Neuronal Process Outgrowth of Human Sensory Neurons on Monolayers of Cells Transfected with cDNAs for Five Human N-CAM Isoforms

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Abstract. Full length cDNAs for a variety of human N-CAM isoforms have been transfected into mouse L-cells and/or 3T3 cells. Three independent clones of each cell line that were shown to express human N-CAM were tested for their ability to support the morphological differentiation of sensory neurons. The cell surface expression of N-CAM isoforms, linked to the membrane directly by an integral transmembrane spanning domain or indirectly via covalent attachment to a glycosyl-phosphatidylinositol moiety, were consistently found to be associated with a significant increase in the morphological differentiation of both human and rat dorsal root ganglion neurons. Modification of the extracellular structure of both classes of N-CAM, consequent to the expression of a glyco-sylated 37–amino acid sequence normally found expressed exclusively in muscle N-CAM isoforms did not obviously affect the ability of transfected cells to support increased neuronal differentiation. 3T3 cells that were transfected with a full length cDNA encoding a secreted N-CAM isoform, and that have previously been shown to secrete N-CAM into the growth media rather than link it to the membrane did not significantly differ from control cells in their ability to support neuronal differentiation. These data provide direct evidence for both transmembrane and lipid-linked N-CAM isoforms being components of the regulatory machinery that determines neuronal morphology and process outgrowth.

ONE of the most intriguing problems in molecular embryology is an understanding of the mechanisms that control the generation of the large numbers of neuronal cell phenotypes, characterized by a complexity and diversity of neuronal morphology, that are found in the vertebrate nervous system. The cytoskeleton has long been recognized as the major determinant of cell shape, and cells of both neuronal and nonneuronal origin are believed to use similar motility based mechanisms to assemble this structure during morphogenesis (Letourneau, 1982; Smith, 1988; Mitchison and Kirschner, 1988). Factors present in the microenvironment have been postulated to control neuronal shape via interactions with the neuronal growth cone, the motile structure of the axon. Modulatory factors may include soluble neurotrophic and neurotropic factors such as nerve growth factor as well as cell adhesion molecules and substrate associated adhesion molecules (Edelman and Thiery, 1985; Dodd and Jessell, 1988; Edelman, 1988).

Antibody perturbation experiments have identified the neural cell adhesion molecule (N-CAM)[1] as a homophilic recognition molecule that can mediate cell–cell adhesion between many different cell types (Edelman, 1986; Cunningham et al., 1987; Rutishauser et al., 1988). N-CAM function has been implicated in a wide variety of events that depend on cell–cell adhesion including the induction of enzymes for neurotransmitter synthesis (Rutishauser et al., 1988; Acheson and Rutishauser, 1988) and the growth of axons over monolayers of several nonneuronal cells (Bixby et al., 1987; Neugebauer et al., 1988; Tomaselli et al., 1988).

It is only recently that a description of the molecular complexity of N-CAM has been established (Nybroe et al., 1988). Antibody reagents and molecular probes have been used to demonstrate that alternative splicing of a single N-CAM gene can generate up to four main classes of N-CAM polypeptides, with differences in the length of their COOH-terminal domains accounting for different modes of association with the cell (Cunningham et al., 1987; Nybroe et al., 1988). Whereas all four classes in general share a common extracellular NH_2-terminal binding domain of ~500 amino acids that shows sequence homology with members of the immunoglobulin superfamily (Williams and Barclay, 1988), two classes have transmembrane domains that differ in the size of their intracellular COOH-terminal tails (large domain [ld] and small domain [sd]). One class has a small surface domain (ssd) that associates the polypeptide with the cell mem-

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1. *Abbreviations used in this paper:* DRG, dorsal root ganglia; ld, large domain; N-CAM, neural cell adhesion molecule; sd, small domain; ssd, small surface domain.

brane via covalent linkage to a glycosyl-phosphatidylinositol anchor, and the final class is encoded by a mRNA that has a stop codon inserted between the coding region for the NH₂-terminal binding domain and that of the membrane-associated domains, with considerable evidence suggesting that this represents a secreted class of N-CAM (Gower et al., 1988). In addition to these four main size classes, subtle differences have also been identified in the primary structures of the extracellular domains, and in particular muscle has been shown to express mRNA species with three additional exons encoding for a 37–amino acid block (termed the MSD1 region) that is inserted between the NH₂-terminal binding domain and the membrane associated domain of the ssd and secreted isoforms (Dickson et al., 1987; Walsh, 1988; Thompson et al., 1989). More recently, an 18-nucleotide exon that corresponds to the first of these three exons identified in muscle has been shown to be alternatively spliced in mouse brain (Santoni et al., 1989; Thompson et al., 1989). Whereas the evolutionary advantages of the generation of a large family of protein isoforms from a single gene via alternative splicing mechanisms has been the subject of considerable discussion (Breitbart et al., 1987), the direct functional consequences of such diversity in the primary structure of a single cell adhesion molecule remains to be determined.

In the present study we have examined the outgrowth of human and rat sensory neurons over monolayer cultures of 3T3 and L cells transfected with cDNAs encoding up to five distinct N-CAM isoforms, specifically the sd and ssd isoforms plus and minus the MSD1 region, and a secreted isoform that also contained the MSD1 region. To date proteins corresponding to four of these isoforms have been identified in muscle or brain (Nybroe et al., 1988; Moore et al., 1987; Gower et al., 1988). The results show that neuronal differentiation as indexed by expression of immunoreactive neurofilament protein is significantly increased on cell monolayers that express both the sd and ssd cell surface–associated N-CAM isoforms. In contrast differentiation is not obviously affected when neurons are grown on monolayers of cells expressing the secreted N-CAM isoform. These results support the proposal that N-CAM can promote neuronal process outgrowth, and show for the first time that this function can be studied at the level of expression of individual isoforms of this CAM.

