Cell Surface Modulation of the Neural Cell Adhesion Molecule Resulting from Alternative mRNA Splicing in a Tissue-specific Developmental Sequence

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Abstract. The neural cell adhesion molecule N-CAM is an intrinsic membrane glycoprotein that is expressed in the embryonic chicken nervous system as two different polypeptide chains encoded by alternatively spliced transcripts of a single gene. Because they differ by the presence or absence of ~250 amino acids in their cytoplasmic domains, these polypeptides are designated ld and sd, for large and small cytoplasmic domain, respectively. We report here that the ld-specific sequences comprise a single exon in the chicken N-CAM gene and that developmental expression of the ld and sd chains occurs in a tissue-specific fashion, with the ld chain restricted to the nervous system. Comparison of the nucleotide sequences from an N-CAM genomic clone with cDNA sequences showed that a single exon of 783 base pairs corresponded to the unique cytoplasmic domain of the ld polypeptide. Sequences from this exon were absent from the single N-CAM mRNA detected in several non-neural tissues by RNA blot hybridization, and immunoblot analysis confirmed that antigenic determinants unique to the ld-specific domain were not expressed in these tissues. Immunohistochemical experiments indicated that only the sd chain was expressed on cell surfaces of non-neural tissues throughout embryonic development. The ld chain was found on cell bodies and neurites of differentiated neurons; it first appeared as neurons began to extend neurites and to express the neuron-glia cell adhesion molecule (Ng-CAM) and it was restricted to definite layers in laminar tissues such as the retina and cerebellum. These results suggest that the control of mRNA splicing may affect the regulation of N-CAM function at specific sites within the nervous system and thus influence the control of neural morphogenesis and histogenesis.

Different cell adhesion molecules (CAMs)1 are expressed in dynamic and complex patterns during embryonic development and appear to be involved in the formation of borders between cell collectives (13). During development, CAMs undergo several types of cell surface modulation including changes in prevalence (cell surface concentration), differential prevalence (concentration relative to other CAMs on the same cell) (27), polarity (unequal distribution at different sites on the same cell), and chemical structure (differences in polypeptide and carbohydrate structure) (14). The presence of a limited number of primary CAMs in dynamically controlled patterns on a wide variety of tissues derived from all three embryonic germ layers (7, 16) and of several secondary CAMs that appear in a more restricted set of tissues later in development (36, 44) suggests that modulation of CAM activity at the cell surface, rather than the expression of very large numbers of different cell-specific recognition molecules, may be responsible for the control of morphogenetically significant tissue interactions during development (11, 12).

The neural CAM (N-CAM) is a primary CAM that appears to play a key role in such dynamic processes. It is an intrinsic cell surface protein that is mobile in the plane of the membrane (19). N-CAM purified from embryonic chicken brain (25) contains two structurally related glycopolypeptide chains, the ld (large cytoplasmic domain) chain of 170 kD and the sd (small cytoplasmic domain) chain of 140 kD (8, 24); in rodents, an ssd (smallest domain) chain of 120 kD is more prominent than in the chicken (15, 41). Both the ld and sd polypeptides are present in varying proportions in different regions of the brain (3). In contrast, in a variety of non-neural tissues (7) including skin (4, 5) and muscle (6, 23, 39), the sd polypeptide of N-CAM is expressed and modulated but the ld polypeptide has not been detected.

The ld and sd polypeptides in the brain are synthesized from two 6–7-kilobase (kb) mRNAs that appear to arise from alternative splicing of transcripts from a single N-CAM gene (10, 33, 34; Owens, G. C., G. M. Edelman, and B. A. Cunningham, unpublished data). Nucleotide sequence analysis

1. Abbreviations used in this paper: CAM, cell adhesion molecule; E, embryonic day; ld, large cytoplasmic domain; N-CAM, neural CAM; Ng-CAM, neuron-glia CAM; sd, small cytoplasmic domain.
of chicken N-CAM cDNA clones derived from the larger brain mRNA (24) indicated that the ld chain differs from the sd chain by the presence of ∼250 additional amino acids carboxy terminal to a membrane-spanning region. These findings are consistent with other evidence that the ld polypeptide differs from the smaller chains on the cytoplasmic side of the cell membrane (8, 20, 21, 38, 40).

