Differential Effects of Over-expressed Neural Cell Adhesion Molecule Isoforms on Myoblast Fusion

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Abstract. We have used a transfection based approach to analyze the role of neural cell adhesion molecule (NCAM) in myogenesis at the stage of myoblast fusion to form multinucleate myotubes. Stable cell lines of myogenic C2 cells were isolated that express the transmembrane 140- or 180-kD NCAM isoforms or the glycosylphosphatidylinositol (GPI) linked isoforms of 120 or 125 kD. We found that expression of the 140-kD transmembrane isoform led to a potent enhancement of myoblast fusion. The 125-kD GPI-linked NCAM also enhanced the rate of fusion but less so when a direct comparison of cell surface levels of the 140-kD transmembrane form was carried out. While the 180-kD transmembrane NCAM isoform was effective in promoting C2 cell fusion similar to the 140-kD isoform, the 120-kD isoform did not have an effect on fusion parameters. It is possible that these alterations in cell fusion are associated with *cis* NCAM interactions in the plane of the membrane. While all of the transfected human NCAMs (the transmembrane 140- and 180-kD isoforms and the 125- and 120-kD GPI isoforms) could be clustered in the plane of the plasma membrane by species-specific antibodies there was a concomitant clustering of the endogenous mouse NCAM protein in all cases except with the 120-kD human isoform. These studies show that different isoforms of NCAM can undergo specific interactions in the plasma membrane which are likely to be important in fusion. While the transmembrane and the 125-kD GPI-anchored NCAMs are capable of enhancing fusion the 120-kD GPI NCAM is not. Thus it is likely that interactions associated with NCAM intracellular domains and also the muscle specific domain (MSD) region in the extracellular domain of the GPI-linked 125-kD NCAM are important. In particular this is the first role ascribed to the O-linked carbohydrate containing MSD region which is specifically expressed in skeletal muscle.

THE fusion of mononucleate myoblast cells to form multinucleate myotubes is a central event in skeletal development and leads ultimately to the formation of mature muscle fibers. The myoblast to myotube transition is also characterized by the expression of a large number of structural proteins required for the correct functioning of the myofibrillar apparatus and other components required for different aspects of terminal differentiation. The process of skeletal muscle fusion has been studied extensively but the exact mechanism is not known (Knudsen, 1990; Wakelam, 1985, 1988). Fusion is clearly complex and multi-stage and involves the operation of both cell–cell and cell–extracellular matrix interactions (Knudsen, 1990; Mege et al., 1992; Dickson et al., 1990). A large number of studies have shown that in vitro, myoblasts express both a calcium-dependent and -independent cell adhesion system, and that both are likely required for fusion to proceed (Pizzey et al., 1988; Gibralter and Turner, 1985; Knudsen, 1985). There are also additional complexities in vivo. For instance, in vivo myogenesis proceeds in two phases called primary and secondary and may involve the use of specific populations of myoblasts (Miller 1991; Stockdale, 1992). After the formation of primary myofibers from the fusion of primary myoblasts the secondary myoblasts attach to the primary myofibers but do not fuse with them. Instead they fuse with other secondary myoblasts.

The molecular basis of cell–cell interactions in skeletal muscle myoblasts is starting to be understood and a number of gene products active in the process identified. The calcium-dependent adhesion system in myoblasts is associated with the expression and operation of a set of cell adhesion molecules (CAMs)[1] called cadherins (Takeichi, 1988, 1991). In skeletal muscle, three cadherins have been identified namely N-cadherin (Knudsen et al., 1990; Mege et al., 1992; Walsh et al., 1990), M-cadherin (Donalies et al., 1991; Moore and Walsh, 1993) and T-cadherin (Ranscht,

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1. *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; CAM, cell adhesion molecule; GPI, glycosylphosphatidylinositol; HS, horse serum; MSD, muscle-specific domain; PIPLC, phosphatidylinositol-specific phospholipase C.

1991; Ranscht and Bronner-Fraser, 1991). The exact role and relative importance of these three cadherins is not clear although synthetic peptides that block N-cadherin-mediated adhesion have dramatic effects on blocking fusion (Mege et al., 1992). Two calcium-independent adhesion systems have been identified in muscle. The Ig superfamily member VCAM-1 has been identified on myoblasts and myotubes and its partner, the β_1 integrin VLA4 on myotubes (Rosen et al., 1992). A scheme has been presented where the specific interaction of this heterophilic CAM pair may account for the specificity of primary and secondary myoblast fusion (Rosen et al., 1992). Antibodies reactive with the β_1 integrins have major inhibitory effects on myoblast fusion in vitro (Menko and Boettiger, 1980) although it is not clear whether these were due to blockade of the interaction of integrins with VCAM-1 or with the extracellular matrix binding sites. The neural cell adhesion molecule (NCAM) is the second Ig superfamily member present on the cell surface of myoblasts (Moore and Walsh, 1985; Sanes et al., 1986). The pattern of expression of NCAM is tightly regulated during development of skeletal muscle (Lyons et al., 1992). In proliferating myoblasts the predominant NCAM isoform is a 140-kD transmembrane species whereas myotubes express a 125-kD glycosylphosphatidylinositol (GPI) linked form (Covault et al., 1986; Moore et al., 1987). There are several consequences of this isoform switch. First, there is a change in membrane anchorage of NCAM and second, due to alternative splicing there is the expression of an extracellular muscle specific domain (MSD-1) which bears an O-linked oligosaccharide (Dickson et al., 1987; Hamshere et al., 1992; Pan et al., 1992; Thompson et al., 1989; Walsh et al., 1989). During myogenesis there is an upregulation of NCAM expression by ~4–5-fold which is due to an increased transcriptional rate (Roubin and Goridis, 1992). A number of pieces of data support a role for NCAM in fusion (Dickson et al., 1990; Knudsen et al., 1990). Calcium-independent adhesion which is a prerequisite for fusion has been shown to be sufficient to allow aggregation in mouse myoblasts and that antibodies to NCAM inhibited this adhesion (Knudsen et al., 1990). Also, phosphatidylinositol-specific phospholipase C (PIPLC) which removes the 125-kD GPI-linked NCAM inhibits myoblast aggregation (Knudsen et al., 1989) while overexpression of 125-kD NCAM by gene transfer enhances fusion (Dickson et al., 1990).

