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IDENTIFICATION OF MAMMALIAN AND INVERTEBRATE ANALOGUES OF THE AVIAN CALCIUM-DEPENDENT CELL ADHESION PROTEIN N-CADHERIN WITH SYNTHETIC-PEPTIDE DIRECTED ANTIBODIES AGAINST A CONSERVED CYTOPLASMIC DOMAIN

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N-cadherin, a 130kD transmembrane adhesive glycoprotein, is a mediator of specific cellular interactions during development. Analysis of N-cadherin at the protein level, to date, has been largely dependent upon monoclonal antibody NCD-2 which recognizes only avian N-cadherin. We produced a monospecific polyclonal antiserum, C-NCAD(838-856), to a synthetic peptide corresponding to a portion of the highly conserved c-terminal cytoplasmic domain of chick N-cadherin. Using polyacrylamide gel electrophoresis and immunoblotting to map tissue distribution we show that the antiserum detects chick N-cadherin with a similar tissue distribution as NCD-2. Unlike NCD-2, however, anti-C-NCAD(838-856) recognizes N-cadherin analogues in a wide variety of species, including mouse, human, fish and drosophila. The results of comparative immunoblot studies demonstrate similar tissue-specific patterns and apparent molecular weight variation in the chick, mouse and human. This indicates that N-cadherin structure and expression, and most likely function as well, have been highly conserved in evolution. The antiserum recognizes an epitope unique to N-cadherin which is conserved among N-cadherins from a variety of species but is absent from other members of the cadherin gene family, as no immunoreactivity was detected with tissues bearing these other cadherins. The antiserum is thus a useful tool for the phylogenetic and biochemical investigation of N-cadherin from a variety of tissue sources.

The cadherins, including N-, P- and E-cadherin, are a multigene family of calcium-dependent cell adhesion proteins which mediate the formation of intercellular bonds among cells from a wide variety of tissues and species (1). N-cadherin is the predominant cadherin of neural and certain non-neural tissues. Our studies have been directed at elucidating the role which this protein plays in ocular development. Originally referred to as gp130/4.8 when first identified in the chick retina (2), we have more recently demonstrated its identity with N-cadherin (3) as defined by the monoclonal antibody (MAb) NCD-2 (4). One major limitation of the use of MAb NCD-2 is that it detects N-cadherin only in tissues of the chick. In order to investigate the role of N-cadherin in other species, we have prepared antibodies against synthetic peptides corresponding to specific regions of N-cadherin as deduced from published cDNA sequences (5). Antibodies to one of these peptides, C-NCAD(838-856), defines an epitope in the cytoplasmic domain of chick N-cadherin which is highly conserved among members of the cadherin gene family from a variety of tissues and species. This report describes the utilization of this antibody for the identification of N-cadherin and its possible analogues in a variety of invertebrate and vertebrate, including human, tissues and species.

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MATERIALS AND METHODS

Immunological reagents and antisera production

The hybridoma cell line NCD-2, which produces a rat IgG MAb recognizing chick N-cadherin, was the generous gift of Dr. Masatoshi Takeichi, Kyoto, Japan and was maintained in EX-CELL 300 medium from J.R.Scientific (Woodland, CA.) for MAb production. This MAb only recognizes avian N-cadherin. Secondary goat anti-rat IgG and secondary goat anti-rabbit Ig conjugated to alkaline phosphatase were purchased from Fisher Scientific. Following the collection of preimmune serum, New Zealand white female rabbits received subcutaneous injections every two weeks for 8 weeks with 1 mg peptide dissolved in 1ml of TBS/CaCl2 (Tris-buffered saline [0.01M Tris, pH 7.5, 0.15M NaCl], 2mM CaCl2). The initial immunization was done with peptide emulsified with 1 ml of Freund's complete adjuvant and all following immunizations were with 1 ml of incomplete adjuvant. Rabbits were bled by the central ear artery at two week intervals and the titer of antisera was tested by SDS/PAGE and immunoblotting as described below.

