Alternative splicing of the neural cell adhesion molecule gene generates variant extracellular domain structure in skeletal muscle and brain

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Myotube mRNA isoforms of the neural cell adhesion molecule (N-CAM) contain a novel sequence block termed muscle-specific domain 1 (MSD1), which is inserted within the extracellular coding region. Here, we report a characterization of the genomic organization of MSD1 and its pattern of expression within cellular N-CAM RNA and polypeptide species. S1 nuclease protection analyses and sequence analysis of an N-CAM human genomic clone containing MSD1 sequences indicated that MSD1 is comprised of three discrete exons of 15, 48, and 42 bp, designated MSD1a, MSD1b, and MSD1c, respectively. Although the MSD1a exon was present in a small proportion of mRNAs from both brain and muscle cells, the entire MSD1 sequence occurred predominantly in mRNAs from differentiated myotube cells. In addition, antiserum raised to a synthetic, MSD1b-encoded peptide sequence was found to stain the cell surface of human skeletal myotubes in culture, whereas myoblasts, fibroblasts, and neural cells were negative. MSD1a, MSD1b, and MSD1c sequences thus arise collectively in N-CAM mRNA and polypeptide isoforms as a result of muscle tissue-specific and developmentally regulated alternative mRNA splicing events. In addition, the occurrence of brain and muscle mRNAs containing only MSD1a indicate that alternative splicing may occur within the MSD region itself to generate further diversity.

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The formation of adhesive interactions between cell surfaces is thought to be essential to the processes of cell recognition, sorting, and migration during development, and for the subsequent stabilization and regeneration of tissues in the adult (Edelman and Thiery 1985). A variety of surface glycoproteins involved in cell adhesion has been identified and grouped into Ca^{2+} -dependent and Ca^{2+} -independent families (Takeichi 1988). The best characterized members of the latter family are a group of high-molecular-weight sialoglycoproteins known collectively as the neural cell adhesion molecules (N-CAMs; for reviews, see Cunningham 1986; Edelman 1986; Rutishauser 1986; Nybroe et al. 1988).

N-CAMs are expressed in a wide range of early embryonic tissues but are present predominantly in cells of the nervous system and developing muscle at later stages of development. They have been implicated in a wide range of morphogenetic events, including cell segregation, axonal bundling (Rutishauser et al. 1978; Thanos et

al. 1984), the migration of neurons along glial pathways (Silver and Rutishauser 1984), and in several aspects of myogenesis, including muscle innervation and synaptogenesis (Rutishauser et al. 1983; Covault et al. 1986; Tosney et al. 1986; Rieger et al. 1988). During these processes, the adhesive properties and function of N-CAMs apparently are modulated by the differential expression of variant N-CAM polypeptide isoforms in specific highly regulated patterns.

N-CAM isoform diversity is generated both by alternative splicing and differential polyadenylation site selection within mRNA products of a single complex gene (Hemperley et al. 1986; Barthels et al. 1987; Santoni et al. 1987; Small et al. 1987; Barton et al. 1988). In addition, N-CAM is subject to a variety of post-translational modifications, including glycosylation, phosphorylation, and sulfation (Finne et al. 1983; Hoffman et al. 1982; Sadoul et al. 1983; Rutishauser et al. 1988). Although the chick N-CAM gene has been found to contain at least 19 exons (Owens et al. 1987), alternative RNA processing has been described mainly in relation to

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exons at the 3' end encoding variable membrane-associated and cytoplasmic polypeptide domains (Cunningham et al. 1987; Small et al. 1987; Barthels et al. 1987). Thus, in chick and mouse brain, three N-CAM isoforms occur with similar extracellular domain structure; two transmembrane isoforms of 180 and 140 kD differ only by the insertion of a domain encoded by a single exon within the cytoplasmic region, whereas a single nontransmembrane species of 120 kD is linked externally to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (Gennarini et al. 1986; He et al. 1986; Barthels et al. 1987).