To analyze the role of NCAM further, we have used a transfection-based approach which has been used previously to define the role of NCAM in recognition events (Dickson et al., 1990; Doherty et al., 1989; Pizzey et al., 1989). We have shown that the C2 myoblast cell line when transfected with a 125-kD GPI-linked NCAM containing the MSD region showed an enhanced rate of myoblast fusion which was due to the introduced transgene (Dickson et al., 1990). In the present study we have extended our analyses to assess the role of membrane association and also alternative splicing in the extracellular domain of NCAM on muscle fusion. We show that the 140-kD transmembrane isoform of NCAM causes a dramatic increase in muscle fusion while the GPI-linked 120-kD isoform which is lacking the MSD-1 region has no effect on C2 fusion. Thus the NCAM component of myoblast fusion can be modulated by the expression of its intracellular domain and also the MSD-1 region in the extracellular domain.

Materials and Methods

Cell Culture and Transfection

The C2 mouse skeletal muscle cell line was grown on 150-mm culture dishes in DME containing 2 mM L-glutamine and supplemented with 10% FCS. Cells were maintained at 37°C in 8% CO_2 and used between passages 22 and 26, always at subconfluent density. To induce fusion, myoblasts were reseeded at 3.2×10^4 cells per cm^2 on collagen (Vitrogen) (Collagen Corp., Palo Alto, CA) coated culture dishes, allowed to adhere overnight and then the media changed to DME containing 5% horse serum (HS).

Populations of C2 cells were transfected singly with eukaryotic expression vectors encoding four different NCAM isoforms. These were transmembrane NCAM of 180 kD (Doherty et al., 1992*a*) or 140 kD (Doherty et al., 1990) and GPI-anchored forms of 125 kD (Dickson et al., 1990; Doherty et al., 1990) or 120 kD (Doherty et al., 1990). For the 120, 125, and 140 kD expressing cDNA NCAM clones the expression vector used was pHβApr-1-neo which contains the β-actin promoter while for the 180-kD construct, pcDNA1-neo which contains the cytomegalovirus promoter was used. C2 cells (1×10^4) in a 60-mm dish were transfected with 3 μg of the expression plasmid using the calcium phosphate protocol provided with the Cell Phect Transfection Kit (Pharmacia Fine Chemicals, Piscataway, NJ). After 16 h, cells were transferred to 150-mm dishes containing DME, 10% FCS, 2 mM glutamine, and 0.5 mg/ml G418. After 10 d of culture, G418 resistant colonies were isolated and analyzed for human NCAM expression.

Characterization of Transfected C2 Cells

Control and transfected C2 cells were characterized for the expression of human NCAM by immunocytochemistry and Western blotting using the human NCAM-specific mAb Eric-1 (Bourne et al., 1991).

For immunocytochemistry cells were incubated sequentially with the Eric-1 mAb (1:100 dilution of ascites fluid), biotinylated anti-mouse Ig and Texas red streptavidin (Amersham International, Amersham, UK) both diluted 1:500. Western blotting of C2 cell extracts was carried out as described previously (Dickson et al., 1990) using the Eric-1 mAb at a 1:100 dilution and the ECL Western blotting reagents from Amersham International (Doherty et al., 1991).

The relative level of human NCAM on individual clones was determined using radioimmunoassay by measuring the binding of a saturating concentration of a ^{125}I-Fab fragment of Eric-1 mAb. Saturating levels of ^{125}I Eric Fab (22.4 μCi/μg) were incubated for 60 min with a range of cell numbers ($4\text{-}10 \times 10^3$) in microtiter plates. Cells were then washed three times with PBS and the bound ^{125}I-Fab extracted with 0.1 M NaOH. Samples were counted in a Beckman 6000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Analysis of Myoblast Fusion

C2 cells were plated at a density of $3.2 \times 10^4/cm^2$ on either 24-mm culture dishes or individual wells of Lab-Tek eight-chamber slides (Lab-Tek, Naperville, IL) coated with collagen. After 1–4 d of culture, samples were analyzed for biochemical differentiation by measuring creatine kinase levels. This was carried out using the UV-CK-NACS kit (Boehringer Mannheim, Lewes, UK). Protein was measured using the Bio Rad Bradford reagent (Bio Rad Laboratories, Richmond, CA). Morphological differentiation was assessed by measuring the total number of nuclei in wells of Lab-Tek slides and also the number of myotube associated nuclei. Nuclei were identified by staining with the Hoescht 33258 dye after permeabilization of cells with 100% methanol for 8 min at −20°C. Samples were viewed under UV light on a Zeiss Axiophot microscope (Zeiss, Carl Ltd., Herts, UK) and generally the cells in three separate fields measured.

Blockade of Fusion with Antibodies to NCAM

The ERIC-1 mAb has been shown previously to block human NCAM-mediated interactions in C2 cells transfected with human NCAM cDNAs (Dickson et al., 1990). Either a monovalent Fab fragment of the ERIC-1 mAb or control non specific mouse Ig was added at a concentration of 300 μg/ml to culture 24 h after plating. The fusion media was changed every 24 h according to the time course of the assay.

Assessment of Cell Mitotic Index

Subconfluent cultures of replicating myoblasts were reseeded at 3.2 × $10^4/cm^2$ on collagen-coated 13-mm-diameter coverslips and allowed to adhere overnight. Fusion was induced by feeding cultures with DME containing 5% HS.

Representative cultures were labeled at fixed time points (0–56 h) with 10 μM bromodeoxyuridine (BrdU) (Sigma Chemical Co., Poole, UK) for 4 h, fixed in methanol at −20°C for 10 min followed by fixation with 0.2% paraformaldehyde for 1 min. Finally cultures were treated with 0.07 M NaOH for 7–10 min. BrdU was visualized with a BrdU antibody (Dako Corp., Carpinteria, CA) and detected by a Texas red–conjugated secondary antibody. Total nuclei counts in the cultures were assessed after Hoechst 33258 staining.

Analysis of NCAM cis Interactions in the Myoblast Membrane

C2 myoblasts expressing transfected human NCAM were initially reacted with ERIC-1 mAb for 90 min at 37°C followed by a fluorescein-conjugated anti-mouse Ig secondary antibody for 90 min at 37°C. Cells were then fixed for 15 min with 4% paraformaldehyde and the position of the endogenous mouse NCAM visualized using the mAb H28 and a rhodamine labeled rat antibody. The position and amount of clustering of the human and mouse NCAM was determined by indirect immunofluorescence analysis.

