Intercellular Adhesion Mediated by Human Muscle Neural Cell Adhesion Molecule: Effects of Alternative Exon Use

John A. Pizzey, Lewis H. Rowett, C. Howard Barton, George Dickson, and Frank S. Walsh

Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom

Abstract. Mouse 3T3 fibroblasts were permanently transfected with cDNAs encoding isoforms of the neural cell adhesion molecule (N-CAM) present in human skeletal muscle and brain. Parental and transfected cells were then used in a range of adhesion assays. In the absence of external shear forces, transfection with cDNAs encoding either transmembrane or glycosylphosphatidylinositol (GPI)–linked N-CAM species significantly increased the intercellular adhesiveness of 3T3 cells in suspension. Transfection of a cDNA encoding a secreted N-CAM isoform was without effect on adhesion. Cells transfected with cDNAs containing or lacking the muscle-specific domain 1 sequence, a four-exon group spliced into the muscle but not the brain GPI-linked N-CAM species, were equally adhesive in the assays used. We also demonstrate that N-CAM–mediated intercellular adhesiveness is in-

hibited by 0.2 mg/ml heparin; but, at higher concentrations, reduced adhesion of parental cells was also seen. Coaggregation of fluorescently labeled and unlabeled cell populations was performed and measured by comparing their distribution within aggregates with distributions that assume nonspecific (random) aggregation. These studies demonstrate that random aggregation occurs between transfected cells expressing the transmembrane and GPI-linked N-CAM species and between parental cells and those expressing the secreted N-CAM isoform. Other combinations of these populations tested exhibited partial adhesive specificity, indicating homophilic binding between surface-bound N-CAM. Thus, the approach exploited here allows for a full analysis of the requirements, characteristics, and specificities of the adhesive behavior of individual N-CAM isoforms.

MORPHOGENETIC events during development and the subsequent maintenance of tissue architecture are largely dependent on cellular interactions involving selective recognition and adhesion between cell surfaces. These interactions are often mediated by cell adhesion molecules which have been identified in a wide range of vertebrate species and tissues (Edelman, 1983, 1986; Buck and Horwitz, 1987). Such molecules have frequently (Urushihara et al., 1977; Brackenbury et al., 1981; Thomas et al., 1981; Gibralter and Turner, 1985) been functionally assigned to one of two broad categories: those whose adhesive interactions require the presence of Ca^{2+} ions, such as the cadherin family (Takeichi, 1987), and those whose do not. The neural cell adhesion molecule (N-CAM)[1] is the best characterized and most prevalent member of the latter category known to date (for reviews see Goridis and Wille, 1988; Jessell, 1988; Nybroe et al., 1988; Walsh, 1988). N-CAM is a member of the immunoglobulin superfamily and is en-

coded by a single gene (Hunkapiller and Hood, 1986; Cunningham et al., 1987), and the observed diversity of the polypeptide is achieved by extensive transcriptional and posttranslational processing (Edelman, 1986; Cunningham et al., 1987; Small et al., 1988; Walsh, 1988). In general, however, four main classes of N-CAM polypeptide are known to exist (Walsh, 1988). Two isoforms are transmembrane variants, one of which possesses a larger intracellular domain than the other, and another class is represented by a nontransmembrane isoform that has a small surface domain responsible for attaching the protein to the cell membrane by a glycosylphosphatidylinositol (GPI) moiety. These have been described as the ld, sd, and ssd isoforms, respectively, by Owens et al. (1987). We have recently identified a fourth major species that although not yet unequivocally proven at the protein level, is apparently a secreted isoform of N-CAM (Gower et al., 1988).

Recently, the complexity of N-CAM expression has been more fully realized by observations (Santoni et al., 1989; Thompson et al., 1989) that at least two exons appear to be spliced into the muscle ssd form (but not into the brain species). These exons form part of a region we have recently (Dickson et al., 1987) named muscle-specific domain 1 (MSD1). The total number of N-CAM variants in brain tissue, known to date, accounts for eight species (Santoni et al.,

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1. *Abbreviations used in this paper:* CFDA, 6-carboxyfluorescein diacetate; GPI, glycosylphosphatidylinositol; HBSS, Hanks' basal salts solution; MEM, modified Eagle's medium; MSD1, muscle-specific domain 1; N-CAM, neural cell adhesion molecule; PIPLC, phosphatidylinositol-specific phospholipase C.

1989). Although the extent of N-CAM diversity is beginning to be elucidated, little is known of the functional consequences of the expression of the various isoforms.

Recent molecular approaches have allowed for isoform diversity to be studied in terms of physiological function. For example, it has been shown that, after transfection of chicken N-CAM cDNAs into mouse L cells (which do not normally express N-CAM), a brain transmembrane isoform can increase the intercellular adhesiveness of these cells and another isoform increases their capacity to bind chick brain vesicles (Edelman et al., 1987). Using a similar strategy, we have recently shown that human cDNAs encoding the sd and ssd muscle N-CAM homologues, when transfected into 3T3 cells, enhanced the ability of these cells to promote morphological differentiation of human and rat dorsal root ganglion neurons (Doherty et al., 1989). Cells transfected with cDNA encoding the secreted species had no effect on this process (Doherty et al., 1989). Since N-CAM isoform expression in muscle is both quantitatively and qualitatively developmentally regulated (Moore and Walsh, 1985; Moore et al., 1987) and may thus be integral in adhesive recognition events during myogenesis, we have now used this molecular approach to characterize the adhesive properties of the major species of human muscle N-CAM isoforms and also the GPI-linked brain isoform of 120 kD. We also present data on the conditions that affect N-CAM–mediated adhesion and on the extent of adhesive specificity between cell populations expressing individual muscle N-CAM species and those lacking the glycoprotein.

Materials and Methods

Construction of Eukaryotic Expression Vectors Incorporating Full-length N-CAM cDNAs

Eukaryotic expression vectors encoding transmembrane, GPI-linked, and secreted N-CAM isoforms as expressed in human embryonic muscle cultures were prepared from the three cDNA clones λ4.4, λ9.5 (Dickson et al., 1987), and λ8.6 (Gower et al., 1988). The NH_2-terminal region of the expression vectors was taken from clone λ5 (Barton et al., 1988), which contains the same coding sequence as CHB1 (Barton et al., 1988) but extends further 5' and features a unique Xmn I site used in the constructions. Human brain N-CAM derivatives were prepared from the same clones as described previously (Walsh et al., 1989a). All constructions were checked for (a) correct orientation by restriction site mapping and (b) maintenance of reading frame by the size of the synthesized protein directed by SP6 RNA polymerase-prepared RNA in a rabbit reticulocyte lysate in vitro translational system (Barton et al., 1988). The full-length cDNAs were used to express N-CAMs after calcium phosphate–mediated transfection into NIH 3T3 cells under the control of the β-actin promoter (Gunning et al., 1987) in pH_β APr-1-neo (Gower et al., 1988).

Cell Culture

Mouse NIH 3T3 and L cell fibroblasts were obtained from Gibco Laboratories Ltd. (Paisley, UK) and maintained in DME, 10% FCS, 2 mM glutamine. Cultures were kept at 37°C in 8% CO_2 in 140-mm Petri dishes and routinely passaged by dissociation in Versene (Gibco Laboratories Ltd.) containing 0.05% trypsin. All assays were performed on cells derived from cultures that had been replated 2–4 d after passage. Cultures were grown to near confluence before being used in the adhesion assays, and preliminary experiments showed that a degree of confluency equivalent to between 2 and 8×10^6 cells per dish had no significant effect on the subsequent adhesive behavior of these cells in the assays described below. Inconsistencies were found in the adhesive properties of cell cultures falling outside of this range, and such cultures are not represented in the data.

