

NCAM Polypeptides in Heart Development: Association with Z Discs of Forms That Contain the Muscle-specific Domain

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Abstract. Previous studies of neural cell adhesion molecule (NCAM) cDNAs have revealed an alternatively spliced set of small exons (12A, 12B, 12C, and 12D) that encode a region in the extracellular portion of the molecule known as the muscle-specific domain (MSD). The entire MSD region can be expressed in skeletal muscle, heart, and skin; only exons 12A and 12D have been found in brain. These studies did not reveal which NCAM polypeptides contain the MSD region or the immunohistochemical distribution of these NCAM molecules. To address these questions, we prepared antibodies against the oligopeptides encoded by exons 12A and 12B and by exons 12C and 12D, and we used these antibodies to study the forms of NCAM containing the MSD region expressed during embryonic chicken heart development. These antibodies recognize certain forms of NCAM found in the heart, but they do not recognize brain NCAM. In the heart, each of the splice variants of NCAM (large cytoplasmic domain, small cytoplasmic domain, and small surface domain) that differ in their mode of attachment to the plasma membrane or in the size of their cytoplasmic domain is expressed in a form that

contains and in a form that lacks the MSD region. No microheterogeneity is observed in the size of NCAM molecules containing the MSD region, even at the level of cyanogen bromide fragments, suggesting that exons 12A–D are expressed as a single unit. Depending on the site and the stage of development, the percent of NCAM molecules containing the MSD region can vary from nearly 0 to 100%. In general, this percentage increases during development. In immunohistochemical studies of hearts from stage 18 embryos, forms of NCAM containing the MSD region colocalized with Z discs. No other adhesion molecules were found in this distribution at this early stage of development. Studies on isolated cells *in vitro* demonstrate that the colocalization with Z discs of NCAM molecules containing the MSD region does not depend on cell–cell contact, and they raise the possibility that this form of NCAM is involved in cell–extracellular matrix interactions. The association of NCAM molecules containing the MSD region with Z discs suggests that this form of NCAM is involved in early myofibrillogenesis.

ADHESION molecules are important in the control of cell behavior during development both directly through their roles in mediating cell migration and the maintenance of tissue borders, as well as indirectly through the transmembrane signal transduction mechanisms their binding initiates (Takeichi, 1990; Edelman and Crossin, 1991; Hynes, 1992; Damsky and Werb, 1992; Juliano and Haskill, 1993). There are many stages in early heart development when changes in the expression or function of cell–cell adhesion molecules (CAMs)¹ may mediate or sta-

bilize changes in morphology or may influence other developmental processes. The primitive heart (precardiac mesodermal epithelium) appears as paired primordia (Romanoff, 1960; Carlson, 1981). NCAM and N-cadherin are expressed on almost all of these cells (Crossin and Hoffman, 1991; Linask, 1992). Some of these precardiac cells downregulate expression of these adhesion molecules, convert into mesenchymal cells, and migrate into the basement membrane. The primordia then fuse to form one double-layered tube consisting of an inner and outer epithelium (the endocardium and myocardium, respectively) separated by a middle acellular basement membrane known as the cardiac jelly. The endocardium is derived from the cells that have undergone an epithelial–mesenchymal transformation, which then convert back to epithelium.

The transformation of the heart from a simple tube to a four-chambered structure containing valves and the appropriate connections to the rest of the circulatory system involves a second epithelial–mesenchymal transformation. This transformation is region specific; it results in the growth of the endocardial cushion tissue (ECT) in the atrioventricu-

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1. *Abbreviations used in this paper:* CAM, cell–cell adhesion molecule; CNBr, cyanogen bromide; GPI, glycosyl-phosphatidylinositol; ECT, endocardial cushion tissue; KLH, keyhole limpet hemocyanin; ld, large cytoplasmic domain; MSD, muscle-specific domain; NCAM, neural cell adhesion molecule; OTG, octylthioglucopyranoside; PIPLC, phosphatidylinositol-specific phospholipase C; sd, small cytoplasmic domain; ssd, small surface domain; VASE, variable domain alternatively spliced exon.

lar region and the outflow tract but not in the atrium or ventricle (Markwald et al., 1984). The ECT arises from cells of the endocardial endothelium that adhere, convert into mesenchymal cells, and migrate into the cardiac jelly (Markwald et al., 1978). Neural cell adhesion molecule (NCAM) is continuously expressed on all cells of the myocardium and endocardial endothelium; in contrast, N-cadherin is expressed only in the myocardium at this stage of heart development (Linask, 1992). Concomitantly with their deadhesion, migrating ECT cells downregulate their expression of NCAM (Mjaatvedt and Markwald, 1989; Crossin and Hoffman, 1991) as assayed immunohistochemically. It remains to be demonstrated, however, whether the levels of NCAM protein actually decrease or whether NCAM becomes masked so that it is not immunohistochemically detectable. Later in development, the growing cushion tissue pads fuse in a manner that subdivides the flow of blood through the heart into two channels. It is likely that the regulation of CAM expression or function plays a role in mediating this sequence of deadhesion and adhesion that occur during the epithelial-mesenchymal transformation and the fusion of cushion tissue pads.

NCAM, the first adhesion molecule to be identified as a member of the IgG superfamily, is a cell surface glycoprotein that contains large amounts of polysialic acid (Cunningham et al., 1987). Several variant forms of NCAM are produced by alternative mRNA splicing from a single gene. The best known of these variants are three forms of brain NCAM that differ in their attachment to the plasma membrane. One form, small surface domain (ssd), is covalently linked to the glycolipid glycosyl-phosphatidylinositol (GPI); two integral membrane forms, large cytoplasmic domain (ld) and small cytoplasmic domain (sd), differ in the size of their cytoplasmic domains (Hemperly et al., 1986; Murray et al., 1986; He et al., 1986). In addition, small inserts have been identified at two distinct sites in the extracellular portion of the molecule. The variable domain alternatively spliced exon (VASE) insert is present between the sequences encoded by exons 7 and 8, and it is found in both the brain and heart (Small et al., 1987; Small and Akeson, 1990). The so-called muscle-specific domain (MSD) region is encoded by four small exons (12A, 12B, 12C, and 12D) that are localized between exons 12 and 13 (Prediger et al., 1988; Thompson et al., 1989), and that appear to be expressed in a variety of splice combinations (Reyes et al., 1991). This region is located between two consecutive fibronectin type III repeats in NCAM and contains site(s) for O-linked glycosylation (Walsh et al., 1989). From this region, only exons 12A and 12D are expressed in the brain (Santoni et al., 1989; Hamshere et al., 1991; Reyes et al., 1993); all four of these small exons are present in heart, skeletal muscle, and skin (Dickson et al., 1987; Prediger et al., 1988; Marsh and Gallin, 1992). While the sequence of these four small exons is highly conserved between humans, rats, and mice, two of the four chicken exons (12A and 12B) are very different in sequence from their mammalian counterparts (Prediger et al., 1988).

NCAM mediates cell-cell adhesion both by a homophilic mechanism (Rutishauser et al., 1982; Hoffman and Edelman, 1983; Sadoul et al., 1983), i.e., NCAM to NCAM, and by a heterophilic mechanism involving the interaction of a heparin-binding domain in NCAM with heparan sulfate pro-

teoglycans (Reyes et al., 1990). Binding experiments on the homophilic mechanism have revealed that the absolute level of expression of NCAM and differential polysialylation strongly affect its adhesive strength (Hoffman and Edelman, 1983). Functional studies on splice variants of NCAM suggest that the expression of the VASE insert inhibits neurite outgrowth, while the MSD region had no effect in these experiments (Doherty et al., 1992). Recent experiments suggest that the MSD region plays a role in myoblast fusion (Peck and Walsh, 1993). Possible functions for the MSD region may be related to the fact that it contains an O-linked carbohydrate acceptor site (Walsh et al., 1989). The mode of association of NCAM with the cell surface may also have functional consequences. Because the ssd form of NCAM is targeted to the apical surface of polarized epithelial cells (Powell et al., 1991), it is possible that this form of NCAM may be localized to the luminal surface of the endocardial endothelium. Recent studies suggest that transmembrane forms of NCAM promote myoblast fusion (Peck and Walsh, 1993) and downregulate the secretion of a 92-kD gelatinase (Edvardsen et al., 1993); the GPI-linked form of NCAM does not have these effects.

In the current study, we have prepared antibodies against two oligopeptides that together comprise the MSD region of chicken NCAM. These antibodies have been used to analyze the expression of forms of NCAM in the developing heart. We find that the ld, sd, and ssd forms of NCAM all can be expressed either containing or lacking the MSD region, that these six forms of NCAM change in their temporal and spatial patterns of expression during development, and that at the protein level, there is no evidence for differential splicing among the small exons 12A, 12B, 12C, and 12D. Most strikingly, we find that forms of NCAM containing the MSD region are colocalized with Z discs, suggesting a role for this region in myofibrillogenesis.