Tissue Sources

Normal human eyes obtained at autopsy were generously supplied by the Delaware Valley Eye Bank, Wills Eye Hospital, Philadelphia. The following tissues were generously contributed by colleagues at Thomas Jefferson University: whole fruit flies (*drosophila melanogaster*), sorted into males and females, from Dr. Alan Cristensen; gerbil (*meriones unguicolatus*) tissue, Dr. Kevin Lee; eel (*anguilla rostrada*) tissue, Dr. August Epple; hydra (*hydra attentuata*), Dr. E. Marshall Johnson. White leghorn chicks and embryos (*gallus gallus*) were obtained from Shaw Hatcheries, Oxford. PA. Balb/c mice (*mus musculus*) were obtained from Charles River.

Tissue dissection and cell preparation

Animals were killed by decapitation, cervical dislocation or CO_2 narcosis as appropriate to the species. Tissues were dissected and used immediately or first dissociated into cells as previously described (6) with the following modifications. Dissection was into HBSG (Hepes-buffered saline containing 10 mM Hepes [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.4, 0.15 M NaCl, 3 mM KCl, 1 mg/ml glucose, and 10 µg/ml phenol red) with an added 1 mM CaCl2 (HBSG/CaCl2). For the preparation of trypsinized (TRP) cells, tissues were washed with HBSG and then incubated in HBSG with 0.25 mg/ml of trypsin (231 units/ mg, Worthington) at 37oC for 30 minutes. Calcium-trypsinized (CAT) cells were prepared similarly except that HBSG/CaCl2 was used for the wash and trypsin incubation. Following tryptic digestion, tissues were dispersed by trituration and were washed in HBSG/CaCl2 with 1 mM PMSF (phenylmethylsulfonyl fluoride).

Peptide synthesis

Four peptides were synthesized by conventional solid phase techniques and purified as described previously (7). Each peptide has been designated according to the origin of its sequence followed by the inclusive amino acid residue numbers of the sequence to which it corresponds. For example, C-NCAD(838-856) designates the peptide comprised of residues 838-856, inclusive, of the cDNA-deduced amino acid sequence of chicken N-cadherin (5). Following this nomenclature, the following peptides were synthesized: C-NCAD(105-124) (TEFVVSARDKETQEEWQMKV), corresponding to the leader peptide region; C-NCAD(165-173) (DWVIPPINL), corresponding to the amino terminus; C-NCAD(541-60) (SKLSDPANWLKIDPVNGQIT), corresponding to an extracellular region near the cell membrane; and C-NCAD(838-856) (IGDFINEGLKAADNDPTAP), corresponding to a cytoplasmic region near the carboxy terminus. These peptides were predicted to be antigenic amphipathic α-helical segments according to the algorithm developed by Margalit and associates (8). The single letter abbreviation code for the amino acids used is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Polyacrylamide gel electrophoresis and western blotting

Polyacrylamide gel electrophoresis was carried out according to Laemmli (9). For one-dimensional SDS/PAGE slab gels, tissues or cells were placed directly into 1X sample buffer (0.0625M Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol), immediately boiled for five minutes, centrifuged at high speed in a tabletop microcentrifuge and then loaded onto 5-15% gradient polyacrylamide gels. Molecular weight markers were pre-stained protein standards from Bethesda Research Laboratories. Following electrophoresis the separated proteins in the gel were transferred to nitrocellulose according to Towbin et al. (10) for immunodetection of proteins. Following transfer the blots were removed and washed briefly in TBS/CaCl2. In some cases the lower portion of the immunoblot was cut off and stained with Amido Black for total protein determination. For immunoblot analysis the nitrocellulose paper was first incubated with "blotto" (5% nonfat dry milk) (11) in TBS/CaCl2 (0.01M Tris, pH 7.5, 0.15M NaCl, 2mM CaCl2) to block non-specific binding sites. The blots were then incubated with primary antibodies diluted in TBS/CaCl2/blotto for twelve hours with gentle shaking. In certain experiments, the specificity of antibody binding was tested by preincubation of the primary antibodies with various synthetic peptides. After washing three times in TBS/CaCl2 the blots were incubated with the appropriate secondary alkaline phosphatase conjugated antibodies at a dilution of 1:4000 in TBS/CaCl2/blotto. Following three washes in TBS/CaCl2, the blots were developed with bromochloro-indolylphosphate (BCIP, 75 µl of a 50 mg/ml stock) and nitroblue

tetrazolium (NBT, 12.5 µl of a 75mg/ml stock) in 50 mls of alkaline phosphatase detection buffer (100mM Tris, 100mM NaCl, 5mM $MgCl_2$, pH 9.5).

