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Activation of the brain-specific neurogranin gene in murine T-cell lymphomas by proviral insertional mutagenesis

Anne Ahlmann Nielsen [a,1,2], Kristín Rós Kjartansdóttir [a,1,3], Mads Heilskov Rasmussen [a], Annette Balle Sørensen [a,4], Bruce Wang [b], Matthias Wabl [c], Finn Skou Pedersen [a,*]

[a] Department of Molecular Biology, Aarhus University, C.F. Møllers Allé, Bldg. 1130, DK-8000 Århus C, Denmark
[b] Picobella, 863 B Mitten Road, Burlingame, CA 94010, USA
[c] Department of Microbiology and Immunology, University of California, 513 Parnassus Avenue, San Francisco, CA 94143, USA

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ABSTRACT

Neurogranin (Nrgn) is a highly expressed brain-specific protein, which sequesters calmodulin at low Ca^{2+}-levels. We report here on retroviral activation of the *Nrgn* gene in tumors induced by the T-cell lymphomagenic SL3-3 murine leukemia virus. We have performed a systematic expression analysis of Nrgn in various mouse tissues and SL3-3 induced T-cell tumors. This demonstrated that insertional activation of Nrgn increased RNA and protein expression levels to that observed in brain. Furthermore, elevated *Nrgn* expression was also observed in some T-cell tumors with no detected provirus integrations into this genomic region. The presented data demonstrate that Nrgn can be produced at high levels outside the brain, and suggest a novel oncogenic role in T-cell lymphomas in mice.

1. Introduction

Neurogranin (Nrgn, also denoted RC3) belongs to the neuron-specific calpacitin family of proteins, and functions as a calmodulin (CaM) storage protein at low Ca^{2+} levels (Baudier et al., 1991; Gerendasy, 1999; Prichard et al., 1999; van Dalen et al., 2003). The focus on Nrgn's function has been primarily in brain tissues, and several studies have demonstrated that the protein plays a role in learning and memory (Pak et al., 2000; Huang et al., 2004, 2006). The expression level of *Nrgn* is highest in cortex, striatum and hippocampus, while lower levels are detected in the olfactory bulb and midbrain as analyzed in rat and human tissue samples (Represa et al., 1990; Watson et al., 1990; Martinez de Arrieta et al., 1997). Additionally, low levels of expression have been reported in rat thymus and spleen (Watson et al., 1990).

Murine *Nrgn* consists of four exons of which the first two encompass the coding sequence for a 78-amino acid protein. It is located in a gene-dense region on chromosome 9 surrounded by *Esam*1 (endothelial cell-selective adhesion molecule), *Vsig*2 (V-set and immunoglobulin domain containing 2 (also known as CTM)), *Ysg*2 (yolk sac gene 2/Sialic acid-specific 9-*O*-acetylesterase (*Siae*)) and *Spa*17 (sperm autoantigenic protein 17). While *Esam*1 and *Spa*17 have been reported to play a role in cancer development, no carcinogenic role has been correlated with *Vsig*2 or *Ysg*2. *Esam*1 belongs to the immunoglobulin receptor family and may play an important role in pathological angiogenic processes such as tumor growth (Hirata et al., 2001; Ishida et al., 2003). *Spa*17 is a member of the cancer/testis antigen family and is expressed in various human cancers including multiple myeloma, ovarian cancer and nervous system tumors (Lim et al., 2001a,b; Chiriva-Internati et al., 2002; Grizzi et al., 2006).

Murine leukemia viruses (MLVs) induce hematopoietic tumors when injected into newborn susceptible mice (for recent reviews see Mikkers and Berns, 2003; Uren et al., 2005). Tumor induction by non-acutely transforming MLVs is a complex process containing multiple steps of which the activation of cooperating genes by retroviral insertional mutagenesis is believed to play an important role in the clonal expansion of target cells into full-blown tumors (Mikkers and

Abbreviations: Actb, beta-actin; CaM, calmodulin; Esam1, endothelial cell-selective adhesion molecule 1; LTR, long terminal repeat; MLV, murine leukemia virus; Nrgn, Neurogranin; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; RIS, retrovirus integration site; RTCGD, Retroviral Tagged Cancer Gene Database; Siae, Sialic acid-specific 9-O-acetylesterase; Spa17, sperm autoantigenic protein 17; UBC, Ubiquitin C; Vsig2, V-set and immunoglobulin domain containing 2; Ysg2, yolk sac gene 2.

