Functional conservation of mouse Notch receptor family members

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Abstract All the known members of the mouse Notch receptor family were examined for their biochemical function by interaction with a DNA binding protein RBP-Jκ. mNotch2, mNotch3 and int3 (= mNotch4) were shown to interact with RBP-Jκ by the GST-fusion pull down assay and dominant negative competition with Epstein Barr virus nuclear antigen 2. Furthermore the intracellular region of int3 was shown to transactivate the Epstein Barr virus TP1 promoter. These results indicate that all mouse Notch family members have biochemical functions similar to mNotch1, which transduces proliferative signal by direct interaction with the DNA binding protein RBP-Jκ.

Key words: Mouse Notch receptor; Biochemical function; DNA binding protein interaction

1. Introduction

Notch is a large transmembrane receptor involved in the regulation of nervous system development [1,2]. The Notch receptor consists of an extracellular region containing 36 EGF repeats and 3 lin12/Notch repeats and an intracellular region containing the RAM region, six cdc10/ankyrin repeats, nuclear localization signals and PEST sequences. EGF repeats are generally involved in protein-protein interaction and ligand binding: EGF repeats 11 and 12 of the Notch receptor are essential and sufficient for binding to the ligand Delta [3]. The RAM region [4] is involved in interaction with RBP-Jκ, the mammalian homologue of *suppressor of hairless*, which binds to DNA directly and also interacts with Epstein Barr virus nuclear antigen 2 (EBNA2). Cdc10/ankyrin repeats are also involved in protein-protein interaction in a number of proteins but the exact function of the Notch ankyrin repeats is not known.

Many members of the Notch receptor family have been shown to exist in vertebrates including zebra fish, frog, rat, mouse and human [5–9]. Three mouse Notch family genes (mNotch1, mNotch2 and mNotch3) have been identified [10–12]. These three genes encode proteins remarkably similar in both length and structure; their amino acid sequences are approximately 50% homologous to each other and also to the *Drosophila* Notch protein, suggesting that they share similar biological functions [1,2,12]. However, there is no evidence that mNotch2 and mNotch3 have biochemical functions similar to mNotch 1. In addition, the int3 gene is also assumed to be a Notch-related gene because of its high homology with Notch [13–17].

To examine whether mNotch2, mNotch3 and int3 are biochemically similar to mNotch1, we tested their abilities to interact with RBP-Jκ and to transactivate the TP1 promoter of EB virus. Our results clearly indicate that mNotch2, mNotch3 and int3 are all able to interact with RBP-Jκ and that int3 can transactivate the TP1 promoter through interaction with RBP-Jκ. These results indicate that mNotch1, mNotch2, mNotch3 and int3 (or mNotch4) are functionally similar.

2. Materials and methods

2.1. Plasmid construction

mNotch1-RAM, mNotch2-RAM, mNotch3-RAM and int3-RAM fragments were generated by polymerase chain reaction (PCR) amplification based on published sequences [10–12]. The cloned fragment is shown in Fig. 1. These fragments were cloned into the GST-fusion vector (pGEX 4T1; Pharmacia). The RAM regions cloned into the GST-fusion vector were added with the c-*myc* tag (5′-AACACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAGAG-3′) at the 5′ end. RAM regions with the c-*myc* tag were obtained as blunt ended fragments using appropriate restriction enzymes (*HpaI-ScaI*) and cloned into the blunt ended pEF-BOS neo vector [18]. A part of the int3-RAMIC fragment (Gln-1465–Ala-1787) was amplified by PCR based on published sequences [16]. PCR was performed on cDNA corresponding to 0.1 μg of total RNA from D3 ES cells. The rest of the int3-RAMIC fragment (Arg-1788–Asn-1964) was derived from an ICR mouse embryo cDNA library (8.5 d.p.c.). To generate the entire int3-RAMIC fragment, these two fragments were ligated at the *SmaI* site. The int3-RAMIC fragment containing the c-*myc* tag at the 5′ end was cloned into the pEF-BOS neo expression vector as described above. pSG5-EBNA2 was obtained from G. Bornkamm [19]. All the PCR amplified DNAs were confirmed by nucleotide sequence determination.

