Identification of a Novel Neural Cell Adhesion Molecule-related Gene with a Potential Role in Selective Axonal Projection*

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We describe here the cloning of mouse complementary DNAs encoding a novel protein, Rb-8 neural cell adhesion molecule (RNCAM), with a predicted extracellular region of five immunoglobulin C2-type domains followed by two fibronectin type III domains. Alternative splicing is likely to generate two RNCAM isoforms, which are differently attached to the cell membrane. These structural features and overall sequence identity identify this protein as a novel member of a cell adhesion molecule subgroup together with vertebrate neural cell adhesion molecule, Aplysia cell adhesion molecule, and Drosophila fasciclin II. In insects, fasciclin II is present on a restricted subset of embryonic central nervous system axons where it controls selective axon fasciculation. Intriguingly, RNCAM likewise is expressed in subsets of olfactory and vomeronasal neurons with topographically defined axonal projections. The spatial expression RNCAM corresponds precisely to that of certain odorant receptor expression zones of the olfactory epithelium. These expression patterns thus render RNCAM the first described cell adhesion molecule with a potential regulatory role in formation of selective axonal projections important for olfactory sensory information coding.

Formation of neuronal connectivity relies on a variety of cellular mechanisms acting on axons such as fasciculation, steering, target recognition, synapse formation, and stabilization (reviewed in Refs. 1 and 2). Guidance of axons to their appropriate targets is controlled by proteins on axons and growth cones that can recognize both attractant and repellent guidance cues. These cues or signals can either be secreted molecules or cell surface proteins. One of the most well characterized cell surface proteins implicated in growth, guidance, and fasciculation of axons is the neural cell adhesion molecule (NCAM). The adhesive properties of NCAMs can be modified as a consequence of alternative splicing of the primary transcript or posttranslational modifications such as glycosylation (3, 4).

NCAM is relatively uniformly expressed in the developing nervous system suggesting that it may not play a primary role in establishing precise patterns of topographic connectivity. An insect homologue to NCAM designated fasciclin II (Fas II) is, however, expressed on longitudinal axons where it controls specific patterns of selective fasciculation among subsets of neighboring axons (5–7).

Axonal navigation from the neuronal cell body in olfactory epithelium (OE) into the olfactory bulb (OB) of the brain provides an interesting system for studies of molecular mechanisms regulating formation of a projection map in vertebrates (reviewed in Ref. 9). The potential of this system for studies of sensory information coding depends on the possibility of using individual odorant receptors (ORs) as molecular probes to detect receptive properties of individual OE sensory neurons. In mammals, this subfamily of G-protein-coupled receptors may include as many as 1000 distinct genes. Analyses of spatial expression of individual OR transcripts indicate that each individual OE neuron expresses a single OR gene, which in turn may determine which particular odorants the neuron will respond to. In rodents OE is divided into four distinct spatial zones in which different sets of OR genes are expressed (10, 11).

A comparison of OR expression zones to results from retrograde labeling experiments and axonal staining patterns obtained with certain antibodies and lectins has led to the hypothesis that neurons in a specific OR-expressing zone of the epithelium project their axons selectively to a corresponding zone of the OB.

While it is conceivable that axonal guidance cues operate in the olfactory system, proteins regulating specificity of primary olfactory projections have not yet been identified. Schwob and Gottlieb (12) used a monoclonal antibody designated Rb-8 and demonstrated that OE has at least two non-overlapping zones, one that stains with Rb-8 and another that does not. The Rb-8-positive neurons project their axons to the ventrolateral olfactory bulb while avoiding the dorsomedial part.

In this paper we report the molecular cloning of cDNAs corresponding to the Rb-8 antigen. This protein, designated RNCAM, is strikingly similar to NCAM with regard to sequence, overall structure, and existence of different membrane-bound splice forms. RNCAM is, however, more similar to Fas II than to NCAM in its expression pattern. RNCAM shows a spatial expression, which suggests a role in forming topographically defined axonal projections in the olfactory and vomeronasal systems.