Results

Expression and Characterization of Human NCAM in Transfected C2 Cells

We have shown previously that transfection of mouse C2 myoblasts with a cDNA encoding a 125-kD GPI-linked human NCAM protein resulted in the expression of immunoreactive NCAM at the cell surface. In the present study a panel of C2 myoblasts expressing additional NCAM isoforms has been generated. These were two transmembrane isoforms of 180 and 140 kD and a GPI-anchored form of 120 kD. Indirect immunofluorescence staining has been used to show coexpression of each of these human isoforms with the endogenous mouse NCAM pool. We have found that for each of the isoforms analyzed (180-, 140-, 125-, and 120-kD NCAMs) there was coexpression of the endogenous mouse NCAM with the transgene on both myoblasts and myotubes. Fig. 1 shows an example of cells expressing a 140-kD NCAM isoform. In each case the distribution of the transgene appeared random on the cell surface. Western blot analysis of the transfected cells was carried out to determine the relative molecular mass of the transfected NCAM. Fig. 2 shows that

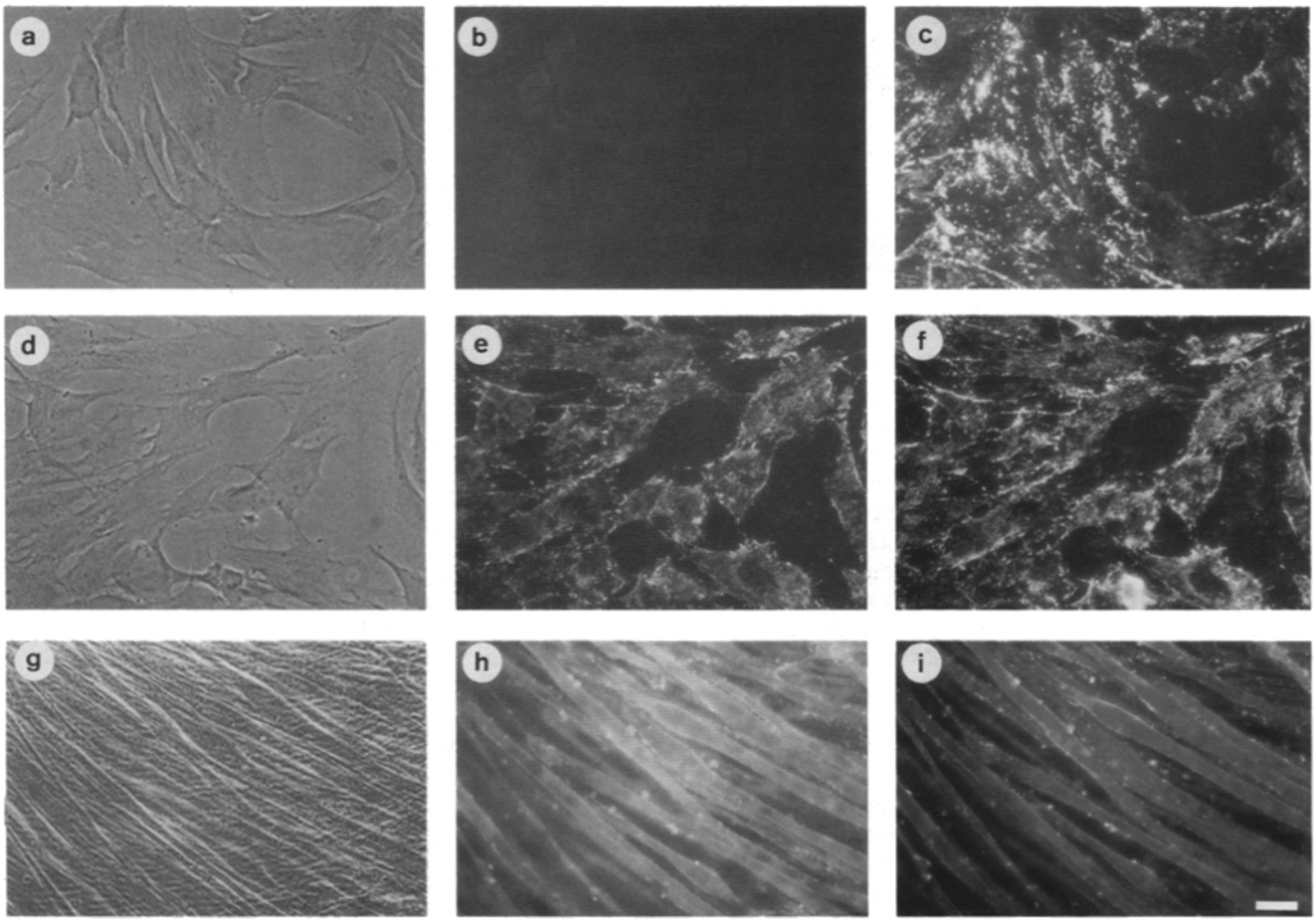

Figure 1. Coexpression of mouse and human NCAM in transfected C2 cells. Cell surface immunostaining of parental C2 myoblasts (*a–c*) and of myoblast (*d–f*) and myotube (*g–i*) cultures of a C2 cell line transfected with a gene construct encoding the 140-kD transmembrane form of human NCAM after double staining with human NCAM specific (*b*, *e*, and *h*) or mouse specific (*c*, *f*, and *i*). Bar, 100 μm.

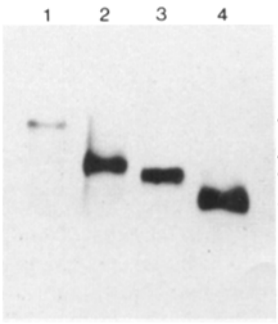

Figure 2. Western blot analysis of transfected human NCAM in C2 cells. C2 cells transfected with various human NCAM isoforms were analyzed for human NCAM using a species specific antibody. Samples were treated with neuraminidase to remove sialic acid sidechains. Lane *1*, 180-kD NCAM transfectant; lane *2*, 140-kD NCAM transfectant; lane *3*, 125-kD NCAM transfectant; lane *4*, 120-kD NCAM transfectant. The molecular weight of the reactive bands were calculated from the molecular weights of proteins of known M_r.

protein bands of 180, 140, 125, and 120 kD could be found specifically in cultures of cells expressing the appropriate NCAM isoform. We have shown previously that overexpression of human NCAM in C2 cells did not alter the expression of the endogenous NCAM pool (Dickson et al., 1990). A similar result was found in the present study (data not shown).