RESULTS

Antisera directed against synthetic peptides recognize N-cadherin

Of the four synthetic peptides used to immunize rabbits only peptide C-NCAD(838-856) elicited a high antibody titer by 8 weeks. This antiserum (hereafter refered to as anti-C-NCAD(838-856) recognizes a protein of 130kD (Figure 1, lanes 4 through 7). In comparison, this band is identical to that defined by MAb NCD-2 (Figure 1, lane 1). Preimmune serum or secondary antibody alone showed no reaction (Figure 1, lanes 3 and 4). The specificity of anti-C-NCAD(838-856) reaction with the 130kD protein on immunoblots was examined by competitive inhibition analysis using the N-cadherin synthetic peptides (Figure 1, lanes 5-8); purified N-cadherin is not available. Of those peptides tested, only C-NCAD(838-856) uniquely and completely inhibited reaction with the 130kD protein (Figure 1, lane 8), demonstrating the specificity of the antiserum and indicating that the 130kD protein and Peptide C are structurally related. These results and the finding that MAb NCD-2 and anti-C-NCAD(838-856) identify identical bands on immunoblots strongly suggest that the 130kD protein recognized by anti-C-NCAD(838-856) is N-cadherin.

The identity of the immunoreactive 130kD protein as N-cadherin was further investigated by examination of its differential susceptibility to proteolysis in the presence and absence of calcium (Figure 2). N-cadherin as detected

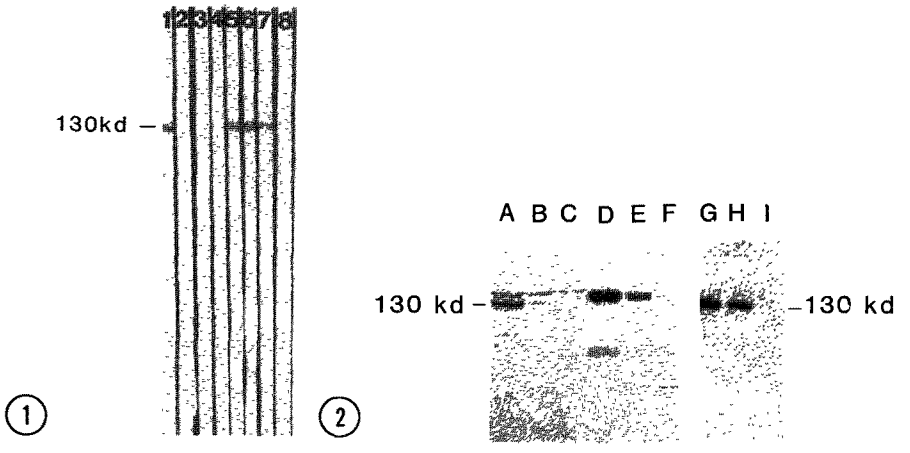


Figure 1. The target of anti-C-NCAD(838-856) comigrates with N-cadherin and is specifically blocked by the corresponding synthetic peptide. Brain tissue from embryonic day 10 chicks was subjected to SDS/PAGE, electroblotting and immunostaining as described in Materials and Methods. Monoclonal antibody NCD-2 was used at a concentration of 1:1000, anti-C-NCAD(838-856) and preimmune serum were used at 1:10,000, and, where indicated, purified peptides were preincubated with antibodies and included during the subsequent incubation at 20µg/ml. Blot strips containing total brain proteins were stained with MAb NCD-2 (Lane 1), preimmune serum (Lane 2), secondary antibody alone (Lane 3), anti-C-NCAD(838-856) (lane 4), or anti-C-NCAD(838-856) and the following purified peptides: C-NCAD(105-124) (Lane 5), C-NCAD(165-173) (Lane 6), C-NCAD(541-60) (Lane 7), and C-NCAD(838-856) (Lane 8). The position of 130kD N-cadherin is indicated.