2.2. In vitro interaction by GST pull down assay

E. coli DH5α was transformed with pGEX4T1 containing the inserts of interest and GST-fusion proteins were extracted according to the standard protocol (Pharmacia). The purification of GST-fusion proteins using glutathione-Sepharose 4B beads (Pharmacia) and the in vitro synthesis of [^{35}S]RBP-Jκ were done as described previously [4]. The same amount of purified GST-fusion proteins was mixed with [^{35}S]RBP-Jκ and incubated at 4°C for 1 h and washed 6 times with RIPA buffer before analysis.

2.3. Cell culture and luciferase assay

COS7 cells were maintained in DMEM supplemented with 10% FCS. 1.0×10^5 cells were seeded in 35 mm tissue culture plate the day before transfection. 2.6 or 3.0 μg of plasmids was transfected into COS7 cells by lipofection. 20 μg of lipofectAMINE reagent (Life Technologies) was used for each lipofection. After incubating the cells with the DNA-lipofectAMINE complex at 37°C in a CO_2 incubator for 6 h, the transfection mixture was removed and replaced with normal growth medium. pGa981-6 is a reporter plasmid ERE-

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TP1 luciferase, and ptk-Luc177 was used as a reporter plasmid for the negative control. The luciferase assay was performed 48 h after lipofection [20]. The values of luciferase activity were normalized by transfection efficiency as measured by β-galactosidase activity. Expression of RAM domains and RAMIC was detected in the nucleus as well as cytoplasm.

3. Results and discussion

3.1. Interaction of Notch members with RBP-Jκ

We have shown that several residues in the RAM region of mNotch1 are critical for interaction with RBP-Jκ [4]. To examine whether mNotch2, mNotch3 or int3 can interact with RBP-Jκ, we first confirmed and corrected DNA sequences of putative RAM regions in mNotch2, mNotch3 and int3 using cDNA obtained from the embryonic stem cell (D3) mRNA. As shown in Fig. 1, the sequences immediately downstream of the transmembrane of the Notch members are not strongly conserved but the critical residues for interaction with RBP-Jκ [4], i.e. Arg-1752, His-1754, Trp-1758 and Pro-1760, are conserved. It is important to stress that the above residues are conserved among almost all the Notch proteins from various species. We then tested for their ability to bind directly to RBP-Jκ. [^{35}S]RBP-Jκ protein was incubated with GST-fusion proteins containing the RAM regions (Fig. 1) of the Notch members. As shown in Fig. 2, [^{35}S]RBP-Jκ was retained on glutathione-Sepharose beads as a complex with GST-mNotch2-RAM, GST-mNotch3-RAM and GST-int3-RAM as well as GST-mNotch1-RAM but not with GST itself. The relative amounts of RBP-Jκ complexed with the same amount of mNotch1, mNotch2, mNotch3 and int3 were 8.6, 5.2, 5.8 and 1, respectively. As a negative control, ^{35}S-labeled in vitro translation product of luciferase was used instead of RBP-Jκ. There was no interaction of luciferase with any of GST-fusion proteins (data not shown).