The level of human NCAM at the cell surface was calculated by binding of a ^{125}I-labeled Fab fragment of the ERIC-1 mAb. Eight different cell lines which by indirect immunofluorescence staining appeared qualitatively to express different levels of NCAM were screened for ^{125}I-ERIC-1 Fab binding. The highest expressing cell line (C2-B11) expressed 9.6 × 10^5 human NCAM sites per cell while the lowest expressed 2.5 × 10^5 sites per cell with intermediate values found for the other cell lines.

The Transmembrane 140-kD NCAM Isoform Promotes Myoblast Fusion to a Greater Extent than the GPI-linked 125-kD Isoform

We have shown previously (Dickson et al., 1990) that transfection of mouse C2 cells with a cDNA encoding a 125-kD GPI-anchored NCAM isoform enhanced myoblast fusion. To determine whether the naturally occurring myoblast NCAM isoform of 140 kD affected myoblast fusion in the same or a different fashion, a comparison of fusion parameters in transfected C2 cell lines expressing 125 or 140 kD human NCAM isoforms was carried out. The 125-kD NCAM synthesizing cell line expressed 2.5 × 10^5 sites per cell while the 140-kD cell line expressed 2.0 × 10^5 sites per cell. Fig. 3 shows the time course of fusion over a 4-d period as indexed by measuring CPK levels for C2 cells and the 125- and 140-kD transfectants. C2 cells expressing 125-kD NCAM fuse more rapidly and to a slightly greater extent as indexed by CPK measurements than wild type C2 cells. The 140-kD NCAM expressing cell line was significantly faster in its rate of fusion and differences could be detected as early as 2 d after plating. These differences increased in magnitude up to the day 4 time point. At that point the 140-kD expressing clone had a CPK value 254% higher than that of C2 cells. Similar results were found when an alternative measurement namely the percent nuclei in myotubes were made (Fig. 3 *B*). Here the 125-kD expressing clone appeared to fuse faster but not to a greater end point than C2 cells, while the 140-kD

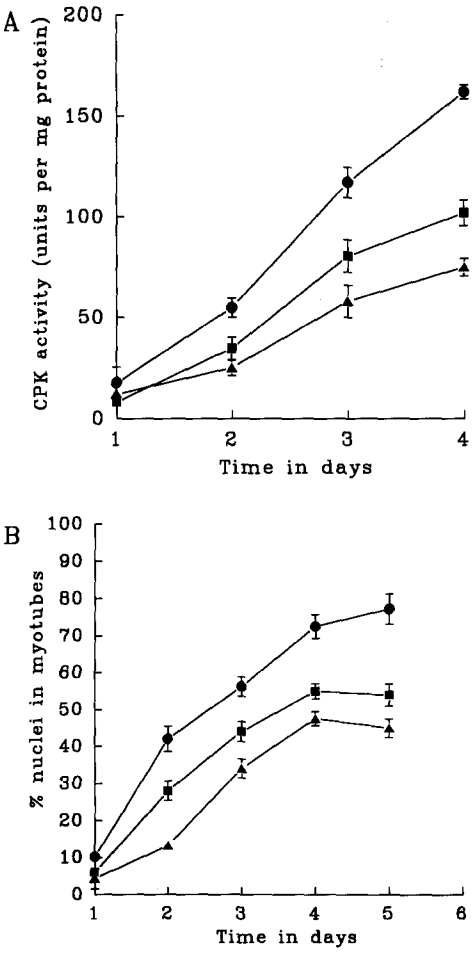

Figure 3. Effect of 140- and 125-kD transfected NCAM isoforms on C2 cell fusion. Parental and transfected C2 cells were placed in culture for various periods of time (1–4 d) before analysis for muscle differentation as assessed by the level of CPK in the cell cultures (*A*). The graphs shows the result for C2 cells (▲), 125-kD NCAM expressing C2 cells (■), and 140-kD NCAM expressing cells (●). Values are means ± sem (n = 3) for independent cultures. (*B*) The same cells except an alternative measurement (the percent nuclei in myotubes) is used. Here cultures were analyzed over a 5-d time period. Samples were C2 cells (▲), 125-kD expressing NCAM transfectants (■), and 140-kD NCAM expressing transfectants (●). Values are means ± sem (n = 3).

clone was dramatically faster and almost doubled the nuclei in myotubes compared with C2 cells.

To determine whether the enhanced rate of fusion found in the 140- and 125-kD transfectants was related to the level of human NCAM in these cell lines a larger number of clones synthesizing different levels of human NCAM were analyzed. Fig. 4 shows that a series of 140-kD NCAM expressing transfectants analyzed 3 d after plating, all expressed levels of CPK significantly above C2 cells with a range from 150 to 290% and a clear relationship between levels of human NCAM and the CPK level was found. In contrast to the 140-kD NCAM transfectants, the 125-kD lines are significantly less active on a sites per cell basis. All of the 125-kD lines fuse to a greater extent than the C2 cells (from 150 to 230%) and there is a relationship between level and

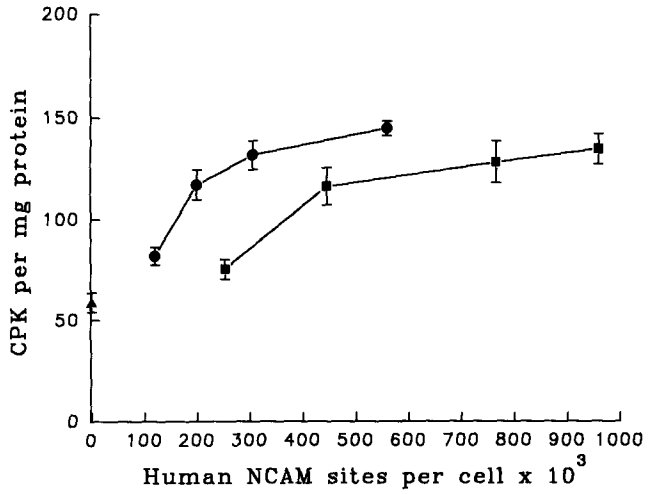

Figure 4. Comparison of the effects of expression of different levels of 140-kD human NCAM and 125-kD human NCAM on C2 cell fusion. C2 cell lines expressing different levels of 140-kD human NCAM (●) or 125-kD human NCAM (■) as assessed by ^{125}I-labeled anti-NCAM Fab binding were analyzed for their fusion competence in terms of CPK levels and compared to C2 cells (▲) at a set 3-d time point. Each value is the mean ± sem from three determinations.