Figure 2. Calcium protects N-cadherin against proteolysis as detected by both MAb NCD-2 and anti-C-NCAD(838-856). Embryonic day 10 neural retina (Lanes A through C and G through I) and lens (Lanes D through F) tissues were used directly (Lanes A, D and G) or following digestion with trypsin in the presence (Lanes B,E and H) or absence (Lanes C,F and I) of calcium for analysis by SDS/PAGE, electroblotting and immunostaining with anti-C-NCAD(838-856) (1:1000, Lanes A through F) or MAb NCD-2 (1:10,000, Lanes G through I) as described in Materials and Methods. The position of 130kD N-cadherin is indicated.

by MAb NCD-2 is present in intact retina tissue (Figure 2, lane G) as well as tissue trypsinized in the presence of calcium (Figure 2, lane H), but is no longer detected in tissue trypsinized in the absence of calcium (Figure 2, lane I). A similar pattern in observed using anti-C-NCAD(838-856), with respect to the presence and absence of the 130kD protein in intact and trypsinized retina (Figure 2, lanes A-C) or lens (Figure 2 lanes D-F) tissues, findings also consistent with the protein recognized being N-cadherin. At the low dilution of antiserum used in this experiment, other minor protein bands are detected. However, they do not share the same pattern of expression expected of intact cell surface N-cadherin. These immunoreactive bands may in some cases represent low molecular weight fragments or high molecular weight precursors of N-cadherin. Consistent with the former possibility is the increase in certain low molecular weight bands under trypsinization conditions where the 130kD band is concommittantly reduced (compare Figure 2, lanes D,E and F). Consistent with the latter possibility is the tryptic insensitivity of the approximately 140 kD band observed in retinal tissues (Figure 2, lanes A-C). We have consistently observed that N-cadherin from the lens is resolved on SDS-PAGE to an apparently higher molecular weight than retinal N-cadherin (compare Figure 2, lanes A and D; Lagunowich and Grunwald, submitted; also, see below).

The expression patterns of chick N-cadherin as defined by MAb NCD-2 and anti-C-NCAD(838-856) are identical

A comparison of the immunoreactivity patterns of anti-C-NCAD(838-856) and MAb NCD-2 demonstrate identical patterns of antigen recognition (Figure 3). The parallel tissue specific levels of N-cadherin expression revealed with both antibody probes include a very high level of expression in the heart (Figure 3 lanes A3 and B3), an intermediate level in neural tissues and the lens (Figure 3, lanes A1,2,4,5 and B1,2,4,5), a low level in the retinal pigment epithelium (Figure 3, lanes A6 and B6), and no detectable N-cadherin in muscle or liver (Figure 3, lanes A7,8 and B7,8). The lack of immunoreactivity with liver indicates that other members of the cadherin gene family are not recognized by anti-C-NCAD(838-856) (see discussion). As detected with both anti-C-NCAD(838-856) and Mab NCD-2, the immunoreactive bands from both lens and heart have a slightly higher apparent molecular weight than that observed in neural tissues (compare lanes 2 and 3 with lanes 1,4 and 5 in both panels A and B of Figure 3). The qualitatively and quantitatively distinct patterns appear identical whether detected with the anti-C-NCAD(838-856) or MAb NCD-2, strongly suggesting that anti-C-NCAD(838-856) specifically recognizes N-cadherin.