Materials and Methods

Materials

Reagents were purchased from the following sources: keyhole limpet hemocyanin (KLH), Calbiochem-Novabiochem Corp. (San Diego, CA); *N*-succinimidyl 3-(2-pyridylthio)propionate and bicinchoninic acid protein assay kit, Pierce Chemical Co. (Rockford, IL); reagents for gel electrophoresis and Western blotting, BioRad Laboratories (Hercules, CA); leupeptin, Boehringer Mannheim Biochemicals (Indianapolis, IN); neuraminidase from *Streptococcus* sp. (catalogue No. 151738; ICN Biomedicals, Inc., Costa Mesa, CA); DE 52, Whatman Lab Sales Inc. (Hillsboro, OR); cyanogen bromide (CNBr), Aldrich Chemical Co. (Milwaukee, WI). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies

Anti-NCAM. NCAM from 14-d embryonic chicken brains, purified as described below or as previously described (Hoffman et al., 1982), was used to immunize rabbits. This antibody is frequently referred to as the pan-NCAM antibody because it recognizes all forms of NCAM.

Anti-12AB and Anti-12CD. Two peptides that together comprise the MSD region in chicken NCAM (Prediger et al., 1988) were synthesized by the Medical University of South Carolina Protein Sequence Facility. Oligopeptide 12AB contains the sequence encoded by exons 12A and 12B (single letter code RIPHSPSAAAPTCLAE); oligopeptide 12CD contains the sequence encoded by exons 12C and 12D (TTQPLPASESTEPPKG). Each peptide was synthesized with two additional cysteine residues at its COOH-terminal. To induce strong immunogenicity, each oligopeptide was con-

jugated with carrier KLH before immunization. First, Cys residues were reduced using 100 mM DTT for 30 min, and the peptides were desalted on G-25 Sephadex equilibrated in 0.1 M sodium phosphate/0.1 M NaCl, pH 7.5. KLH was desalted using this same buffer, then activated with SPDP at a molar ratio of 1:2,500 for 1 h, then desalted again. Finally, the reduced oligopeptides were incubated with the activated KLH at a 500:1 molar ratio for 1 h, then desalted. These oligopeptide-KLH conjugates were dialyzed against water, lyophilized, and resuspended in PBS.

Inoculations (50 µg NCAM or 1 mg oligopeptide-KLH conjugates per rabbit per injection) were performed, and IgG was purified from serum by standard methods (Thiery et al., 1977).

Monoclonal antibodies 4d (specific for the 1d form of NCAM), V2E9, and JG22 (each specific for the β 1-integrin subunit) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Monoclonal antibody NCD-2 (specific for N-cadherin) was a gift from Dr. Jack Lilién (Clemson University, Clemson, SC). Monoclonal antibodies against vinculin and α -actinin (sarcomeric) were obtained from Sigma Chemical Co. (product Nos. V-9131 [clone hVIN-1] and A-7811 [clone EA-53], respectively).

Western Blots

Samples were resolved by SDS-PAGE under reducing conditions on minigels containing 6% acrylamide, except in the experiment on CNBr fragments of NCAM, in which 12% acrylamide was used and nonreducing conditions were used to enhance antigenicity. Proteins were transblotted to nitrocellulose using a semidry transfer method (Trans-Blot Semi-Dry Electrophoretic Transfer Cell; BioRad). The nitrocellulose was blocked, incubated with primary antibodies, incubated with secondary antibodies (goat anti-rabbit or anti-mouse IgGs conjugated to alkaline phosphatase), and color developed using bromochloroindoyl phosphate and nitroblue tetrazolium as described by BioRad.

Preparation of Membranes/NP-40 Extraction/Neuraminidase Treatment/Phosphatidylinositol-specific Phospholipase C (PIPLC) Digestion

Purified membranes were prepared from 14-d embryonic chicken brains and 14-d embryonic and adult chicken hearts. Brains in cold PBS containing protease inhibitors (*N*-ethylmaleimide, 10 mM; benzamide, 5 mM; leupeptin, 50 µg/ml; aprotinin, 2 µg/ml; phenylmethylsulfonyl fluoride, 2 mM; pepstatin A, 5 µg/ml) were homogenized using an ultrasonic generator (Sonic 2000U; Braun Biotech International, Allentown, PA). Homogenates were overlaid on PBS containing 42% sucrose, then ultracentrifuged at 100,000 g for 30 min at 4°C. The membrane fraction that appeared above the 42% sucrose was harvested, washed by dilution with cold PBS, and collected by centrifugation at 8 krpm for 20 min in a rotor (JA10; Beckman Instruments, Inc., Fullerton, CA) in a Beckman J2-HS high speed centrifuge. For heart membrane preparation, tissue was sonicated in ice-cold hypotonic buffer (200 mM sucrose/5 mM sodium phosphate, pH 7.5/1 mM EGTA) in the presence of protease inhibitors. Low speed centrifugation, 300 g for 10 min, separated the membranes from nuclei and large debris. Membranes in the supernatant were then collected by ultracentrifugation at 100,000 g for 30 min.

Crude membranes were prepared from 4-, 7-, and 10-d embryonic chicken hearts and 5-d embryonic chicken ventricles and cushion tissue pads. Tissue in PBS was homogenized in the presence of protease inhibitors, then washed with PBS and harvested by two rounds of centrifugation (14,000 rpm for 3 min) in a microfuge.

To prepare NP-40 extracts for Western blotting and peanut lectin chromatography, membranes were pelleted in a microfuge (14,000 rpm for 3 min), resuspended in 10 vol of 0.5% NP-40/PBS, and the extracts clarified in the microfuge (14,000 rpm for 5 min). The protein content of NP-40 extracts was quantitated using the BCA assay.

To treat membranes with neuraminidase, pelleted membranes were resuspended in 3 vol of PBS containing 0.3 U/ml of neuraminidase, incubated for 1 h at 37°C, then washed with PBS and harvested by centrifugation. To neuraminidase treat NP-40 extracts, neuraminidase was added to NP-40 extracts to a final 0.3 U/ml and incubated for 1 h at 37°C.

To PIPLC digest membranes, pelleted membranes were resuspended in 3 vol of PBS containing 0.02 U/ml of PIPLC (from *Bacillus cereus*, Sigma catalogue No. P 8804), incubated for 30 min at 37°C, and the PIPLC-released molecules in the supernatant were harvested by centrifugation in a microfuge (14,000 rpm for 3 min). The residual membrane pellet was NP-40 extracted as described above.

Immunoprecipitation of NCAM Molecules Containing the MSD Region

Purified membranes from 14-d embryonic chicken hearts (50 µl packed volume) were resuspended in 0.5 ml PBS containing 1 mg/ml anti-12AB or anti-12CD IgG and incubated for 1 h at 4°C with agitation. Membranes were washed with PBS and harvested by two rounds of centrifugation in a microfuge (14,000 rpm for 3 min). The membranes were extracted with NP-40 as described above, and antigen-antibody complexes were collected by incubation with 50 µl of protein A-Sepharose for 1 h at 4°C. The beads were then washed several times with 0.5% NP-40/PBS. Bound NCAM was eluted by boiling the beads in SDS-PAGE sample buffer and Western blotted using the pan-NCAM antibody as the primary antibody for detection. This procedure allowed immunoprecipitation experiments to be performed using anti-12AB and anti-12CD. Immunoprecipitation experiments that were performed with these antibodies, in which the antibodies were added to proteins that had already been NP-40 extracted, gave uniformly negative results.

Peanut Lectin Chromatography

1.5 ml of NP-40 extract from purified membranes from 14-d embryonic chicken hearts was incubated with 50 µl of peanut lectin-agarose (Sigma catalogue No. L 2507) for 1 h at 4°C with agitation. The beads were then washed several times with 0.5% NP-40/PBS, and bound proteins were eluted with 0.5 ml of 0.5% NP-40/PBS/0.1 M galactose.

Purification of NCAM/CNBr Digestion

Packed brain or heart membranes were resuspended in 10 vol of 0.1 M Tris base to elute weakly attached proteins. Membranes were collected by ultracentrifugation and extracted in 10 vol of 0.5% NP-40/0.2 M sodium phosphate, pH 6.3, containing protease inhibitors. Extracts were clarified by ultracentrifugation and applied to DE 52 equilibrated in 0.5% NP-40/0.2 M sodium phosphate, pH 6.3 (10 ml of extract per ml of beads). The column was washed with 0.2 M sodium phosphate, pH 6.3, until no NP-40 could be detected in the eluate. Contaminants were then eluted with 1 M NaCl/20 mM sodium phosphate, pH 6.5. The column was then washed with 25 mM octylthioglucoopyranoside (OTG)/20 mM sodium phosphate, pH 6.5. Finally, NCAM was eluted from the DE 52 using 0.55 M NaCl/25 mM OTG/20 mM sodium phosphate, pH 6.5. The eluate was dialyzed versus water and lyophilized. NCAM was further purified by HPLC gel filtration on a column (G4000SW; TosoHAAS, Philadelphia, PA) equilibrated with 25 mM OTG/20 mM sodium phosphate, pH 6.5. This procedure yielded brain NCAM that was essentially 100% pure, as evaluated by SDS-PAGE, and heart NCAM that was ~30% pure.

Purified NCAM was dialyzed versus water, adjusted to a final 70% formic acid, and treated with a large excess of CNBr under N₂ overnight, then dried in a Speed-Vac (Savant Instrument, Inc., Hicksville, NY). This material was then dissolved in a small volume of 25 mM OTG/PBS and neuraminidase treated as described above.