Adhesion Assays

Cell cultures were washed briefly with Hanks' basal salts solution (HBSS) and incubated with 5 ml Versene (which contains 7 mM EDTA) for 5 min at ambient temperature. Cells were then collected into 20 ml HBSS and centrifuged for 10 min at 4°C. Cells were then washed in HBSS and centrifuged once more before resuspension in modified Eagle's medium (MEM). To ensure a single cell suspension, cells were passed twice through a 21-gauge needle and filtered through a 20-μm mesh. Cells were then brought to a concentration of 1–1.5 × 10^6 cells/ml in MEM and introduced into a battery of adhesion assays. For couette viscometry (Curtis, 1969), the cell suspension was supplemented with 5% ficoll, and assays were conducted as described previously (Pizzey et al., 1988). This technique allows cells to come into contact under a constant fluid laminar shear rate (10^{-1} in this case; generated by a rotational speed of 2 rpm at 37°C), and the intercellular adhesiveness of the cells is represented by the collision efficiency which is quantitated by the number of predicted collisions that result in stable adhesions (Curtis, 1969). Alternatively, cell suspensions were subjected to higher rotational forces using a rotator or orbital shaker to generate more intercellular collisions in assays similar to those used by several other laboratories (see references in Frazier and Glaser, 1979; Garrod and Nicol, 1981; Roseman, 1985). These rotational speeds corresponded to 30, 90, and 270 rpm. The least shear force placed upon the cells in this study was by using an assay in still medium similar to those previously described for a variety of other cell types (Weiss, 1968; Rosenberg et al., 1969; Garrod and Born, 1971; Skehan, 1975). Briefly, cells are allowed to collide under no forces other than those imposed by gravity on a cell suspension. After resuspension in MEM, cell suspensions were agitated, counted, and incubated at 37°C for various time periods as indicated. After incubation, samples were inverted gently several times, before aliquots to be counted were taken, to ensure sampling from a homogenous population. Multiple samples were taken from each suspension. For this assay, a total of 24 separate cultures were used, representing the parental and four transfected cell lines studied.

In all of the assays described above, intercellular adhesiveness was measured as a function of particle number decrease with time. As cells are recruited into multicellular aggregates, the number of particles in suspension decreases. Particle number was determined using an electronic particle counter (ZM; Coulter Electronics Ltd., Luton, UK) or by hemocytometry (which additionally visually confirmed the viability of the cells under study). Suspensions were examined by dark-field and phase–contrast microscopy.

In some experiments, the aggregation medium was divided into two aliquots, one of which was supplemented with 10% FCS or 0.2–1 mg/ml heparin. Analysis of the GPI-linked isoform was also performed by allowing these cells to aggregate after phospholipase C treatment (see below). For these experiments, cell monolayers were incubated with 1 U/ml of the enzyme for 15 min at 37°C, washed in HBSS, dissociated, washed again, and resuspended in MEM for the adhesion assays as described above. Phospholipase C was then also included in all of the media changes before aggregation.

Adhesive Specificity of N-CAM Isoforms

The hypothesis that N-CAM–mediated adhesion involves homophilic binding was tested directly by coaggregation experiments using labeled and unlabeled cell suspensions using methods similar to those described previously (Pizzey and Jones, 1985; Pizzey et al., 1988). Briefly, two different cell suspensions were prepared for the adhesion assays as described above and were resuspended in MEM at a density of 2.5 × 10^5 cells/ml. To one suspension, 5 μl/ml 6-carboxyfluorescein diacetate (CFDA) in acetone was added (CFDA was obtained from Molecular Probes Inc., Junction City, OR); to the other, 0.005% acetone (control) was added. Cell suspensions were then incubated at 37°C for 15 min washed in HBSS at 4°C, resuspended in MEM, filtered and syringed to ensure a single cell suspension, and, finally, brought to a concentration of 10^6 cells/ml. Equal volumes of the cell suspension were then mixed together, and 1 ml of the mixed suspension was then incubated at 37°C for 30 min, as described above. Aliquots were then taken, and the distribution of labeled and unlabeled cells within 50 aggregates of the four-cell aggregate class was determined by phase–contrast and fluorescence microscopy, as described previously (Pizzey et al., 1988). In each individual experiment, 50 aggregates were measured.

Immunofluorescence Studies

N-CAM was visualized on the cell surface by indirect immunofluorescence

using techniques similar to those described earlier (Walsh et al., 1983) and using the human-specific anti-N-CAM mAb 5.1H11 (Walsh et al., 1989b). Cells were either incubated with mAb 5.1H11 (at a dilution of 1:100) as a monolayer or in suspension. In the latter case, cells or aggregates were incubated with mAb 5.1H11 for 45 min at 37°C in MEM, centrifuged at 400 g for 5 min, washed twice in HBSS, resuspended in MEM, and incubated with fluorescein-conjugated anti-mouse IgG. For detection of N-CAM before and after phosphatidylinositol-specific phospholipase C (PIPLC) (ICN Biochemicals Ltd., High Wycombe, UK) treatment, cells were washed in HBSS, dissociated in Versene, washed in HBSS once more, and resuspended in 100 μl MEM. Aliquots were then removed for immunofluorescence before adding the enzyme at a concentration of 1 U/ml. Remaining cells were then incubated for 30 min at 37°C before being washed and incubated with mAb 5.1H11 as described above.

Statistical Analyses

Values for the percentage aggregation for the parental and transfected cell lines were found to fit a Gaussian distribution, and, thus, these populations were compared using Student's t-test. For measurements of adhesive specificity, deviations from binomial distributions (which assume no adhesive specificity) were calculated using modifications (Pizzey and Jones, 1985) of the technique of Sieber and Roseman (1981). Briefly, in a sample (N) of aggregates of the same size, consisting of A and B cells, the variance (s^2) is given by (Σ $[A - \bar{A}]^2$)/$N - 1$), where A is the content of A cells and $\bar{A}$ is the mean content of A cells within the aggregates. If the relative frequencies of A and B cells are m and n, respectively, then the binomial distribution which assumes nonspecific (random) adhesion between the cells in, for example, the four-cell aggregate class is described by $P(A) = N(4!)/(A![4 - A]!)$ (m^A) (n^{1-A}) with a variance (σ^2) of $4 \times m \times n$. The deviation of the observed distribution from that calculated on the basis of no adhesive preference between the two cell populations can then be measured by a chi-square test: $\chi^2 = N \times s^2/\sigma^2$ with v ($v = N - 1$) degrees of freedom.

Results

N-CAM Expression in Transfected 3T3 Cells

We have previously shown that transfection into 3T3 cells of all four of the cDNAs used in this study results in the expression of high levels of immunoreactive N-CAM (Doherty et al., 1989). For all of the isoforms, except the secreted variant, this was determined by ELISA and Western blotting. The secreted species was not detectable by these analyses, but we have shown that, in contrast to the other transfectants, significant levels of N-CAM can be detected in media conditioned by cells transfected with a cDNA encoding this isoform (Gower et al., 1988). No N-CAM can be detected on parental 3T3 cells using mAb 5.1H11 (Doherty et al., 1989). The same clonal lines used by us in that earlier study (Doherty et al., 1989), and thus characterized for relative N-CAM expression, were used here.