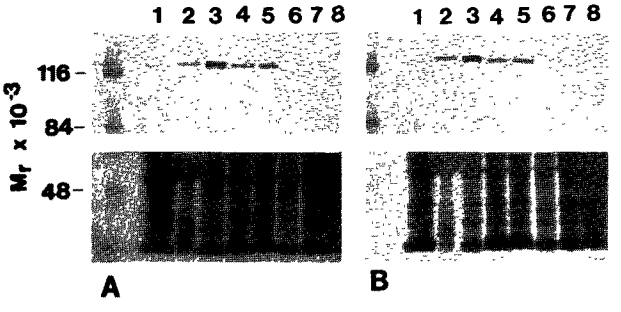


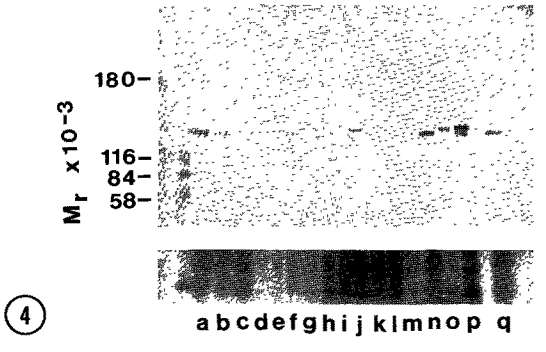
Figure 3. Comparison of MAb NCD-2 and anti-C-NCAD(838-856) immunoreactivity with various embryonic chick tissues. Proteins from 10-day embryonic chick tissues were resolved by SDS-PAGE, electroblotting and immunostaining with MAb NCD-2 (1:1000, Panel A) or anti-C-NCAD(838-856) (1:10,000 Panel B) as described under Materials and Methods. The lower half of the blots was cut off and stained for total protein prior to immunostaining. Tissues represented in both panels are neural retina (Lane 1), lens (Lane 2), heart (Lane 3), cerebral lobe (Lane 4), optic lobe (Lane 5), retinal pigment epithelium (Lane 6), skeletal muscle (Lane 7) and liver (Lane 8). Molecular weight standards are indicated in the lane at the left of each panel.

The expression pattern of murine N-cadherin detected with anti-C-NCAD(838-856) is similar to the chick pattern

MAb NCD-2 does not recognize N-cadherin from organisms outside the class *Aves*. However, anti-C-NCAD(838-856) does recognize a similar 130kD protein in the mouse (Figure 4). This protein has similar electrophoretic properties to chick brain N-cadherin (Figure 4, lane Q). It is also detected in neural tissue from embryonic and adult mouse brain, spinal cord and eye (Figure 4, lanes A,B,C,F,G,M,N). Embryonic and adult mouse heart, as is the case for the chick heart, both possess an immunoreactive band of higher apparent molecular weight than the neural tissues (Figure 4, lanes O and P). These results, which parallel N-cadherin patterns in the chick, are consistent with the recognition of a murine analogue of chick N-cadherin by anti-C-NCAD(838-856). Among the mouse tissues tested, no immunoreactive bands are detected in embryonic or adult tongue, embryonic or adult intestine, embryonic liver, or placenta (Figure 4, lanes D,E,H,I,K,L), indicating that anti-C-NCAD(838-856) does not recognize other murine cadherins such as P-cadherin and E-cadherin. Unlike the embryonic liver, however, anti-C-NCAD(838-856) does detect a protein in adult mouse liver with an apparent molecular weight intermediate between neural and cardiac N-cadherin (Figure 4, lane J). These results suggest that, unlike MAb NCD-2, anti-C-NCAD(838-856) recognizes murine N-cadherin.