Immunohistochemistry

Hamburger and Hamilton (1951) stage 18 chicken embryos were briefly rinsed in PBS and placed in plastic molds with Tissue-Tek compound media (ICN Biomedicals). Samples were quick frozen using liquid nitrogen. 5-µm frozen sections were cut and mounted on poly-L-lysine-coated slide glasses. After rinsing off the embedding medium with distilled water and PBS, the sections were incubated at room temperature with 10% normal goat serum/PBS for 30 min to block nonspecific binding. Sections were incubated with primary antibodies (25 µg/ml for rabbit antibodies, 10 µg/ml for monoclonal antibodies, except for antivinculin and anti- α -actinin, which were used at 1:400 and 1:800 dilutions, respectively, as per the manufacturer's instructions) in 1% BSA/PBS in a humid chamber at 4°C for 4–16 h, then rinsed with 1% BSA/PBS. Appropriate fluorescein-conjugated secondary antibodies (10 µg/ml of goat anti-rabbit IgG or goat anti-mouse IgG [Organon Teknica Corp., Durham, NC] in 1% BSA/PBS) were applied for 1 h, followed by rinsing and mounting in 90% glycerol, 0.2 M *n*-propyl gallate (Giloh and Sedat, 1982). Stained sections were examined by epifluorescent illumination using an Axioscope (Carl Zeiss, Inc., Thornwood, NY).

Two experiments were performed to verify that the only molecules recognized by anti-12CD are NCAM molecules containing the peptide sequence used as an immunogen. In one, the diluted antibody (25 µg/ml) was

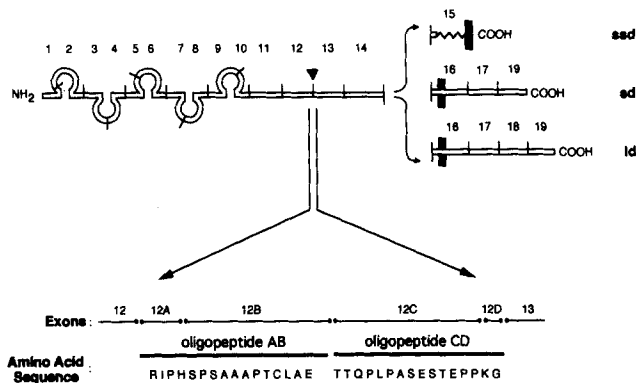


Figure 1. Schematic model of forms of NCAM produced by alternative mRNA splicing in the MSD and membrane attachment regions. Exon numbers encoding the various regions within the molecule are indicated. Id, sd, and ssd forms differ in their attachment to the membrane through the expression of transmembrane and cytoplasmic exons 16, 17, 18, and 19, or through the expression of exon 15 encoding a peptide that becomes covalently linked to GPI. The MSD region comprised of exons 12A, 12B, 12C, and 12D is expanded, and the sequences of the peptides from this region used to immunize rabbits are shown in single-letter code.

preabsorbed by mixing with 1.25 $\mu\text{g/ml}$ of the immunogenic peptide for 30 min at room temperature, followed by clarification at 13,000 g. In the second, tissue sections were preincubated with the pan-NCAM antibody (25 $\mu\text{g/ml}$) for 4 h before the addition of anti-12CD (25 $\mu\text{g/ml}$). Both treatments completely blocked anti-12CD staining.

To examine the distribution of anti-12CD staining in cultured cells, cells were released from stage 18 hearts as described by Krug et al. (1987) and depleted of fibroblasts as described by Lau (1994). The enriched cardiomyocytes were plated on slides coated using a 20 $\mu\text{g/ml}$ solution of fibronectin

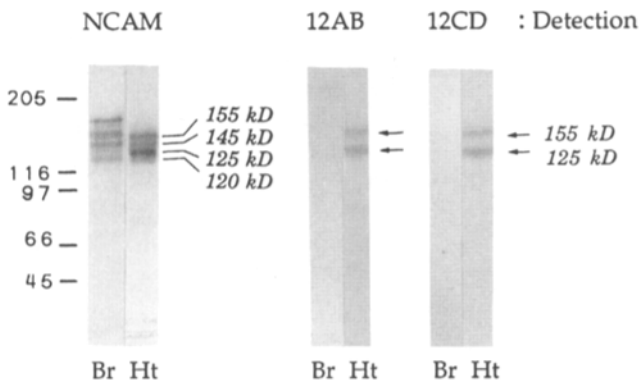


Figure 2. Specificity of antibodies against the MSD region of NCAM. As described in Materials and Methods, NP-40 extracts from 14-d embryonic chicken heart and brain membranes were neuraminidase treated, then Western blotted using antibodies prepared against the indicated antigens as the primary antibodies for detection. Lanes containing heart extract (*Ht*) were loaded with 5 μg of protein for detection with the pan-NCAM antibody and 15 μg of protein for detection with anti-12AB or anti-12CD; lanes containing brain extract (*Br*) were loaded with 1 μg of protein for detection with the pan-NCAM antibody and 3 μg for detection with anti-12AB or anti-12CD. This 5:1 heart extract/brain extract ratio results in similar levels of total NCAM detected with the pan-NCAM antibody. The four forms of heart NCAM recognized by the pan-NCAM antibody (155, 145, 125, and 120 kD) and the two forms of heart NCAM recognized by anti-12AB and anti-12CD (155 and 125 kD) are indicated. The migration of standard proteins is indicated by their relative molecular masses $\times 10^{-3}$.

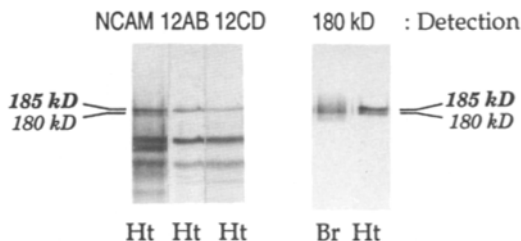


Figure 3. Forms of NCAM in 7-d embryonic heart tissue. As described in Materials and Methods, NP-40 extracts of membranes were neuraminidase treated, then Western blotted using antibodies specific for the indicated antigens as the primary antibodies for detection (180 kD indicates the use of monoclonal antibody 4d specific for the ld form of NCAM). Lanes containing 7-d embryonic heart extract (*Ht*) were loaded with 5 μg of protein for detection with the pan-NCAM antibody or 4d and with 15 μg of protein for detection with anti-12AB or anti-12CD. The lane containing 14-d embryonic brain extract (*Br*) was loaded with 1 μg of protein. The 185- and 180-kD forms of heart NCAM recognized by 4d are indicated.

(New York Blood Center, New York) and incubated for 3–7 d in the “complete M199 medium” of Krug et al. (1987). Cells were fixed with cold methanol (10 min, -20°C), then rinsed briefly with 50% methanol/50% PBS followed by a PBS rinse. Cells were stained as described above and examined by confocal microscopy.

Confocal Microscopy

As described above, 10- μm frozen sections were prepared and stained with anti-12CD as the primary antibody and with FITC-conjugated goat anti-rabbit IgG as the secondary antibody. The sections were then double stained by further incubation with either monoclonal anti- α -actinin (sarcomeric) followed by rhodamine isothiocyanate-conjugated goat anti-mouse IgG or the pan-NCAM antibody followed by rhodamine isothiocyanate-conjugated goat anti-rabbit IgG. Stained sections were examined by confocal microscopy using a BioRad MRC-1000 Laser Scanning Confocal Imaging System equipped with a Zeiss Axioscope. The confocal iris was set at the smallest value to obtain the thinnest possible optical sections ($<0.25 \mu\text{m}$).

Results

Antibodies Specific for the MSD Region in NCAM

A subset of NCAM molecules from skeletal muscle, heart, and skin contain a polypeptide sequence that is encoded by a series of four small alternatively spliced exons (12A–D) (Dickson et al., 1987; Prediger et al., 1988; Marsh and Gallin, 1992). This portion of the molecule was originally (Dickson et al., 1987) named the muscle-specific domain; this nomenclature will be used here. The localization and sequence of this region of NCAM is shown in Fig. 1.

Two antibodies were prepared against the MSD region of NCAM; one (anti-12AB) against the polypeptide encoded by exons 12A and 12B, the second (anti-12CD) against the polypeptide encoded by exons 12C and 12D (see Materials and Methods for details of antibody production). The ability of these antibodies to recognize brain and heart NCAM in extracts from 14-d embryos was compared to that of antibodies prepared against the entire NCAM molecule (Fig. 2). Whereas the pan-NCAM antibody recognizes all forms of NCAM present in brain or heart, anti-12AB and anti-12CD recognize only heart NCAM. Moreover, only two (155 and 125 kD) of the four forms of heart NCAM recognized by the

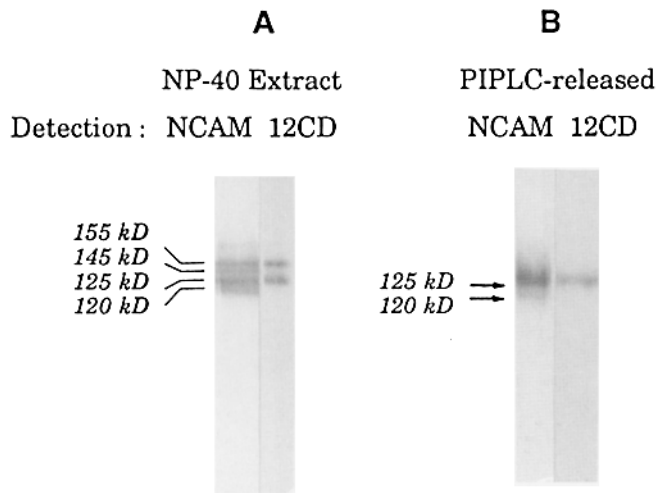


Figure 4. Transmembrane and GPI-linked forms of NCAM. As described in Materials and Methods, 14-d embryonic heart membranes were neuraminidase treated, treated with PIPLC to release GPI-linked proteins, and the residual membranes were extracted with NP-40. NP-40 extracts (5 μ g for detection with the pan-NCAM antibody; 15 μ g for detection with anti-12CD) and PIPLC-released material (from equivalent amounts of membranes as the NP-40 extracts) were Western blotted using antibodies prepared against the indicated antigens as the primary antigens for detection. The four forms (recognized by the pan-NCAM antibody) of heart NCAM in NP-40 extracts (155, 145, 125, and 120 kD) and the two PIPLC-releasable forms of heart NCAM (125 and 120 kD) are indicated.

pan-NCAM antibody at this stage of development (155, 145, 125, and 120 kD) are recognized by anti-12AB and anti-12CD.