All of these observations prompted us to examine the structure of the N-CAM gene and to determine the order of expression in development of the N-CAM polypeptides. We report here that the ld-specific sequences comprise a single exon in the chicken N-CAM gene and that the expression of the ld and sd chains occurs in a tissue-specific fashion, with the ld chain restricted to the nervous system.

Materials and Methods

Isolation and Sequencing of the ld Chain–specific Exon

A 735-base pair (bp) PstI cDNA fragment (indicated by the hatched bar in Fig. 1) that specifically hybridized to the larger N-CAM mRNA (24, 34) was used as a probe to screen an EMBL3B (18) chicken genomic DNA library (kindly supplied by Dr. J. D. Engel, Northwestern University, Evanston, IL) according to Benton and Davis (1). Phage DNA from positive plaques was prepared by the method of Yamamoto et al. (46). Restriction mapping and Southern blotting followed standard procedures (30). A 1.5-kb HindIII/BamHI restriction fragment to which the probe hybridized was sub-

Figure 1. Structure of N-CAM polypeptides and nucleic acid probes. The open bars represent the sd (top) and the ld (bottom) polypeptides of chicken N-CAM. The 735-bp PstI cDNA fragment used for the isolation of the N-CAM genomic clone and the corresponding ld chain–specific polypeptide region are indicated by the hatched bars. Solid shading indicates the proposed membrane-spanning sequence (24). The extent of cDNA clone pEC208 (which contains the 3′-most 2892 bp of ld chain coding sequence and 664 bp of 3′ untranslated sequence) and the positions of EcoRI (R), PstI (P), and PvuII (V) restriction sites are shown (24). Bacteriophage λN208 contains the same cDNA insert in the EcoRI site of bacteriophage λgt11 (34). The cDNA sequences present in fragments A–D and in the cDNA insert in bacteriophage λN252 (34) are also indicated.

Figure 2. Sequence of the N-CAM exon found only in the larger N-CAM mRNA. HindIII, PstI, PvuII, and BamHI restriction sites are underlined and labeled. The predicted protein sequence for the exon is indicated above the nucleotide sequence. The 5′ and 3′ splice sites (determined by comparison with the sequence of pEC208) are underlined and the single base difference between the exon and the cDNA sequence in pEC208 is indicated at amino acid position 55.
cloned into plasmid pUC9 (45) and sequenced by the chemical degradation method (31).

**RNA Blot Hybridization Analysis**

RNA was extracted from tissues by homogenization in guanidine thiocyanate (2), electrophoresed in 0.8% agarose denaturing gels containing formamide, blotted to nitrocellulose, and hybridized with 32P-labeled DNA probes as described previously (33, 34).

**Immunoprecipitation and Immunoblot Analysis**

Membranes were prepared from organs of 12-d embryonic White Leghorn chickens, extracted with nonionic detergent, immunoprecipitated using monoclonal antibody anti-N-CAM No. 1 covalently attached to agarose beads (25), and treated with neuraminidase as described (8, 42). Immunoblot analysis using antibodies purified by binding to lysates of the bacteriophage λgt11-based expression clones λN208 and λN252 was performed as previously described (34).

**Preparation of ld-Specific Antibodies and Immunofluorescence Analysis**

Antibodies specifically directed against the ld chain of N-CAM were prepared by injecting rabbits with N-CAM-β-galactosidase fusion protein purified (43) from a lysogen of bacteriophage LN252 (34). Rabbits were injected subcutaneously at intervals of several weeks with polyacrylamide gel-purified and electroeluted (28) fusion protein in PBS was injected subcutaneously without adjuvant. Rabbits were bled 10-14 d after each injection and anti-N-CAM antibodies were affinity-purified on columns of chicken N-CAM covalently coupled to Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ). Alternatively, lysates of lytic bacteriophage cultures containing N-CAM-β-galactosidase fusion proteins were immobilized on nitrocellulose disks and used to affinity purify ld-specific antibodies from rabbit polyclonal anti-N-CAM IgG (34). Preparation of frozen sections, staining with antibodies, and visualization using fluorescein- or rhodamine-conjugated second antibody were as described (7, 9).