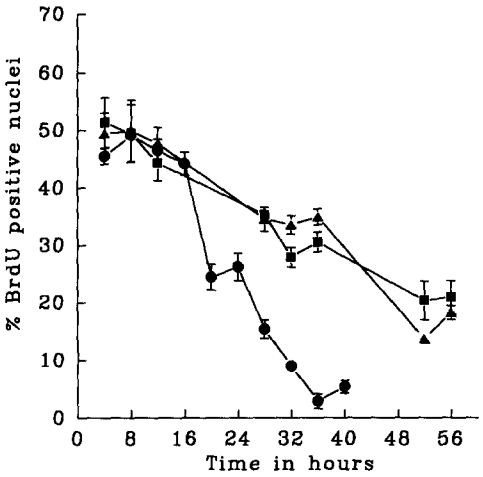

Figure 5. Comparison of the number of postmitotic cells with time in cultures of C2 cells versus transfected C2 cells expressing 140-kD human NCAM. C2 cells (▲), 125- (■) and 140-kD human NCAM transfected C2 cells (●) were cultured on slides and at set periods of time (0–56 h) were pulsed with BrdU for 4 h. At that point samples were fixed and reacted with anti BrdU. The percentage of BrdU positive nuclei is plotted against time. Each value represents the mean ± sem of three determinations.

response. However on a comparable sites per cell basis the 125-kD construct clearly has less efficacy than the transmembrane 140-kD construct.

The 140-kD NCAM Expressing Cell Lines Become Postmitotic before C2 Cells

One of the steps before myoblast fusion is withdrawal from the cell cycle and the appearance of postmitotic cells. BrdU incorporation can be used as a sensitive index of the proliferative capacity of cell populations. We therefore wished to compare the proliferative behavior of NCAM transfected and C2 cells to determine whether this might show an alteration in parameters earlier than cell fusion. Fig. 5 shows the kinetics of cell cycle withdrawal in C2 cells, and in a transfectant expressing the transmembrane 140-kD NCAM isoform. Over the 56-h period of analysis C2 cells progressively leave the cell cycle and become postmitotic. This is in line with the culture kinetics where myotubes are starting to appear by day 2 in the C2 cultures. (Fig. 3 *b*). The same graph (Fig. 3 *B*) shows that the 140-kD NCAM expressing transfectants have >40% of nuclei in myotubes at day 2. Thus the rapid rise in fusion is accompanied by a dramatic decrease in the mitotic index (Fig. 5) such that there is a major difference between the C2 cells and the 140-kD expressing line. The 140-kD expressing C2 cell line contains virtually no mitotic cells after 36 h of culture while the C2 cells have 35% mitotic cells at this time point. We also found that the 180-kD NCAM transfectants are similar to the 140-kD transfectants (data not shown). However both the 120- and 125-kD GPI-linked NCAM isoforms were identical to the C2 profile and Fig. 5 shows the result for the 125-kD transfectant. It remains to be determined whether these results indicate a difference in mechanism of action or sensitivity of assay systems under study.

The Transmembrane 180-kD NCAM Isoform Enhances Myoblast Fusion in a Similar Manner to the 140-kD Transmembrane Isoform

The largest NCAM isoform of 180 kD is identical to the 140-kD isoform except that it has a longer cytoplasmic domain. It is expressed transiently in myotomes around day 9 in the mouse embryo both by in situ hybridization (Lyons et al.,

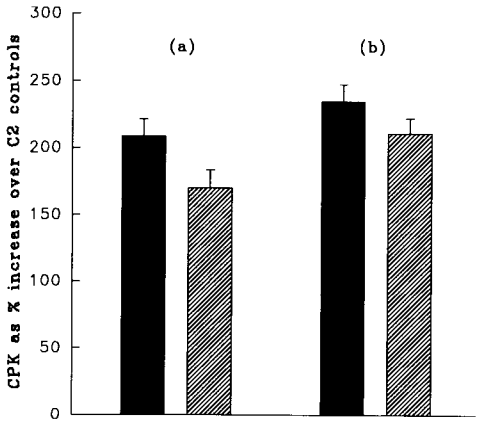

Figure 6. The transmembrane 180-kD NCAM isoform enhances fusion to the same degree as the 140-kD isoform. Comparison of fusion in two pairs of NCAM transfectants that synthesize roughly equal amounts of human NCAM. Cells were placed in culture and analyzed for CPK at day 3. *a* shows the result for 140-kD expressing NCAM (C2C5 cells, 2.0 × 10^5 sites per cell of NCAM; ■) and 180-kD expressing (C2R5 cells; 1.9 × 10^5 sites per cell of NCAM; ▨). Each value is the mean sem pooled for three determinations. *b* shows the result for the second pair of transfectants. These were the 140-kD expressing cells (C2C6 cells, 2.9 × 10^5 sites per cell of NCAM; ■) and 180-kD expressing transfectant (C2R2 cells, 3.0 × 10^5 sites per cell of NCAM, ▨). Each value is the mean ± sem pooled from three determinations.

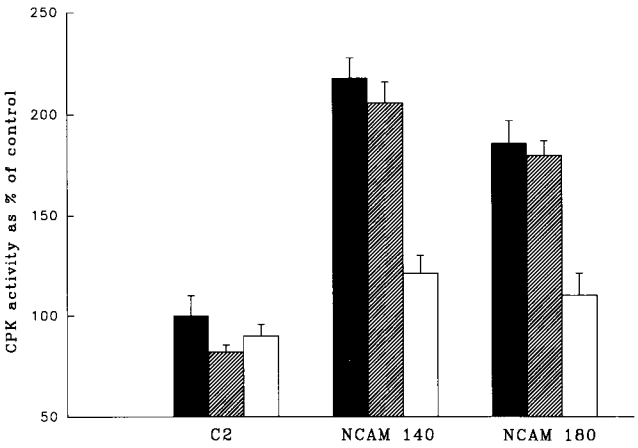

Figure 7. Human NCAM antibodies specifically block the enhancement of fusion induced by transfected NCAM. C2 cells and a line of 140- and 180-kD transfectants were cultured in the presence and absence of the blocking ERIC-1 mAb and the non blocking 5.1 H11 mAb that both react specifically with human NACM. Samples were analyzed for CPK activity after 3 d in culture either in control cultures (■). Nonblocking 5.1 H11 mAb (▨) and blocking ERIC-1 mAb (□). Values are expressed as the mean ± sem (n = 3). 100% values relate to 58.98 U CPK.