The specificities of anti-12AB and anti-12CD were also examined in extracts of 7-d embryonic hearts (Fig. 3) in which six forms of NCAM are detected using the pan-NCAM antibody (185 and 180 kD, in addition to the four forms observed in 14-d heart extracts). Anti-12AB and anti-12CD recognized the 185-kD form, as well as the two forms recognized in 14-d extracts.

ld, sd, and ssd Forms of NCAM All Can Contain the MSD Region

Three forms of NCAM (Cunningham et al., 1987) are defined by their mode of attachment to the plasma membrane (Fig. 1). Two forms, ld and sd, are transmembrane proteins containing large and small cytoplasmic domains, respectively. The third form, ssd, is GPI linked to the plasma membrane. ld NCAM has a molecular mass of \sim 180 kD and had previously been observed conclusively only in neural tissues. To determine whether the 185- and 180-kD forms of NCAM observed in 7-d embryonic heart are authentic ld NCAM, extracts were Western blotted (Fig. 3) using a monoclonal antibody (4d; Watanabe et al., 1986) specific for this form of NCAM. Both the 185- and 180-kD forms of heart NCAM, as well as a form of brain NCAM of similar molecular mass, were recognized by this antibody. These results indicate that two forms of ld NCAM (185 and 180 kD) are present in these heart extracts, and that one of these forms (185 kD) also contains the MSD region.

To determine which of the forms of NCAM we observe are GPI linked to the plasma membrane, membranes from 14-d

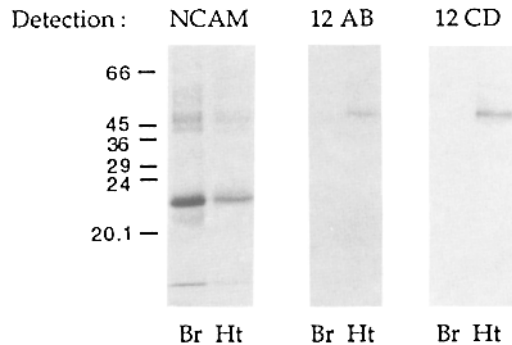


Figure 5. MSD region in CNBr digests of NCAM. As described in Materials and Methods, NCAM was purified from 14-d embryonic brain and heart membranes, digested with CNBr, neuraminidase treated, and Western blotted using antibodies prepared against the indicated antigens as the primary antibodies for detection. Lanes containing brain NCAM CNBr fragments (*Br*) were loaded with 10 μ g of protein for detection with the pan-NCAM antibody and 30 μ g of protein for detection with anti-12AB or anti-12CD; lanes containing heart NCAM CNBr fragments (*Ht*) were loaded with 30 μ g of protein for detection with the pan-NCAM antibody and 90 μ g of protein for detection with anti-12AB or anti-12CD. This ratio of brain and heart NCAM CNBr fragments was chosen to provide similar levels of signal using the pan-NCAM antibody for detection. The migration of standard proteins is indicated by their relative molecular masses $\times 10^{-3}$.

embryonic heart tissue were treated with PIPLC to release the GPI-linked forms. NP-40 extracts of the residual membranes (Fig. 4 A) or the PIPLC-released material (Fig. 4 B) were then Western blotted with either the pan-NCAM antibody or anti-12CD. Only the 125- and 120-kD forms of NCAM were released by PIPLC, and of these, only the 125-kD form was recognized by anti-12CD. As in Fig. 2, the 155-, 145-, 125-, and 120-kD forms of NCAM were present in the residual membranes, of which only the 155- and 125-kD forms were recognized by anti-12CD. Thus, it appears that the 185- and 180-kD forms of NCAM are ld, the 155- and 145-kD forms are sd, and the 125- and 120-kD forms are ssd. In each pair, there is a higher molecular mass species containing the MSD region and a lower molecular mass species lacking the MSD region.

The MSD Region Does Not Show Heterogeneity in Molecular Weight

PCR amplification studies of rat heart cDNAs suggested that alternative splicing occurs among exons 12A–D that should lead to multiple forms of NCAM with MSD regions of different sizes (Reyes et al., 1991). In contrast, our results show no evidence for size heterogeneity in the MSD regions of ld, sd, or ssd NCAM from either 7-d or 14-d embryonic hearts (Figs. 2–4). To evaluate whether we do not observe size heterogeneity because the differences in the sizes of the variants are small compared to the size of the intact NCAM molecule, CNBr fragments of heart NCAM were prepared and Western blotted with the pan-NCAM antibody, anti-12AB, and anti-12CD (Fig. 5). As expected from the localization of the MSD region within NCAM (Dickson et al., 1987), anti-12AB and anti-12CD recognized a single CNBr fragment of

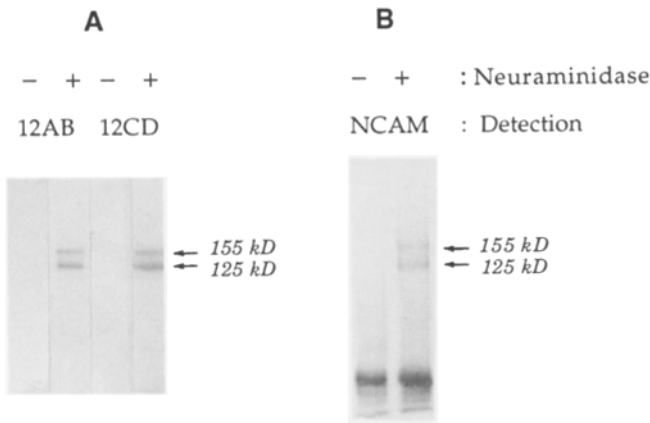


Figure 6. Effect of polysialic acid on the recognition of NCAM by anti-12AB and anti-12CD. (A) Western blots: as described in Materials and Methods, NP-40 extracts of 14-d embryonic heart membranes (15 μ g aliquots) either without (–) or with (+) neuraminidase treatment were Western blotted using antibodies against the indicated antigens as the primary antibodies for detection. (B) Immunoprecipitation: as described in Materials and Methods, NCAM from 14-d embryonic heart plasma membranes, either without (–) or with (+) neuraminidase treatment, was immunoprecipitated using anti-12CD IgG. This material was Western blotted using the pan-NCAM antibody as the primary antibody for detection. Similar results were obtained in immunoprecipitation experiments using anti-12AB IgG (data not shown). The heavy band near the bottom of both lanes represents IgG heavy chain. The migration of the 155- and 125-kD forms of NCAM containing the MSD region is shown in both A and B.

~45 kD. No size heterogeneity in the MSD region was seen, even in these CNBr digests. In control experiments, no CNBr fragments of brain NCAM were recognized by either anti-12AB or anti-12CD, even though the profiles of CNBr fragments of brain and heart NCAM recognized by the pan-NCAM antibody were similar. These results indicate that, at the protein level, only one form of the MSD region can be detected in our extracts, presumably that encoded by mRNAs containing the transcripts from all of exons 12A, 12B, 12C, and 12D.

Polysialic Acid on NCAM Inhibits the Ability of Anti-MSD Antibodies to Recognize the Molecule

NCAM molecules bear unique, massive polysialic acid-containing oligosaccharide(s) in close proximity to the MSD region (Cunningham et al., 1987). To determine whether this structure interferes with the ability of anti-MSD region antibodies to bind to the molecule, the ability of anti-12AB and anti-12CD to recognize NCAM molecules before and after neuraminidase treatment was compared (Fig. 6). Whereas polysialic acid does not interfere with the ability of the pan NCAM antibody to recognize NCAM, anti-12AB and anti-12CD do not recognize NCAM in Western blots (Fig. 6 A) or by immunoprecipitation (Fig. 6 B) unless the polysialic acid is first removed from the molecule. Interestingly, anti-12CD does recognize NCAM equally well before and after neuraminidase treatment in immunohistochemical experiments (data not shown).