Transmembrane (140 kD) and GPI-linked (125 kD and 155 kD) N-CAM isoforms also have been identified in skeletal muscle (Covault et al. 1986; Dickson et al. 1987; Moore et al. 1987; Barton et al. 1988). However, cDNA sequencing studies have predicted that the membrane-proximal coding regions of muscle GPI-linked mRNA forms contain an additional novel 108-bp region, demonstrating for the first time a tissue-specific variation within the extracellular domain (Dickson et al. 1987). This additional sequence block was found to be expressed in muscle but not brain tissue and hence was termed the muscle-specific domain 1 (MSD1). MSD1 is inserted in human mRNA at a position corresponding to a splice site defined within the chick N-CAM gene, suggesting that it is an alternatively spliced exon. In addition, S1 nuclease protection studies in the mouse have indicated the presence of an alternative splice site in brain N-CAM mRNAs in a similar location to MSD1 in muscle (Barthels et al. 1987). Candidate exon sequences have now been described in this instance (Santoni et al. 1989).

In the present study, we have analyzed the organization of MSD1 within the human N-CAM gene and have correlated its pattern of expression in cellular RNAs with that in N-CAM polypeptides on muscle cell surfaces. Here, we present evidence that MSD1 sequence indeed occurs collectively within N-CAM muscle mRNA species as the result of a tissue-specific alternative splicing event and is expressed, as an entire domain, specifically by skeletal muscle myotubes in cell culture. We also report that the MSD1 sequence is encoded by three distinct exons within the N-CAM gene and provide evidence that additional diversity in brain and muscle involving specific N-CAM species also may be generated by alternative splicing of individual exons encoding sections of the domain.

Results

Tissue and mRNA-specific expression of MSD1 results from alternative RNA splicing

Previous cDNA sequencing studies have indicated that the MSD1 region is inserted into mRNA-coding sequences at a discrete site (Dickson et al. 1987) that can be identified as a splice junction by comparison with the chick N-CAM gene structure (Owens et al. 1987). However, direct evidence showing its presence or absence as a complete unit in cellular mRNA and, hence, its formal

identity as an alternatively spliced region is lacking. Therefore, we examined the pattern of MSD1 expression in N-CAM RNAs extracted from human embryonic brain and primary cultures of embryonic muscle by S1 nuclease protection analysis.

A 521-nucleotide single-stranded probe spanning MSD1 was prepared from the previously reported cDNA clone λ 9.5 (Dickson et al. 1987), hybridized to total RNA, and treated with S1 nuclease. The digestion products then were examined on sequencing gels (Fig. 1A). In line with previous Northern and Western blot studies (Dickson et al. 1987; Moore et al. 1987), a general increase in N-CAM mRNA levels occurred during myogenesis in vitro; however, distinct patterns of MSD1 expression were observed between brain, myoblast, and myotube RNAs. A completely protected band of 521 nucleotides, indicating the presence of mRNAs colinear with the whole probe including the MSD1 region, was observed only with differentiated myotube cells in culture. In contrast, human embryonic brain tissue (Fig. 1A, lane 3) and skeletal myoblasts (lane 4) produced predominantly two fragments of 325 and 91 nucleotides, arising as a result of the nonprotection and S1 nuclease digestion of the MSD1 region of the probe. The slight laddering observed with the 91-nucleotide fragment is most likely due to slight S1 nuclease overdigestion at the ends of this small product. Small amounts of an additional band of 340 nucleotides also were observed in both brain and muscle tissues corresponding to the protection of the 5' end of the probe and the first 15 bp of MSD1. At later stages of fusion (Fig. 1A, lane 6), two additional bands of 388 and 430 nucleotides were apparent. As shown schematically in Figure 1B, these additional bands correspond to the protection of the 5' end of the probe plus the first 63 bp or the whole of MSD1, respectively. The occurrence of the latter fragment indicates a sequence continuity break in some myotube RNAs compared with the λ 9.5 probe sequence and therefore must represent either a novel inserted sequence or a partial deletion in these mRNAs, at this point immediately 3' of MSD1. Because another alternatively spliced exon (SEC; Gower et al. 1988) indeed occurs immediately 3' i.e., downstream of MSD1, it seems likely that the 430-nucleotide fragment may represent usage of this alternative splicing pathway that leads to production of a secreted N-CAM isoform.

Therefore, these results provide evidence that MSD1 is either present in or absent from mRNAs generated from the single-copy N-CAM gene as a result of alternate exon splicing. In agreement with results from previous Northern blot analyses (Dickson et al. 1987), MSD1 was not found as a complete domain within human embryonic brain mRNAs but was expressed specifically in skeletal muscle mRNAs coincident with the appearance of myotubes. The analysis also predicts that splice junctions exist within MSD1, suggesting that the genomic sequences encoding this region exist as three or more alternatively spliced exons, the first of which, at least, may be individually alternatively spliced into both brain and muscle N-CAM species.