Materials and Methods

Construction of Full-Length N-CAM cDNAs

Full-length cDNA constructs were produced from cDNA clones previously described (Dickson et al., 1987; Barton et al., 1988; Gower et al., 1988) to the three major human N-CAMs described to date in muscle, ssd + MSD1, sd, and secreted isoforms according to the nomenclature of Cunningham et al. (1987) and Gower et al. (1988). Two other isoforms (sd + MSD1 and ssd) were generated by deleting or adding the previously described MSD1 region to the above clones (Gower et al., 1988; Walsh et al., 1989). All constructs were checked for correct orientation and maintenance of reading frame by restriction site mapping and by in vitro translation of RNA synthesized using SP6 RNA polymerase. The five full-length coding sequences were used to express N-CAMs after transfection into L cells and/or 3T3 cells under the control of the β-actin promoter (Gunning et al., 1987) in pH β Apr-1-neo as previously described (Gower et al., 1988).

Cell Culture

Both control and transfected clones of 3T3 cells and L cells were routinely

grown on culture dishes in DME containing 10% FCS (DME/10% FCS). Monolayers for co-cultures were established by seeding 3T3 cells at 500 cells/mm² and L cells at 250 cells/mm² both in 96-well microtitre plates (Nunclon®) and eight chamber tissue culture slides (Lab-Tek®). For the 3T3 cells, the wells were coated with Vitrogen 100® (purified collagen from the Collagen Corp., Palo Alto, CA) immediately before use. Monolayers of 3T3 cells were maintained for 24–48 h and L cells for 3 d before addition of neurons.

Dorsal root ganglia (DRG) were dissected from human fetal spinal cords of approximately 8–13 wk gestation and dissociated as previously described (Doherty and Walsh, 1988). Briefly, for each preparation 12–20 DRGs were incubated for 30 min in Versene solution (Gibco Laboratories, Grand Island, NY) containing 0.05% trypsin and 0.05% DNAse I (wt/vol). The DRG were washed and resuspended in 7–10 ml SATO media (see Doherty et al., 1987) for 3T3 co-cultures or DME/10% FCS for L cell co-cultures and dissociated by gentle trituration. The dissociate was passed over a 35 μm filter and the single cell suspension taken to the equivalent of 1 DRG/ml in the appropriate media which also contained NGF at 50 ng/ml. Co-cultures were established by seeding 100 and 200 μl of the respective dissociates onto monolayers of 3T3 cells and L cells established on 96-well plates or eight-chamber slides. The final media composition for the co-cultures was DME, 5% FCS, 50% SATO, and 25 ng/ml NGF for 3T3 monolayers, and DME, 10% FCS, and 25 ng/ml NGF for L cell monolayers.

We have previously shown that all cells exhibiting a neuronal morphology in cultures of human DRG stain for neurofilament protein (Doherty and Walsh, 1988). To determine the numbers of neurons seeded onto the monolayers, the cell dissociate was seeded into collagen-coated chambers at the same density. In six representative experiments the average neuronal density was 22 ± 16 neurons/mm² (mean ± SD, n = 6). This corresponds to ∼900 neurons per microwell. When cultures were scored for process outgrowth at 24 h, ∼70–80% of the cells had extended neurites of more than two cell diameters.

In three representative experiments co-cultures of human neurons on monolayers of control and transfected 3T3 cells were fixed and double stained with antibodies to neurofilament (see below) and the Hoechst 33258 dye. The pattern of nuclear staining with the latter can be used to distinguish between human and rodent cells. In this series of experiments no evidence was found for the incorporation of human nonneuronal cells into the cellular monolayers, and neuronal growth cones were always found in association with control and transfected 3T3 cells.

In two experiments reported in the present study, DRG from embryonic day 5 rats were dissociated as above and seeded onto monolayers of control and transfected 3T3 cells at a density of 0.5 DRG per microwell.

Immunoprecipitation and Western Blotting of N-CAM Polypeptides

Immunoprecipitation of N-CAM polypeptides from NP-40 extracts of control and transfected 3T3 cells and growth media from cells transfected with the secreted N-CAM isoform was carried out essentially as previously described, with the exception that a mouse mAb to N-CAM was used rather than a rabbit anti–mouse muscle N-CAM antibody (Moore et al., 1987; Gower et al., 1988). After immunoprecipitation, samples were resuspended in 25 mM acetate buffer, pH 5.0, with 0.1 U neuraminidase (Type X; Sigma Chemical Co., St. Louis, MO) incubated at 37°C for 1 h, boiled in SDS sample buffer, and analyzed by SDS-PAGE and Western blotting as previously described (Moore et al., 1987; Gower et al., 1988).

Determination of the Relative Expression of N-CAM

The relative level of N-CAM expression was determined by use of an enzyme-linked immunoabsorbent assay as previously described (Doherty et al., 1988). Briefly, 20,000 cells were plated in individual wells of a collagen-coated microtiter plate for 18 h. Culture media was carefully removed and the cells were fixed by treatment with methanol at −20°C for 10–15 min. Cultures were washed and incubated with 0.5% gelatin in PBS for 60 min to block nonspecific protein binding sites. Cultures were then incubated for 60 min at 20°C with a 1:500 dilution of up to three individual anti–N-CAM mAbs. Cultures were washed three times with PBS containing 1% (wt/vol) BSA, and incubated for a further 60 min with a 1:1,000 dilution of horseradish peroxidase–conjugated F(ab')₂ fraction of anti–mouse IgG (Sigma Chemical Co.). Cultures were washed four times with PBS and twice with distilled H₂O, and finally incubated with 50 μl of 0.2% (wt/vol) O-phenylenediamine and 0.02% (vol/vol) H₂O₂ in citrate buffer. After 10–30 min the conversion of O-phenylenediamine to its oxidized product was stopped

by the addition of 50 μl H_2SO_4 and OD of the final reaction product measured at 490 nm using a Dynatech MR700 microtiter plate reader.

The relative binding of all three mAbs to individual clones of transfected cells did not differ, and a mean value was taken. Binding of all three antibodies to control 3T3 cells as compared with a typical transfected cell line was consistently <5%.