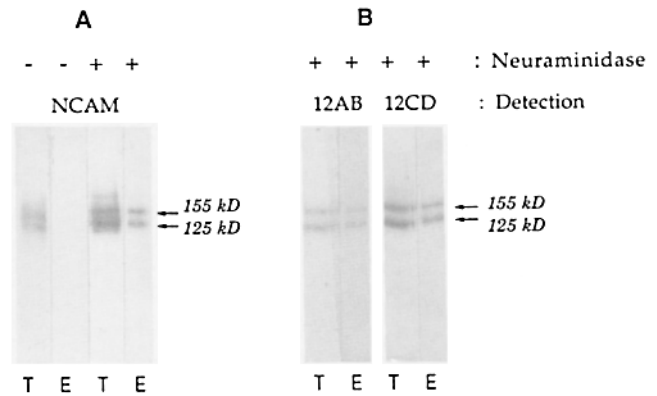


Figure 7. Binding of NCAM to immobilized peanut lectin. As described in Materials and Methods, NP-40 extracts of 14-d embryonic heart membranes, either untreated (–) or treated (+) with neuraminidase, were incubated with peanut lectin-agarose. Aliquots of the total extract (T) and the 0.1-M galactose eluate (E) were Western blotted using antibodies against the indicated antigens as the primary antibodies for detection. Lanes containing total extract were loaded with 5 μ g of protein for detection with the pan-NCAM antibody and with 15 μ g of protein for detection with anti-12AB or anti-12CD; lanes containing the galactose eluate were loaded with the material purified from 20 μ g of extract for detection with the pan-NCAM antibody and with the material purified from 60 μ g of extract for detection with anti-12AB or anti-12CD. The migration of the 155- and 125-kD forms of NCAM that are specifically precipitated by immobilized peanut lectin and are specifically recognized by anti-MSD region antibodies are indicated.

Forms of NCAM Containing the MSD Region Also Bind to Peanut Lectin

NCAM from a skeletal muscle cell line consists of a 155-kD transmembrane form that contains the MSD region and binds to peanut lectin and a 125-kD GPI-linked form that does not contain the MSD region and does not bind to peanut lectin (Walsh et al., 1989). To characterize the peanut lectin binding of heart NCAM, NP-40 extracts of plasma membranes from 14-d embryonic heart tissue were fractionated on immobilized peanut lectin; the forms of NCAM in the total and eluate were then detected by Western blotting with the pan-NCAM antibody, anti-12AB, and anti-12CD (Fig. 7). NCAM molecules bind to peanut lectin only after neuraminidase treatment; even then, only the two polypeptides (155 and 125 kD) recognized by anti-12AB and anti-12CD bind to peanut lectin. These results are consistent with the facts that peanut-lectin binding oligosaccharides are believed to be O-linked and that the MSD region contains two putative acceptor sites for O-glycosylation.

Developmental Changes in the Expression of Forms of NCAM

The forms of NCAM present in extracts from 4-, 7-, 10-, and 14-d embryonic heart tissue and adult heart tissue were compared (Fig. 8). Dramatic shifts were observed in the expression of ld, sd, and ssd NCAM, as well as in the proportions of each of these forms containing the MSD region. ld NCAM was present at 4 d, peaked at 7 d, was still present at 10 d,

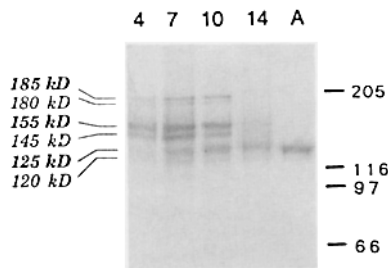


Figure 8. Forms of NCAM present in the developing heart. As described in Materials and Methods, 5- μ g aliquots of NP-40 extracts of membranes from 4-d (lane 4), 7-d (lane 7), 10-d (lane 10), and 14-d (lane 14) embryonic heart tissue, and from adult (A) heart tissue were neuraminidase treated, then Western blotted using the pan-NCAM antibody as the primary antibody for detection. The migrations of the 185-, 180-, 155-, 145-, 125-, and 120-kD forms of NCAM are indicated; those containing the MSD region (185, 155, and 125 kD) are in bold. The identity of these forms of NCAM was confirmed in parallel experiments using anti-12AB and anti-12CD for detection (data not shown). The migration of standard proteins is indicated by their relative molecular masses $\times 10^{-3}$.

but was absent thereafter. The sd form of NCAM is expressed in a similar time course as ld, while the ssd is expressed later in development and is essentially the only form observed in adult tissue.

The ratio of MSD positive to MSD negative forms of NCAM varies during development. For ld, sd, and ssd NCAM, the percentage of the molecules containing the MSD region always increases during development (Fig. 8). For example, in 7-d tissue, the MSD-positive 185- and 155-kD forms of ld and sd NCAM are present in similar concentrations to the MSD-negative 180- and 145-kD forms of ld and sd. By 10 d, these MSD-positive forms are present at clearly higher concentrations than the corresponding MSD-negative forms. Similarly, the 120-kD MSD-negative form of ssd NCAM is present in 7-d tissue but is below detection thereafter, while the 125-kD MSD-positive form of ssd NCAM continues at high concentration throughout development.

Spatial variations in the expression of forms of NCAM are also readily detected. Ventricular tissue (primarily myocardium) from 5-d embryos contains a similar set of forms of NCAM (Fig. 9) to that observed in the 4- or 7-d heart (Fig. 8). In contrast, atrioventricular cushion tissue from 5-d embryos contains primarily one form of NCAM, the MSD-negative form of sd. It contains much less ld, ssd, or even the MSD-positive form of sd NCAM than does the ventricular extract from the same stage embryos. Moreover, the small amount of MSD-positive sd present may result from myocardial contamination of the cushion tissue, given that cultured endocardial or mesenchymal cells contain only one form of NCAM, MSD-negative sd (Mironov, V., R. R. Markwald, and S. Hoffman, unpublished observations). Thus, the percentage of MSD-positive NCAM in heart tissue can vary from almost 0 to 100%, depending on the stage of development and region of the heart.

NCAM Molecules Containing the MSD Region Colocalize with Z Discs

To determine the distribution at the immunohistochemical

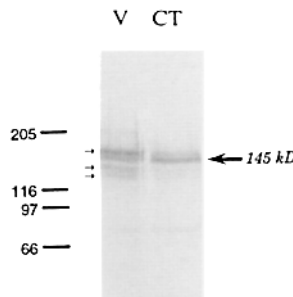


Figure 9. Endocardial cushion tissue is deficient in GPI-linked and MSD region-containing forms of NCAM. Ventricles (V) and cushion tissue (CT) pads were dissected from 5-d chicken embryos. As described in Materials and Methods, 5- μ g aliquots of NP-40 extracts of membranes were neuraminidase treated, then Western blotted using the pan-NCAM antibody as the primary antibody for detection. Small arrows on the left indicate the forms of NCAM (155, 125, and 120 kD) present at relatively high levels in the ventricle and low levels in cushion tissue; the large arrow on the right indicates the form of N-CAM (145 kD) present at relatively high levels in both samples. The migration of standard proteins is indicated by their relative molecular masses $\times 10^{-3}$.

level of NCAM molecules containing the MSD region, sections of stage 18 embryonic heart tissue were stained using anti-12CD (Fig. 10). Anti-12AB was not useful in immunohistochemical studies. As predicted from the results of Fig. 9, the myocardium was stained with anti-12CD, while little or no staining was seen in the endocardium or endocardial cushion tissue. When the myocardial portion of these sections was observed at high magnification (Fig. 11), a striking subcellular distribution of NCAM molecules containing the MSD region was observed; staining with anti-12CD appeared to coincide with the position of Z discs. This staining was specific; no staining was observed if the antibody was preincubated with the peptide used to produce anti-12CD (Fig. 11 b), and no staining was observed with nonimmune IgG (Fig. 11 f). Although anti-NCAM antibodies did stain the surface of myocardial cells, they did not highlight Z discs (Fig. 11 c). Other adhesion molecules, N-cadherin and β 1-integrin, have previously been shown to be associated with Z discs later in development (Hilenski et al., 1992; Knudsen, K. A., personal communication). We examined the expression of these proteins in stage 18 myocardium; while both molecules were present, neither N-cadherin (Fig. 11 d)

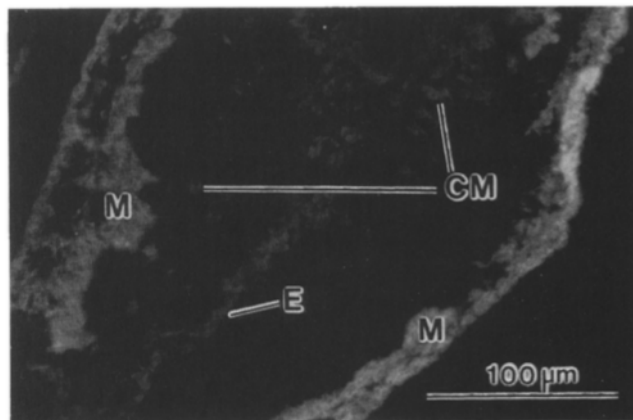


Figure 10. Anti-12CD immunostaining of the stage 18 chicken heart. Anti-12CD immunostaining was detected in the myocardium (M), whereas little staining was revealed in either endocardium (E) or in cushion mesenchyme (CM). Bar, 100 μ m.