It is important to examine interaction of the RAM regions with RBP-Jκ in vivo. EBNA2 is a pleiotropic activator of viral and cellular genes and responsible for transformation of human B cells by the virus. It is known that EBNA2 has no DNA binding site and its transcriptional activity is at least in part mediated by RBP-Jκ [21–25]. We next tested the interaction of the RAM regions with RBP-Jκ in vivo by their dominant negative inhibition of the transactivation activity by EBNA2 because the RAM region is expected to compete for binding to RBP-Jκ with EBNA2. EBNA2 and the RAM regions of mNotch2, mNotch3 or int3 were cotransfected into COS7 cells and its transcriptional activity was detected by the luciferase assay. The RAM regions of mNotch1, mNotch2, mNotch3 and int3 inhibited the transactivation activity of the TP1 promoter by EBNA2 to variable extents (Fig. 3A). mNotch2-RAM seems to be the most effective inhibitor among them. The extents of inhibition by various RAM regions are not in parallel with their relative affinities to RBP-Jκ described above. There are several reasons that can explain the discrepancy: the RAM region might compete with not only RBP-Jκ but also other coactivator proteins required for transactivation; the relative efficiency of the nuclear transport may be different between RAM regions. The transactivation activity of the TP-1 promoter by mNotch1-RAMIC is also inhibited by the RAM regions of mNotch1, mNotch2, mNotch3 and int3 (data not shown). These in vivo as well as

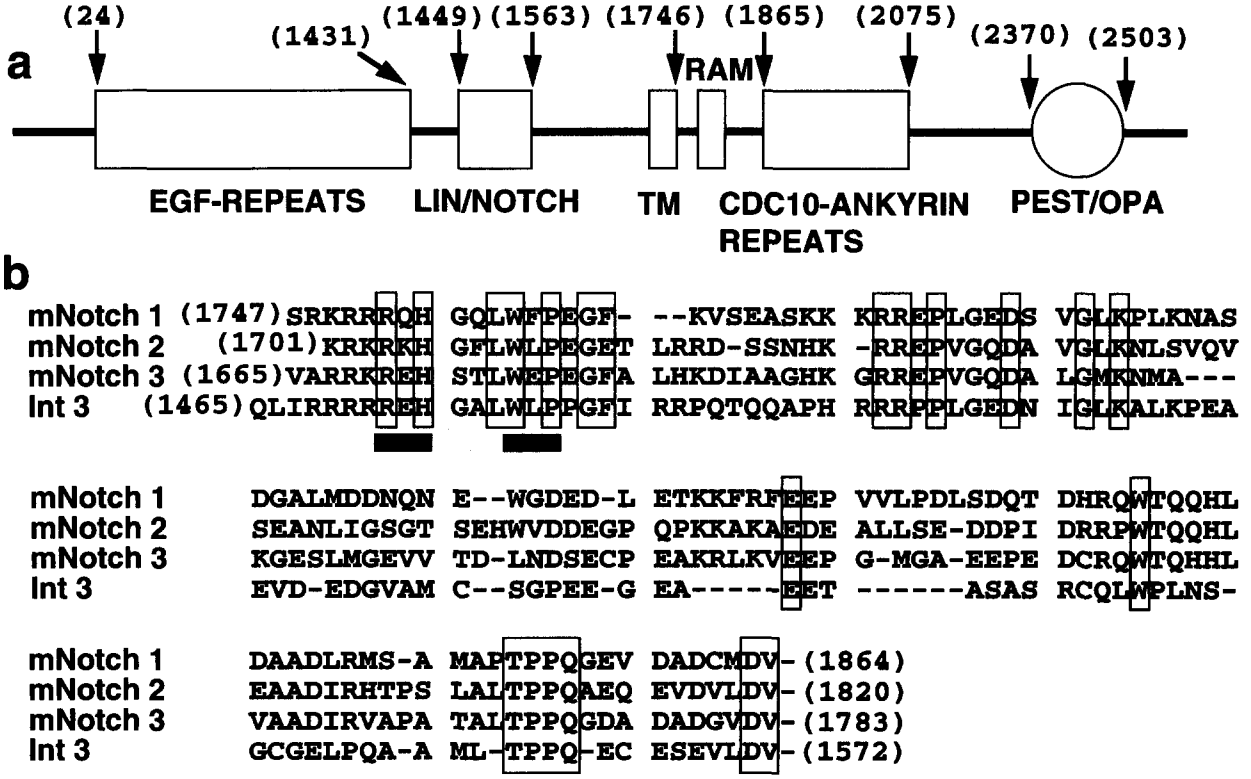

Fig. 1. (a) The structure of Notch is schematically shown. Numbers indicate positions of amino acid residues of mNotch1. (b) Structure of Notch RAM regions. Alignment of the deduced amino acid sequences for RAM regions of mNotch1, mNotch2, mNotch3 and int3. The underlined residues are those responsible for the interaction with RBP-Jκ [4]. Conserved residues are boxed.

