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Research Article

An 8-bp deletion in mNOTCH4 intron 10 leads to its retention in mRNA and to synthesis of a truncated protein

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Abstract

Notch signaling participates in the development of multicellular organisms by maintaining self-renewal potential or inducing differentiation of numerous tissues. In this study, we characterized Notch4, the evolutionary most distant and least studied Notch family member. We identified a Notch4 inter-strain polymorphism with a previously undescribed mRNA variant. This longer Notch4 mRNA, which represented up to one-third of total Notch4 mRNA, resulted from intron 10 retention. Analysis of Notch4 intron 10 revealed that an 8-bp deletion, reducing its length from 68 to 60 bp, strictly correlated with its retention. Further experiments demonstrated that intron length was the only cause of the mis-splicing. Moreover, this mRNA variant resulted in a truncated protein containing half the extracellular domain of Notch4, including the ligand-binding domain.

Keywords: Notch; Intron length; mRNA splicing; Polymorphism; Truncated protein

Introduction

The description in 1917 of a *Drosophila* strain with notches at the end of the wing blades and subsequent cloning of the defective gene 70 years later were milestones in the study of Notch receptors [1,2]. Mammals express four Notch genes located in paralogous regions on distinct chromosomes. Although synthesized as single-pass transmembrane precursors, Notch proteins are cleaved into two subunits within the trans-Golgi network, yielding a heterodimeric protein expressed at the cell membrane. Notch receptors consist of an extracellular subunit that binds Notch ligands, linked non-covalently to a transmembrane subunit containing several functional domains (in the intracellular part) that mediate notch signal transduction [3]. Notch proteins have been highly conserved through evolution and differ mainly by the number of EGF-like repeats (29 to 36) in their extracellular domain.

The main structural motif of the Notch extracellular portion is the EGF-like repeat composed of about 40 amino acids, including six cysteine residues and a mainly β-sheet structure. Only the eleventh and twelfth EGF-like repeats contained in the Notch extracellular domain are involved in ligand binding [4]. The Notch extracellular subunit interacts with ligands of the DSL (Delta, Serrate and Lag2) family (Delta and Jagged in mammals), which are also transmembrane proteins carrying EGF-like repeats [5–9]. Recent studies show that this receptor–ligand interaction is strongly regulated by post-translational glycosylation of EGF-like repeats [10].

Notch-ligand interaction occurs between two neighboring cells and leads to two successive proteolytic cleavage steps that release the cytoplasmic portion of Notch (Notch-IC) from the membrane. Notch-IC enters the nucleus and binds to the transcriptional repressor CSL (CBF1/RBPJκ, Su(H), Lag1), that is subsequently converted into a transcriptional activator [10].

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Notch signaling participates in the development of multicellular organisms by maintaining self-renewal potential and inducing differentiation of numerous tissues. Among the known functions of Notch receptors, their involvement in cell fate decisions is one of the best documented, and its importance has been demonstrated in a wide array of developmental processes, from neurogenesis to hematopoiesis [11,12]. Mutations in Notch proteins have been implicated in a wide variety of diseases [13–15].

Most of our knowledge of Notch functions and signaling comes from loss-of-function studies of individual Notch family members or from studies of Notch1 functions [16]. Moreover, Notch signaling has generally been studied with Notch-IC, a constitutively active form of the receptor. Despite the clear functional redundancy of Notch family members, each member may be involved in specific processes.

Among the four Notch proteins described in mammals, Notch4 appears to be the most distant, in evolutionary terms, from other Notch members, bearing fewer EGF repeats, a shorter intracellular domain, and no cytokine-responsive elements. Notch4 also differs from the other Notch receptors by its expression pattern in mice [17]. The most extensively characterized function of Notch4 is its involvement in vascular development and remodeling [18,19]. To our knowledge, this is the only Notch4-specific function that has been precisely studied.

In order to further characterize Notch4, we sequenced and cloned the entire mouse cDNA. We identified a Notch4 mRNA splice variant produced in some but not all mouse strains, as well as the molecular mechanism underlying its generation. This mRNA variant is translated into a truncated protein containing half the extracellular domain of Notch4, including the ligand-binding domain.

Materials and methods

Mice

AKR, Biozzi, CBA, MRL, NZB, NZW and SWR mice were from Harlan (Gannat, France); BALB/c, C3H, DBA/2, C57Bl/6 and SJL mice were from Centre d'élevage Janvier (Le Genest Saint Isle, France); NOD mice were from Centre de Développement des Techniques Avancées (Orléans, France); 129/Sv mice were maintained in our own animal facilities, and PWK mice were kindly provided by P. Avner (Institut Pasteur, Paris, France). At 8–9 weeks of age, mice were sacrificed, and organs were harvested and stored in liquid nitrogen. All animal studies were approved by our institutional review board.

Cell culture

NIH3T3 cells (ATCC CRL-1658, American Tissue Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen). Cultures were maintained at 37°C in a humidified air–5% CO_2.

RNA purification

Total RNA was isolated from frozen tissues and cell lines by using the RNAble kit (Eurobio, Les Ulis, France) and the Absolutely RNA microprep kit (La Jolla, CA), respectively, following the manufacturers' instructions. RNA was quantified by absorbance at 260 nm and checked for integrity by electrophoresis on agarose gel with ethidium bromide staining.

RT-PCR and genomic analysis

Total RNA (2.5 μg) was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) using 100 ng of Random Hexamers or 0.2 μM gene-specific primer (5′-CCGCCACTGTGCTGGATATCT-3′) in Minigene experiments.

Notch4 cDNA analysis

PCR reactions were performed with 0.2 μM each primer (forward primer (5′-TGCCTCTGCCTTCCTGGATTC-3′) and reverse primer (5′-TTCTCGCAGTGTGGCCCTTC-3′)) in a 25-μl reaction mix containing 1.5 μl cDNA, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.2 mM each dATP, dCTP, dGTP and dTTP, and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA). After initial denaturation at 94°C for 3 min, samples were amplified for 35 cycles at 94°C for 30 s and 68°C for 30 s, followed by a final extension step at 72°C for 10 min. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons. The expected size of the PCR product was 155 bp.

Notch4 intron 10 analysis

PCR amplification reactions were performed as described above, using forward primer 5′-CGTGTTGCCCA-AACGCCTAGAGTCT-3′ and reverse primer 5′-CTCACA-CACCCTCCCAGTTCTTTCTCTT-3′ in a 25-μl reaction mix containing 100 ng of DNA. PCR conditions comprised 1 cycle of 94°C for 2 min followed by 30 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, and 1 cycle at 72°C for 10 min. Ten-microliter aliquots of PCR product was digested with 5 U *Ava*II in a total volume of 15 μl for 1 h at 37°C. The expected size of the digestion products was 250 and 200 bp.

PCR products were separated on 4.0% agarose gels and stained with ethidium bromide.

Plasmid constructs

Full-length Notch4 constructs were generated in a five-step protocol. Five fragments were generated by PCR from