Genomic organization of MSD1

The organization of MSD1 within the N-CAM gene was defined by DNA sequence analysis of clones that were isolated from a human genomic library in Charon 35 using the N-CAM cDNA probe λ 9.5 [Dickson et al. 1987]. Figure 2A shows the restriction map of a 14.4-kb clone, CH11a, which hybridized exclusively with an *NheI*–*ApaI* fragment of λ 9.5, corresponding to 83 bp of the 3' region of MSD1. The region of hybridization was localized further to a 5-kb *HindIII* subfragment at the 3' end of the clone that was subcloned for sequence analysis.

Two regions of MSD1 sequence homology were identified, demonstrating that this domain is present within

the genome as more than one exon. Sequences of 48- and 42-bp designated exons MSD1b and MSD1c were found to be 100% identical with bases 16–63 and 64–105 of MSD1, respectively. The sequences of the two exons, together with adjacent intron sequences, are shown in Figure 2B. Intron/exon boundaries were identified by comparison with cDNA sequences, and in both cases, donor and acceptor splice junctions were found to conform with statistically predicted consensus sequences [Shapiro and Senepathy 1987]. All splice junctions occur between the second and third base of a codon, indicating that omission of any exon by alternative splicing within the MSD1 region would not alter the reading frame of more 3' sequences. In addition, further sequence analysis has indicated that MSD1c is situated 0.7 kb, 3' of the next N-CAM exon—the recently reported N-CAM SEC exon [Gower et al. 1988].

Previous cDNA sequence data [Dickson et al. 1987] have predicted that an additional 15-nucleotide sequence is present at the 5' end of MSD1, and the results from S1 nuclease protection analyses presented here have indicated further that a 15-nucleotide sequence corresponding exactly to this region is spliced alternatively as a discrete unit into brain and muscle N-CAM RNA species. Therefore, it is most likely that these 15 nucleotides exist as one or more small exons, which have been termed MSD1a. We emphasize, however, that the MSD1a sequence is not, in itself, expressed in a muscle-specific manner but only so in the context of the MSD1 region as a whole. Due to its small size and high GC content, we have been unable to detect sequence counterparts specifically in Ch11a or in genomic DNA

Figure 1. S1 nuclease protection analyses of MSD1 expression in human N-CAM mRNAs. (A) Autoradiograph of a polyacrylamide gel showing separation of protected probe fragments. A single-stranded antisense DNA probe spanning MSD1 was hybridized to total RNA from various sources or to control tRNA and digested with S1 nuclease. (Lane 1) Intact probe; (lane 2) digestion of unprotected probe; (lane 3) probe protection with 50 μ g total RNA from human brain; (lanes 4, 5, 6) unfused, early fusion, and late fusion human primary muscle cultures, respectively. The ladder observed with the smallest, 91-nucleotide fragment is not a consistent feature in other analyses and most likely represents slight overdigestion by S1 nuclease. (Left) Lengths (in nucleotides) of the various protected probe fragments; (right) size markers. (B) Schematic representation of the probable location of the various protected fragments (solid bars) relative to the intact probe (open bar) and the internal MSD1 sequence block (dotted region).

Figure 2. Genomic organization of MSD1. (A) Restriction map of Ch11A (a) and a 5-kb subclone of Ch11 (b) showing the positions of exons MSD1b and MSD1c. Arrows show the direction and extent of sequencing beginning at the indicated restriction enzyme site. Cleavage sites for restriction enzymes: *Bam*HI (B); *Eco*RI (E); *Hind*III (H); and *Hinc*II (Hc). (B) Intron/exon junction sequences of MSD1b and MSD1c are shown in lower case and uppercase, respectively; 5' and 3' splice sites, determined by comparison with λ 9.5 cDNA sequences, are underlined. Predicted protein sequences encoded by the two exons are indicated below nucleotide sequences.

using synthetic oligonucleotide hybridization probes. The preceding exon to MSD1a, i.e., the human equivalent of exon 12 as defined in the chick N-CAM gene (Owens et al. 1987), is not present on Ch11a, hence, the MSD1a sequence occurs within a large genomic DNA region >12 kb. Recent studies have identified a homolog of the putative human MSD1a exon in an appropriate location in the mouse N-CAM gene and described its alternative splicing in brain N-CAM mRNAs (Santoni et al. 1989). Therefore, we conclude that splice junctions are present within the MSD1 sequence at bases 15/16 and 63/64 and that MSD1 is most likely composed of three exons of 15, 48, and 42 bp at the genomic level.