**Results**

**Structure of an ld-specific Exon in the N-CAM Gene**

To determine the number of exons encoding the sequences that differ between the two N-CAM mRNAs in the brain, a cDNA probe (Fig. 1) was used to isolate a genomic clone containing this region of the N-CAM gene. The sequence of a restriction fragment homologous to the probes covering the difference region was determined (Fig. 2) and, by comparison with the sequence of cDNA clone pEC208 (24), was found to contain a single exon of 783 bp bounded by consensusesplice sites (32). The sequence of this exon and the corresponding sequence in pEC208 were obtained using independent methods and differed by only a single neutral base change, which we attribute to a strain difference between the sources of the cDNA and the genomic DNA. The molecular mass of the 261 amino acid peptide predicted to be encoded by the exon (25,793 D) is sufficient to account for the observed difference in the molecular masses of the ld and sd chains (8).

Both the 5′ and 3′ splice sites are located between the second and third positions of codons for glutamic acid residues (Fig. 2); thus the exclusion of the exon from the mRNA would preserve the reading frame and leave a glutamic acid residue at the spliced position in the sd chain. From the sequence of pEC208 (24), the cytoplasmic domains would contain 362 and 101 amino acids in the ld and sd polypeptides respectively, with the C-terminal 21 amino acids common to both chains (Fig. 1).

All of these results indicate that a major if not the only difference between the ld and sd mRNAs involves the inclusion or exclusion of a single exon from the mRNA during RNA splicing. Whether additional variations in splicing also occur remains to be determined.

**Expression of Different N-CAM RNAs in Neural and Non-neural Tissues**

Previous biochemical studies indicated that the ld polypeptide is not expressed in several non-neural tissues, including muscle (6, 26, 39, 42) and skin (4, 5), but the generality of this result and the structural relationships between the N-CAM polypeptides and mRNAs in the various tissues were not determined. Therefore RNA was prepared from different tissues of 12-d embryonic chickens and probed with the N-CAM cDNA probe pEC208 (Fig. 3). Two large N-CAM mRNAs were detected in brain RNA but not in liver RNA, as observed in earlier studies (33). Skin, breast muscle, heart, and gizzard RNA, however, all contained a single major reactive component that co-migrated with the smaller brain mRNA. Similar results were obtained with lung and kidney RNA (data not shown). Small amounts of mRNA co-migrating with the larger brain mRNA were detected in gizzard, apparently reflecting the presence of the ld chain in the large enteric ganglia in this organ; this is consistent with im-

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**Figure 3.** RNA blot hybridization analysis of N-CAM mRNA. Poly(A)* RNA (2.5 μg/lane) from the indicated organs of 12-d embryonic chicken embryos was electrophoresed in a 0.8% agarose denaturing gel, transferred to nitrocellulose, and probed with the cDNA insert from pEC208. The heterogeneous hybridization in the lower region of the blot was not seen in other experiments and may reflect a small amount of RNA degradation. Positions of 28S and 18S ribosomal RNAs are indicated. Br, brain; Li, liver; He, heart; BM, breast muscle; Sk, skin; Gz, gizzard; Tot Br, 5 μg total brain RNA.
Figure 4. Expression of difference exon sequences in neural and non-neural N-CAM mRNAs. Blots of total RNA (10 µg/lane) from brain (Br), skin (Sk), or heart (He) of 12-d embryonic chicks were probed with fragments A-D (Fig. 1) as indicated. Positions of 28S and 18S ribosomal RNA are shown.

Expression of the Different N-CAM Polypeptides

The N-CAM polypeptides from neural and non-neural tissues were examined using antibodies that recognize both N-CAM polypeptides or only the ld-specific domain. Because embryonic N-CAM is highly sialylated and hence polydisperse in these tissues, immune precipitates of N-CAM were treated with neuraminidase before being resolved on SDS-containing polyacrylamide gels and immunoblotted to nitrocellulose.