Determination of the Relative Level of Immunoreactive Neurofilament Protein

We have previously measured immunoreactive levels of neurofilament protein in co-cultures of PC12 cells on monolayers of fibroblasts, muscle, and C6-glioma (Doherty et al., 1987) and the same methods were used in the present study. Briefly, cultures were carefully fixed by a 50% medium exchange with 4% paraformaldehyde in DME for 30 min at 20°C, followed by a total medium exchange for a further 30 min. Cells were permeabilized with methanol (−20°C) and the binding of a 1:2,000 dilution of anti-neurofilament protein antibodies BF10 and RT97 (for human cultures) or a 1:2,000 dilution of RT97 (for rat cultures) determined as above. For each data point the mean level of antibody binding to at least four independent monolayers was determined and this value subtracted from the level of binding to the respective co-culture.

Indirect Immunofluorescence

Co-cultures of human neurons on monolayers of control and transfected 3T3 cells were fixed and processed for BF10/RT97 binding as above with the exception that a rhodamine-conjugated anti-mouse IgG was substituted for the peroxidase-conjugated reagent. After incubation with this reagent, the cultures were washed four times with PBS and mounted for indirect immunofluorescence as previously described (Doherty and Walsh, 1988).

Results

N-CAM Expression in Transfected Cells

Fig. 1, a–e shows a Western blot analysis of neuraminidase-treated NP-40 extracts of control 3T3 cells as compared with cells transfected with cDNAs encoding four membrane associated N-CAM isoforms which have been described previously (Gower et al., 1988; Thompson et al., 1989; Walsh et al., 1989). Cells transfected with the ssd, ssd + MSD1, sd, and sd + MSD1 constructs expressed desialo–N-CAM immunoreactive proteins with respective molecular masses of 120, 125, 145, and 155 kD. None of these bands were observed in extracts of untransfected 3T3 cells. When a cell line transfected with a cDNA encoding a secreted N-CAM isoform was analyzed, no major immunoreactive bands were found associated with the NP-40 cell extracts, however an immunoreactive band at 115 kD was found in media conditioned over these cells (Fig. 1 f); this protein was not present in media conditioned over untransfected 3T3 cells or cells transfected with the ssd N-CAM isoform (Gower et al., 1988). Immunoprecipitation of the above samples with the anti–N-CAM serum, followed by electrophoresis and Western blotting using peroxidase labeled peanut lectin, demonstrated the presence of O-linked carbohydrate only in N-CAM isoforms from cells transfected with cDNAs containing the MSD1 coding region (Walsh et al., 1989). A similar analysis of L cells transfected with the sd and sd + MSD1 cDNAs revealed the specific synthesis of desialo–N-CAM bands of 145 and 155 kD, respectively, with only the latter binding peanut lectin (data not shown). Cells transfected with the cDNAs for the ssd ± MSD1 and sd ± MSD1 isoforms showed cell surface immunoreactivity with four independent anti–N-CAM mAbs and two independent rabbit anti–N-CAM sera (data not shown). An antiserum raised
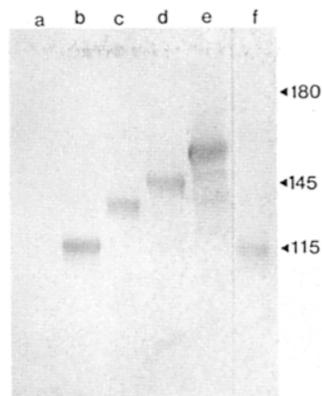

Figure 1. Immunoblot analysis of immunoprecipitated N-CAM from NP-40 cell extracts (lanes *a–e*) or conditioned media (lane *f*) from control 3T3 cells (*a*) or cells transfected with the cDNA constructs for the following N-CAM isoforms: (lane *b*) ssd, (lane *c*) ssd + MSD1, (lane *d*) sd, (lane *e*) sd + MSD1, and (lane *f*) the secreted isoform.

against a synthetic peptide corresponding to a region of the MSD1 domain specifically labeled the surface of cells transfected with cDNAs containing the MSD1 coding region (Thompson et al., 1989). None of the above reagents labeled untransfected 3T3 cells or L cells.

Consistent with the observations of Edelman et al. (1987) there was no obvious difference in the morphology of the cells expressing the sd and ssd N-CAM isoforms and we found no obvious difference in the rate of cell division (data not shown).

Morphology of Human Sensory Neurons on Monolayers of Control 3T3 Cells and Cells Expressing the sd N-CAM Isoform

Dissociated cells from human dorsal root ganglia were plated onto monolayers of control and transfected 3T3 cells. Fig. 2 shows typical examples of the morphology of neurofilament positive cells cultured for only 18 h on monolayers of control 3T3 cells (*a–c*) or alternatively on monolayers expressing the sd N-CAM isoform (*d–f*). A considerable degree of morphological differentiation was apparent for neurons grown on untransfected monolayers and there was a wide heterogeneity in neuronal morphology. The lack of neurite fasciculation is consistent with the untransfected monolayers being a relatively adhesive substratum for neuronal growth. When the relative amount of differentiation was compared with that of cells grown on monolayers expressing the sd N-CAM isoform, two independent "blind" investigators scored the transfected cells as clearly the better substratum. Examples of typical neurons on the latter are shown in Fig. 2 (*d–f*). This analysis was carried out using several independent preparations of human neurons grown in co-cultures for up to 64 h, and cells grown on monolayers of transfected cells consistently showed a greater extent of morphological differentiation than neurons grown on control monolayers.

The Expression of the sd Isoform of N-CAM Is Associated with Significant Increases in the Level of Immunoreactive Neurofilament Protein