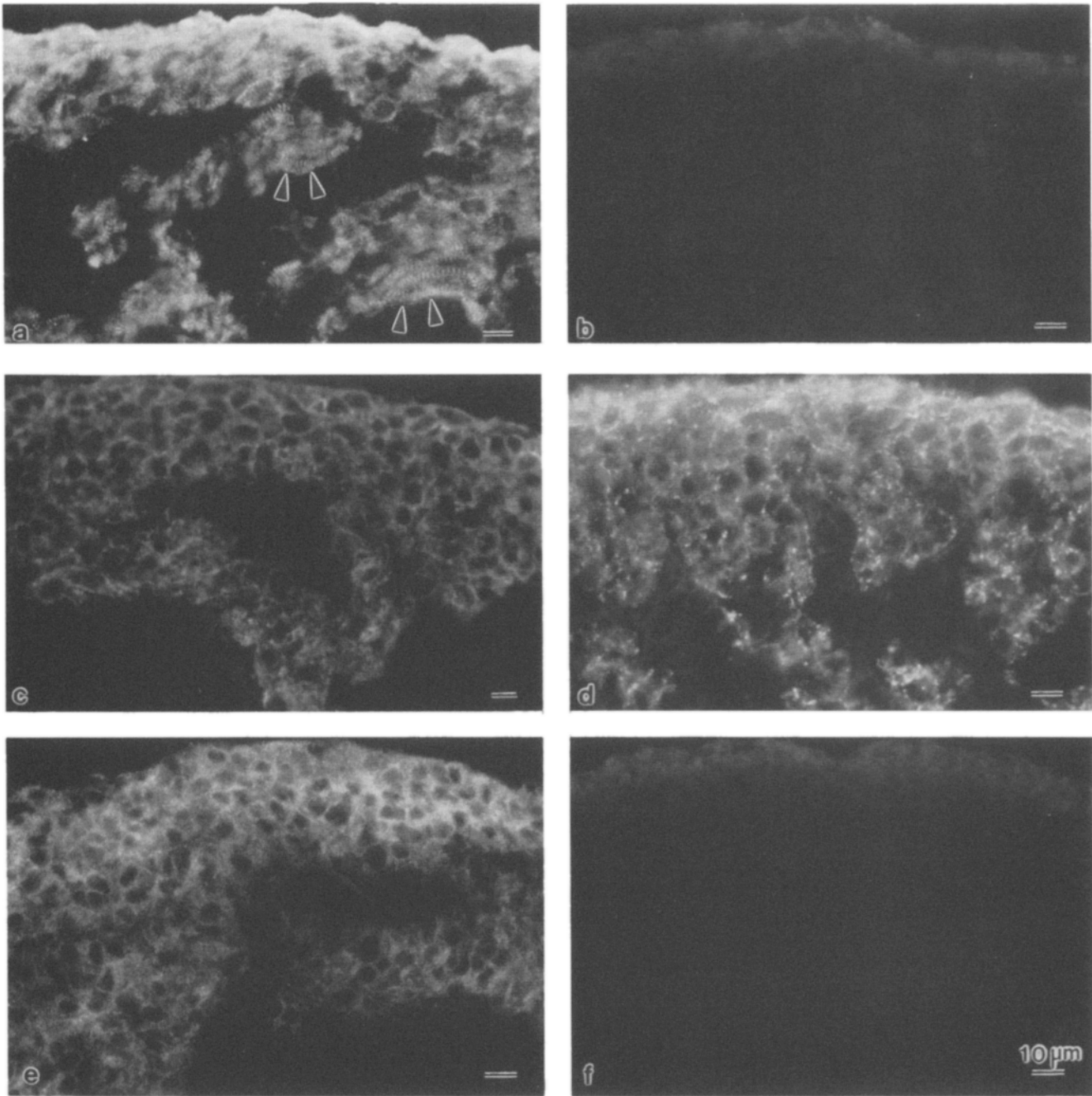


Figure 11. Comparative immunostaining of different adhesion molecules in the stage 18 chicken heart. (a) High magnification photomicrograph depicts a striking subcellular distribution of anti-12CD staining (*arrowheads*) that coincides with the position of Z discs. (b) A section adjacent to that shown in *a* in which anti-12CD was preabsorbed with the immunogenic peptide as described in Materials and Methods. Note that this treatment prevented the immunostaining seen in *a*. (c) Immunostaining with the pan-NCAM antibody was localized at the periphery of the myocardial cells. (d) Anti-N-cadherin (NCD-2) immunostaining was found in a punctate pattern at the periphery of the myocardial cells. (e) Anti- β 1-integrin (V2E9) immunostaining labeled the cell periphery. Similar results were obtained with anti- β 1 integrin antibody JG22. (f) No staining was observed with nonimmune rabbit IgG. Bar, 10 μ m.

nor β 1-integrin (Fig. 11 *e*) colocalized with Z discs. Thus, at this early stage in development, NCAM molecules containing the MSD region appear to colocalize with Z discs, while N-cadherin, β 1-integrin, and NCAM molecules lacking the MSD region do not exhibit this specific association.

Confocal microscopy was used to further compare the localization of Z discs and forms of NCAM. When tissue sections were double labeled using anti-12CD and anti- α -

actinin (a marker for Z discs), an almost complete coincidence between the two signals was observed (Fig. 12, *a* versus *b*). In two regions (indicated by brackets), periodic anti- α -actinin staining was observed, but no staining with anti-12CD. These observations suggest that most Z discs in these cells are located close to the cell surface in the same confocal section (thickness $<0.25 \mu$ m) as the MSD region-containing NCAM on the cell surface. Only the occasional

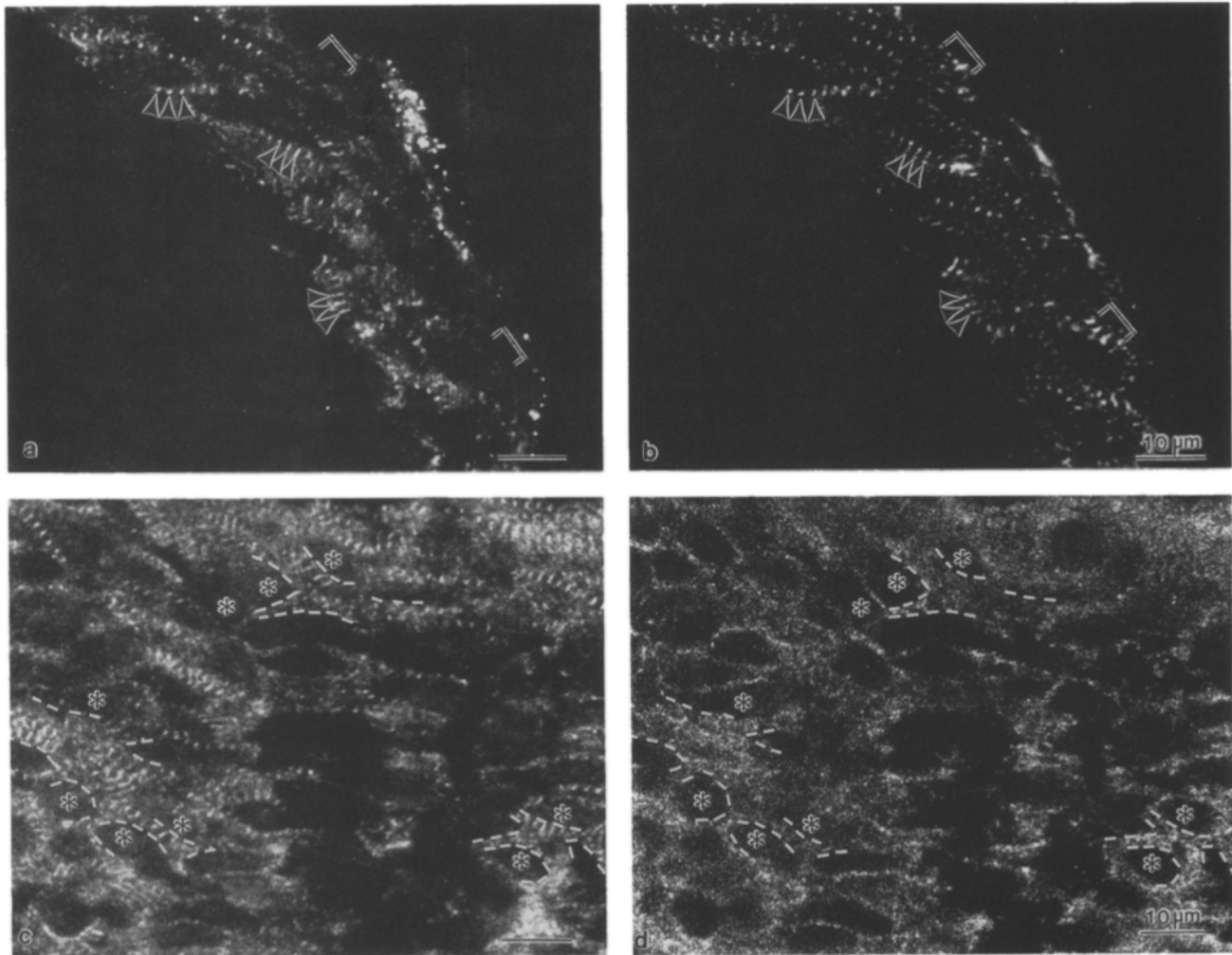


Figure 12. Confocal microscopic observation of stage 18 chicken heart doubly stained with anti-12CD and anti- α -actinin (sarcomeric) or anti-12CD and anti-NCAM. Double immunostaining routinely reveals a coincidence between anti-12CD (*a*, arrowheads) and α -actinin (*b*, arrowheads) staining in association with Z discs. Occasionally anti- α -actinin staining is observed in the absence of anti-12CD staining (compare brackets in *a* and *b*). In contrast, there is only limited overlap between anti-12CD staining (*c*) and staining with the pan-NCAM antibody (*d*). Cells in which the plane of focus captured the cell surface (regions between broken lines) showed almost uniform staining with the pan-NCAM antibody (*d*), whereas anti-12CD staining appeared in the characteristic pattern (*c*). Cells cut in equatorial section (asterisks inside) showed almost uniform circumferential staining with the pan-NCAM antibody (*d*) and frequently show periodic circumferential staining with anti-12CD (*c*). Bar, 10 μ m.

set of Z discs is located either deeper in the cell, or it is not associated with NCAM molecules containing the MSD region.