Anti-N-CAM antibodies that had been purified by binding to lysates of bacteriophage λN208 (Fig. 1) reacted with both the ld and sd chains of brain N-CAM and recognized components that co-migrated with the sd chain of brain N-CAM in all of the non-neural tissues (Fig. 5 a). In addition, these antibodies recognized two different components (~150 and 125 kD) present in heart and breast muscle, but not in skin, gizzard, or brain. Total polyclonal anti-N-CAM antibodies gave identical results (data not shown). In contrast, ld-specific antibodies prepared by binding polyclonal anti-N-CAM antibodies to lysates containing the ld-specific LN252 fusion protein recognized only the ld chains found in brain and gizzard (Fig. 5 b). None of the smaller polypeptides, including the 150- and 125-kD chains in heart and breast muscle, reacted with these antibodies. The same results were obtained with ld-specific antibodies raised in rabbits directly against the λN252 fusion protein (Fig. 5 c, and data not shown). Traces of the ld polypeptide occasionally were observed in heart and breast muscle samples, probably reflecting the innervation of these tissues.

These results agree with the RNA hybridization data and confirm that the predominant N-CAM polypeptides found in the non-neural tissues lack the ld-specific large cytoplasmic domain. Furthermore, the unambiguous correlation between polypeptide and mRNA expression reported here strengthens the evidence that the ld and sd polypeptide chains are translated from the larger and the smaller N-CAM mRNA, respectively.

Spatiotemporal Distribution of the ld and sd Polypeptides during Development

To analyze the sequences of expression of the different N-CAM polypeptides during development, we examined frozen sections of embryos of different ages using immuno-
Figure 5. Immunoblot analysis of neuraminidase-treated N-CAM. Immune precipitates of nonionic detergent extracts from the indicated tissues were treated with neuraminidase, extracted, and electrophoresed in 7.5% polyacrylamide gels. Material in lanes -N was not treated with neuraminidase. Proteins were transferred to nitrocellulose and probed with anti-N-CAM antibodies that had been purified by binding to filters carrying lysates of cultures of λN208 (a) or λN252 (b). In a separate experiment, blots were probed with rabbit polyclonal antibodies raised against the β-galactosidase–N-CAM fusion protein synthesized from λN252 (c). Migration positions of molecular mass markers (kD) are indicated at the left of each panel. Tissue abbreviations are given in the legend to Fig. 3.

Discussion

Our results indicated that the difference between the ld and sd polypeptides from chicken brain N-CAM can be ac-
Figure 6. Immunofluorescence analysis of N-CAM distribution in neural tissues. Transverse frozen sections of stage 8 (four somite) embryos were stained with total polyclonal anti-N-CAM antibodies (a) or Id-specific antibodies raised against the λN252 fusion protein (b). Stage 26 (E5) neural tubes were stained with total polyclonal anti-N-CAM antibodies (c), Id-specific antibodies (d), or polyclonal anti-Ng-CAM antibodies (e). Cerebella (f and g) or retinas (h and i) of E18 embryos were stained with total polyclonal anti-N-CAM antibodies (f and h) or Id-specific antibodies (g and i). Bar, 100 μm. NG, neural groove; N, notochord; S, somite; NE, neuroepithelium; VH, ventral horn; EGL, external granular layer; ML, molecular layer; FT, fiber tract; OFL, optic fiber layer; IPL, inner plexiform layer; NL, nuclear layer; PE, pigmented epithelium.
Figure 7. Immunofluorescence analysis of N-CAM distribution in non-neural tissues. Frozen sections through the trunks of stage 26 (E5) embryos at the level of the lung buds (a and b) or of the kidney (c and d) were stained with total polyclonal anti-N-CAM antibodies (a and c) or with Ld-specific antibodies raised against the ~N252 fusion protein (b and d). Sections of E12 gizzard were stained with total polyclonal anti-N-CAM antibodies (e) or with anti-N-CAM antibodies that had been purified by binding to lysates of ~N208 (f), ~N252 (g), or ~gtll (h). Bars, 100 μm. LB, lung bud; E, esophagus; MT, mesonephric tubule; GR, genital ridge; G, ganglion; M, muscle. Arrows, nerves in all panels.
counted for by a single exon of 783 bp bounded by consensus splice sites and encoding a peptide of 261 amino acids. In support of this hypothesis, we have recently obtained a cDNA clone that resembles pEC208 but that exactly lacks the sequences corresponding to the exon in Fig. 2 (unpublished observations). Our data also showed that the ld chain is expressed only in neural tissues, whereas the sd chain is expressed in both neural and non-neural tissues. These findings are consistent with previous biochemical work (4–6, 26, 35, 38, 39, 42); in addition, they indicate that the observed differences in N-CAM polypeptide structure in different tissues reflect variations in the splicing of the same exon. Although differential splicing of this exon appears to account for the entire difference between the mRNAs for the ld and sd polypeptides and between the mRNAs in neural and non-neural tissues, we cannot rule out the possibility that more complicated patterns of alternative splicing might occur, similar to those described for fibronectin (29, 37, 43). Characterization of additional cDNA clones should resolve this question.