For all of the assays described below, cells were dissociated in Versene (7 mM EDTA in PBS). This was performed since it is well documented in a range of cell types that such dissociation in the absence of trypsin will inactivate Ca^{2+}-dependent adhesive mechanisms, leaving Ca^{2+}-independent systems functionally active (Urushihara et al., 1979; Gibralter and Turner, 1985; Pizzey et al., 1988). Thus, any differences in adhesion due to the presence of N-CAM in the transfected cells should be maximal after this means of dissociation because of the inactivation of Ca^{2+}-dependent adhesion systems which, if present, may reduce or mask the
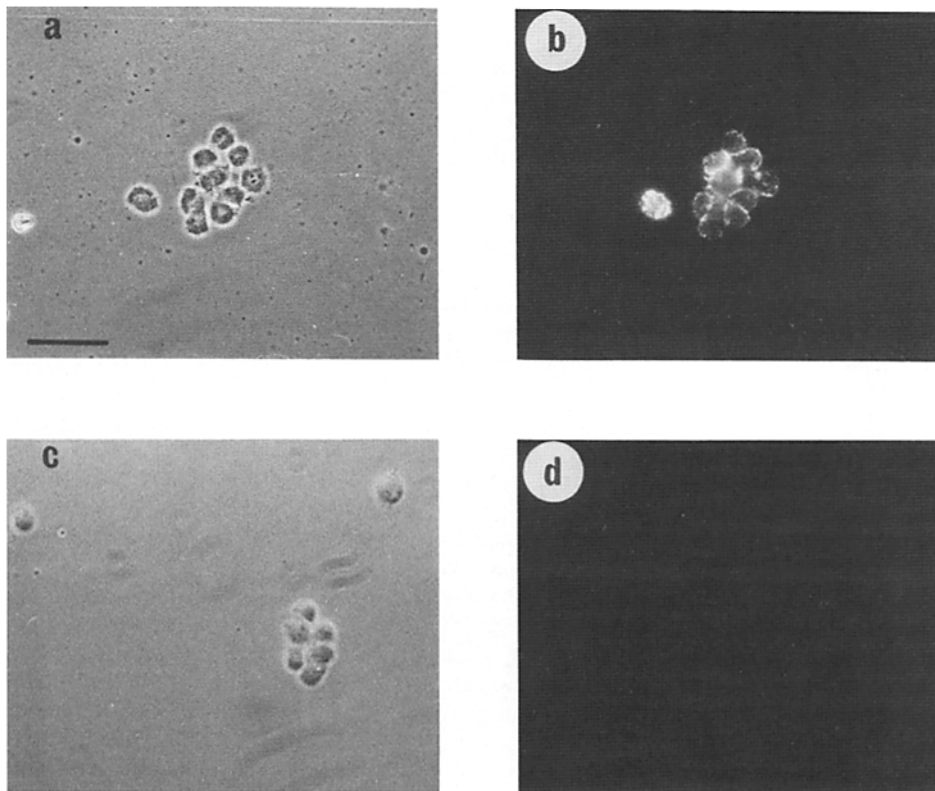

Figure 1. Staining of 3T3 fibroblasts transfected with a cDNA encoding the GPI-linked (with MSD1) N-CAM isoform (*a* and *b*) with the anti-human N-CAM antibody, mAb 5.1H11. Identical fields were viewed by phase-contrast (*a* and *c*) and epifluorescence (*b* and *d*) microscopy after allowing cells to aggregate in still media for 30 min. Parental cells (*c* and *d*) are negative for human N-CAM, while aggregates and single cells from the transfected population react strongly. Bar, 50 μm.

contribution made by N-CAM in subsequent intercellular adhesions. However, since all methods of dissociation of a cell monolayer are known to perturb the cell surface (Garrod and Nicol, 1981), it was important to determine that immunoreactive N-CAM remained there after dissociation, washing, and preparation for the assays described below. It was found that N-CAM could be detected in 3T3 cells transfected with cDNAs for all N-CAM isoforms except the secreted species when these cells were incubated with mAb 5.1H11 just before the adhesion assays; N-CAM reactivity was clearly seen to be localized to the cell membrane (not shown). After 30 min of aggregation (see below), N-CAM was still seen on cells within aggregates formed from GPI-linked N-CAM transfectants, and most reactivity was localized to the cell surface (Fig. 1, a and b). Aggregates and single cells from parental 3T3 populations showed no evidence of human N-CAM immunoreactivity (Fig. 1, c and d).

Adhesive Strength of N-CAM Transfectants

Transfected and parental cell populations were subjected to a variety of adhesion assays involving intercellular collisions between cells in suspension. In assays in which intercellular collisions were generated by rotational forces of 30–270 rpm, the aggregation kinetics of the two populations were similar (Fig. 2, a–c) for any individual assay.

Generally, the number of collisions between cells in suspension is proportional to the rotational speed imposed upon that suspension. However, as the speed increases, the shear forces present to disrupt new adhesions also become greater. Thus, it may be expected that only the effects of surface molecules that are initially involved in mediating relatively strong adhesive interactions will be detected by such assays using relatively high shear. For this reason, the technique of couette viscometry (Curtis, 1969) was additionally used to investigate the intercellular adhesive properties of the paren-
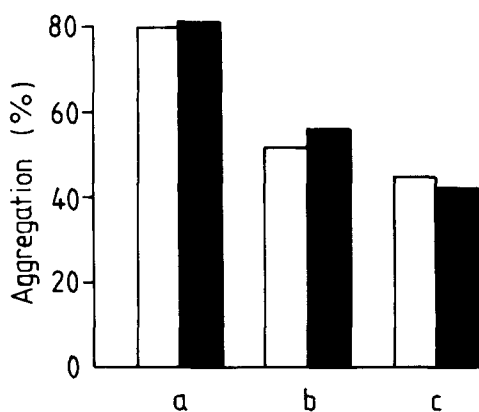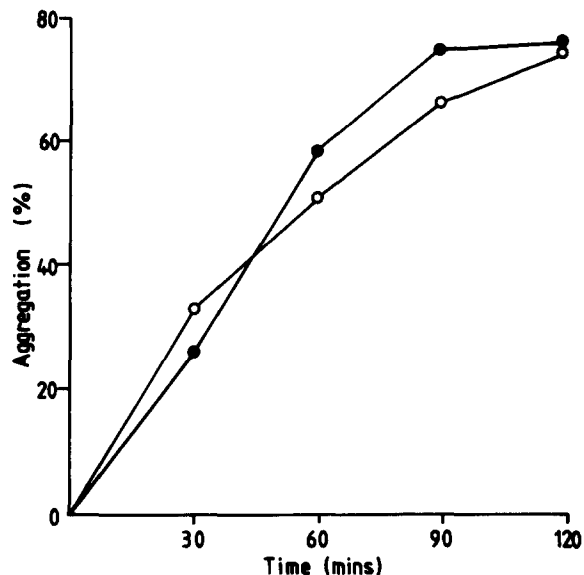

Figure 3. Aggregation kinetics of parental (○) and GPI-linked N-CAM transfectants (●) determined by couette viscometry. Although the initial rate of adhesion of the transfected cells appears greater than that of the parental population, the final values are similar. This technique also allows for calculations of collision efficiency (α), which is an index of intercellular adhesiveness. Values of α were higher for the transfected cells ($\alpha = 6.33 \pm 0.83$) than for parental 3T3 cells ($\alpha = 4.61 \pm 0.48$) from six determinations, but this also failed to reach significance ($P > 0.05$).

tal and transfected cell lines. This technique has recently been used by us to identify some of the requirements for Ca^{2+}-independent adhesion in a mouse myoblast cell line (Pizzey et al., 1988). However, although this technique generates very low laminar shear, it also failed to detect a consistent difference between the parental and transfected cell populations (Fig. 3). It should be noted here that all of these techniques allowed for the formation of stable, multicellular aggregates and that the kinetics of aggregate formation varied with the assay conditions. For example, using couette viscometry, 33% of parental 3T3 cells were recruited into aggregates within 30 min. This represented ~40% of the total adhesion seen with these cells in this system. However, after agitation at 30 rpm, 80% (~100% of the final value) were recruited into aggregates at the same point using these cells. Cell number was also found to affect aggregation kinetics, but, again, parental and transfected cells responded in a similar manner (data not shown).