When tissue sections were double labeled using anti-12CD and the pan-NCAM antibody, cells in which the plane of focus captured the cell surface showed almost uniform staining with the pan-NCAM antibody, whereas anti-12CD staining appeared in the characteristic striped pattern (Fig. 12, *c* versus *d*, regions between broken lines). Only at scattered sites did the pan-NCAM antibody show any hint of highlighting NCAM molecules associated with Z discs. Similarly, cells in which the plane of focus cut an equatorial section (Fig. 12, *c* and *d*, asterisks inside) showed circumferential staining that was relatively uniform with the pan-NCAM antibody and that was frequently periodic with anti-12CD. These results strongly suggest that NCAM molecules containing the MSD region are colocalized with Z discs, while

NCAM molecules lacking the MSD region are uniformly distributed over the entire cell surface.

Despite the fact that the anti-12CD antibody recognizes only NCAM in Western blotting experiments, we were concerned that it might recognize an additional protein in immunohistochemical studies. To rule out this possibility, tissue sections were sequentially incubated with the pan-NCAM antibody and anti-12CD antibodies. This pretreatment with the pan-NCAM antibody totally blocked anti-12CD staining (data not shown), indicating that the pan-NCAM antibody and anti-12CD recognize the same protein, i.e., NCAM.

Structures known as costameres have been identified (Pardo et al., 1983) in which cell surface proteins appear to be linked to Z discs by vinculin. However, when we examined the distribution of vinculin in tissue sections from stage 18 myocardium, the staining patterns observed did not coincide

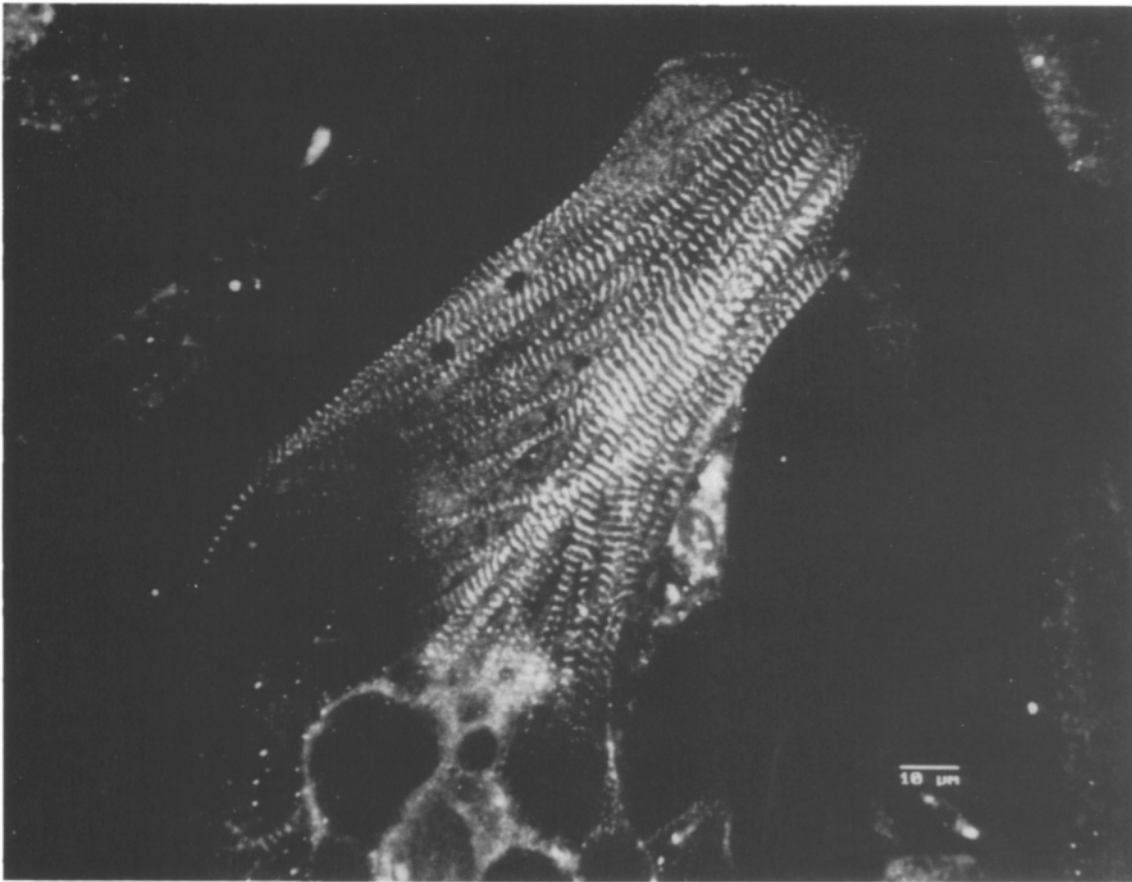


Figure 13. Confocal microscopic observation of cultured stage 18 myocardial cells stained with anti-12CD. Anti-12CD staining of cultured stage 18 myocardial cells is associated with Z discs. Bar, 10 μm .

with the patterns obtained with anti-12CD or anti- α -actinin (data not shown). Because previous studies of costameres were performed later in development, it is likely that the association of vinculin with Z discs has not yet occurred at stage 18.

The anti-12CD staining pattern was also examined in cultured stage 18 myocardial cells. As in vivo, anti-12CD staining was in register with Z discs (Fig. 13). Moreover, the fact that this staining pattern is exhibited by isolated cells suggests that the maintenance of this pattern does not depend on cell-cell adhesion.

Discussion

We have prepared antibodies against two oligopeptides (12AB encoded by exons 12A and 12B; 12CD encoded by exons 12C and 12D) that together comprise the entire alternatively spliced MSD region in the extracellular portion of chicken NCAM. Using these antibodies, we have made the following novel observations: (a) All three splice variants of NCAM (ld, sd, and ssd) that differ in their mode of attachment to the plasma membrane are expressed during heart development both in forms that contain and in forms that lack the MSD region. (b) At the protein level, the MSD region encoded by exons 12A, 12B, 12C, and 12D appears either to be absent or to be expressed in its entirety, i.e., we see no evidence for alternate splicing among these four small exons.

(c) The six forms of NCAM detected in this study each change dramatically in their expression during development. In particular, the percentage of the ld, sd, or ssd form expressed that is also MSD region positive can vary between 0 and 100% in a temporally and spatially controlled manner. (d) At the immunohistochemical level, forms of NCAM containing the MSD region are specifically associated with Z discs early in heart development, suggesting a role for this region of NCAM in myofibrillogenesis.

Our previous studies on forms of NCAM expressed in the developing chicken heart revealed 180-, 150-, 140-, and 130-kD polypeptides (Prediger et al., 1988; Hoffman et al., 1990). The 130-kD polypeptide was found to be GPI linked to the plasma membrane and to be the predominant form of NCAM in adult heart, while the 150- and 140-kD forms were predominant earlier in development. In the current study, we have been able to resolve and unambiguously identify six forms of NCAM during heart development. 185- and 180-kD polypeptides have been shown to be variants of the large cytoplasmic domain form of NCAM because they are recognized by monoclonal antibody 4d. 125- (previously referred to as 130) and 120-kD polypeptides are variants of the small surface domain form of NCAM as evidenced by the fact that they are released from membranes by PIPLC. 155- and 145- (previously referred to as 150 and 140) kD polypeptides are variants of the small cytoplasmic domain form of NCAM. In each of these pairs, the higher molecular mass forms (185,

155, and 125 kD) are recognized by anti-MSD region antibodies, while the lower molecular mass forms (180, 145, and 120 kD) are not recognized by these antibodies. Thus, each splice variant of NCAM that differs in its attachment to the plasma membrane can also be expressed either containing or lacking the MSD region. This interpretation of the identity of NCAM polypeptides is in agreement with the studies of Yoshimi et al. (1993) on the 155- and 145-kD forms of NCAM expressed during skeletal muscle development.