EXPERIMENTAL PROCEDURES

Cloning Procedure—Polymerase chain reaction (PCR) was carried out according to the Taq polymerase supplier (Boehringer Mannheim) using 2 μl of each primer with 1 μl of 10× plaque-forming unit pools of a OE cDNA phage library per 10 μl of reaction mixture prepared using C57BL/6J OE poly(A)+ RNA and AZAPII vector (Stratagene). PCR was performed according to schedule: 96 °C for 45 s, 45 °C for 3 min, and 72 °C for 3 min for 50 cycles. Reverse (3′) primers specific for the X88357 sequence (14) were: X88.3R, 5′-TTCTCCAACACTAAGCTCTAC-3′; X88.4R, 5′-CTAAGTGAAATTGTCACTT-3′. These primers were used in anchored PCR using oligonucleotides corresponding to T3 and T7 promoters present in the vector as forward (5′) primers. DNA sequencing confirmed that the PCR product contained the X88357 sequence plus a 200-base pair (bp) additional sequence. 1×106 plaque-forming units of the cDNA library were screened.

Sequence comparison was done to mouse NCAM (GenBank accession no. X88357) and Drosophila fasciclin II (GenBank accession no. P34082). Ig domains were measured as including 20 amino acids outside the two characteristic cysteine residues; fibronectin type III domains were measured as including 20 amino acids outside the characteristic tryptophan and tyrosine residues.
aligned at amino acid no. 694 and onward. The NH2-terminal sequence AF016620. The unique COOH termini of the short (L) and long (S) forms of RNCAM are shown. The deduced amino acid sequence of the long and short forms of RNCAM is shown.

NGGCGC...IIAIGFYTGFLVTHK...GRCAM (L)

NGGCGC...IIAIGFYTGFLVTHK...GRCAM (S)

RNA/DNA Preparation and Northern/Southern Analysis—Total RNA was prepared and analyzed by Northern blotting according to standard procedures (15). For Southern blots, genomic DNA prepared from C57BL/6J mouse spleen was analyzed. A 1.4-kbp fragment corresponding to the first 462 amino acid residues of RNCAM was 32P-labeled by random priming and used as a probe.

In Situ Hybridization—The procedure was as described previously using paraformaldehyde-fixed and paraffin-embedded tissues to generate sections that were subsequently hybridized to 35S-labeled RNA probes, and exposed for 10–13 days (10). The RNCAM probe corresponded to the same 1.4-kbp region of RNCAM as was analyzed by Northern blot hybridization. OR probes used (M40, K20, L45, M50) and probes specific for Geo and Gu2 were as described previously (10, 16, 17). Images were processed using Adobe Photoshop 4.0.

RESULTS

In 1986 a monoclonal antibody termed Rb-8 was reported to detect an axonal membrane protein (12). Further characterization and purification using rat brain homogenate revealed that Rb-8 recognized a glycoprotein with an NH2-terminal amino acid sequence with no significant homology to any known proteins (Fig. 1 and Ref. 19). We performed a computer-aided data base search with the most ambiguous DNA sequence corresponding to the NH2-terminal peptide. This procedure revealed a match to a 75-bp DNA sequence (GenBank accession no. X88357) with potential to encode the NH2-terminal sequence of Rb-8. X88357 was identified among 559 putative exon sequences localized on human chromosome 21 (14). We designed oligonucleotides matching different regions within these 75 bp and performed anchored PCR on pools of λ-phages from an OE cDNA library. A 235-bp PCR product was cloned, and sequence determination confirmed that it contained the correct sequence. This PCR product was subsequently used as a probe to screen an OE cDNA library. Of eight isolated clones we sequenced two cDNA inserts of 3.5 and 4.5 kbp, respectively, that showed different restriction enzyme digestion patterns (data not shown).