These data suggested that, in these assay systems at least, N-CAM may be mediating initial, weak adhesions that are being disrupted by the assay conditions. To test this possibility, cell suspensions were subjected to no external shear forces and allowed to aggregate in still medium. Under these conditions, a preliminary study of six independent cultures indicated that the GPI-linked muscle N-CAM species conferred greater intercellular adhesiveness to 3T3 cells (data not shown). Thus, a full study of parental and N-CAM transfectants was carried out after aggregation in still medium, routinely measured after 30 and 60 min, as described below.

Adhesive Characteristics of N-CAM Isoforms

Parental 3T3 and 3T3 cells transfected with cDNAs encoding

Figure 2. Aggregation of parental (*open bars*) and GPI-linked N-CAM cDNA transfectants (*solid bars*) after 30 min of incubation. Cells were allowed to aggregate under different shear forces, generated by 30 (*a*), 90 (*b*), and 270 (*c*) rpm. Aggregation in this and subsequent figures is calculated as the percentage decrease in particle number with time, reflecting adhesion between cells as they are recruited into aggregates. Greatest adhesion was seen at 30 rpm (*a*), although in all cases similar rates of adhesion were seen between parental and transfected cells. Values are shown as means (in all cases, SEM ≤ 5% of the mean) from a total of 15 independent cultures.

Treatment	Transfectants	Aggregation	
		30 min	60 min
		%	%
None	Parental	11.8 ± 2.6	26.4 ± 1.3
	Sec	8.2 ± 3.6	14.2 ± 5.9
	GPI	27.9 ± 1.8*	42.6 ± 2.0*
	GPI (-MSD1)	21.8	ND
	TM	23.8 ± 1.7*	35.6 ± 1.7*
10% FCS	Parental	22.2	44.2
	GPI	38.2	59.4
0.2 mg/ml heparin	Parental	12.0 ± 1.3	25.2 ± 1.2
	GPI	12.0 ± 3.3	25.4 ± 2.8
0.5 mg/ml heparin	Parental	3.9	19.6
	GPI	0.0	17.9
1 mg/ml heparin	Parental	0.0	3.8
	GPI	0.0	9.5
PIPLC	GPI	15.2 ± 2.3‡	26.4 ± 2.6‡
	TM	28.5	40.2

Aggregation is expressed as the percentage of cells recruited into aggregates (percent decrease in cell number) after 30 and 60 min of incubation at 37°C in still medium. The N-CAM transfectants used were the transmembrane (*TM*), secreted (*Sec*), and GPI-linked, which can either include (*GPI*) or lack (*GPI [-MSD1]*) the MSD1 region. All values represent the mean ± SEM from three to nine cultures except where no SEM is indicated and the value instead represents the mean of duplicate determinations.
* Significantly different from parental 3T3 cells after equivalent period of aggregation in the absence of FCS; $P < 0.005$.
‡ Significantly different from GPI transfectants not treated with PIPLC; $P < 0.005$.

the transmembrane, GPI-linked (with and without MSD1), and secreted isoforms of N-CAM were introduced into the assay described above. The highly transformed mouse L cell line was shown to be very weakly adhesive in this assay; only 7.6% were recruited into aggregates by 30 min. In contrast, parental 3T3 cells aggregated much more rapidly (Table I). In a series of experiments involving independent cultures of cells transfected with the cDNA encoding the GPI-linked (with MSD1) N-CAM species, it was found that these cells aggregated significantly more rapidly than parental 3T3 cells (Figs. 4 and 5 and Table I).

The N-CAM transmembrane transfectants were also found to be significantly ($P < 0.005$) more adhesive then the parental cells (Figs. 4 and 5 and Table 1). This increase was slightly less than that seen in cells expressing the GPI-linked N-CAM species and may be related to quantitative, rather than qualitative, differences in N-CAM expression. Although clones were matched as nearly as possible for N-CAM expression, the clones expressing the transmembrane isoform of N-CAM expressed slightly lower levels based on ELISA data than those containing the GPI-linked species (Doherty et al., 1989). It is noteworthy that in that study it was found that the former species were also marginally less efficient in promoting neuronal differentiation.

In contrast to the ability of these two isoforms to significantly increase the adhesiveness of 3T3 cells, cells trans-

fected with the cDNA encoding the secreted isoform of N-CAM responded to our assay procedure in a similar manner to parental cells. No differences could be seen between the two populations either in the size or the number of aggregates formed (Figs. 4 and 5 and Table I).

The GPI-linked 125-kD N-CAM isoform was further studied by two approaches: first, the contribution of the MSD1 region of the protein to intercellular adhesiveness was investigated; second, removal of the entire N-CAM isoform from the surface of the transfected cells was performed, and the adhesive behavior of the cells was reanalyzed. For the first approach, a cDNA was generated that encodes the 125-kD muscle N-CAM species but from which the sequence encoding the MSD1 region had been deleted (Doherty et al., 1989). This reduces the relative molecular mass of the protein to 120 kD and renders it identical to one of the known brain N-CAM isoforms (Walsh, 1988; Thompson et al., 1989). Clonal cells transfected with this isoform exhibited a significant increase in intercellular adhesiveness compared with parental 3T3 cells, and this increase was indistinguishable from that shown by transfected cells expressing the same isoform but containing MSD1 (Fig. 5 and Table I). Thus, the MSD1 region, although it may modulate adhesive events in muscle in vivo, is not capable, on its own, of changing the adhesiveness of 3T3 fibroblasts within the limits of detection of the assay used here.

Second, to confirm the specificity of the increased adhesiveness of cells expressing the GPI-linked (with MSD1) N-CAM isoform, these, and cells expressing the transmembrane N-CAM variant, were treated with 1 U/ml PIPLC be-
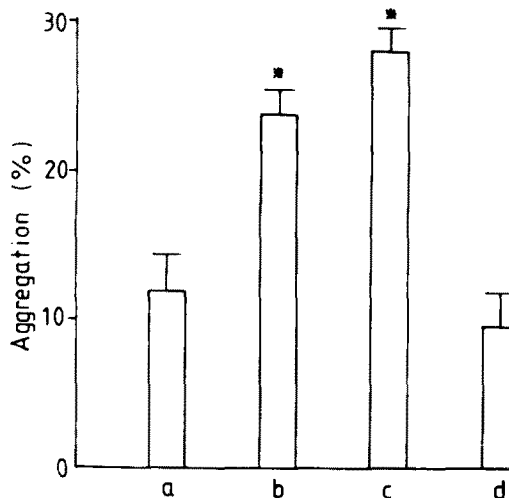