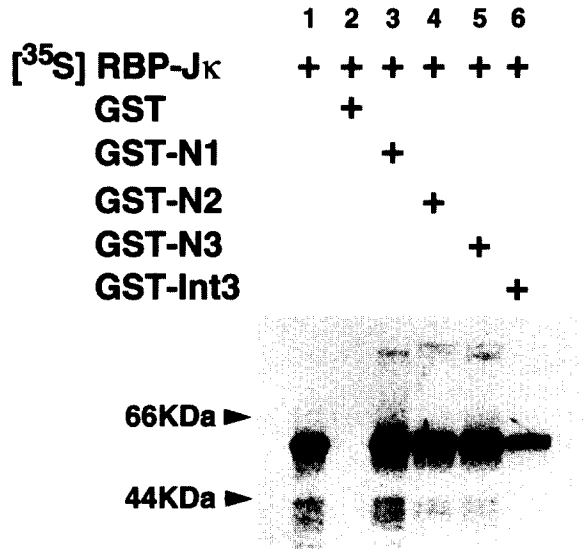

Fig. 2. The interaction of the RAM regions of mNotch1, mNotch2, mNotch3 and int3 with RBP-Jκ. After the incubation of GST-fused RAM regions of mNotch1 (N1), mNotch2 (N2), mNotch3 (N3) and int3 with in vitro translation products of ^{35}S-labeled RBP-Jκ, the retention of RBP-Jκ on GST-fused proteins was examined. Lane 1, positive control for RBP-Jκ. All the other lanes contain RBP-Jκ and GST proteins as indicated.

in vitro studies clearly show that the RAM regions of mNotch2, mNotch3 and int3 can directly bind to RBP-Jκ.

3.2. *The intracellular region of int3 has transactivation activity of the ERE-TP1 promoter*

The intracellular region of mNotch1 has a transactivation activity of the genes regulated by RBP-Jκ [26]. The intracellular region of mNotch1 transactivates the EBNA2 response element of the EB virus TP1 promoter (ERE-TP1 promoter) (T. Sakai and T. Honjo, in preparation). In order to examine whether other Notch members have a similar transactivation activity, we chose int3, which has the weakest interaction activity with RBP-Jκ. We transfected the intracellular region of int3 including the RAM region (int3-RAMIC) with a reporter plasmid ERE-TP1-luciferase (pGa 981-6) into COS7 cells. The luciferase assay clearly demonstrated the transactivation activity of the ERE-TP1 promoter by int3-RAMIC although it is slightly less efficient than mNotch1-RAMIC (Fig. 3B). When the ptk-luciferase reporter plasmid with no binding site for RBP-Jκ was cotransfected as a negative control reporter plasmid, the transcriptional activity was not detected by either mNotch1 or int3 (data not shown).

To further confirm the involvement of RBP-Jκ in the transcriptional activity of int3, we tested whether the transactivation activity of int3-RAMIC is blocked by overexpression of RBP-Jκ. The transactivation activity of mNotch1-RAMIC and int3-RAMIC is inhibited by RBP-Jκ (Fig. 3B). The result not only indicates that the transactivation by int3-RAMIC is mediated by RBP-Jκ but also suggests that free RBP-Jκ traps a putative cofactor in a limited supply.