The two clones represented two putative splice forms with potential to code for proteins of 727 (short form) and 837 amino acids (long form), respectively (Fig. 1). Both clones had open reading frames that generate a common 693-amino acid long NH2-terminal region with a putative signal peptide having the most likely cleavage site between positions 19 and 20 (GQA-LL). Cleavage at this position would create a NH2-terminal sequence identical to that of purified Rb-8 antigen (Fig. 1), and thus the cloned gene most likely encodes proteins recognized by the Rb-8 antibody. At the COOH terminus of the short form there is a run of hydrophobic amino acids and no cytoplasmic domain, which is compatible with attachment to the cell membrane by a phosphatidylinositol anchor (18). The long form contained a 25-amino acid potential transmembrane region and a 106-amino acid intracellular domain. The amino acid sequence of the complete open reading frame showed highest similarity to the extracellular domain of different NCAM isoforms, e.g., the long form showed an overall identity of 45% to the 140-kDa splice product of NCAM whereas the short form was 42% identical to the glycosylphosphatidylinositol-anchored 120-kDa splice product of mouse NCAM. The Hidden Markov Model program (19) for structural consensus domains revealed the presence of five Ig C2-type and two fibronectin type III domains arranged as in NCAM. The aligned sequences from both mouse NCAM and RNCAM showed that these molecules have approximately 36–56% amino acid identity over all seven of their extracellular domains. RNCAM showed the second highest degree of similarity to Drosophila Fas II with an overall identity of 27% whereas individual domains showed 25–36% sequence identity. Interestingly, a comparison between NCAM and Fas II gave a similar degree of sequence identity: 27% overall and 23–36% between domains. Due to the history of being recognized by the Rb-8 antibody, sequence/structural similarity to NCAM, and the neuronal expression pattern (see below) we tentatively termed the protein RNCAM (Rb-8 neural cell adhesion molecule). The Rb-8 antigen binds certain lectins, and thus glycosylation might explain the discrepancy in calculated mass of RNCAM isoforms (91 and 79 kDa) and reported sizes of the two proteins recognized by the Rb-8 antibody (125 and 94 kDa, Ref. 13).

We next performed Northern and Southern blot analyses using a probe specific for the major part of the extracellular domain present in both long and short forms of RNCAM. Using this 1.4-kbp probe a single hybridizing species was recognized in restriction enzyme-digested mouse genomic DNA suggesting that the two isolated cDNAs were generated from a single copy gene as a result of alternative splicing (Fig. 2A). Fig. 2B shows that the same probe generated a signal of about 9.5 kb using total RNA from brain as well as from OB. A probe specific for the short form of RNCAM generated a hybridization signal of approximately the same size, and reverse transcriptase-PCR analyses indicated that both forms are present in brain and OE (data not shown). Interestingly, no hybridization was detected to RNA from non-neuronal tissues such as lung, liver, gut, heart, kidney, and testis. This result indicated that RNCAM is preferentially expressed in neuronal tissue.

The Rb-8 antigen was reported to be expressed specifically in the ventrolateral two-thirds of OE as well as on the axons terminating in the ventrolateral OB but not in the dorsomedial part (12). This prompted us to compare the expression pattern
of RNCAM to OR expression in OE since Rb-8 staining in relation to the OR zones was not investigated before. Any given OR gene is expressed by a low percentage of randomly dispersed OE neurons distributed within only one out of four spatially segregated zones (10, 11). In mouse OE these zones are referred to as zones 1–4, with zone 1 being most dorsal/medial located and zone 4 most ventral/lateral located. OR genes M40, K20, L45, and A16 were previously shown to be expressed in zones 1, 2, 3, and 4, respectively (10, 16). Importantly, in situ hybridization revealed that RNCAM expression was restricted in OE to cover exactly zones 2, 3, and 4 but not zone 1 (Fig. 3). Fig. 4A shows that RNCAM hybridization exhibited sharp boundaries as opposed to a gradient of increased RNCAM expression between zones 1 and 2. No hybridization above background was detected in zone 1 even after prolonged exposure times. RNCAM expression in zones 2, 3, and 4 was confined to cell layers containing immature and mature OE neurons whereas no expression was detected in the most superficial cell layer containing supporting cells. The neuron-specific expression was similar to that of NCAM although NCAM is expressed throughout OE in all OR zones (20, 21). RNCAM transcripts were detectable in the mitral cell layer of OB containing one type of OE target cells, which are also the principal output neurons of the bulb (layer 4 in Fig. 4B). In addition the granule cell layer with interneurons showed intense expression of RNCAM (layer 5 in Fig. 4B).