Figure 4. Mean values for the intercellular adhesion of parental (*a*) and 3T3 cells expressing variant N-CAM isoforms (*b–d*) after 30 min of incubation at 37°C. Parental 3T3 cells show significantly less aggregation in still medium than cells transfected with cDNAs encoding either the transmembrane (*b*) or GPI-linked (*c*) human muscle N-CAM isoforms. However, 3T3 cells expressing the secreted N-CAM species do not show this increase in adhesiveness (*d*). There is also no significant difference in the extent of aggregation between cells expressing transmembrane or GPI-linked N-CAM. For these experiments, >20 independent cultures were used. Further details of each distribution are given in Table I. Bars represent SEM. (*) Significantly different from parental cell distribution; $P < 0.005$.
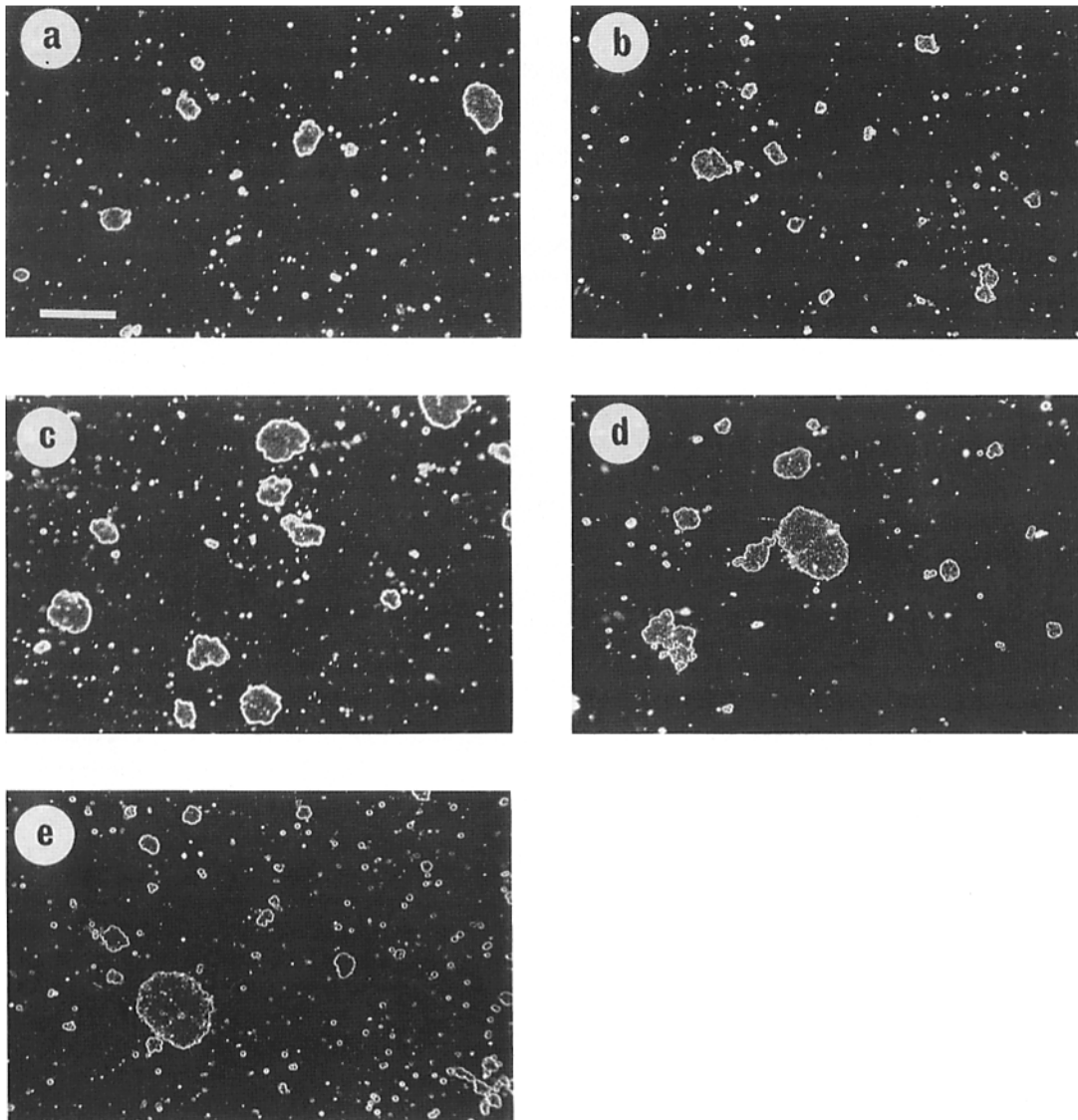
Figure 5. Aggregation of parental 3T3 cells and N-CAM transfectants after 30 min of aggregation. Although many of the parental cells (*a*) have aggregated by 30 min, the size of these aggregates is relatively small. A similar frequency of small aggregates is seen after aggregation of 3T3 cells transfected with a cDNA encoding a secreted form of N-CAM (*b*). In contrast, the number and size of aggregates is much larger after aggregation of 3T3 cells expressing either the GPI-linked (*c*) or transmembrane (*d*) N-CAM muscle isoforms. A similar increase in cell adhesiveness is seen in populations of cells expressing the GPI-linked isoform that lacks the MSD1 region (*e*). Low magnification fields were viewed by dark-field microscopy. Bar, 380 μm.

fore being allowed to aggregate. After enzyme incubation for 15 min at 37°C, which had no detectable effect on cell viability, immunoreactive N-CAM was removed completely from the surfaces of the former cell population. Levels of N-CAM on transfected cells expressing the transmembrane isoform appeared unchanged by this treatment (see below). When both populations of such phospholipase C–treated cells were then allowed to aggregate for 30 min, those containing the transmembrane isoform aggregated at the same rate as untreated cells. In contrast, the rate of adhesion of the GPI-linked N-CAM transfectants fell markedly and was indistinguishable from that of the parental 3T3 cells (Table I). Incubating aggregates with mAb 5.1H11 after PIPLC treatment confirmed that N-CAM had not been replaced at the surface of the GPI-linked transfectant and that levels of sur-

face N-CAM on the transmembrane transfectants were unaffected by the enzyme (Fig. 6). In this latter case, it can also be seen that the most intense reactivity was frequently seen on apposing membranes of cells within the aggregates. This pattern is similar to that previously described by Covault and Sanes (1986) who noted a similar pattern of N-CAM distribution in vivo in developing myotube clusters in 14-d embryonic chicken muscle. The specificity of the enzyme and the lack of its effect on adhesion in cells expressing the transmembrane N-CAM species demonstrates that the reduced adhesiveness of phospholipase C–treated GPI-linked N-CAM transfectants was neither due to a general loss of all phosphatidylinositol-linked membrane proteins nor to nonspecific proteolysis at the cell surface.
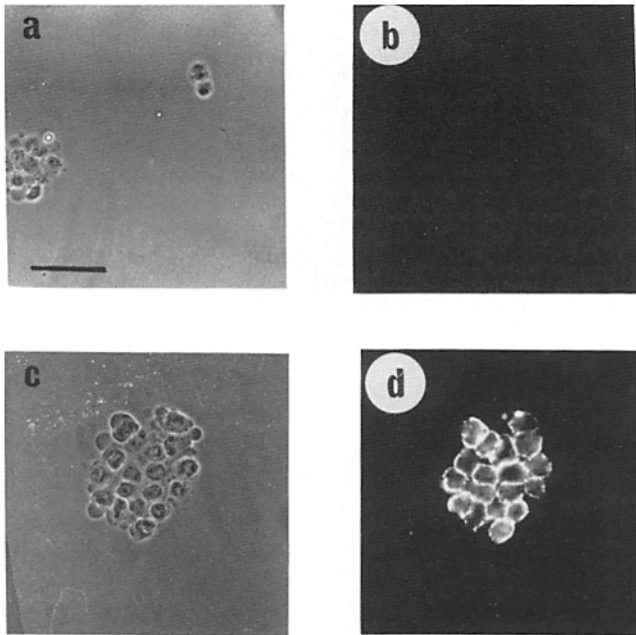
Figure 6. Distribution of human muscle N-CAM in aggregates of transfected 3T3 cells after incubation with PIPLC. Cells were treated with the enzyme before being allowed to aggregate for 30 min, and identical fields were viewed by phase–contrast (*a* and *c*) and epifluorescence (*b* and *d*) microscopy. The size and number of aggregates formed from PIPLC-treated GPI-linked N-CAM transfectants are smaller than those seen in untreated cultures (see text) and also lack immunoreactive N-CAM at their surface (*b*). In contrast, the extent of aggregate formation of transmembrane N-CAM transfectants is unaffected by the enzyme. The cells in these aggregates are seen to express high levels of N-CAM at their surface, particularly on apposing cell membranes (*c* and *d*). Bar, 50 μm.