Anti-C-NCAD(838-856) recognizes N-cadherin analogues in a variety of species

The immunoreactivity pattern of anti-C-NCAD(838-856) was also tested with tissues from organisms representing various vertebrate and invertebrate classes (Figure 5). Immunoblot analysis indicates that proteins with similar electrophoretic mobility to chick N-cadherin (Figure 5A, lane F) are recognized by anti-C-NCAD(838-856) in neural tissues from a variety of vertebrates including gerbils, eels, and humans (Figure 5A, lanes B,D,E). As in



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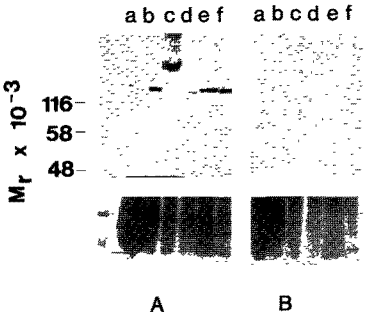

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Figure 4. Immunoreactivity of anti-C-NCAD(838-856) with tissues of the mouse. Proteins of tissues from embryonic day 15 and adult mice were resolved by SDS-PAGE, electroblotting and immunodetection with anti-C-NCAD(838-856) (1:10,000) as described in Materials and Methods. Murine tissues represented are a) embryonic whole brain (a), adult cerebrum (b), adult cerebellum (c), embryonic tongue (d), adult tongue (e), embryonic spinal cord (f), adult spinal cord (g), placenta (h), embryonic liver (i), adult liver (j), embryonic intestine (k), adult intestine (l), embryonic whole eye (m), adult whole eye (n), embryonic heart (o), adult heart (p). Lane q contains day 10 embryonic chick brain. The lower portion of the blot was cut off and stained for total protein prior to immunostaining the upper portion.

Figure 5. Immunoreactivity of various vertebrate and invertebrate tissues with anti-C-NCAD(838-856). Tissue samples prepared from various organisms were analyzed by SDS-PAGE, electroblotting and immunostaining with anti-C-NCAD(838-856) (1:10,000, Panel A) or preimmune serum (1:10,000, Panel B) as described in Materials and Methods. The tissues and species represented in both panels are adult human lens (a), adult human neural retina (b), whole male drosophila (c), adult eel brain (d), adult gerbil brain (e) and 10 day embryonic chick brain (f) The lower portion of the blot was cut off and stained for total protein prior to immunostaining the upper portion.

ocular tissues of the chick, the human protein recognized on immunoblots differs in electrophoretic mobility between the retina and lens, with the lens protein possessing a higher apparent molecular weight than the retina protein (Figure 5A, lanes A and B). Anti-C-NCAD(838-856) is also immunoreactive with two Drosophila proteins, one with a molecular weight close to that of the vertebrate N-cadherins, and the other having a considerably higher apparent molecular weight (Figure 5A lane C). None of the above tissues demonstrated significant immunoreactivity with preimmune rabbit serum (Figure 5B). In contrast, no immunoreactive bands were detected with anti-C-NCAD(838-856) in extracts of hydra (data not shown).

DISCUSSION

That the protein recognized by anti-C-NCAD(838-856) is N-cadherin is indicated by 1) similar apparent molecular weights, 2) inhibition of binding when competed with the original N-cadherin peptide fragment, 3) similar calcium-dependent tryptic sensitivity profiles, 4) similar quantitative differences in the level of expression between various tissues, and 5) similar variations in apparent molecular weight between different tissues, when compared to reaction with MAb NCD-2. When used at the optimal concentration, anti-C-NCAD(838-856) recognizes a single protein band in most immunoreactive tissues. The data also indicate that anti-C-NCAD(838-856) does not recognize other members of the cadherin family in the chick such as L-CAM (12) and CRM-L (13) which are found in the embryonic chick liver. The molecular weight differences observed between N-cadherin from different chick tissues, whatever their nature, must involve biochemical differences in the protein molecules which do not affect recognition by either MAb NCD-2 or anti-C-NCAD(838-856).

When used at a low dilution, and especially when directed against chick tissues, anti-C-NCAD(838-856) is immunoreactive with an array of minor protein bands with higher and lower molecular weight than authentic N-cadherin. The lower molecular weight bands most likely correspond to breakdown products of N-cadherin. Such products can occasionally be revealed with MAb NCD-2 as well, and are found primarily in trypsinized tissues but occasionally in intact tissue samples as well. The higher molecular weight protein bands may correspond to precursors of mature N-cadherin, as the reported cDNA sequence for chick N-cadherin suggests the presence of both a signal peptide as well as a large N-terminal open reading frame region which is not detected in the mature protein (5). The apparent resistance to trypsinization of these higher molecular weight bands is consistent with the possibility that they represent such a precursor, since such a molecule would be expected to be found intracellularly and not be accessible to exogenously added trypsin. These bands were not observed in all tissues and species, and further experiments will be required to determine the relationship of these immunoreactive bands to N-cadherin.