The vomeronasal organ (VNO) is a tubular structure at the base of the nasal septum that represents a second site for receiving olfactory information in rodents (22). VNO appears to specifically detect pheromones and contains two neuronal populations, apically located neurons that express Goo2 and basal neurons that express the Goo subunit of heterotrimeric G-proteins (17). Axons of the Goo2 and Goo positive VNO subpopulations terminate in two spatially separate regions of the accessory OB (23). Fig. 4C shows a representative in situ hybridization of consecutive sections of VNO using Goo2, Goo, and RNCAM-specific probes. A comparison between hybridization signals obtained suggested that RNCAM is restricted to VNO sensory neurons that express Goo2.

**DISCUSSION**

We describe here identification and molecular cloning of two splice products coding for an antigen (Rb-8) present on axons of a topographically defined subpopulation of OE neurons (12). DNA sequencing revealed two splice forms that are likely to encode different cell attachment regions, a glycosylphosphatidylinositol anchor and a transmembrane-spanning region, respectively. We designate the Rb-8 antigen RNCAM (Rb-8 NCAM) because of its similarity to NCAM, which is evident both with regard to overall sequence identity (45%) and putative structural features, notably five Ig C2-type domains followed by two fibronectin type III domains. Thus identification of RNCAM expands the subgroup of cellular adhesion molecules having this structural arrangement in which hitherto NCAM, Aplysia cellular adhesion molecule, and Fas II have been exclusive members. Intriguingly, RNCAM shows an equal degree of amino acid identity to Drosophila Fas II as does NCAM. This raises the possibility that a gene duplication sometime during the evolution from invertebrates to vertebrates resulted in two Fas II-related cell adhesion molecules. Moreover, studies by Chen et al. (14) indicate that NCAM and RNCAM are located on different human chromosomes.

It is noteworthy that even though several tissues normally express NCAM, most appear unaffected in mice that are homozygous null for NCAM (24, 25). One possible explanation for this observation is that some functions of NCAM may over-

**FIG. 2. Southern and Northern blot analyses of RNCAM.** A, mouse spleen DNA (15 μg) digested with KpnI (lane 1), EcoRI (lane 2), HindIII (lane 3), and PstI (lane 4) was analyzed by Southern blot using a 1.4-kbp RNCAM probe specific for 466 amino acids of the NH2-terminal sequence of the short and long form of RNCAM. Single bands detected in all digests (except for PstI), a hybridization pattern which indicates that the probe recognizes one single gene. B, total RNAs (15 μg) obtained from mouse OB (lane 1), brain without OB (lane 2), lung (lane 3), gut (lane 4), liver (lane 5), heart (lane 6), testis (lane 7), and kidney (lane 8) were analyzed by Northern blot. The 1.4-kbp RNCAM probe (same as in A) recognized a band with the apparent size of about 9.5 kb specifically in RNA prepared from brain (lane 1) and OB (lane 2). Rehybridization with a probe recognizing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control for loading and confirmed that the RNAs were intact.

