Skeletal Muscle Neural Cell Adhesion Molecule (N-CAM): Changes in Protein and mRNA Species during Myogenesis of Muscle Cell Lines

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Abstract. Qualitative and quantitative changes in neural cell adhesion molecule (N-CAM) protein and mRNA forms were measured during myogenesis in G8-1 and C2 cell lines. Indirect immunofluorescence assay showed that N-CAM was constitutively expressed by myoblasts in culture and that myotubes appeared to be stained more strongly. These changes were quantified using a dot blot assay. N-CAM levels increased almost 4-fold in G8-1 cells and 15-fold in C2 cells during myogenesis. The kinetics of accumulation of N-CAM were not coordinate with other muscle markers such as creatine kinase or acetylcholine receptor levels, since N-CAM accumulated significantly ahead of these markers. Immunoblotting showed that myogenesis was not associated with changes in the extent of sialylation of N-CAM. However, distinct changes in desialo forms were observed after neuraminidase treatment. Myogenesis was accompanied by increases in 125- and 155-kD desialo forms with minor changes in 120- and 145-kD forms. Biosynthetic labeling studies showed that myoblasts specifically expressed a transmembrane isoform of 145 kD that was phosphorylated and was down-regulated in myotubes. Pulse-chase analysis of myotubes showed that the 120-kD isoform and an isoform of 145 kD that co-migrated with, but was distinct from, the 145 kD transmembrane isoform of myoblasts were precursors of the 125- and 155-kD isoforms, respectively, that accumulated in myotubes. The 125- and 155-kD isoforms in myotubes are linked to the cell membrane via phosphatidylinositol linkage and can be released by phospholipase C. Indirect immunofluorescence analysis showed that phosphatidylinositol specific phospholipase C specifically released N-CAM from the myotube membrane generating N-CAM-free myotubes, while myoblasts were unaffected by this treatment. Three N-CAM mRNA species were observed in mouse muscle cell lines. Myoblasts were characterized by their expression of 6.7- and 5.2-kb transcripts while myotubes express 5.2- and 2.9-kb transcripts. Thus, myogenesis is qualitatively associated with a down regulation of the 6.7-kb transcript and an up regulation of the 5.2- and 2.9-kb transcript.

NEURAL cell adhesion molecule (N-CAM)[1] is the best characterized cell adhesion molecule and is likely to be of crucial importance in regulating cell-cell interactions in the brain and other tissues (11, 34). It is well placed to play a pivotal role in such systems because it is expressed in a variety of alternatively spliced (10, 29) molecular forms of different sizes that can be differentially glycosylated (11, 12, 34) and because subpopulations of the molecule may express unique antigenic determinants (31). A number of studies have been reported on the in vitro and in vivo expression of N-CAM in skeletal muscle (6–9, 17, 26, 27, 30, 32, 33, 38, 43, 44, 46) and a number of roles have been proposed for N-CAM in this tissue. These include myoblast fusion (8, 39), the interaction of nerve and muscle during in vitro and in vivo neuromuscular synaptogenesis (33), and the interaction of primary and secondary myoblasts during histogenesis (20, 21). However, it will be necessary to have a full molecular profile of N-CAM expression and regulation in skeletal muscle before it will be possible to address precisely the question of whether or not this molecule is involved in specific muscle functions. As most of the assay systems available for study of N-CAM function use cells in culture, it is necessary in the first instance to have a clear picture of N-CAM expression in appropriate in vitro models. A number of skeletal muscle cell culture systems have indeed been analyzed in this respect. These include primary cultures of human (26, 44), rat (7, 8), and chicken muscle (17), and several rodent cell lines (7, 46). In human muscle cell cultures N-CAM has been shown to be expressed by cycling myoblasts (26) but in neonatal rat cultures N-CAM has been shown to be present on myosin-positive myocytes and another uncharacterized population, probably myoblasts (7). Differing results have been found with respect to N-CAM molecular forms in muscle, probably due to the different types of analyses carried out. Immunoprecipitation of bio-

1. *Abbreviations used in this paper*: AchR, acetylcholine receptor; CK, creatine kinase; GPDH, glyceraldehyde 3-phosphate dehydrogenase; N-CAM, neural cell adhesion molecule; T4, thyroxine.

synthetically labeled N-CAM from primary cultures of rat muscle yielded a single polypeptide of 135 kD (30) while Western blotting of NP-40–extractable proteins from similar cells showed a greater degree of polydispersity (7). Cell surface lactoperoxidase labeling of rat L6 cells identified the following after immunoprecipitation: N-CAM polypeptides of 140 and 120 kD, and small amounts of a 180-kD component (46).