In previous studies of forms of NCAM expressed during heart development, the ld form observed was considered likely to be of neural origin. The current study suggests, however, that the ld form of NCAM may be expressed by heart tissue. First of all, the 185- and 180-kD forms of NCAM are present at significant levels in 4-d embryos, and they peak in expression in 7-d embryos. If these forms of NCAM were expressed by the innervation of the heart, they might be expected to appear later and to be maintained throughout development. Second, the 185-kD form of NCAM is recognized by anti-MSD region antibodies, even though these antibodies do not recognize any form of NCAM in the brain. Assuming that the innervation of the heart is similar to the brain in not expressing forms of NCAM recognized by anti-MSD antibodies, then the data would suggest that at least the 185-kD form of NCAM is produced within the heart. It is tempting to speculate that the ld form of NCAM may be preferentially expressed in the conduction system, given that the polysialic moieties found on NCAM, as well as the carbohydrate epitopes recognized by monoclonal antibody HNK-1 and found on NCAM and other neural adhesion molecules, are also preferentially expressed in this region of the heart (Watanabe et al., 1992; Nakagawa et al., 1993).

Data from PCR amplification of cDNAs prepared from developing rat heart mRNAs have suggested that alternative splicing occurs between exons 12A, 12B, 12C, and 12D, and that these four small exons are present together in only a small percentage of NCAM mRNAs (Reyes et al., 1991). Our results provide a very different picture for NCAM molecules at the protein level. Even at the level of CNBr fragments, we see that anti-12AB and anti-12CD recognize the same polypeptide and that there is no apparent heterogeneity in the size of this polypeptide. Because exons 12A and 12D are very small, we may not have been able to detect their alternative splicing at the protein level; however, we should have been able to detect alternative splicing of exons 12B and 12C because they encode sufficient mass (11 or 14 amino acids) to noticeably affect the migration of a 45-kD CNBr fragment. The PCR data of Reyes et al. (1991) indicate that ~70% of the NCAM mRNA obtained from perinatal rat hearts lacks both exons 12B and 12C, and that only 8–15% (depending on whether ld, sd, or ssd molecules are being considered) contains both 12B and 12C. In contrast, at the protein level, we find that for ld, sd, and ssd NCAM, forms of the molecule containing the MSD region represent ≥50% of the NCAM molecules present in the heart from 4-d embryos through adults. Moreover, the great majority of NCAM molecules contain the MSD region in 10- and 14-d embryos and in adults. This difference between our results and the results of Reyes et al. (1991) may be caused by species differences, differences in message stability or the ability of messages to be translated, or to differences in the half-life of the various polypeptides.

A striking difference in the regional expression of forms of NCAM containing and lacking the MSD region was seen in early heart development in both Western blotting and immunohistochemical experiments. Extracts of atrioventricular endocardial cushion tissue included very little NCAM containing the MSD region. Moreover, all the MSD region-containing NCAM in these samples may be derived from myocardial contamination; only the sd form of NCAM lacking the MSD region is present in cultured endocardial and mesenchymal cells (Mironov, V., R. R. Markwald, and S. Hoffman, unpublished observations). In contrast, extracts of ventricle showed equal levels of NCAM molecules containing and lacking the MSD region. Similarly, immunohistochemical analyses showed NCAM molecules containing the MSD region to be present in the myocardium, but they were either absent or present at only low levels in tissues of endocardial origin.

The subcellular distribution of NCAM molecules containing the MSD region was even more striking. Anti-12CD staining colocalized with the Z discs of myofibrils (detected by staining with anti- α -actinin [sarcomeric]). Curiously, although the pan-NCAM antibody uniformly stained the cell surface, it showed little, if any, enhanced staining in register with Z discs. Nevertheless, it is clear that the structures recognized by anti-12CD that colocalize with Z discs must be NCAM molecules containing the MSD region because anti-12CD staining is blocked by preincubation of the antibody with the peptide against which it was made or by preincubation of the tissue section with the pan-NCAM antibody.

Other adhesion molecules (namely β 1-integrin [Hilenski et al., 1992] and N-cadherin [Knudsen, K. A., personal communication]) have previously been localized to Z discs at later stages of development; however, we do not detect anti- β 1-integrin and anti-N-cadherin staining in association with Z discs at stage 18 when we do detect anti-12CD staining. These observations make NCAM molecules containing the MSD region the earliest-appearing cell surface component to be detected in association with Z discs. Therefore, NCAM molecules containing the MSD region are particularly good candidate molecules to play a role in the initiation of myofibrillogenesis during early heart development.

This study provides two striking insights regarding the role of MSD region-containing NCAM in cellular interactions and in the mechanism of its linkage to the cytoskeleton. (a) NCAM is well known to be a cell-cell adhesion molecule and is usually observed at sites of cell-cell contact in immunohistological studies. Nevertheless, our observations raise the possibility that cell-cell adhesion does not play a role in the colocalization of MSD region-containing NCAM with Z discs. In vivo, MSD region-containing NCAM is localized in register with Z discs and not in a uniform distribution on the cell surface or in intercalated discs, as would be expected for a molecule involved in cell-cell adhesion. In vitro, MSD region-containing NCAM can become colocalized with Z discs in isolated cells where little, if any, of the cell surface is involved in cell-cell contact. These observations raise the possibility that MSD region-containing NCAM is involved in cell-ECM adhesion. This would be consistent with scanning electron microscopic studies that show collagen-containing "struts" of connective tissue that interact with the myocardial cell surface in register with Z discs (Borg et al., 1985). (b) NCAM molecules containing

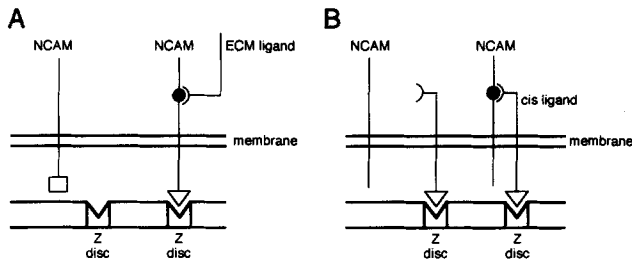


Figure 14. Models for the mechanism whereby NCAM containing the MSD region is specifically associated with Z discs. The MSD region and its covalently attached O-linked oligosaccharide is represented by a filled circle. Ligands recognizing the MSD region are depicted with a complementary shape. (A) the interaction of an ECM ligand with the MSD region induces a conformational change in the cytoplasmic tail of NCAM, allowing it to bind to the Z discs. NCAM molecules lacking the MSD region cannot bind this ECM ligand and, therefore, cannot undergo the conformational change that allows them to dock in Z discs. (B) A *cis* ligand (present in the plasma membrane of the same cell) binds both to the MSD region and to the Z disc. NCAM molecules lacking the MSD region do not bind to this *cis* ligand and do not become associated with Z discs.

the MSD region are identical in the primary structure of their cytoplasmic region with NCAM molecules lacking the MSD region. Nevertheless, the MSD region-containing molecules appear to interact with the cytoskeleton in a manner that results in their colocalization with Z discs, while molecules lacking the MSD region do not interact with the cytoskeleton in this manner. This suggests two models depicted in Fig. 14. Either the interaction of MSD region-containing NCAM with an ECM ligand alters the conformation of the cytoplasmic tail of NCAM, resulting in its ability to bind to a component Z discs (Fig. 14 A), or MSD region-containing NCAM molecules can interact with a protein in the same cell (*cis* ligand) that possesses the ability to bind to a component of Z discs (Fig. 14 B). Of course, more complicated models are also possible; for example, the interaction of MSD region-containing NCAM with a *cis* ligand might induce the ability of the NCAM or of the *cis* ligand to interact with Z discs. Whether MSD region-containing NCAM interacts with an ECM ligand or a *cis* ligand, the interactive portion of the MSD region may lie in its amino acid sequence or in the O-linked carbohydrate (recognized by peanut lectin after neuraminidase treatment of the molecule [see above and Walsh et al., 1989]) associated with the MSD region.

The current study demonstrates the feasibility and utility of using antibodies against alternatively spliced regions in NCAM to evaluate their identity and distributions *in vivo*. The existence of the MSD region in NCAM was deduced from molecular biological studies. However, such studies did not indicate precisely which mRNAs detected on Northern blots using MSD region-specific probes encode which NCAM polypeptides observed on Western blots. Molecular biological studies also did not and cannot indicate the subcellular distribution of forms of NCAM containing the MSD region. The antibodies prepared in the current study have allowed the identification and characterization on Western blots of the forms of NCAM containing the MSD region.

Moreover, these antibodies have allowed us to make the striking observation that forms of NCAM containing the MSD region are colocalized with Z discs and, thus, are likely to be involved in myofibrillogenesis. Antibodies specific for other splice variants of NCAM (e.g., 1d and ssd forms and forms containing the VASE insert) may be equally useful for determining the distribution *in vivo* of particular forms of NCAM, thereby suggesting specialized functions for each of these forms.

Monoclonal antibodies 4d (specific for the 1d form of NCAM, developed by U. Rutishauser, Case Western Reserve University, Cleveland, OH) and V2E9 and JG22 (each specific for the β 1-integrin subunit, developed by A. Horwitz, University of Illinois, Urbana, IL, and D. Gottlieb, Washington University, St. Louis, MO, respectively) were obtained from the Developmental Studies Hybridoma Bank, which is maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract N01-HD-2-3144 from the National Institute for Child Health and Human Development. Monoclonal antibody NCD-2 (specific for N-cadherin) was a gift from Dr. Jack Lilien (Clemson University, Clemson, SC).

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