Perhaps the most important conclusion to be drawn from these studies is with regards to the highly conserved nature of N-cadherin expression in mammalian tissues as compared to avian tissues. The presence of N-cadherin in mammalian tissue had been originally suggested by studies using antibody-mediated cell lysis, although the responsible protein was not identified (1). More recently, a cDNA corresponding to murine N-cadherin has been described (14), although there is no published data regarding the protein or its distribution in the mouse or other mammals. Thus the studies reported here are the first to our knowledge describing the distribution of N-cadherin in the mouse. It is of interest that the tissue distribution pattern of murine N-cadherin, both in terms of level of expression and molecular weight differences, parallels that of avian N-cadherin, suggesting that not only the stucture of the protein but its genetic regulation, molecular heterogeneity and presumably function have all been remarkably conserved over long evolutionary periods. The observation of an immunoreactive protein in adult mouse liver was unexpected as no such immunoreactivity was detected with embryonic mouse liver and the only cadherins described to date in liver have been E-cadherin in the mouse and L-CAM and CRM-L in the chick. Future studies will be

directed at distinguishing the following possibilities: 1) adult chicken liver indeed expresses avian N-cadherin, and the mouse and chick patterns of N-cadherin expression are therefore in complete register; 2) the adult chicken liver does not, while the adult mouse liver does, express N-cadherin, suggesting that cadherins are utilized differently in the development of homologous tissues between different species; or 3) the protein observed in adult mouse liver is not N-cadherin but is a distinct, however related, gene product.

The previously identified members of the murine cadherin gene family are E-cadherin, generally found in non-neural epithelial tissues (15), and P-cadherin, originally identified in placenta (16). The immunoreactivity patterns of anti-C-NCAD(838-856) with tissues of the mouse indicate that neither E-cadherin nor P-cadherin are recognized by this antiserum. This result, together with the similar lack of immunoreactivity with chick tissues that contain L-CAM, suggest that despite the high degree of sequence conservation of the C-terminal domain of all known members of the cadherin gene family, anti-C-NCAD(838-856) is a unique tool which fortuitously recognizes an epitope unique to N-cadherin that is conserved among N-cadherins from a variety of species but is absent from other members of the cadherin gene family.

Immunoreactivity of anti-C-NCAD(838-856) extends to human tissues, revealing another degree of structural conservation between vertebrate N-cadherins. In chicks and humans, there is a consistent difference in apparent molecular weight between retinal and lens N-cadherins, with the lens protein displaying an apparent molecular weight approximately 5kD higher than the retinal form in both species. In chicks and mice, there is a similar conservation of a relatively higher apparent molecular weight among cardiac N-cadherins as compared to neural N-cadherins. Experiments are currently under way to determine whether these differences are possibly due to alternative mRNA processing or posttranslational modifications. With respect to the source of the original sequence information, the most distantly related organisms probed with anti-C-NCAD(838-856) were fish, insects and coelenterates. The inability to detect an N-cadherin analogue in hydra may reflect either a true absence of an N-cadherin related protein in these primitive organisms, or rather the inability to recognize such a protein due to sequence divergence. Two protein bands are recognized by the antiserum in both eel brain and whole drosophila. The increasing biochemical divergence of the immunoreactive species may reflect increasing phylogenetic divergence of the organisms.

In conclusion, our studies demonstrate the highly conserved nature of N-cadherin expression throughout evolution and suggests that N-cadherin plays a key role in the development of a wide variety of tissues and species. Further experiments directed at analyzing the function of N-cadherin analogues in these species as well as molecular genetic analyses of the related genes in these organisms will ultimately enhance our understanding of these molecules.

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