1992) and immunocytochemistry (Thiery et al., 1982). Small levels have also been transiently found in some muscle cells in culture (Tassin et al., 1991). It was therefore of interest to determine whether this isoform was as active as the 140-kD isoform. Fig. 6 shows that in two independent 180-kD NCAM expressing cell lines tested 3 d after plating there was a roughly similar enhancement of fusion. There was no difference statistically between the 180-kD cell line C2R5 and the 140-kD expressing C2C5 line or between the C2C6 and C2R2 cell lines where both pairs synthesized roughly the same amount of transfected NCAM. Thus both a large and a small cytoplasmic domain seems to be capable of mediating the positive effects on myoblast fusion.

Antibodies to Human NCAM Reverse the 140- and 180-kD NCAM-mediated Enhancement of Fusion

To show that the enhanced fusion seen in the 140- and 180-kD NCAM transfectants was due to the expression of the transgene, a blocking antibody approach using species-specific antibodies was used. We have shown previously that Fab fragments of the human specific NCAM blocking mAb called ERIC 1 but not other NCAM antibodies would block the enhanced fusion due to transfected 125-kD NCAM (Dickson et al., 1990). Fig. 7 extends this approach to the 140- and 180-kD isoforms. The addition of 300 μg/ml of ERIC-1 Fab led to a significant reversal of the enhanced fusion found to be due to the 180- and 140-kD isoforms while non blocking NCAM antibodies had no effect as found previously. In addition to the biochemical marker of differentiation (CPK) shown in Fig. 7 similar effects were found for morphological differentiation. ERIC-1 antibody at 300 μg/ml resulted in a 35% decrease in the percent nuclei in myotubes in a 140-kD NCAM expressing cell line at day 3 of fusion. The positive effects of NCAM expression are specific and seem to be directly related to greater expression of NCAM rather than some general perturbation of the plasma membrane.

The GPI-linked NCAM Isoform of 120 kD Has No Effect on C2 Cell Fusion

The GPI-anchored isoform of NCAM found on skeletal muscle myotubes is of 125 kD with no evidence of the 120-kD isoform which is found on other cells such as glial cells (Walsh and Doherty, 1991). The only difference between these isoforms is the presence of the MSD1 region which is a sequence of 37 amino acids between the type III fibronectin repeats in the extracellular domain (Walsh and Doherty, 1991). To determine whether the MSD1 region is important in the process of fusion the 120-kD isoform which is lacking in this region only has been analyzed. Three cell lines expressing 3.53 × 10^5, 4.18 × 10^5, 4.86 × 10^5, sites per cell of human NCAM were screened for their effects on fusion and compared with the positively acting 125-kD NCAM clone which expresses 4.3 × 10^5 sites per cell of human NCAM. In all three cell lines expressing the 120-kD NCAM isoform there was no enhancement of fusion but a small inhibition which was not statistically different from C2 cell fusion (Fig. 8). Thus the 120-kD isoform neither enhances or inhibits fusion while the 125-kD control clone significantly enhances fusion. Thus it seems likely that the MSD-1 region is a positive modulator of myoblast fusion.

Co-clustering of Transfected NCAM and the Endogenous NCAM Pool in C2 Myoblasts

The overexpression of transmembrane and the 125-kD GPI NCAM isoform but not the 120-kD GPI isoform in C2 cells leads to enhanced fusion. One mechanistic possibility is that there are *cis* interactions occurring in the cell membrane, in addition to the obvious *trans* interactions between NCAM isoforms that mediate these effects. The availability of species specific NCAM antibodies allows for the study of possi-

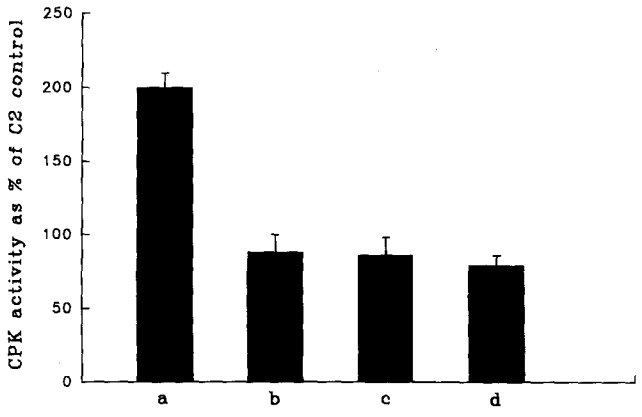

Figure 8. Transfection of C2 cells with 120-kD NCAM has no effect on fusion. Three C2 cells lines cells synthesizing different amounts of human NCAM ([*b*]3.53 × 10^5 sites per cell; [*c*] 4.18 × 10^5 sites per cell; and [d] 4.86 × 10^5 sites per cell) were compared with a 125-kD NCAM expressing cell line ([*a*] 4.30 × 10^5 sites per cell). Cells were placed in culture and analyzed at day 3 when CPK levels were measured and compared to control C2 cells. Values are means ± sem (n = 3).