Perturbation of Adhesion in Parental 3T3 Cells and N-CAM Transfectants

During morphogenesis, a wide range of adhesive systems are operative, and it is common for adhesive mechanisms to be modulated by a variety of secondary factors and other adhesion systems (see Discussion). To investigate such mediation in our assay system, we introduced two reagents that are candidates for mediating N-CAM–associated intercellular adhesion. First, it has been reported that N-CAM contains a domain involved in the substratum binding of heparan sulfate (Cole and Glaser, 1986). To determine whether the presence of exogenous heparin could block N-CAM–mediated intercellular adhesion, N-CAM transfectants and parental cells were allowed to aggregate in the presence of 0.2–1 mg/ml heparin for up to 60 min. In a series of experiments involving determinations from cultures using parental 3T3 cells and those expressing the GPI-linked N-CAM isoform, it was found that the inclusion of 0.2 mg/ml heparin in the aggregation medium reduced the adhesiveness of the latter cells to values equivalent to those of the untreated parental cell suspension (Figs. 7 and 8). The levels of aggregation seen in the parental cell suspension were unaffected by this concentration of heparin. Thus, although nearly 50% of the N-CAM transfectants were recruited into aggregates in heparin-free media by 60 min, this value was reduced to ~75% in the presence of 0.2 mg/ml heparin (Fig. 7 *a*). At higher concentrations of heparin, intercellular adhesion of both parental

and N-CAM–transfected 3T3 cells was reduced such that 20% of cells in the original suspension were recruited into aggregates by 60 min in the presence of 0.5 mg/ml heparin (Fig. 7 *b*). When the concentration of heparin was increased to 1 mg/ml, this value fell further to ~10% for both cell populations (Fig. 7 *c*). This indicates that, at the lowest concentration used here, heparin is capable of specifically blocking N-CAM–mediated intercellular adhesion. At higher concentrations, other adhesion-promoting heparin-binding sites on the surface of 3T3 cells may be blocked, and this effect masks that of the specific contribution of N-CAM to the promotion of intercellular adhesion. This finding represents the first report that heparin can directly block N-CAM–mediated intercellular adhesion of cells in suspension.

Aggregation assays were also performed in the presence of 10% FCS. This greatly increased the rate of cell aggregation and aggregate size compared with cells incubated in serum-free media (Figs. 4 and 9). However, this increase in adhesion was similar for both parental and transfected cells, thus maintaining the relative difference between the two cell populations; the number of particles in the parental cell suspension decreased to 78% by 30 min and to 62% for cells expressing the GPI-linked N-CAM isoform (Fig. 9). Because of absorption of FCS at the cell surface possibly masking adhesive interactions due to N-CAM alone, FCS was not routinely included in the aggregation media in comparison of the adhesive behavior of individual N-CAM isoform transfectants or the specificity assays described below.

Adhesive Specificity of N-CAM Isoforms

Specificity of adhesion between N-CAM isoforms was measured by coaggregating two populations of cells, one of which was previously labeled with CFDA (Fig. 10). The distribution of cells within aggregates of the four-cell class were
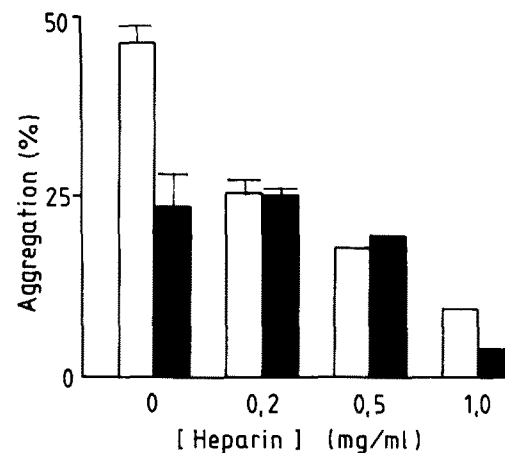

Figure 7. Effect of heparin on the intercellular adhesion of parental 3T3 cells (*solid bars*) and 3T3 cells expressing the muscle GPI-linked N-CAM isoform (*open bars*). Cells were allowed to aggregate for 60 min and in the absence or presence of exogenous heparin. In the absence of heparin, the characteristic increase in the adhesiveness of GPI-linked N-CAM is seen, although this is inhibited by the introduction of 0.2 mg/ml heparin. At higher heparin concentrations, reduced adhesion of both parental and transfected cells is seen. Values represent means and SEM. Where no SEM is indicated, the mean represents four determinations from two independent experiments.
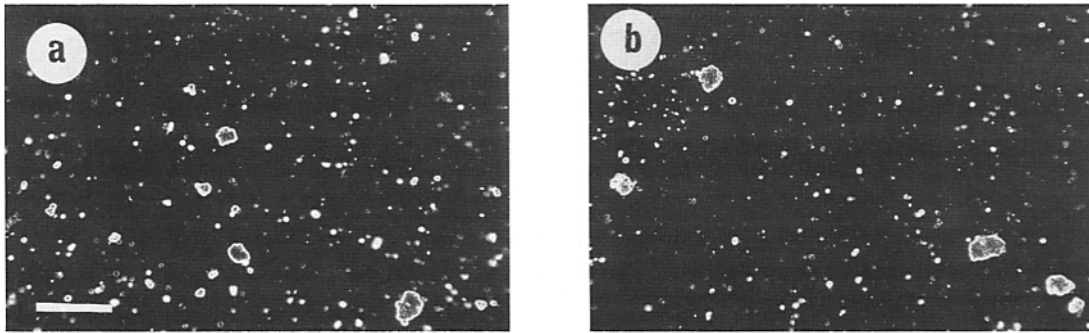
Figure 8. Aggregation of GPI-linked N-CAM transfectants (*a*) and parental 3T3 cells (*b*) in the presence of 0.2 mg/ml heparin. In both cases, only a few, small aggregates are seen. Compare this with Fig. 5, *a* and *c*. Dark-field microscopy. Bar, 380 μm.

then compared with distributions that assume random adhesion. No morphological differences in the aggregates were seen between those formed from any pairs of cell suspensions under study. Furthermore, we have previously shown that the concentration of CFDA or acetone (control) used here is without significant effect on 3T3 cell viability after incubation at 37°C for 30 min (Pizzey et al., 1988).