* Corresponding author. Tel.: +45 89422614; fax: +45 86196500.
E-mail address: fsp@mb.au.dk (F.S. Pedersen).

[1] AAN and KRK share first-authorship.
[2] Present address: Technical University of Denmark, National Veterinary Institute, Hangøvej 2, DK-8200 Aarhus N., Denmark.
[3] Present address: The Scientific Unit, Horsens Hospital, DK-8700 Horsens, Denmark.
[4] Present address: The State and University Library, Universitetsparken, DK-8000 Aarhus C, Denmark.

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Berns, 2003; Uren et al., 2005). Large-scale screenings in various virus/host systems have identified thousands of insertion sites of which several hundred represent genes or loci with putative oncogenic potential (recently reviewed in Uren et al., 2005; Touw and Erkeland, 2007). Many of these sites are accessible online in the Retroviral Tagged Cancer Gene Database RTCGD (http://rtcgd.abcc.ncifcrf.gov/) (Akagi et al., 2004). The murine leukemia virus SL3-3 is a potent inducer of T-cell lymphomas in susceptible mice with a mean latency between two and four months (Sørensen et al., 2004; Ejegod et al., 2009). Previously, we reported on the enhancer mutant SL3-3(turbo) (Ethelberg et al., 1997b; Nielsen et al., 2005). SL3-3(turbo) has an extra LTR repeat in combination with deletion of two binding site sites for nuclear factor 1, which significantly shortened the mean latency time of T-cell lymphoma induction in mice (Ethelberg et al., 1997b).

In the present work, we report on insertional activation of *Nrgn* as a result of SL3-3(turbo) and SL3-3 wt integration into a novel retroviral insertion site, the gene-dense *Esam*1/*Vsig*2/*Nrgn*/*Ysg*2/*Spa*17-locus, and give a comprehensive expression analysis of *Nrgn* in mouse T-cell tumors as well as in normal tissue.

2. Materials and methods

2.1. Tumor and control material

Tumor material from the SL3-3(turbo)/inbred NMRI model originates from a previous study (Ethelberg et al., 1997b). Tumor material from the SL3-3 wt/BALB/c model originates from a study described in Glud et al. (2005) and Wang et al. (2006). Control tissues were isolated from mock-injected and non-treated NMRI and BALB/c mice, respectively. Mice were kept according to approved regulations and monitored on a daily basis. Upon signs of illness or development of tumors of defined sizes mice were terminated and relevant organs removed and stored at −80 °C.

2.2. Extraction of total RNA and genomic DNA

Total RNA and genomic DNA were extracted from frozen control or tumor tissues using the TRIzol® Reagent (Invitrogen™) or DNeasy® Blood & Tissue Kit (Qiagen), respectively, following the manufacturer's protocol.

2.3. PCR detection of proviral integrations

Identification of SL3-3(turbo) integration sites in NMRI mice was done using the two-step PCR approach described in Sørensen et al. (1993). Screening and validation of *Nrgn* promoter insertion sites and orientations were done by PCR using the provirus-specific primers 2620 (5′-GATCCCCGGTCATCTGGG-3′; specific for the minus strand of the U3 region of the long terminal repeat) or 6197 (5′-CCCAGATGACCGGGGATC-5′; specific for the plus strand of the U3 region) in combination with either of the *Nrgn* promoter-specific primers 5′-CTCATAAGCCCCTCCTCTTTCCAT-3′ (plus strand) and 5′-CCCACTCATTCTCCCTTTAACA-3′ (minus strand). PCR amplification products were sequenced (ABI™ BigDye Terminators, Applied Biosystems) with retrovirus primers 793 (5′-CTCTGGTATTTTTCCCATG-3′) or 540 (5′-TCCGAATCGTGGTCTCGCTGATCCTTGG-3′) (Nielsen et al., 2005). Primers were purchased from DNA Technology A/S. PCR was performed with Taq DNA polymerase (Invitrogen™) using reaction conditions as described previously (Nielsen et al., 2005). Provirus 1423 and 3427 were detected by a splinkerette-based PCR method (Mikkers et al., 2002), and is described in Wang et al. (2006).