The S1-nuclease and genomic sequence data of the present study indicate that MSD1 is composed of 105 bp in total, rather than 108 bp, as predicted previously from comparison of λ 9.5 and λ 4.4 cDNA sequences (Dickson et al. 1987). Analysis of the MSD1 sequence in genomic DNA has now revealed that the final 3 bp originally assigned to MSD1 are not contained within exon MSD1c and therefore would be predicted to occur in association with the human equivalent of exon 13 (as defined in the chick N-CAM gene). However, analysis of N-CAM and genomic clones has indicated that these 3 bp cannot be localized to the 5' end of exon 13 (Gower et al. 1988), but they are expressed variably at the cDNA sequence level (Dickson et al. 1987; Gower et al. 1988). We would conclude here that either allelic variation exists at the start of exon 13 or possibly an unusually small 3 bp exon that is nonuniformly incorporated into N-CAM mRNAs. In this respect, Santoni et al. (1989) recently have described a similar situation in the mouse N-CAM gene where a potential trinucleotide exon with appropriate flanking consensus sequences has been identified.

N-CAM isoforms containing MSD1 antigen are present specifically on myotubes

As an approach to analyzing the specific cellular expression of N-CAM isoforms containing MSD1-encoded domains, a synthetic peptide corresponding to the 15 amino acids within the MSD1b exon (Fig. 2B) was prepared, conjugated to bovine serum albumin (BSA) and antiserum raised in rabbits. To determine the molecular specificity of the MSD1b antiserum, use was made of mouse 3T3 fibroblast lines that were transfected with full-length human N-CAM cDNA gene constructs, encoding either a 125-kD muscle N-CAM isoform containing MSD1 or a 120-kD deletion mutant lacking this region (Gower et al. 1988; F.S. Walsh et al., unpubl.). Both these exogenous genes were introduced into the mouse cells under control of the human β -actin promoter, yielding constitutive, high-level expression and encoding GPI-linked membrane glycoproteins. A schematic representation of the cDNAs and the derived eukaryotic expression vectors is shown in Figure 3. Transfected 3T3 cells expressing MSD1-containing human N-CAM (G4 cells) and the corresponding deletion mutant (A10 cells) were examined for reactivity with the MSD1b synthetic peptide antiserum by immunostaining.

For immunostaining, live cells were subjected to dual-label analysis with a monoclonal mouse antibody recognizing a common human N-CAM epitope (Fig. 4B,E,H,K) and with the rabbit anti-MSD1 serum (Fig. 4C,F,I,L). In addition, because N-CAMs are glycosylated extensively and the MSD1 region has been shown recently to carry specific carbohydrate side chains (Walsh 1988), cells were also stained following treatment with highly purified neuraminidase to remove sialic acid polymers that

Figure 3. Construction of human N-CAM eukaryotic expression vectors. The expression vector pH β APr-1-neo contains 4 kb of the human β -actin gene, including 5'-flanking, 5'-untranslated (UT), and the first intron (IVS1), linked at the 3' splice site to a short polylinker (*SalI*, *HindIII*, and *BamHI*) and followed by an SV40 polydenylation signal (SVpolyA), in addition to ampicillin (*Amp^R*) and neomycin (SV-neo) resistance genes (Gunning et al. 1987). Sense-orientated full-length human N-CAM cDNAs were inserted in the *HindIII* site, as indicated. Splice sites are indicated with an asterisk (*); and initiation (ATG) and termination (TAG) codons of the cDNAs are shown. A linear representation of the encoded polypeptides is given at the base of the diagram. Sequences of MSD1 (solid block) and those encoding hydrophobic carboxy-terminal domains (hatched block) are shown. Restriction enzyme sites indicated are *EcoRI* (E); *HindIII* (H); *KpnI* (K); *SalI* (S); *BamHI* (B); and *XmnI* (X). Full details of cloning substeps are given in Materials and methods.