The distributions of cells within aggregates formed from a mixture of cells expressing either the transmembrane or GPI-linked N-CAM isoforms were not significantly different from binomial distributions calculated on the basis of no adhesive preference (Fig. 11). This suggests that, in this assay at least, these two N-CAM species have similar specificities for heterotypic and homotypic binding. However, it was also possible that although the assay is sensitive to quantitative changes in adhesion mediated by N-CAM (Fig. 4 and Table I) it is not able to detect qualitative differences between individual isoforms. This was tested directly by a further series of experiments; transfected cells expressing the GPI-linked N-CAM isoforms were allowed to coaggregate either with those expressing the secreted form or with parental cells. In both cases, partial specificity was observed; the observed distributions of cells within aggregates was significantly skewed from that predicted on the basis of no adhesive preference between the cell populations (Fig. 11). In

neither case was specificity complete: i.e., bimodal distributions were not seen and this probably reflects the existence of other functional adhesive systems which are present on parental 3T3 cells. When parental 3T3 cells were coaggregated with transfected cells expressing the secreted species of N-CAM, these populations were found to aggregate randomly (Fig. 11). Taken together, these data strongly suggest that binding to the GPI-linked or transmembrane N-CAM isoform on one cell with either molecule on another cell is responsible for the distributions observed and that, in terms of N-CAM adhesive specificity at the cell surface, the behavior of 3T3 cells transfected with a cDNA encoding the secreted N-CAM variant is indistinguishable from that of the parental cell population.

Discussion

N-CAM is known or suspected to mediate a wide range of events during embryogenesis and development of the neuromuscular system. These processes include intercellular adhesion between neuronal and astrocyte populations (Rutishauser et al., 1976; 1978; Keilhauer et al., 1985), muscle innervation by motor neurons (Grumet et al., 1982; Covault and Sanes, 1986; Tosney et al., 1986), axonal growth (Bixby et al., 1987), and control of the synthesis of some of the en-
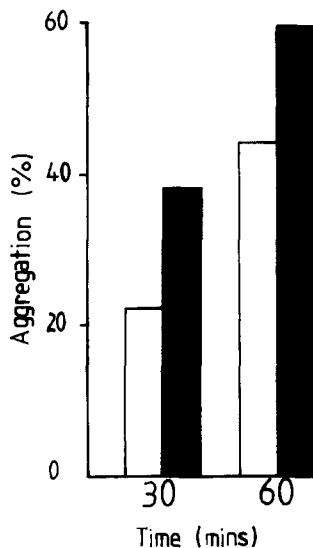

Figure 9. Mean aggregation of parental (*open bars*) and GPI-linked N-CAM transfectants (*solid bars*) in the presence of 10% FCS. Aggregation rate and aggregate size are increased by the presence of FCS compared with cells incubated in serum-free media. However, the relative difference between the two populations is unchanged (see Fig. 4). Similar results were seen with transfectants expressing a transmembrane N-CAM isoform. Values represent the mean of two experiments.
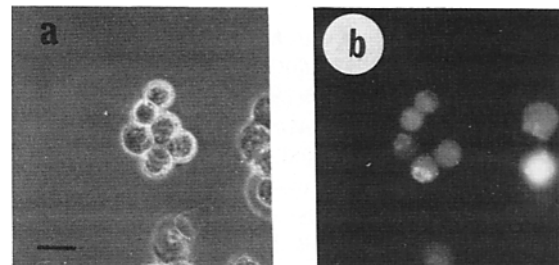

Figure 10. Coaggregation of parental and GPI-linked N-CAM transfectants. In the example shown, transfected cells were labeled with CFDA and allowed to coaggregate with unlabeled parental cells. Both cell populations cooperate in aggregate formation, and no evidence of the transfer of CFDA to unlabeled cells was seen. In the figure, a six-cell aggregate can clearly be seen to contain one parental and five transfected cells. Bar, 17 μm.
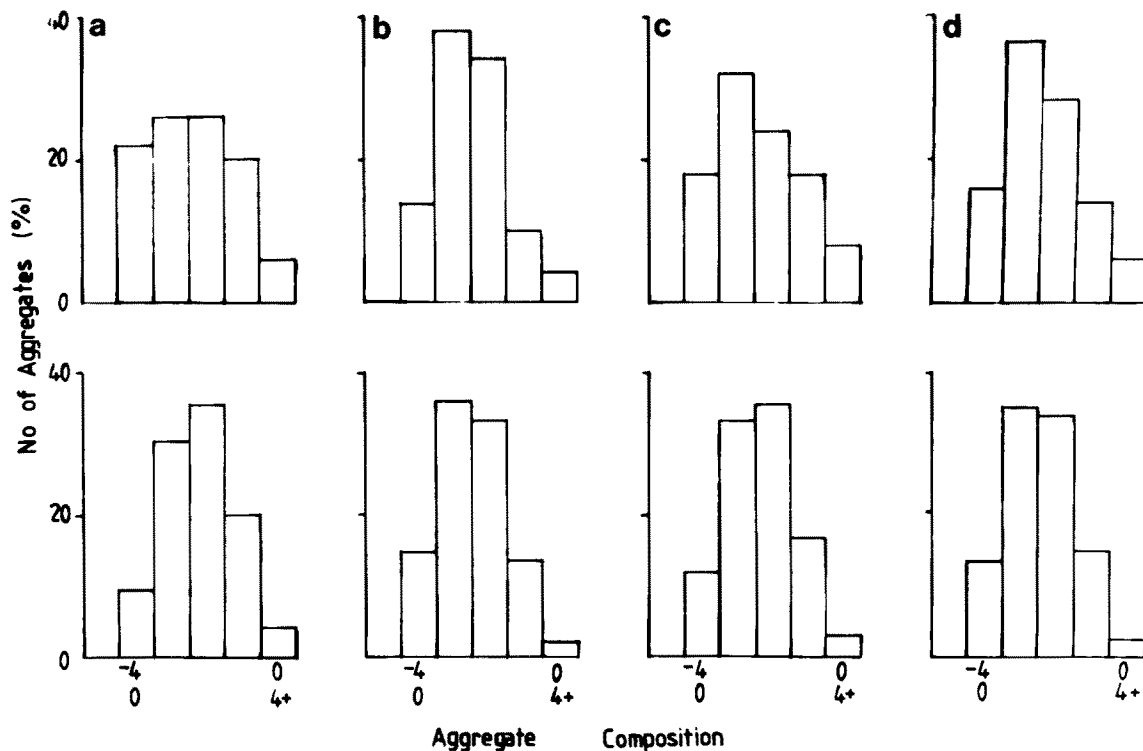
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Figure 11. Analysis of the four-cell aggregate composition from mixed suspensions of parental cells and N-CAM transfectants. Upper panels represent observed distributions, and below are shown the equivalent binomial distributions predicted on the basis of random (nonspecific) adhesion between the two cell suspensions. In each histogram, the first bin represents the class of four-cell aggregates consisting of only unlabeled cells. The second bin represents those consisting of one CFDA-labeled and three unlabeled cells, and so on. In *a*, parental cells and GPI-linked N-CAM transfectants were allowed to coaggregate. In this case, the observed distribution is significantly different ($P < 0.05$) from that predicted on the basis of no adhesive specificity. In contrast, no differences were seen between observed and predicted distributions from mixtures of GPI-linked and transmembrane N-CAM transfectants (*b*) or secreted N-CAM transfectants and parental cells (*d*). However, coaggregation of secreted N-CAM and GPI-linked transfectants (*c*) exhibit partial adhesive specificity ($P < 0.05$). For each experiment, $v \geqslant 49$.

zymes involved in neuromuscular transmission (Acheson and Rutishauser, 1988).

A major problem in studies of morphogenesis and development concerns the mechanisms by which molecules such as N-CAM can perform such diverse functions. Major N-CAM diversity is due to alternatively spliced N-CAM mRNA which generates transmembrane, GPI-linked, and secreted forms (for reviews see Nybroe et al., 1988; Walsh, 1988). Further subdivisions of these species are introduced by mRNAs that encode transmembrane forms that differ in the length of their cytoplasmic domains (Murray et al., 1986) and by the exclusion of some exon groups from N-CAM in specific tissues; we have recently shown that a previously undetected four-exon block (MSD1) is inserted into the GPI-linked and secreted N-CAM variants in skeletal muscle (Dickson et al., 1987; Thompson et al., 1989). Further variation in N-CAM diversity is introduced by differential processing of the polypeptide, such as in the extent of N-CAM sialylation, and this is known to affect the adhesiveness of the molecule (Sadoul et al., 1983; Rutishauser et al., 1988).