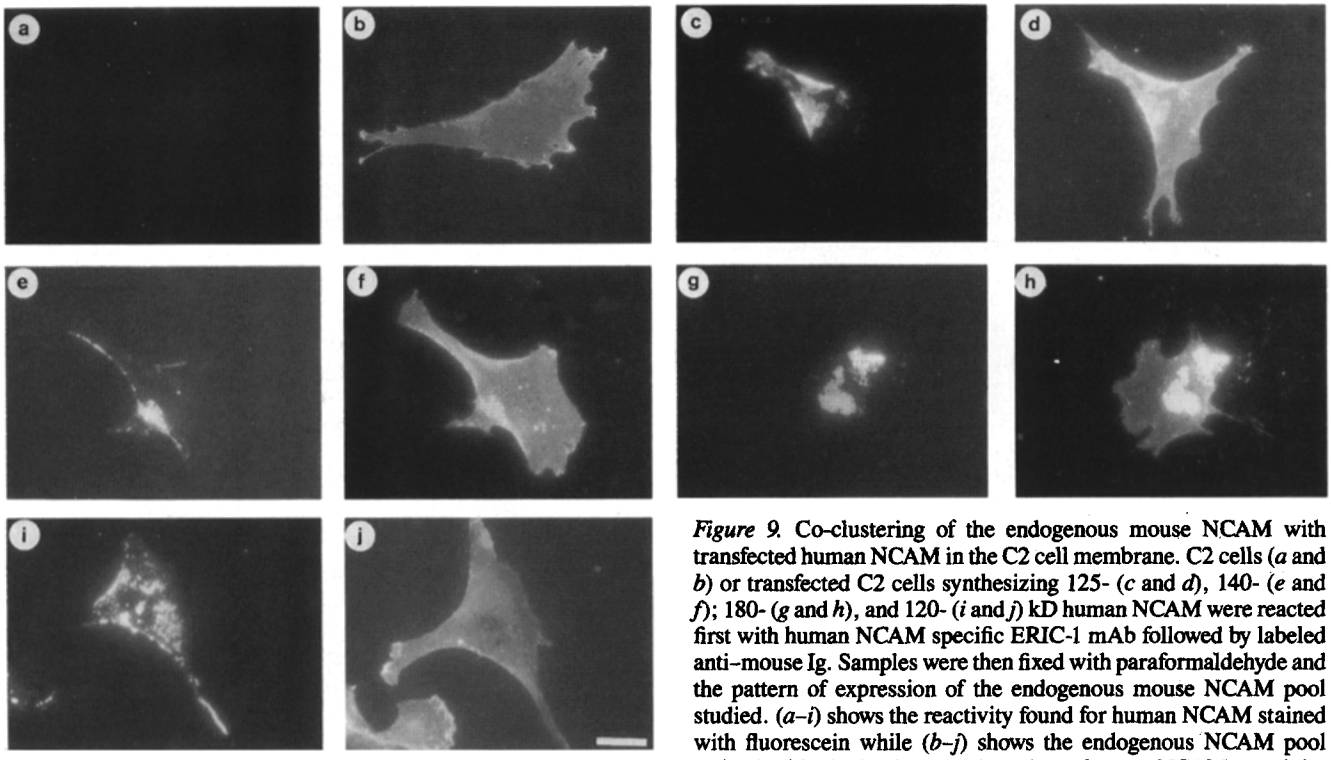

Figure 9. Co-clustering of the endogenous mouse NCAM with transfected human NCAM in the C2 cell membrane. C2 cells (*a* and *b*) or transfected C2 cells synthesizing 125- (*c* and *d*), 140- (*e* and *f*); 180- (*g* and *h*), and 120- (*i* and *j*) kD human NCAM were reacted first with human NCAM specific ERIC-1 mAb followed by labeled anti-mouse Ig. Samples were then fixed with paraformaldehyde and the pattern of expression of the endogenous mouse NCAM pool studied. (*a–i*) shows the reactivity found for human NCAM stained with fluorescein while (*b–j*) shows the endogenous NCAM pool stained with rhodamine. As there is no human NCAM reactivity in the C2 cells (*a*) there is no reactivity in this panel. Bar, 100 μm.

ble *cis* interactions between human NCAM and the endogenous NCAM in the same cells. The transfected human NCAM can be clustered in the membrane of C2 myoblasts by a double antibody binding method. The NCAM reactive ERIC-1 mAb was bound to cells followed by rabbit anti-mouse Ig. This led to the formation of micro-clusters in the cell membrane due to crosslinking (Fig. 9). It was found that the majority (>90%) of the human NCAM could be clustered by this method and was a reproduceable phenomena being found in six independent experiments. The phenomena was observed in the four cell lines that synthesized 180-, 140-, 125-, and 120-kD NCAM (Fig. 9). An assessment was also made as to whether there was any mouse NCAM in the human NCAM clusters. Fig. 9 shows that in human NCAM clusters from the 180-, 140-, and 125-kD transfectants there was a co-clustering of mouse NCAM. The 120-kD human NCAM expressing cell line was different because it was unable to co-cluster the endogenous NCAM pool. However a mouse NCAM antibody was able to cluster this pool showing that transfection with the 120-kD isoform did not irreversibly alter the ability of the endogenous NCAM pool to cluster. Thus there appears to be *cis* interactions between human and mouse NCAM occurring in the plane of the plasma membrane. The fact that the 125-kD GPI-linked NCAM can co-cluster the endogenous NCAM pool but the 120-kD expressing transfectant cannot suggests that this phenomenon can be determined by sequence in the extracellular part of the NCAM protein.

Discussion

At the cell surface CAMs have been shown to be crucial components in the process of myoblast fusion to form multinucleate myotubes. CAM families are likely involved at both the initial recognition events that may lead to transmission of specific signals and also the strong stable adhesive events that may be required before membrane union. It also seems likely that CAMs do not operate in isolation and that there are functional interactions between CAMs. For instance in the myoblast membrane there are close associations between NCAM and N-cadherin that can be identified by immunocytochemical means (Knudsen et al., 1990; Mege et al., 1992).

A detailed picture of NCAM expression in skeletal muscle has been produced. Myoblasts express predominantly the transmembrane 140-kD isoform while myotubes express a GPI-linked 125-kD form (Covault et al., 1986; Moore et al., 1987). During the myoblast to myotube transition the transcriptional rate of the gene also increases (Roubin and Goridis, 1992). These studies have tended to suggest a role for NCAM in myoblast fusion although how or where it is acting has not been clear. We have previously analyzed the function of the 125-kD GPI-linked NCAM isoform in myoblast fusion by gene transfer (Dickson et al., 1990). Constitutive overexpression of this isoform caused an enhancement in the rate of fusion as assessed by both morphological and biochemical parameters. In the present study we have analyzed the effect of introducing additional forms of NCAM into the myoblast membrane. These include two transmembrane forms of 140 and 180 kD and a GPI-linked form of 120 kD. While the 140-kD isoform is the main NCAM of myoblasts (Moore et al., 1987) and the 180-kD isoform is expressed transiently in somites (Lyons et al., 1992) the 120-kD isoform is never found in skeletal muscle but is highly expressed on cells such as glia (Walsh and Doherty, 1991). A comparison of the 120 versus the 125-kD isoforms does