might mask core MSD1b peptide epitopes. As shown in Figure 4 (B,E,H,K), both the G4 and A10 cell transfectants express human N-CAM at their surfaces with the level of staining unaffected by neuraminidase digestion. In contrast, only the MSD1-expressing G4 cells were immunostained with anti-MSD1b serum (Fig. 4C,F). In addition, a much increased immunofluorescence signal on G4 cells was observed following neuraminidase treatment (cf. Fig. 4C,F). As would be predicted, the A10 cells that were transfected with the MSD1-deletion gene construct showed no reactivity with the anti-MSD1b peptide serum (Fig. 4I,K). Parental 3T3 fibroblasts or cells transfected with the wild-type expression vector did not stain with either the mouse anti-N-CAM monoclonal or the rabbit anti-MSD1b antibodies (data not shown). Thus, the anti-MSD1b antiserum exhibited high molecular specificity in terms of immunoreactivity with MSD1-containing N-CAM species and additionally was detecting a neuraminidase-sensitive epitope in the native molecule.

At the RNA level, previous Northern blotting studies (Dickson et al. 1987) and the present S1 nuclease protection analyses have indicated that MSD1 expression is regulated in a tissue- and developmental-stage-specific manner. Thus, mRNAs containing all three MSD1 exons are present in differentiated myotube cultures but not in proliferating myoblast cultures or in brain tissue. To attempt to confirm this observed specificity at the level of cellular polypeptide expression, a further series of dual-label immunofluorescence staining experiments

were performed on neuraminidase-treated cultures of human skeletal muscle cells, skin fibroblasts, and dorsal root ganglia (DRG). Human muscle cell cultures were examined at pre- and post-fusion stages and thus contained populations of N-CAM-positive myoblasts and multinucleate myotubes and N-CAM-negative fibroblasts (Fig. 5A,B,D,E). As shown in Figure 5, C and F, only differentiated myotubes, but not myoblast or fibroblasts, showed immunoreactivity with the anti-MSD1b peptide antiserum.

In the case of human DRG, these cultures have a complex cellular composition, containing populations of neurite-bearing sensory neurons with rounded-phase dense cell bodies and prominent nuclei and nucleoli (Fig. 5G), as well as a complement of substrate-attached 'fibroblast-like' cells, which represent predominantly Schwann cells (Dickson et al. 1982; Doherty and Walsh 1989). As shown in Figure 5 (G,H,I), although the majority of both these DRG cell types express N-CAM at their surface, no reactivity was observed with the anti-MSD1b antiserum. Finally, no immunoreactivity of anti-MSD1b peptide antiserum was observed with cultures of human skin fibroblasts (Fig. 5J,K,L) that were stained strongly in dual-label analyses with antifibronectin monoclonal antibody (Walsh et al. 1981), thus controlling the specificity of both the MSD antiserum and the fluorochrome-conjugated anti-rabbit Ig antibodies used as the detecting second antibody.

Thus, at the cellular level, expression of MSD1-bearing N-CAM isoforms clearly was restricted to mul-

Figure 4. Cells transfected with MSD1 deletion mutants of N-CAM do not stain with antiserum to a synthetic MSD1 peptide. NIH-3T3 fibroblasts transfected with wild-type MSD1-containing (A–F) or MSD1 deletion (G–L) N-CAM cDNAs (G4 and A10 cell lines, respectively) were subjected to dual-label immunofluorescence staining with monoclonal anti-N-CAM (B, E, H, K) or polyclonal anti-MSD1b peptide antibodies (C, F, I, L). Cells were examined with (D–F, J–L) or without (A–C, G–I) prior exposure to neuraminidase. Scale bar, 100 μ m.

tinucleated myotube cells and absent from myoblasts, fibroblasts, and primary cell cultures containing neurons and peripheral glial cells.

Discussion

Studies in a variety of species have concluded that alternative splicing of a single N-CAM primary transcript gives rise to a number of N-CAM isoforms that differ only within the membrane-associated and cytoplasmic polypeptide domains (Gennarini et al. 1986; Barthels et al. 1987; Cunningham et al. 1987; Small et al. 1987).

However, recent examination of human N-CAM cDNAs has identified a discrete region called MSD1 (Dickson et al. 1987), which is expressed in the extracellular domain of a subpopulation of GPI-linked isoforms. In this study we show that the MSD1 sequence is composed of three distinct alternatively spliced exons within the human N-CAM gene, of 15, 48, and 42 nucleotides, now designated MSD1a, MSD1b, and MSD1c, respectively.