2.4. Southern and Northern blot analyses

Southern blot (Ethelberg et al., 1997a) and Northern blot (Rasmussen et al., 2005) analyses were done with random labeled DNA probes as described in Sørensen et al. (2000). Hybridization conditions were either ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion) or Na_2HPO_4/NaH_2PO_4 buffer (Ethelberg et al., 1997a). Probe A was a PCR product from mouse DNA using primers 5′-CCCACTCATTCTCCCTTTAACA-3′ and 5′-CTCATAAGCCCCTCCTCTTTCCAT-3′. Probe B-D were PCR amplification products from brain total cDNA using the following primers: 5′-GAAAGTGTCTTCTGATTGGCTTCGAG-3′ and 5′-CACAGTAGGGAAGTCTTGTCACTGCG-3′ (Probe B), 5′-CTCTTCAGTCTAACGTGGTCTCCT-3′ and 5′-CGCAGAGATTAAAAACCTTCCAGCCA-3′ (Probe C), 5′-CAACCACCAAGTCCTTTCGT-3′ and 5′-GGTAACATGCACACGCAGAG-3′ (Probe D). Northern-blot hybridization of the multiple-tissue Northern filter containing poly(A)+ RNA (Clontech Laboratories, Inc.) was performed according to the manufacturer's protocol.

2.5. Reverse transcriptase PCR

For each reverse transcriptase PCR (RT-PCR) reaction, cDNA (First-Strand CDNA Synthesis Kit (GE Healthcare)) originating from 1.5 ng of total RNA was used as template. The different RT-PCR products (Fig. 1A) were amplified with Taq DNA polymerase (Invitrogen™) using the following primers: 5′-GCTCAAAGTGCTGGTTCCTC-3′ and 5′-GAGACACTGGGTGTGGGAGT-3′ (*Esam*1), 5′-ACTGGGACCTACCTCTGCAA-3′ and 5′-CATCCTCCCGAAGGTCACTA-3′ (*Vsig*2), 5′-CCCTGAGCTGCCACCCAGCAT-3′ and 5′-ATCTTCTTCCTCGCCATGTG-3′ (*Nrgn*), 5′-CAACCACCAAGTCCTTTCGT-3′ and 5′-GGTAACATGCACACGCAGAG -3′ (*Nrgn*, acc. no. NM_022029), 5′-ATGTCGATTCCTTTCTCCAACAC-3′ and 5′-GGGGGTAAAACCTGTGGTCT-3′ (*Spa*17), 5′-GGCCTGTGTTTGGGATAGTG-3′ and 5′-AAAGGACATGAGGACTCCTCAC-3′ (*Ysg*2), 5′-GAAACCTCTCTTCTGGACAAG-3′ and 5′-AAAGGACATGAGGACTCCTCAC-3′ (AF156856), and 5′-TCAACACCCCAGCCATGTACGTAGCCATCC-3′ and 5′-ACATCTGCTGGAAGGTGGACA-3′ (β-actin, *Actb*). The integrity and size of the amplification products were validated by agarose gel electrophoreses and sequencing. Prior to sequencing using the employed PCR primers and ABI™ BigDye Terminators (Applied Biosystems), amplicons were excised from agarose gels and purified using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

2.6. Quantitative real-time PCR

For each quantitative real-time PCR (qRT-PCR) reaction, cDNA (First-Strand cDNA Synthesis Kit (GE Healthcare)) originating from 1.5 ng of total RNA was used as template. qRT-PCR was performed on a Stratagene MX3005 apparatus (AH Diagnostics), using TaqMan probes, assays-on-Demand™ (Applied Biosystems) (Nrgn: Mm00480741_m1, exons 1–2 and UBC: Mm01201237_m1, exons 1–2), and run in 20 μl using TaqMan Universal PCR Master Mix as specified by the manufacturer. Relative quantification was performed using a standard curve method on cDNA isolated from wild-type mouse brain (Nrgn amplifications) and thymus (UBC amplifications), and presented as normalized to Ubiquitin C (UBC) signal. All samples were performed in duplicate. The amplification PCR program was: 95 °C for 10 min (1 cycle), 95 °C for 15 s and 60 °C for 1 min (40 cycles). Data were analyzed by using Mx3005 software.

2.7. Purification of proteins and Western blot analysis

Whole-cell extracts were isolated from frozen tissue samples by lysis in 360 μl lysis buffer (0.1 M NaCl, 0.01 M Tris–HCl (pH 8.0), 0.5 mM EDTA (pH 8.0) and 0.5 mM PMSF) followed by 30 min of incubation on ice and 10 min of centrifugation at 20,000 ×*g*. Samples equivalent to 10 μg of total proteins (BCA™ Protein Assay Kit, Pierce Biotechnology) were resolved on a 12.5% polyacrylamide gel and transferred to an Immobilon™-P transfer membrane (Millipore A/S). The Western blot was probed with primary antibodies Anti-