however allow an assessment as to whether the MSD region in the extracellular domain of NCAM is involved in the fusion process. In the present study we provide evidence that the transmembrane NCAM isoforms of 140 and 180 kD enhance muscle fusion compared to the 125-kD isoform. Interestingly the 120-kD isoform when expressed in C2 cells is neutral and does not alter fusion although there was some evidence of a slight inhibition which in the present model was not significant. The effect of overexpressing the 140-kD isoform was dramatic. There was a doubling of the percentage of nuclei in myotubes and about a threefold increase in the level of creatine kinase in the culture after 4–5 d. These effects are more dramatic than our previous study with the 125-kD isoform (Dickson et al., 1990). In both cases the rate of fusion was greater than in C2 cells. That the differences between the 140- and 125-kD isoforms were not just due to different levels of expression of the transgene was shown by comparing the fusion competence of a panel of 140- and 125-kD expressing cell lines. There was a clear relationship between the fusion competence of cell lines and the level of 140 or 125 kD NCAM expressed. However, in all cases the 140-kD NCAM was always more active on the basis of specific levels. The positive effects on fusion were also blocked by species-specific antibodies that reacted with the transfected human NCAM. It therefore appears likely that the effects on fusion are due to expression of the transfected NCAM. One other index of the culture dynamics in the C2 and transfected cell lines was the analysis of the mitotic index via BrdU incorporation. Here there was a marked difference between the behavior of the 140-kD NCAM transfectants and C2 cells with the 140-kD expressing cells were becoming postmitotic ~24 h before the C2 cells. It also appeared that the 125- and 120-kD NCAM transfectants behaved in the same manner as the C2 cells. It is not clear at present whether this is due to some fundamentally different mechanism operating or to differences in the sensitivity of the different assay systems. The 180-kD NCAM protein is an alternatively spliced form of the 140-kD isoform with a larger intracellular domain. The 120-kD isoform is also similar to the 125-kD isoform except that the former does not use the MSD exons between exon 12 and 13 encoding the extracellular domain. The 180-kD transfectants behave in a very similar manner to the 140-kD transfectants. In contrast, the 120-kD transfectants behave very differently. Overexpression of the 120-kD isoform in the C2 membrane does not alter any of the parameters associated with fusion in a significant manner. This is in contrast to PC12 cells where the expression of the same cDNA construct led to an inhibition of NCAM mediated signaling events and acts like a dominant negative mutant (J. Saffell, unpublished observations). These results suggest that although the 120 kD NCAM has the capacity to become involved in homophilic interactions in C2 cells it may not alter the ability of the endogenous pool to be involved in homophilic interactions. There may also be other compensatory mechanisms involving other CAMs that mask the effect of some loss of NCAM function in these cells.

The enhanced morphological and biochemical events associated with NCAM-mediated fusion may indicate an inherent plasticity associated with NCAM-mediated interactions. Transfection of the calcium-dependent CAM N-cadherin into C2 cells leads to blockade of fusion at a number of different expression levels (Peck, D., and F. S. Walsh, unpublished observations). The exact mechanism of NCAM mediated fusion enhancement is not clear at present. In the growth cone of neurons we have shown that NCAM-mediated homophilic binding results in activation of a signaling pathway which ultimately leads to calcium influx via L- and N-type calcium channels (Doherty and Walsh, 1992; Doherty et al., 1992*b*). Interestingly only transmembrane NCAM isoforms appear to be able to participate in the process suggesting that either the transmembrane regions or intracellular domains are important. The observation that a number of CAMs appear to be able to activate this pathway has led to the suggestion that they may be operating through some form of "adaptor" molecule (Doherty and Walsh, 1992; Doherty et al., 1992*b*; Williams et al., 1992). The clustering of NCAM in the cell membrane via *cis* interactions may be controlled at the level of the membrane anchoring mechanism. Bloch (1992) has also recently shown that NCAM can be clustered in the membrane of C2 myoblasts. Additionally we have found (Walsh, F. S., and K. Jacobson, unpublished observations) that GPI-linked NCAM is more mobile in the plane of the cell membrane of neuronal PC12 cells than transmembrane NCAM isoforms. Thus GPI-anchored NCAM in these cells may be able to interact with NCAM in the monolayer in co-culture experiments but because of mobility considerations is unable to form interactions that will allow co-clustering of adaptor molecules. In contrast the 140- and 180-kD transmembrane isoforms move much slower in PC12 cells and allow stable associations to occur. A similar situation may occur during myogenesis where the NCAM isoforms on both bilayers that are interacting are identical. We have found that the exogenous transmembrane NCAM isoforms and the 125-kD GPI isoforms will co-cluster with the endogenous NCAM pool. However the 120-kD isoform never co-clusters. This data is paralleled by mobility measurements where the transmembrane NCAM isoforms move slowest in the C2 membrane, followed by the 125-kD GPI-linked isoform containing the MSD sequences while the 120-kD GPI-linked isoform is fastest (unpublished observations). Whether there are any transmembrane signaling events associated with NCAM-mediated interactions in fusion is not clear. What is clear from the present study is that the transmembrane and/or the cytoplasmic domains of NCAM are important for the enhancement of fusion as is the MSD region and it is possible that positive effects can be interpreted in terms of NCAM mobility in the plasma membrane. This study is the first indication as to the function of the MSD region on skeletal muscle NCAM. The region is rich in the amino acids serine, threonine, and proline and is known to be a site of O-linked carbohydrate attachment (Walsh et al., 1989). It is possible that the O-linked carbohydrate alters the mobility of NCAM via specific *cis* interactions but it is also possible that the protein core is also involved. Some evidence has been presented that NCAM may interact with the L1 CAM and this interaction involves carbohydrate residues on the L1 glycoprotein (Kadmon et al., 1990).

One of the unusual features of the NCAM gene has been the relatively large number of isoforms that can be produced from alternative splicing of the single copy NCAM gene. Skeletal muscle is characterized by the orderly progression of isoform expression associated with alternative splicing. However a role for the different NCAM isoforms has not been apparent. The present study using gene transfer of different NCAM isoforms has identified roles for the major

NCAM isoforms present in tissue. In particular the MSD set of exons which are specific to skeletal muscle when used as a block (Dickson et al., 1986; Hamshere et al., 1992; Pan et al., 1992; Thompson et al., 1988) and which is a site of O-linked carbohydrate attachment, have a role in the process of muscle fusion. The strategies presented here however provide one way forward to dissecting the role of specific cell surface components in this process.

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