In agreement with previous RNA hybridization studies, S1 nuclease protection analyses showed that mRNAs containing all three MSD1 exons are the predominant form expressed in differentiated myotubes and that human brain tissue and undifferentiated myoblasts

Figure 5. Human skeletal myotubes stain specifically with antiserum to a synthetic MSD1b-encoded peptide. Human skeletal myotubes (A–C), myoblasts (D–F), DRG cells (G–I), and skin fibroblasts (J–L) were treated with neuraminidase and subjected to dual-label immunostaining with monoclonal antibodies to human N-CAM (B, E, H) or fibronectin (K), in conjunction with anti-MSD1b peptide antiserum (C, F, I, L). Scale bar, 100 μ m.

do not express these species. However, small amounts of mRNAs containing MSD1a only were observed in all three circumstances, indicating that alternative splicing of this small exon is apparently not a tissue-specific phenomenon. In contrast, however, inclusion of MSD1a and MSD1b together or the entire set of three exons in human N-CAM mRNA represent developmentally regulated and tissue-specific alternative splicing pathways.

Using antibodies raised to a synthetic peptide antigen corresponding to sequence encoded by MSD1b, we have shown directly that the expression of N-CAM polypeptides containing MSD1 also is restricted to the cell surface of skeletal myotubes in culture. Previous studies

have shown that myotubes express predominantly a GPI-linked N-CAM isoform of 125 kD [Moore et al. 1987], and a full-length MSD1-containing cDNA encoding this species has been characterized recently [Barton et al. 1988]. Because the results reported here indicate that the cellular expression of MSD1 within both N-CAM mRNA and polypeptide isoforms is indeed myotube specific, it is likely that MSD1 is associated predominantly with the GPI-linked isoform found on the surface of myotubes. Of interest, however, is the observation that the MSD1a exon is spliced individually into subpopulations of both muscle and brain N-CAM mRNAs. In the case of brain, MSD1a may correspond to

the alternative splicing event identified by Barthels et al. (1987) using S1 nuclease analysis. Indeed, the presence of a mouse homolog of MSD1a, alternatively-spliced in mouse brain mRNAs encoding different N-CAM species, has been described (Santoni et al. 1989).

The biological consequences of MSD1 expression on N-CAM ligand properties and myotube function in general remains, as yet, undefined. However, the region has structural characteristics similar to the so-called hinge region of immunoglobulin (Walsh 1988). It is rich in the amino acids proline, serine, and threonine and is located topographically membrane proximal to the array of five immunoglobulin homology units that run consecutively from the amino terminus of N-CAM polypeptides (Barton et al. 1988). Therefore, MSD1 may act as a hinge region in N-CAM, resulting in the reorientation or altered conformation of the extracellular ligand binding domains. Indeed, direct electron microscopical visualization of embryonic chick brain N-CAM molecules using rotary shadowing techniques has revealed a 'dog-leg' structure suggestive of a hinged molecule (Hall and Rutishauser 1987). In particular, the specific presence of the MSD1a region in both brain and muscle N-CAMs and its predicted amino acid sequence producing a run of four proline residues is highly suggestive of such an open flexible structure. Furthermore, recent evidence has suggested that MSD1 may be a site of O-linked oligosaccharide attachment similar to the immunoglobulin hinge region (Walsh 1988). Alternatively, because the extent of N-CAM glycosylation influences N-CAM mediated cell aggregation profoundly (Rutishauser et al. 1988), it is possible that the introduction of O-linked oligosaccharide to isoforms expressing MSD1 may modulate directly N-CAM adhesive function or turnover of these forms in the membrane.

In conclusion, therefore, we have provided evidence here that diversity can be generated within the extracellular regions of myotube N-CAM isoforms as a result of developmentally regulated and tissue-specific alternative splicing events that may allow modulation of N-CAM function in coordination with muscle differentiation. Although the precise function of MSD1 remains to be elucidated, the pattern of expression of MSD1-containing N-CAMs in myotube cells is compatible with a role for these isoforms in motor axon outgrowth and neuromuscular junction formation, either during development or nerve regeneration. Comparative functional studies using eukaryotic gene transfer techniques and appropriate *in vitro* and *in vivo* models offer attractive approaches to examine the biochemical and cellular consequences of site-specific and deletion mutations of MSD1-bearing N-CAMs. In this way, hypotheses on the functional contribution of the N-CAM family in skeletal myogenesis may be put to the test directly.