At the polypeptide level, the 140-kD sd chain but not the 170-kD ld chain was detected in all non-neural tissues. Heart and breast muscle contained additional polypeptides of 150 and 125 kD. These polypeptides reacted with anti-N-CAM antibodies but not with antibodies specific for the ld chain; furthermore, cDNA probes that hybridized to the ld-specific exon did not hybridize to N-CAM mRNAs from these tissues. Recent studies (Hoffman, S. H., G. M. Edelman, and B. A. Cunningham, unpublished observations) suggest that the differences in molecular masses are not attributable to asparagine-linked carbohydrate. These findings leave open the possibility that additional RNA splice sites may be used in these organs or that other posttranslational modifications of N-CAM chains may occur.

The immunohistochemical experiments indicated that the sd and ld chains appear at the cell surface in a definite tissue-specific sequence during embryogenesis. In the early embryo, only the sd chain of N-CAM was expressed. During the development of the nervous system, the sd chain continued to be expressed while the ld chain was associated with differentiating neurons of the central and peripheral nervous systems in a definite sequence at several sites, including the ventral horn of the spinal cord, the molecular layer and internal granular layer of the developing cerebellum, and the inner plexiform and optic fiber layers of the retina. A similar pattern of ld chain expression in the nervous system has been reported in the mouse (38). Biochemical studies have indicated that the ld chain is expressed in cultured rodent neurons but not glia (35). In each of the cases examined here, the expression of the ld chain was associated with the expression of Ng-CAM, and in at least one case (the ventral horn), Ng-CAM expression clearly preceded ld chain expression. In contrast, ld chain expression was not detected outside of the nervous system, even though the sd chain was expressed in complex and defined spatiotemporal patterns at many non-neural sites.

These results are consistent with the expression sequence diagrammed in Fig. 8, which hypothesizes that the separation of neural and non-neural tissues early in development is associated with a developmentally regulated event that enables the differential splicing of the ld-specific exon into N-CAM mRNAs in neural tissues only. The actual splicing of this exon would appear to be facultatively controlled later in development, providing the basis for different relative levels of expression of the ld and sd chains in particular neural regions or layers. Whether this sequence of CAM expression occurs in vivo in individual cells can be determined only by higher resolution analysis using immunoelectron microscopy or in situ hybridization. Some rodent cell lines express both the ld and sd chains of N-CAM (17, 21, 22), and alterations in the proportion of the sd and ld polypeptides can occur upon differentiation in vitro (38). This indicates that such a facultative control of N-CAM splicing in individual neurons is possible.

Although the control of N-CAM expression appears to be important in border formation in a variety of neural and non-neural developmental processes (see for example references 4, 5, 7), the results presented here depict a striking dichotomy between the modes of expression of N-CAM in neural and non-neural tissues. It is possible that the sd chain of N-CAM represents the initial evolutionary form of the molecule, whereas the ld chain reflects the acquisition of an additional exon to perform specific functions relating to neural patterning and activity. Such functions remain to be defined in detail. Within the nervous system, the ld chain (probably together with the sd chain) appears in characteristic layers in laminar organs, whereas other layers contain only the sd chain. These observations prompt the hypothesis that the expression of the ld chain on the surface of differentiated neurons may alter the interactions of such subpopulations of N-CAM molecules with the cytoskeleton or with other cell surface molecules such as Ng-CAM, possibly bringing about changes in CAM function that could modulate movement and thus affect layer formation. The roles of such interactions in the control of neural morphogenesis and histogenesis can now be investigated by molecular genetic means.

Figure 8 Schematic representation of expression sequences of N-CAM mRNA splicing in neural and non-neural tissues.

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