For many neurons examined as early as 18 h, the complexity of differentiation was considerably greater than that shown in Fig. 2. At the magnification that was required to clearly identify them, many axons extended in and out of the focal
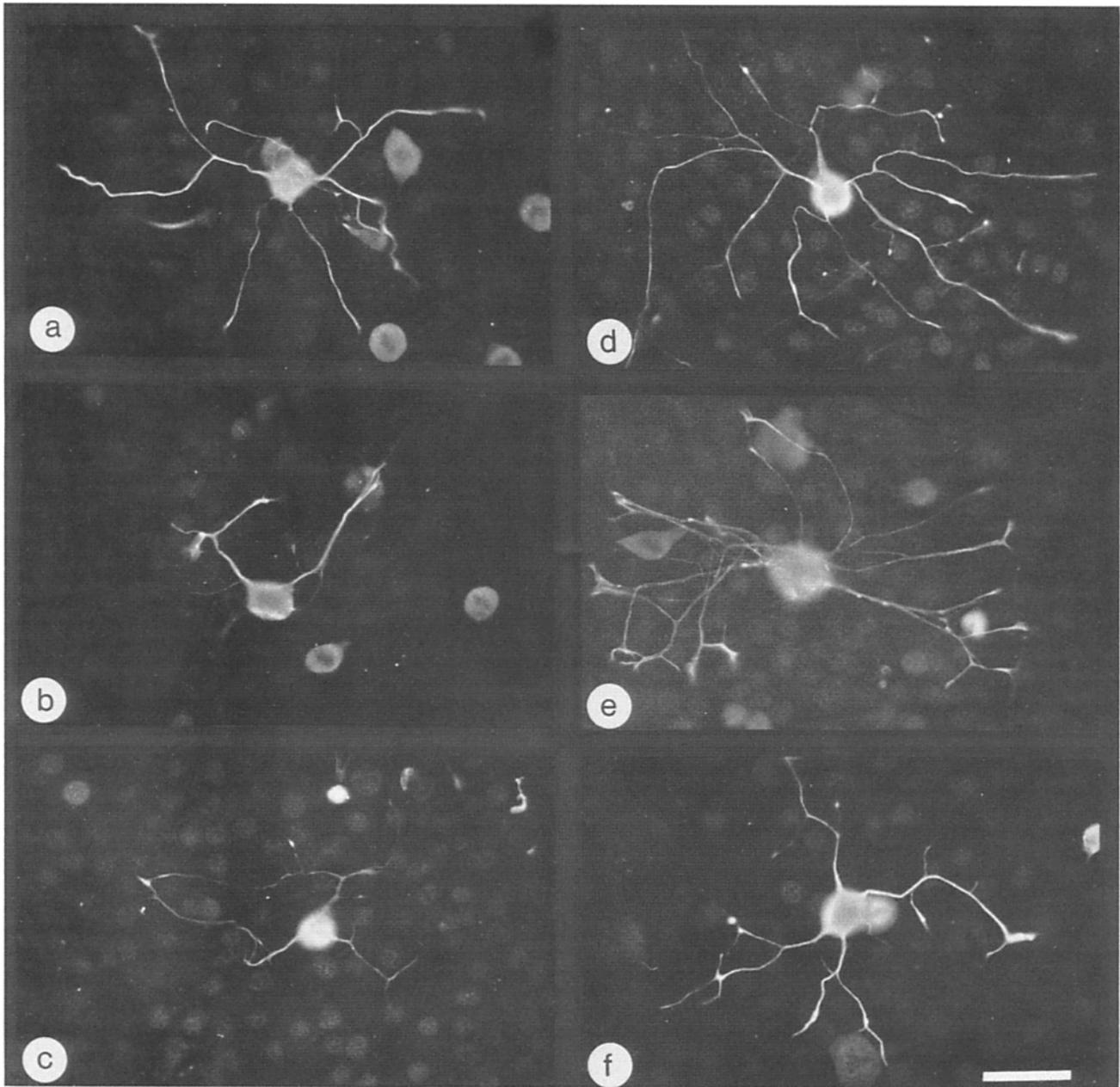
Figure 2. Localization of immunoreactive neurofilament protein for human neurons grown for 18 h on monolayers of nontransfected 3T3 cells (*a*, *b*, and *c*) and transfected 3T3 cells that express the sd N-CAM isoform at the cell surface (*d*, *e*, and *f*). After fixation and staining (see Materials and Methods), cultures were viewed and photographed with an automatic camera under epifluorescence optics. Bar, 50 μM.

plane in several directions well beyond individual visual fields. Thus a simple morphological analysis in terms of axonal length is not a practical measurement to make on these neurons. The degree of morphological differentiation that is apparent to the eye is directly related to the relative expression of immunoreactive neurofilament protein (see Fig. 2 and Doherty et al., 1984a,b, 1987; Doherty and Walsh, 1988). Therefore to critically test the hypothesis that the expression of N-CAM in a transfected cell can promote an increased morphological differentiation of dorsal root ganglion neurons, we have measured the relative expression of neurofilament protein in the respective co-cultures.

Table I summarizes the results obtained at 40 h (*n* = 1) or 62 h (*n* = 9) for 10 consecutive and independent prepara-

tions of human neurons seeded onto confluent monolayers of control 3T3 cells or cells expressing the sd N-CAM isoform. For 9 out of the 10 preparations the relative level of expression of immunoreactive neurofilament protein was significantly greater for neurons cultured on the monolayers expressing the transfected human N-CAM. For three preparations where neurofilament protein levels were determined as early as 17 h after co-culture, smaller but equally significant (*P* < 0.05) increases were apparent for neurons cultured on the transfected cell monolayers (percentage increase over control measured as 17 ± 2, group mean ± SEM, *n* = 3).

Expression of the sd N-CAM Isoform in L Cells

To further test the hypothesis that the expression of the sd

Table I. Percent Increase in the Binding of Anti–Neurofilament Protein Antibodies to Human DRG Neurons Grown on 3T3 Monolayers Expressing the sd N-CAM Isoform

Preparation	Percentage difference	Preparation	Percentage difference
a	58 ± 21*	f	17 ± 4*
b	33 ± 4§	g	33 ± 4§
c	38 ± 6*	h	39 ± 3§
d	19 ± 3§	i	18 ± 2.3§
e	−2 ± 4^n.s.	j	25 ± 4‡

10 independent preparations of human neurons (a–j) were grown on monolayers of control 3T3 cells or monolayers of transfected 3T3 cells expressing the sd N-CAM isoform. The cultures were fixed after 40 (j) or 64 h and the specific binding of BF10/RT97 was determined as described in Materials and Methods for 8 or 16 (preparation g and h) independent neuronal cultures grown on both control and transfected cell monolayers. The results show the percentage difference in the binding of anti–neurofilament protein antibodies to the human neurons grown on monolayers of transfected relative to control cells. Each value is the mean ± SEM.
* $P < 0.05$; ‡ $P < 0.01$; § $P < 0.001$.
n.s., not significantly different from control.