**FIG. 3. In situ hybridization analysis of RNCAM expression in olfactory epithelium and comparison to odorant receptor expression zones.** Sequential coronal tissue sections through adult mouse OE were hybridized with 35S-labeled cRNA probes for RNCAM as well as for four different ORs (M40, K20, L45, and M50) known to be expressed specifically in zones 1–4 (Z1–Z4), respectively. RNCAM showed intense hybridization to neurons corresponding to zones 2–4 of the OE. No hybridization was evident in zone 1, the restricted distribution of which is evident after hybridization to the M40 odorant receptor. Note that each odorant receptor is expressed in a limited number of cells within a zone whereas RNCAM is expressed in all cells in zones 2–4. Photographs were processed to show hybridization signals in white after darkfield illumination whereas the nuclear counterstain (Hoechst 33258, blue) was visualized by UV.
FIG. 4. RNCAM expression in relation to zones 1 and 2, expression in OB and vomeronasal organ. A, shown are high magnification views of an in situ hybridization experiment performed as described in legend to Fig. 3. The RNCAM probe hybridization demarcates a sharp boundary between zone 1 (Z1) and 2 (Z2). B, in situ hybridization analysis of RNCAM expression in the coronal tissue section of OB is shown. 1, olfactory nerve layer; 2, glomerular layer; 3, external plexiform layer; 4, mitral cell layer; 5, granule cell layer; 6, ependymal layer. RNCAM expression was confined to the olfactory nerve, mitral cell, and granule cell layers. C, sequential coronal sections through VNO were hybridized with probes specific for RNCAM and the G-protein α-subunits, Goα and Goβ. Goα positive neurons are in a basal location and Goβ2 in a more apical location of the neuroepithelium. The hybridization patterns indicated that RNCAM and Goβ2 expression spatially coincide. A white line demarcates the basal extent of the neuroepithelium, and positive hybridization signal is in black.

lap with that of e.g. RNCAM. On the other hand, the only major tissue malformation caused by lack of NCAM is in the OB, and the underlying cellular mechanism is a defect in migration of precursor cells to the OB resulting in loss of a large pool of granular interneurons. We detect expression of RNCAM in the graneule cell layer of the OB suggesting differential function of NCAM and RNCAM in this cell type. Expression of RNCAM in mitral cells, which are one type of target neurons for OE neurons, suggests that homophilic binding may contribute to this synapse formation. Identification of RNCAM will enable these hypotheses to be tested.

One evident difference between vertebrate NCAM and Drosophila Fas II is their patterns of expression. Fas II is (in contrast to NCAM) present on a restricted subset of axon fascicles (5–7). Studies in Drosophila have defined Fas II as a neuronal recognition molecule that controls selective axon fasciculation and synaptic stabilization but not other aspects of axonal outgrowth and directional guidance (5, 8, 26). Like Fas II, expression of RNCAM was first visualized in an antibody screen aimed to identify proteins expressed on subsets of axons that target a topographically defined region (7). Our in situ hybridization studies show that this pattern of antibody staining may be explained by the zonally restricted presence of RNCAM mRNA transcripts in OE. Such an expression pattern associated with specific axonal projections in a phenotypically unique subset of neurons has not been reported for NCAM; however, it does resemble the expression pattern of Fas II in Drosophila.

Neuronal maps develop when one set of neurons projects the axons to form connections with one or more neuronal sets so that the spatial order of the elements of the first set is preserved in the spatial order of connections to the next set. This is exemplified by specific axonal projections of neurons in different OR expression zones to spatially restricted regions of the OB (9, 11). Zonal specific expression of subfamilies of ORs divides the olfactory epithelium into four different zones, and it has been hypothesized that each zone projects to a corresponding zone in OB (10, 11). Indeed, such zone-zone projection is in part demarcated by expression of RNCAM in OR expression zones 2, 3, and 4 but not 1. Our results, together with previously published data, obtained using Rh-8 antibodies suggest that lack of RNCAM expression correlates with expression of zone 1 ORs and projection to the dorsal-medial part of the bulb. Moreover, RNCAM-positive OE neurons express zones 2, 3, and 4 receptors and project their axons to a region complementary and ventromedial to zone 1-expressing neurons. The finding that RNCAM is specifically expressed in a subpopulation of vomeronasal sensory neurons, which selectively project to a specific region of accessory OB, further suggests that RNCAM is involved in formation of specific projections and raises the question whether RNCAM expression delineates topographic maps in additional sensory systems as well.

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