally transient, and persistent

Abbreviations: CNS, central nervous system; GUSB, glucuronidase-beta; HEK293, human embryonic kidney 293; qRT-PCR, quantitative real-time PCR; RNF11, RING finger protein 11; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tax1, human T-cell leukemia virus type I; TAX1BP1, Tax1 binding protein 1; TNFAIP3, tumor necrosis factor, alpha-induced protein 3; TRAF, tumor necrosis factor receptor-associated factor.

* Corresponding author at: Emory University, 615 Michael Street, Suite 500, Atlanta, GA 30322, United States. Tel.: +1 404 727 6136; fax: +1 404 727 3728.
E-mail addresses: epransk@emory.edu (E.L. Pranski), cvansan2@gmail.com (C.D. Van Sanford), nvdalal@emory.edu (N.V. Dalal), aorr25@gmail.com (A.L. Orr), karmali.dipan@gmail.com (D. Karmali), dscoope@emory.edu (D.S. Cooper), ncosta@emory.edu (N. Costa), cheilma@emory.edu (C.J. Heilman), mgearin@emory.edu (M. Gearing), jlah@emory.edu (J.J. Lah), alevey@emory.edu (A.I. Levey), rbetarb@emory.edu (R.S. Betarbet).

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NF-κB activation is associated with several autoimmune diseases and cancer in the peripheral system [25] and in both acute (i.e., stroke, seizures) and progressive (Parkinson's disease, Alzheimer's disease, Amytropic Lateral Sclerosis) neurodegenerative disease [12]. However, inhibition of NF-κB signaling can prevent adult neurogenesis in the dentate gyrus [14]. Dysregulation of NF-κB signaling has been cited as a potential source of synaptic pathology in AD and PD due to the importance of NF-κB transcriptional regulation of spine density in mature neurons [6]. Thus, duration of NF-κB activation is tightly regulated causing genes that play key roles in amplification and effector functions to be actively repressed under basal conditions [28]. One such regulator of NF-κB activation is A20 (also known as TNFAIP3), an ubiquitin-editing protein, which regulates NF-κB activation in a negative feedback loop [28]. Specifically, A20 contains several NF-κB binding sites within the promoter of the human gene to induce its expression in response to NF-κB signaling [16]. Recent experiments have established that A20 mediates its inhibitory function in a complex with three other proteins, Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1, also called TXBP151 or T6BP), and two E3 ubiquitin ligases, Itch (also known as AIP3) and RING finger protein 11 (RNF11) [23,24]. The inhibitory role of A20 is critically dependent on its interaction with other complex members, TAX1BP1, Itch and RNF11. TRAF6, a member of the tumor necrosis factor receptor-associated factor (TRAF) family, and a known substrate of A20 is also an E3 ligase [23,24]. To better understand the role of A20 complex in the CNS we determined the presence and distribution of RNF11, TAX1BP1, Itch and TRAF6 proteins in normal human brain. Since A20 is an inducible protein [5], we have determined A20 mRNA expression levels in various normal human brain regions.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293 (HEK293) were purchased from American Type Culture Collection and were maintained at 37 °C and 5% CO_2. HEK293 cells were cultured in DMEM with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Bio Whittaker).

2.2. Human tissue

Post-mortem, control (pathologically clean) brains ($n = 3$) were obtained through Emory University's Brain Bank. The average age at death of the samples was 64 years. Blocks of formalin fixed tissue at the level of cerebral cortex, midbrain, pons, or inferior olive were sectioned on a freezing microtome. Free-floating sections (50 μm) were stored at −20 °C in ethylene glycol cryopreservative.

2.3. Immunohistochemistry

Immunohistochemistry for single-label light microscopy and double-label fluorescence microscopy has been described previously [4]. For light microscopy, 3,3′-diaminobenzidine was used as chromophore. Routine hematoxylin was used to counter-stain nuclei in hippocampal sections following single labeling. For fluorescent microscopy, appropriate secondary antibodies with fluorescent chromophores were used. For control experiments, primary antibodies were omitted. Immunostained sections were imaged using bright-field Olympus microscope or using a Zeiss LSM 510 laser scanning confocal microscope. Images were processed using Adobe Photoshop 7.0 software.

2.4. Antibodies

Antibodies used were: A20 (ab13597, Abcam), A20 (NBP1-40684, Novus), A20 (550859, BD Pharmingen), A20 (sc-166692, Santa Cruz), GFAP (MAB360, Millipore), Iba1 (ab5076, Abcam), Itch (611198, BD Transduction), Olig2 (MABN50, Millipore), rabbit polyclonal RNF11 (described in [1]), TAX1BP1 (ab22049, Abcam), and Traf6 (ab13853, Abcam).

2.5. Plasmids and transfections

Flag-A20, Flag-Itch, Flag-TAX1BP1, and Flag-TRAF6 were a kind gift from Dr. Edward Harhaj (University of Miami). Transient transfections of HEK293 cells were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol.

2.6. Quantitative real-time PCR

Total RNA was isolated from approximately 100 mg of human brain tissue that was homogenized and sonicated before using a RNeasy extraction kit (Qiagen). cDNA was created using a High Capacity cDNA reverse transcription kit (Ambion). Real-time PCR was performed on a 7500 Fast RT-PCR instrument (Applied Biosystems) using TaqMan PCR master mix (Applied Biosystems) and gene-specific TaqMan probes (Applied Biosystems) against RNF11 (Hs00702517_s1), A20 (Hs00234712_m1) and glucuronidase-beta (GUSB) (Hs99999908_m1). RNA samples were run in triplicate. Gene expression was normalized to the house-keeping gene GUSB and relative expression was calculated for each gene using $2^{-\Delta\Delta Ct}$ method.

2.7. Immunoblotting

Immunoblotting was performed as previously described [1]. Cells were transfected for 24 h with indicated plasmids and harvested in PBS with 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.1% Triton 100×, 1% NP-40, Halt phosphatase inhibitor cocktail (Pierce), and protease inhibitor cocktail (Roche Diagnostics). Samples were spun at 14,000 rpm before being separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were scanned using the Odyssey Image Station (LiCor).

3. Results

Sections of adult normal brain tissue were immunostained following characterization of commercial antibodies, against RNF11, TAX1BP1, Itch and TRAF6. The regional distribution and expression levels of different proteins and A20 mRNA are described below.

3.1. Antibody characterization

To determine specificity of the antibodies used for immunohistochemistry, we looked at endogenous expression of complex members and overexpression of FLAG-tagged constructs in HEK293 cells (Supplemental Fig. S1). We tried numerous commercially available antibodies against A20 but they either did not label human brain tissue or the specificity could be confirmed by pre-adsorption. However, in HEK293 cells, endogenous levels of A20 were detectable at the predicted molecular weight for this protein using immunoblotting (Supplemental Fig. S1A). Endogenous expression levels of Itch, TAX1BP1, and Traf6 were also detectable with a band near their predicted molecular weight (Supplemental Fig. S1B–E). With confirmation that our antibodies were specific for the different A20 complex members, we set out to explore the distribution of the complex in different regions of the human brain.