N-CAM isoform can promote an increase in the morphological differentiation of human sensory neurons, the relative level of neurofilament protein expression was determined for neurons grown for 18 h on monolayers of control and transfected L cells. Two clones transfected with the sd N-CAM isoforms differed in their cell surface expression of human N-CAM by ~60%. A third clone transfected with the sd + MSD1 isoform expressed ~20% more N-CAM than the clone expressing the greater amount of the sd isoform. The results of one experiment are shown in Fig. 3. In all three instances there was significantly more neurofilament protein expression for neurons grown on the monolayers expressing human N-CAM. There was a significantly greater response (by ~70%) for neurons grown on the monolayers expressing the higher levels of N-CAM. The expression of the glycosylated MSD1 domain (Walsh et al., 1989) did not obviously affect the ability of N-CAM to promote neuronal differentiation. In a second independent experiment immunoreactive neurofilament protein was again significantly greater for human neurons grown on monolayers of all three transfected clones as compared with control L cells.

Species Specificity of the Response

Using functional adhesion assays N-CAMs expressed on cells of one species have been shown to bind via a homophilic mechanism to N-CAM of a second species, and this has been demonstrated for chick and rat (Edelman, 1986). These data suggest that the functional binding domains are highly conserved. In the present study we have carried out some experiments with embryonic rat dorsal root ganglion neurons grown on monolayers of control and transfected 3T3 cells. Fig. 4 shows an example of the timecourse of expression of immunoreactive neurofilament protein for cells grown on monolayers expressing the sd N-CAM isoform relative to untransfected monolayers. As with human neurons, the expression of the sd N-CAM isoform was associated with a significantly greater increase in this parameter, suggesting a considerably greater morphological differentiation of neurofilament positive cells.

Expression of the Secreted N-CAM Isoform Does Not Modulate Neuronal Differentiation on 3T3 Monolayers

Six independent preparations of human dorsal root ganglion neurons were seeded onto monolayers of a clone of 3T3 cells transfected with cDNA encoding a secreted isoform of N-CAM (Gower et al., 1988), and the level of expression of neurofilament protein determined after 64 h of co-culture and compared with that for neurons grown on monolayers of untransfected 3T3 cells. The transfected cells synthesize human N-CAM, but rather than linking it to the membrane, they secrete it directly into the culture media (Gower et al., 1988). In all six experiments the level of immunoreactive neurofilament protein was not significantly different for both sets of co-cultures; in three instances small increases (7, 9, and 10%) were apparent whereas in the other three cases small decreases (3, 7, and 11%) were apparent on transfected monolayers (see Table II). These data clearly show that transfection and expression of N-CAM is not sufficient to promote an increased morphological differentiation of sensory neurons.

GPI-linked Isoforms of N-CAM Can Also Promote Axonal Outgrowth

To determine if the manner in which N-CAM is linked to the cell membrane is important for its ability to promote neurite outgrowth, we have cultured neurons on monolayers of two clones of transfected 3T3 cells expressing the ssd N-CAM isoform (one of which also expressed the MSD1 region). These clones differed in their expression of human N-CAM by <30%, and they expressed comparable levels to the clone transfected with the sd isoform described in Table I. Up to
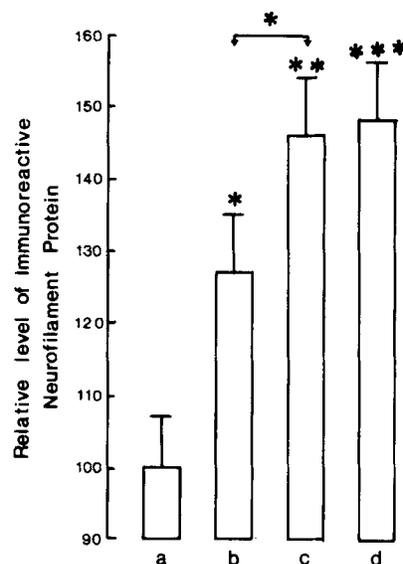

Figure 3. Human neurons were grown for 18 h on monolayers of control L cells (a) transfected L cells expressing relatively low (b) and high (c) amounts of the sd N-CAM isoform (see text) or transfected L cells expressing the sd + MSD1 isoform (d). The cultures were fixed and the specific binding of BF10/RT97 determined as described in Materials and Methods. The results show the absolute binding of anti–neurofilament protein antibodies to the human neurons, and each value is the mean ± SEM of eight independent determinations. 100 arbitrary units = 0.099 OD units. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
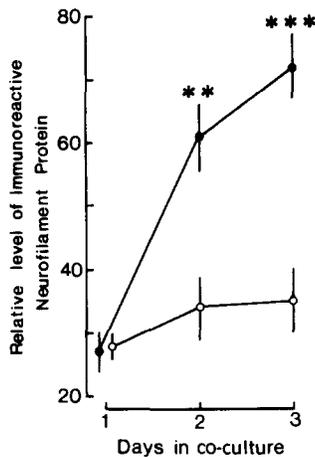
Figure 4. Rat neurons were cultured on monolayers of control 3T3 cells (O) or transfected 3T3 cells expressing the sd N-CAM isoform (●). Cultures were fixed after 1, 2, or 3 d and the specific binding of RT97 determined as described in Materials and Methods. The results show the absolute binding of RT97 to the rat neurons grown on the respective monolayers, and each value is the mean ± SEM of eight independent determinations. 100 arbitrary units = 0.028 OD units. **, $P < 0.01$; ***, $P < 0.001$.

eight independent preparations of dissociated neurons (six human, 2 rat) were plated onto monolayers of control and transfected cells (ssd, $n = 4$; ssd + MSD1, $n = 7$) and the levels of immunoreactive neurofilament protein sampled at 17 h ($n = 1$), 40 h ($n = 7$), or 62 h ($n = 3$). In 10 of the 11 trials, the level of immunoreactive neurofilament protein was significantly ($P < 0.05$) greater for neurons grown on transfected as compared to control monolayers. In the remaining trial there was a small, but insignificant increase for cells grown on the monolayer transfected with the ssd isoform. In three instances where the same preparation of neurons were grown on all three monolayers, there was no significant difference in the magnitude of response for cells grown on the monolayers expressing the ssd as compared to the ssd + MSD1 isoform. The group results summarized in Table II clearly show that as compared with the control transfectants (expressing the sec isoform) cells transfected with both the transmembrane and GPI-linked N-CAM isoforms can promote a significant increase in the morphological differentiation of sensory neurons.