There are still, however, a number of unanswered questions concerning the precise mode of expression of N-CAM in skeletal muscle. We have attempted to answer some of these using mouse muscle cell lines G8-1 and C2. In particular, we have attempted to address the following questions. When does N-CAM appear on myoblasts and are there any changes in N-CAM levels during in vitro myogenesis? Is it possible to modulate N-CAM levels in culture? What are the changes in N-CAM isoforms during myogenesis, and are the different bands found on Western blots the products of the same or different mRNAs? What is the temporal pattern of change of N-CAM–specific mRNA species and how do they change quantitatively and relate to other markers of muscle differentiation?

Materials and Methods

Cell Cultures

The mouse muscle cell lines G8-1 (5) and C2 (4, 36, 47) were obtained from Dr. M. Nirenberg, National Heart, Lung, and Blood Institute, Bethesda, MD, and Dr. H. Blau, Stanford University, Stanford, CA, respectively. They were grown on collagen-coated (Vitrogen; Collagen Corp., Palo Alto, CA) culture dishes in DME containing 10% FCS for G8-1 cells and 20% FCS and 0.5% chick embryo extract for C2 cells. To induce myotube formation in G8-1 cells the above media was changed to DME containing 5% horse serum and 0.5% FCS while C2 cells were treated with DME containing 2% horse serum. In some experiments, fused cultures of G8-1 cells were treated with tetrodotoxin or thyroxine (T4). Tetrodotoxin (0.5 μg/ml) or T4 (0.89 μg/ml) was added for 4 d, after which cells were analyzed for N-CAM levels.

Antibodies, Indirect Immunofluorescence, and Immunoblotting

Two anti-N-CAM antibodies were used in the present study. These were the anti-N-CAM mAb H28 (15) and rabbit anti-mouse muscle N-CAM antisera. The latter reagent was produced as follows. Mouse G8-1 cells were grown to confluence and extracted with 1% NP-40 in Tris-buffered saline (TBS), pH 7.4, containing trasylol (2 U/ml) and phenylmethylsulfonyl fluoride (PMSF) (1 mM). The extract was spun at 100,000 *g* for 1 h at 4°C and the soluble fraction frozen at −20°C until use. The thawed extract was applied first to a nonspecific column containing rabbit immunoglobulin bound to Sepharose 4B and the unretarded extract was applied to a second column containing H28 mAb bound to Sepharose 4B. After extensive washing, bound N-CAM was eluted from the H28 mAb column with 0.05 M diethylamine, 1% NP-40 (pH 11.5) and neutralized with solid glycine. Rabbits were injected with purified N-CAM (50 μg/injection) on four occasions over 8 wk to induce a strong immune response. The third bleed was used in the present experiments and had a titre of 1:6,000 in an ELISA using mouse brain homogenate bound for 2 h to microtitre plates.

Indirect immunofluorescence staining was carried out as described previously (42). A 1:100 dilution of H28 mAb ascites fluid was used for these experiments and, as detecting antibody, a 1:100 dilution of fluorescein-labeled anti-rat immunoglobulin was used. The polyclonal rabbit anti-N-CAM was used at a 1:100 dilution and the detecting antibody was sheep anti-rabbit labeled with fluorescein.

Immunoblotting was carried out as described previously with some modifications (26). 50 μg of NP-40–extracted cellular protein was added per gel track and after electrophoresis and transfer to nitrocellulose, nonspecific binding sites were blocked with PBS containing 2% casein (pH 7.2). Antibodies used were either H28 mAb or rabbit anti-N-CAM. The detecting antibodies were rabbit anti-rat peroxidase for H28 mAb and sheep anti-rabbit peroxidase for rabbit anti-N-CAM. These were visualized with color development reagent (HRP; Bio-Rad Laboratories, Richmond, CA) in the presence of 0.02% H_2O_2.

Neuraminidase was used to generate desialo N-CAM. Neuraminidase (type X, final concentration 0.5 U/ml; Sigma Chemical Co., St. Louis, MO) was added to NP-40 extracts from muscle cell cultures (1 mg/ml protein) in 2.5 mM sodium acetate buffer, pH 5.0, for 1 h. At the end of the reaction time samples were treated as for immunoblotting.