Material and methods

S1 nuclease protection analysis

A deletion clone corresponding to 1–521 bp of λ 9.5 in M13 mp18 (Dickson et al. 1987) was produced using exonuclease III

and S1 nuclease (Henikoff 1984). A labeled probe was prepared subsequently from the deletion clone by extension from the universal priming site of the vector in the presence of [³²P]dCTP. Probes were isolated following *Eco*RI digestion by alkaline agarose electrophoresis and electroelution (Maniatis et al. 1982). Probe (10⁶ cpm) was annealed to total RNA (50 μ g) from various sources (Dickson et al. 1987), digested with S1 nuclease, and analyzed on 4% wedge-gradient sequencing gels. Product sizes were determined using an end-labeled ladder (123 bp multimer) of molecular weight standards (BRL, Paisley, Scotland).

Isolation and sequencing of genomic DNA clones

A human genomic library in Charon 35 (provided by Dr. A. MacLeod) was screened by plaque hybridization with N-CAM cDNAs, and phage clones were isolated by standard procedures. Physical mapping of restriction site and MSD1 sequences was performed by single and double digestion of phage DNA, gel electrophoresis, and blot hybridization analyses (Maniatis et al. 1982). Nick-translated cDNA inserts or a 3'-end-labeled oligonucleotide were used as probes (Davies et al. 1986). Appropriate restriction fragments from genomic clones were subcloned into M13mp18 and 19 vectors and sequences as described previously (Dickson et al. 1987).

Transfection of 3T3 cells with N-CAM cDNA gene constructs

A full-length cDNA (CHB1) encoding a MSD1-containing 125 kD, GPI-linked human N-CAM has been described (Barton et al. 1988). To produce the corresponding MSD1 deletion construct, use was made of the previously described cDNA, λ 4.4, which naturally contains such a deletion within a single *Eco*RI–*Kpn*I fragment. The 3' *Eco*RI–*Hind*III fragment of CHB1 was first subcloned into pUC12 and its MSD1-containing E–K fragment substituted for that from λ 4.4. The remaining 5' region of the gene contained within a single *Eco*RI fragment was then replaced, and appropriate recombinants selected by restriction site mapping.

For cell transfection studies, the entire coding regions of both wild-type and the deletion construct were excised as *Xmn*I–*Hind*III fragments and blunt-end ligated into the *Hind*III site of the eukaryotic expression vector pH β APR-1-neo (Gunning et al. 1987). This vector drives inserted genes from the strong human β -actin promoter and contains appropriate splicing and polyadenylation signals and a linked neomycin-resistance gene. Sense-orientated N-CAM gene constructs were introduced into NIH-3T3 mouse fibroblasts as CaPO₄ precipitates using the Pharmacia Cellfect Kit, and transfected clones selected in 500 μ g/ml Geneticin (Sigma) and screened for human N-CAM expression by immunostaining (Gower et al. 1988).

Production and use of synthetic peptide antiserum

A peptide corresponding to the predicted sequence of part of the MSD1 region of human N-CAM cDNA λ 9.5 (342–393 bases) was constructed using solid-phase procedures using Fmoc chemistry (Burns et al. 1987). Peptide was conjugated to BSA emulsified in Freund's complete adjuvant and injected into rabbits at multiple intradermal and subcutaneous sites. Sera were tested by ELISA against peptide absorbed to microtiter plate wells. For immunostaining, cultures of human muscle cells were passaged and cultured in the presence of 10% fetal calf serum or 2% horse serum for 4 days to yield myoblast and myotube cultures, respectively (Walsh and Ritter 1981). Human skin fibroblasts and DRG cells (Dickson et al. 1982; Doherty

and Walsh 1989) were cultured as described previously. Transfected mouse 3T3 cells were produced as described above. Cultures growing on glass coverslips were rinsed with serum-free SATO medium (Doherty and Walsh 1989) and incubated in a 10% CO₂, 90% air environment at 37°C for 60 min in the presence and absence of neuraminidase (1 U/ml; Sigma type X). Primary antibodies were then added directly at appropriate dilution and the immunostaining analyses completed as described previously (Walsh and Ritter 1981). The anti-MSD1b peptide serum was used at a 1 : 50 dilution in combination with mouse monoclonal antibodies either to human N-CAM (1 : 500 dilution of ascites fluid) or human fibronectin (1 : 100 dilution of ascites; Walsh et al. 1981).

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