Differential Inhibition of Neurite Outgrowth on Monolayers of Control and Transfected 3T3 Cells

Antibodies to integrin receptors will inhibit neurite outgrowth on monolayers of fibroblasts but not astrocytes, Schwann cells or myotubes (e.g., see Tomaselli et al., 1986; Bixby et al., 1987). This can be explained by the fact that in addition to extracellular matrix components the latter cells express other CAMs including N-CAM. We have tested the ability of an antiserum raised against a mouse muscle glycoprotein fraction for its ability to inhibit neurite outgrowth on monolayers of control 3T3 cells and monolayers expressing the sd isoform of N-CAM. The antiserum reacts with many components common to muscle and fibroblasts, including fibronectin, but shows no reactivity to human N-CAM (Doherty, unpublished observation). Fig. 5 shows the relative level of expression of neurofilament protein for two typical preparations of human neurons grown in the presence and absence of the antiserum. In both instances the antiserum inhibited the expression of neurofilament protein for neurons grown on monolayers of control 3T3 cells but had no effect on neurons grown on monolayers of transfected cells. In the examples shown the overall effect of the antiserum was to in-

crease the magnitude of the response attributable to N-CAM from a percentage increase of 33–39% to one of 76–94%. Similar results were obtained in two additional experiments.

Discussion

Ongoing mutational changes in the structure of DNA have been recognized as a major driving force behind the evolution of animal form. Common features have been identified in the genes encoding many proteins, suggesting that whole families of proteins have evolved from single ancestral genes. For example N-CAM has been identified as a member of the immunoglobulin gene superfamily that also includes in the nervous system Thy-1, L1, Po, and myelin associated glycoprotein (Edelman, 1987; Williams and Barclay, 1988; Moos et al., 1988). Although the functions of proteins in the immunoglobulin superfamily appear to be relatively diverse, many of them fall into the general category of molecules involved in adhesion and/or recognition. The exons of individual genes can also be alternatively used to generate families of protein isoforms that have both common and specific regions in their polypeptide chains (Breitbart et al., 1987). Differences in the functions of these proteins may however be quite subtle, and are likely to reflect the evolution of fine tuning mechanisms. The latter are likely to be of immense importance in the generation of the complex cellular morphologies that are found in the vertebrate nervous system. In the case of N-CAM, if all of the potential alternative splice patterns that have been identified to date were used, a single N-CAM gene could encode up to 18 different N-CAM isoforms in the mouse brain (Santoni et al., 1989). To date eight distinct mRNA species encoding N-CAM proteins have in fact been identified in this tissue (Santoni et al., 1989). In human muscle the expression of three distinct isoforms has been identified at both the protein and mRNA levels (Dickson et al., 1987; Moore et al., 1987; Gower et al., 1988). However S1 nuclease analysis predicts the presence of at least five isoforms in this tissue (Thompson et al., 1989). In both brain and muscle, individual N-CAM isoforms differ in their spatio-temporal pattern of expression (Edelman, 1986; Walsh, 1988) suggesting that structural variations in N-CAM may be important in the expression of its function. This no-

Table II. Comparison of the Effects of Different N-CAM Isoform Expressed in 3T3 Cells on Neurofilament Protein Expression from DRG Neurons

N-CAM isoform	Mean percent increase	P
sec	0.83 ± 3.7 (6)	—
sd	34.9 ± 8.5 (11)	<0.005
ssd + MSD1	56.4 ± 16.4 (7)	<0.005
ssd	34.7 ± 15 (4)	<0.05

Human and rat dorsal root ganglion neurons were grown on monolayers of control 3T3 cells or monolayers of cells expressing up to four distinct isoforms of human N-CAM (*sec, sd, ssd, ssd + MSD1*). In each of up to 11 independent trials the percentage difference in the level of expression of immunoreactive neurofilament protein was calculated for neurons grown on monolayers of untransfected and transfected 3T3 cells (see text for details). The results show the mean increase (±SEM) for the given numbers of independent preparations tested on the respective clones of transfected cells. The expression of the secreted N-CAM isoform had no effect on neurofilament protein expression (see text). Compared with this control group, expression of three independent membrane-linked N-CAM isoforms can be clearly seen to be associated with highly significant increases in the expression of neurofilament protein.
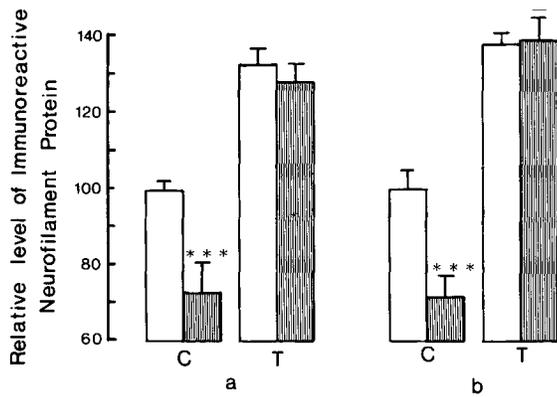
Figure 5. Two independent preparations of human neurons (a and b) were cultured on monolayers of control 3T3 cells (C) or cells expressing the sd isoform of N-CAM (T) in both the absence (□) and presence (▦) of a rabbit antiserum raised against a muscle glycoprotein fraction (1% [vol/vol]). The cultures were fixed after 62 h and the specific binding of BF10/RT97 determined as described in Materials and Methods. The results show the absolute binding of anti-neurofilament protein antibodies to the human neurons, and each value is the mean + 1 SEM of 8-16 independent determinations. 100 arbitrary units = 0.087 and 0.111 OD units for a and b, respectively.

tion is further supported by the clear demonstration that changes in posttranslational processing in terms of glycosylation and sialylation can have pronounced effects on the adhesive properties of N-CAM (Hoffman and Edelman, 1983) and that the alternatively spliced exons found in the extracellular domain of some muscle N-CAM isoforms represent a site of tissue specific O-linked glycosylation (Walsh, 1988; Walsh et al., 1989).