The present study demonstrated the following results: (a) amino acid residues involved in the interaction with RBP-Jκ are conserved among mNotch1, mNotch2, mNotch3 and int3; (b) mNotch1-RAM, mNotch2-RAM, mNotch3-RAM and int3-RAM directly bind to RBP-Jκ; (c) mNotch1-RAM, mNotch2-RAM, mNotch3-RAM and int3-RAM can inhibit the transactivation activity by EBNA2 and mNotch1-RAMIC by competing for binding to RBP-Jκ; (d) the intracellular region of int3 (int3-RAMIC) as well as the intracellular region of mNotch1 (mNotch1-RAMIC) has the transactivation activity of the ERE-TP1 promoter. These data indicate that mNotch1, mNotch2, mNotch3 and int3 have biochemically related functions in vitro as well as in vivo. Int3 may be regarded as Notch4 based on its structural and functional similarity in agreement with the recent report from another group [17]. It remains to be seen whether all Notch members interact with the ubiquitous RBP-Jκ or with other putative RBP-Jκ family members which might be expressed in a tissue-specific manner.

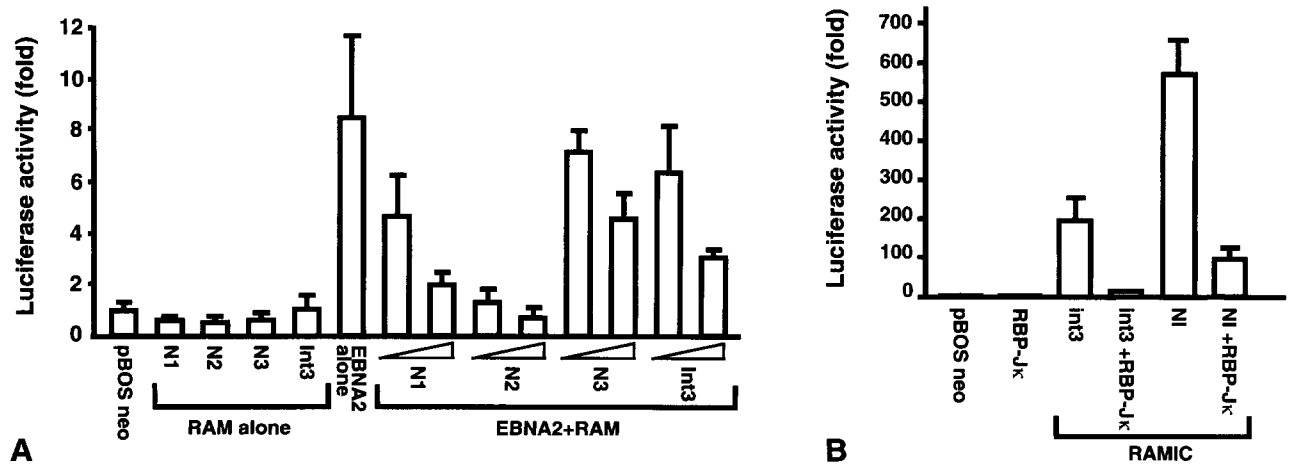

Fig. 3. (A) Transcriptional activity of the TP1 promoter by EBNA2 is inhibited by the RAM regions of mNotch1, mNotch2, mNotch3 and int3. 1.0 μg or 1.75 μg of pEF-BOS neo containing the RAM regions was transfected into COS7 cells together with 0.25 μg of pSV-βGal, 0.5 μg of pGa981-6 and 0.5 μg of pSG5-EBNA2. The total amount of DNA was adjusted to 3.0 μg using the pEF-BOSneo vector. An increased amount of RAM regions resulted in more efficient inhibition. The results are presented as the mean ± S.D. for triplicate determinations. (B) Int3-RAMIC has transcriptional activity of the ERE-TP1 promoter. 0.5 μg of pEF-BOS neo containing Notch1-RAMIC, Int3-RAMIC and RBP-Jκ was transfected into COS7 cells together with 0.25 μg of pSV-βGal and 0.5 μg of pGa981-6. The total amount of DNA was adjusted to 2.6 μg using the pEF-BOS neo vector. The results are presented as the mean ± S.D. for triplicate determinations.

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