An approach we (Doherty et al., 1989) and others (Edelman et al., 1987) have used to investigate the functions of individual N-CAM isoforms has been to transfect cells with full-length cDNAs that encode different N-CAM species. In this way, we have recently carried out a study on the ability

of a range of human N-CAM isoforms to promote neuronal differentiation (Doherty et al., 1989). Edelman et al. (1987) have also shown by transfection studies that one of the known chick brain transmembrane isoforms can enhance the ability of L cells to bind chick brain vesicles and that another form can increase the intercellular adhesiveness of L cells.

In developing the assay for identifying differences in intercellular adhesion due to the presence of N-CAM, we found that, although a trend for increased adhesiveness of GPI-linked and transmembrane N-CAM transfectants could be seen under many conditions, only when dissociated cells were allowed to aggregate in still medium was a consistent difference seen between these transfected and parental cell populations. This suggests that N-CAM may be mediating early, weak adhesions rather than secondary processes that strengthen initial adhesive mechanisms (Garrod and Nicol, 1981). However, it should be noted that it cannot be excluded that secondary factors may augment the native adhesiveness of N-CAM in cells of neural or myogenic origin.

The transmembrane and GPI-linked muscle N-CAM isoforms are differentially regulated during myogenesis; the transmembrane isoform is abundant in myoblasts, but this is replaced by the GPI-linked species which becomes the major N-CAM variant in myotubes (Moore et al., 1987). Thus, it may have been expected that if the transmembrane form

is involved in mediating myoblast fusion and muscle GPI-linked N-CAM has a role in later events in myogenesis, then the former species may exhibit a greater capacity for the promotion of intercellular adhesion. However, no significant difference was found between the increased adhesiveness conferred by either of these two isoforms. Therefore, either the qualitative changes in N-CAM expression during early myogenesis are not directly related to changes in relative adhesive strength between these two isoforms or such differences require other factors present in myogenic cells for physiological significance. It is important to note here that although great homology in the extracellular region exists between these two isoforms (Nybroe et al., 1985; Rougon and Marshak, 1986) that is not predictive per se of similarities in adhesive behavior. For example, in addition to different modes of N-CAM membrane attachment possibly conferring different adhesive properties to the molecule, it has also been shown that for the liver cell adhesion molecule, which is the chicken homologue of mouse E-cadherin (Ogou et al., 1983), a region of the cytoplasmic domain of the polypeptide is required for its normal adhesive properties (Nagafuchi and Takeichi, 1988).

The third major N-CAM species analyzed in the present study was the secreted isoform of N-CAM in human muscle (Gower et al., 1988). Consistent with our earlier study of neuronal differentiation using N-CAM transfectants (Doherty et al., 1989), it was found that expression of the secreted form of N-CAM in 3T3 cells was without effect on adhesion. The physiological consequences of N-CAM secretion are as yet undefined. This species may modulate intercellular adhesion in vivo, mediate other nerve–muscle interactions, or function as a component of the extracellular matrix. To elucidate the role of secreted N-CAM in vivo, it is necessary first to define its target and temporal distribution. Studies are therefore currently being performed using antibodies specific to the predicted amino acid sequence of this isoform.

The sensitivity of the aggregation assay used here allowed us to investigate such subtleties as the contribution of the MSD1 sequence in N-CAM–mediated adhesion. This region may be a candidate for modulating adhesive interactions during myogenesis since, in addition to showing structural similarities to the contact site A protein of *Dictyostelium*, the hinge region of immunoglobulins (Walsh, 1988), it is also a site of O-linked oligosaccharide attachment (Walsh et al., 1989a). This may further affect the three-dimensional structure of the molecule with subsequent modification of its adhesive properties. However, we were unable to detect any difference between adhesiveness of cells expressing the N-CAM GPI-linked polypeptide with, or without, MSD1 present. This may be because (a) this region is not primarily involved in early adhesive interactions in vivo (the spatial and temporal distribution of MSD1 expression demonstrates that it is not required for myoblast fusion), (b) functional expression of MSD1 may require additional factors that are present in myotubes (or other cells) but not in the fibroblast background of the transfectants, or (c) although MSD1 may quantitatively modulate intercellular adhesion, its effects are extremely subtle and beyond the sensitivity of the assay used here.

The presence of a heparin-binding domain of N-CAM in-volved in cell–substratum adhesion has recently been established (Cole and Glaser, 1986). Although the functional significance of this region is unknown, it is possible that this region allows for interactions between heparan sulfate in the extracellular matrix and N-CAM. If so, N-CAM–substratum adhesion may be similar to that of fibronectin-mediated adhesion in fibroblasts in which fibronectin has been shown to bind to the extracellular matrix by both protein–protein and protein–glycosaminoglycan interactions (Laterra et al., 1982). Alternatively (or in addition), such interactions may involve N-CAM binding to the basal lamina, which is also abundant in heparin. We have now extended the earlier observations of neural retina cells binding to N-CAM–coated substrata (Cole et al., 1985; Cole and Glaser, 1986) to the intercellular level. By showing that exogenous heparin can inhibit adhesion (which is specifically mediated by N-CAM) between whole cell populations should facilitate the design of strategies to elucidate the nature and function of interactions between N-CAM and heparin in vivo. The nonspecific inhibition of adhesion in both parental and N-CAM–transfected cells is probably due to saturation of heparin-binding sites, which are common to both cell surfaces. For example, Laterra and Culp (1982) have shown that 50% of total 3T3 cell surface heparan sulfate binds to both plasma and cellular fibronectin. Such adhesions also appear to be Ca^{2+} independent (Laterra et al., 1983).

However, although N-CAM can bind heparin, most data on the binding properties of N-CAM have concerned homophilic N-CAM binding based on antibody perturbation studies (Edelman, 1986). The generation of transfectants that express specific isoforms of N-CAM now allows for direct studies of homotypic adhesion by measurements of adhesive specificity and also for investigations into possible differences in specificity between individual isoforms. By using a quantitative approach to measure specific (nonrandom) adhesions, we have shown that partial adhesive specificity exists between populations of parental 3T3 cells and those of 3T3 cells transfected with cDNAs encoding the GPI-linked or transmembrane (but not the secreted) human muscle N-CAM species. The findings that such adhesive specificity is partial is probably due to the presence of other active Ca^{2+}-independent adhesive mechanisms, such as fibronectin–glycosaminoglycan binding as mentioned above, present on both parental and transfected cells. Coaggregation of cells expressing the GPI-linked and transmembrane N-CAM species generated populations of aggregates in which no specificity (total or partial) could be detected. It should be noted that these analyses of specificity relate to the early recognition/adhesion interactions between parental cells and N-CAM transfectants. The process of cell sorting and cell spreading within multicellular aggregates occurs over a much longer time period and represents a model for different histogenetic and developmental processes (Garrod and Nicol, 1981). At present, a separate series of experiments are being conducted to assess the possibility of such sorting of cells expressing different N-CAM variants, similar to those recently performed on cells expressing different species of the cadherin family (Nose et al., 1988).

cular Dystrophy Group of Great Britain, and the Motor Neuron Disease Association. F. S. Walsh is a Wellcome Trust Senior Lecturer.

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