As no reagents that can specifically perturb the function of individual N-CAM isoforms have yet been identified, alternative strategies have to be developed to understand the role that individual isoforms may play during development. Molecular genetic approaches based on transfection with specific cDNA constructs allows for the generation of a panel of cells that express individual N-CAM isoforms. Edelman et al. (1987) have recently described the expression of three major N-CAM isoforms of the chick brain in L cells (the ld and sd isoforms) and in COS cells (the ssd isoform). These workers reported an increase in intracellular adhesion between transfected cells, providing additional support for the homophilic binding hypothesis. In the present study we have expressed four human N-CAM isoforms in 3T3 cells (sd, ssd ± MSD1, and secreted) and two in L cells (sd ± MSD1). We have subsequently assayed monolayers of control and transfected cells for their ability to support the relatively complex function of neuronal process outgrowth. Briefly, glycoconjugates that support growth cone adhesion can be expected to either directly (via a receptor-mediated physical interaction with cytoskeletal elements) or indirectly (via activation of second messenger pathways) stabilize the structure of the growth cone, a step that is believed to be an essential prerequisite for axonal growth (Smith, 1988). Monolayers of both untransfected 3T3 cells and L cells supported neuronal cell adhesion and neurite outgrowth. Although this high level of differentiation was unexpected, it is advantageous as we can exclude the possibility of indirect effects on neurite out-

growth due solely to changes in neuronal cell body adhesion. We are therefore analyzing whether the expression of an additional CAM can increase the extent of neurite outgrowth over an already highly adhesive cellular substratum.

When neurons were grown on monolayers of control cells and cells transfected with membrane associated N-CAM isoforms, there was always an obvious increase in the degree of morphological differentiation on the latter monolayers (e.g., see Fig. 2). There is at present no simple morphological index that can take into account all of the complex facets of axonal growth. For example, measurements of length do not take into account axonal caliber, and such measurements can be difficult to make in an objective manner at later stages of culture due to neurite fasciculation and complex patterns of axonal growth. Although not a primary measurement of neurite outgrowth, the relative expression of immunoreactive neurofilament protein has been established in many systems to be directly related to the degree of morphological differentiation (Doherty et al., 1984a,b; Doherty et al., 1985,1987; Doherty and Walsh, 1989). When studying the differentiation of a population of neurons that show a considerable degree of heterogeneity in their morphology, measurement of immunoreactive neurofilament protein has several advantages over simple morphological indices. For example it provides an objective index of the relative degree of morphological differentiation. It also samples the total population of neurons that express immunoreactive neurofilament protein and it is a measurement that can readily be made on several hundred independent cultures over a relatively short period of time. Other workers carrying out similar types of studies (Bixby et al., 1988; Tomaselli et al., 1988) have used alternative markers such as the cell surface marker A2B5 (Eisenbarth et al., 1979) in conjunction with morphometric analyses.

For 9 out of 10 preparations of human neurons we have found that 3T3 cells expressing the sd N-CAM isoform can support enhanced neuronal process outgrowth, as indexed by immunoreactive levels of neurofilament protein, to a significantly ($P < 0.05$) greater extent than control cells. Similar results were found for two independent clones of L cells transfected with the same cDNA, in which case the difference in ability of these clones to induce neuronal differentiation was correlated with a difference in the relative amount of expression of this particular N-CAM isoform.

In contrast to the above results, the expression of a secreted N-CAM isoform by transfected 3T3 cells had no obvious effect on neuronal differentiation. The most parsimonious explanation of the data is that the ability to increase neuronal differentiation is directly associated with an increased expression of an N-CAM isoform that is anchored to the cell membrane, in the above instance by a transmembrane-spanning domain. Consistent with this contention we know that neurite outgrowth is an anchorage-dependent phenomenon (Smith, 1988; Mitchison and Kirschner, 1988) and we know that the neuronal population under study expresses N-CAM at the growth cone region (Doherty, unpublished observations). These data do not however preclude a role for soluble N-CAM in modulating neurite outgrowth. For example, the expression of function of a soluble N-CAM isoform may be dependent upon the ability of an extracellular matrix component to bind and anchor this molecule, or alternatively soluble N-CAM may be capable of inhibiting neurite outgrowth over a cellular substratum that contains N-CAM as a func-

tional component. We are currently developing experimental strategies to test both possibilities.

On average, ~900 neurons were seeded into each microwell, and we can estimate the cultures to be at least 70% neuronal. Allowing for a 50% increase in the number of 3T3 cells plated to generate the monolayers, total contamination with human nonneuronal cells can be calculated at ~1%. The neurons under study showed a very complex morphology with the vast majority having considerably >10 branches (see Fig. 2), we can therefore estimate the ratio of, for example, Schwann cells to axonal branches at considerably <1:25. In support we never found any evidence for incorporation of human nonneuronal cells into the monolayer, and growth cones were always found in association with control or transfected 3T3 cells. We can therefore conclude that transfected N-CAM acts directly on neurons to promote an increase in the expression of neurofilament protein.

It is only very recently that direct evidence has been obtained for diversity in the primary structure of the extracellular domain of N-CAM (Walsh, 1988; Santoni et al., 1989; Thompson et al., 1989). The most predominant N-CAM isoform in myotubes (the ssd isoform), was found to contain an amino acid block close to the membrane region that is not found in any brain isoform (Dickson et al., 1987). The expression of this domain both in muscle and in transfected cell lines is correlated with a posttranslational modification that introduces an O-linked carbohydrate group (Walsh, 1988; Walsh et al., 1989). In view of the well-characterized observation that carbohydrate, and in particular polysialic acid can inhibit N-CAM function (Rutishauser et al., 1988) we tested whether the introduction of the MSD1 domain to the sd isoform would inhibit its ability to promote neurite outgrowth. Two clones of L cells were selected that expressed similar levels of immunoreactive N-CAM at the cell surface, the only difference being that one specifically expressed the MSD1 region. In two independent experiments we found no difference in their ability to enhance neurite outgrowth, suggesting that the glycosylated MSD1 region exerts no gross effects on function in this co-culture model.