Biosynthesis of N-CAM and Effect of Tunicamycin and Phospholipase C

Muscle cells at myoblast or myotube stages were labeled with either L-[^{35}S]methionine or [^{32}P]inorganic phosphate. Cells were washed twice in PBS and methionine or phosphorus-free DME followed by either 50 μCi/ml L-[^{35}S]methionine or 500 μCi/ml [^{32}P]inorganic phosphate (Amersham International, Amersham, United Kingdom). Cultures were routinely incubated for 4 h and the cells washed three times in PBS and harvested as described for immunoprecipitation analyses. For pulse-chase studies, cells were treated as above, with chase incubations in DME containing 10% FCS.

When radiolabeling was done in the presence of tunicamycin, the drug (5 μg/ml) was added 30 min before radiolabeling and fresh drug was included in the labeling media.

Phospholipase C release of N-CAM was carried out on cultures labeled for 4 h with L-[^{35}S]methionine and after three washes in PBS the labeled cells were incubated in DME containing 10 U/ml (12 U/mg) phospholipase C (*B. cereus*, type III; Sigma Chemical Co.) for 1 h at 37°C. The media was then collected, filtered through a 0.22-μm filter, and N-CAM immunoprecipitated as below.

Phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* (a gift from Dr. M. Low) was used to assess which cells in culture expressed N-CAM isoforms that were sensitive to the action of this enzyme. C2 myotubes were grown on coverslips and phosphatidylinositol-specific phospholipase C added at a level of 12 U/ml in PBS for 45 min at 37°C. Samples were then processed for immunofluorescence analysis of N-CAM as described above.

Immunoprecipitation of N-CAM Polypeptides

Muscle cells labeled as above were washed three times in PBS and harvested into ice-cold extraction buffer containing 10 mM Tris-HCl, pH 7.4, 1% NP-40, 2.5 mM EDTA, 1 mM PMSF, and 2 U/ml aprotinin (buffer A). The cell lysate was left on ice for 20 min and then centrifuged for 10 min at 10,000 rpm. The supernatants (0.5 ml) or media were then cleared with 50 μl of a 10% solution of preimmune rabbit Ig–coated *Staphylococcus aureus* bacteria for 30 min at 4°C. The supernatants were cleared by centrifugation and 20% vol/vol of 2% casein in 5 M NaCl was then added followed by 2 μl of rabbit anti-N-CAM antisera. The supernatants were left at 4°C overnight and then centrifuged for 10 min before being transferred to a new centrifuge tube. To this was added 50 μl of a 10% suspension of *Staphylococcus aureus* cells. After 60 min at 4°C with occasional mixing the suspension was washed six times in 0.5 ml buffer A containing 0.5 M NaCl and twice more with buffer A without NP-40 but with 0.1% SDS. The resulting pellets were then either suspended in 25 mM acetate buffer, pH 5.0, for neuraminidase treatment, or boiled in SDS sample buffer for polyacrylamide gel electrophoresis analysis.

Creatine Kinase Assay and α-Bungarotoxin Binding

Two well characterized biochemical markers were used to follow the kinetics of fusion in G8-1 and C2 cultures, namely creatine kinase (CK) levels (39) and acetylcholine receptor (AchR) levels as assessed by α-bungarotoxin binding (14). For CK assay, samples were harvested at specific time points into PBS, sonicated for 15 s, and their enzyme levels measured with the UV kit (Boehringer Mannheim, Lewes, United Kingdom). Protein levels were measured by the Lowry protein assay using BSA as standard. For assessment of α-bungarotoxin binding, samples were harvested as above into PBS containing 1% BSA. Binding of ^{125}I–α-bungarotoxin (Amersham International) was determined in suspension, and otherwise according to Franklin et al. (14), and the specific binding found by adding an excess of unlabeled α-bungarotoxin (Sigma Chemical Co.).