An unusual feature that some N-CAM isoforms share with a number of proteins is a linkage to the membrane via a GPI-tail (Ferguson and Williams, 1988). It would be predicted that these isoforms would be considerably more mobile in the plane of the membrane than isoforms that express a transmembrane-spanning region. As neurite outgrowth may be dependent upon forces of tension generated by adhesion of the growth cone to the substratum, and as such forces may be influenced by the mode of association of adhesion molecules to the membrane, it was of interest to determine if GPI-linked (ssd) N-CAM isoforms could also promote neuronal differentiation.

Two 3T3 cell clones expressing the ssd and ssd + MSD1 N-CAM isoforms were found to induce increases in immunoreactive neurofilament protein to a very similar extent as a clone that expressed approximately the same levels of the sd N-CAM isoform (see Table II). As well as directly demonstrating for the first time that the expression of a lipid-linked cell adhesion molecule can promote the morphological differentiation of both human and rat sensory neurons, these data also further support the contention that expression of the glycosylated MSD1 region exerts no gross effect on this N-CAM function.

Antibody perturbation experiments suggest that neurite outgrowth over cellular substrata in vitro is a multicomponent system that involves neuronal expression of integrin receptors for various extracellular matrix molecules as well as the expression of receptors for the cell adhesion molecules N-CAM, N-Cadherin, and L1 (Bixby et al., 1987, 1988; Seilheimer and Schachner, 1988; Tomaselli et al., 1986, 1988). The contribution of each component can differ for both the nature of the cellular substrata, as well as the type and developmental age of the neuron. For example, whereas anti-N-CAM antibodies have no detectable inhibitory effects on neurite outgrowth from E7 retinal neurons grown on an astrocyte monolayer, they strongly inhibited neurite outgrowth from E11 retinal neurons grown on the same substratum (Neugebauer et al., 1988). In several other models the sole addition of blocking antibodies to N-CAM fails to inhibit neurite outgrowth, e.g., as in the case of neurite outgrowth over axons (Chang et al., 1987) and neurite outgrowth over myotubes (Bixby et al., 1987). A difficulty in interpreting data from these studies is the spare capacity that many complex substrata have for supporting neurite outgrowth in vitro. For example, whereas the sole addition of antibodies to N-CAM, N-Cadherin, and neuronal integrin receptors has little effect on neurite outgrowth for ciliary ganglion neurons cultured on myotubes, maximal inhibition of neurite outgrowth required the addition of all three reagents (Bixby et al., 1987). An alternative approach to the study of individual cell adhesion molecules has led to conflicting results. Lagenaur and Lemmon (1987) have purified both N-CAM and the 8D9 antigen (the chick homologue of L1/Ng-CAM) and found that when attached to a nitrocellulose-coated substrate, the latter but not the former has pronounced effects on cell adhesion and neurite outgrowth. The authors have suggested that the failure of N-CAM to promote adhesion and differentiation may reflect their inability to purify and/or attach this molecule in an active form. In this context it is worth noting that there are fundamental differences between N-CAM and Ng-CAM; whereas N-CAM is believed to mediate interactions solely through homophilic binding to itself, considerable evidence suggests that Ng-CAM can bind via a heterophilic mechanism (Hoffman et al., 1986). Thus the expression of a homophilic binding CAM in a cellular system clearly represents a more valid and critical model to test its function as a neurite outgrowth promoting molecule.

In the present study we have shown that the expression of cell membrane-linked isoforms of N-CAM in fibroblasts increased their capacity to support neurite outgrowth, as indexed by neurofilament protein expression, by an average of 42% (calculated from the data in Table II, $n = 22$). The magnitude of this response should be viewed in the context of the normal differential ability of fibroblasts and other cell types to support neurite outgrowth. For example astrocytes express high levels of several CAMs, including N-CAM and N-Cadherin, yet they are only twofold better than fibroblasts in supporting neurite outgrowth from cerebellar neurons (Noble et al., 1984) or ciliary ganglion neurons (Bixby et al., 1988). The magnitude of response should also be viewed in comparison with the relative ability of antibodies to putative neurite outgrowth promoting molecules to perturb this function. For example, two neural cell surface antigens involved in the extension of neurites on axons (defined by two mono-

clonal antibodies named G4 and F11) were identified on the basis that polyclonal antibodies raised against them could maximally inhibit neurite outgrowth by ~20% (F11) and ~40% (G4) (Chang et al., 1987).

The observation that the expression of immunoreactive neurofilament protein can be differentially inhibited on monolayers of control and transfected 3T3 cells supports the contention that the expression of N-CAM has fundamentally changed the ability of 3T3 cells to support neuronal differentiation. The differential inhibition is similar to that seen for neurons grown on fibroblast as compared to cells that constitutively express other CAMs including N-CAM. For example, whereas anti-integrin receptor antibodies can block ciliary ganglion neurite outgrowth over fibroblasts, they will only block outgrowth over myotubes after neutralization of N-CAM and N-Cadherin function (Bixby et al., 1987).

In the present study we have critically tested the hypothesis that transmembrane and lipid-linked isoforms of N-CAM can promote the morphological differentiation of sensory neurons. Although the results have clearly shown this to be the case, more detailed studies will be required to determine the relative efficacy of individual isoforms and the relationship between quantity of N-CAM expression and magnitude of the neuronal response. The ability of blocking antibodies to increase the resolution between control and transfected cells will be useful for answering these questions and should also facilitate the study of the structure-functional relationship of individual N-CAM isoforms.

In summary, we have shown that the molecular genetic approach can be used to study the function of individual N-CAM isoforms. Our results provide direct evidence for both transmembrane and GPI-linked N-CAM isoforms being important components of the cellular recognition machinery involved in controlling the morphological differentiation of neurons as assessed by an analysis of neurite growth. N-CAM can therefore be considered as a candidate to be one of the CAMs involved in specific pathways of axonal guidance. The diversity of structure of this cell adhesion molecule, together with its spatio-temporal pattern of expression in both the developing and regenerating nervous system (Edelman, 1986) suggests that N-CAM may play a highly dynamic and perhaps subtle role in controlling neuronal shape, and as such in defining the spatial relationship between cells of the nervous system.

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