Quantitation of N-CAM Levels

N-CAM levels were quantitated using a dot blot ELISA. Serially diluted aliquots (50 μl) of G8-1 and C2 cell homogenates of known protein concentra-

tions were spotted onto nitrocellulose (0.45 μm, Schleicher & Schuell, Inc., Keene, NH) using a Bio Dot apparatus (Bio-Rad Laboratories). The filter was then transferred to PBS containing 2% casein for 30 min to block nonspecific binding sites. A 1:1,000 dilution of H28 mAb in PBS, 2% casein was added for 1 h at 20°C. The filter was washed and a 1:1,000 dilution of peroxidase-conjugated rabbit anti-rat Ig added for 1 h at 20°C. The filter was washed extensively and finally treated with an aliquot of HRP color development reagent in the presence of 0.02% H_2O_2. Dots were scanned with a Chromoscan 3 apparatus (Joyce, Loebl and Co. Ltd., Gateshead, England) and the relative level of N-CAM protein calculated from the linear region of the graph of protein concentration versus signal intensity.

Isolation of mRNA

RNA was extracted from cells at various time points after plating by extracting cells overnight at 4°C in 3 M LiCl, 6 M urea, 0.5% wt/vol SDS, 0.1% wt/vol heparin, 0.1 M sodium acetate, pH 5.2 (1). Samples were spun at 10,000 *g* for 30 min, resuspended in 3 M LiCl and 6 M urea, and recentrifuged. The precipitated pellet was resuspended in 0.2% wt/vol SDS, 0.1 M sodium acetate, pH 5.2, extracted twice with phenol/chloroform (1:1), once with chloroform, precipitated with ethanol, and resuspended in 10 mM Tris, 0.5 M EDTA.

Poly(A)$^+$ RNA was isolated by one round of affinity chromatography on oligo dT-cellulose (Sigma Chemical Co.) (2) and resuspended in H_2O.

Northern Blot Analysis of RNA

Samples of Poly(A)$^+$ RNA were denatured with glyoxal (37), separated by electrophoresis on 1% agarose gels, and transferred to a membrane (Genescreen; New England Nuclear, Boston, MA) in 15 mM NaH_2PO_4 (pH 6.5).

Filters were submerged in 10 mM Tris HCl (pH 8) at 100°C for 30 min and then prehybridized for 18 h at 42°C in 50% formamide, 0.04% polyvinylpyrollidone, 0.04% BSA, 0.04% Ficoll, 5× saline sodium citrate (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1% SDS, 100 μg/ml sheared, denatured calf thymus DNA (Sigma Chemical Co.) and 2 μg/ml Poly A and 1 μg/ml Poly G (Sigma Chemical Co.). Hybridization was carried out in the same solution at 42°C for 18 h with 3.9×10^6 cpm/ml of electrophoretically purified insert from the N-CAM cDNA probe pM1.3 (16) labeled by nick translation (16).

Filters were washed with 2× SSC, 0.1% SDS twice for 15 min at 20°C followed by washes at 0.2× SSC, 0.1% SDS twice for 30 min at 65°C. For autoradiographic analysis filters were exposed to Fuji x-ray film at −70°C in the presence of an intensifying screen.

In some experiments the level of N-CAM hybridizing bands was compared with a control probe whose level did not change during myogenesis. For these experiments we used cDNA probe to glyceraldehyde-3-phosphate dehydrogenase (GPDH) (24). Blots were treated as follows. The N-CAM probe pM1.3 was removed by washing for 1 h at 65°C in 5 mM Tris-HCl (pH 8), 0.2 mM Na_2 EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinylpyrollidone, 0.002% BSA, and 0.002% Ficoll, and then prehybridized and hybridized as above in 50% formamide, 0.04% polyvinylpyrollidone, 0.04% BSA, 0.04% Ficoll, 5× SSC, 1% SDS, 100 μg/ml sheared denatured calf thymus DNA, and 1 μg/ml Poly A/Poly G with 3.5×10^6 cpm/ml of cDNA probe to chicken GPDH (24), labeled by nick translation.

Filters were washed in 2× SSC, 0.1% SDS twice for 15 min each at 20°C and then twice for 30 min at 65°C, and autoradiographed as before.

Densitometric analysis of autoradiographs was carried out on a Chromoscan 3 apparatus.

Results

Qualitative and Quantitative Changes in N-CAM Levels during Myogenesis in Culture

The mouse muscle cell lines G8-1 and C2 were chosen to analyze the expression of N-CAM during myogenesis. These cell lines form myotubes reproducibly and with high frequency (4, 5). In addition, G8-1 cells will synapse with appropriate neural cell lines (5) and we have shown specific changes in cell surface proteins during myogenesis (41). G8-1 and C2 cells were grown in cell culture for varying periods of time and allowed to go through their myogenic program.

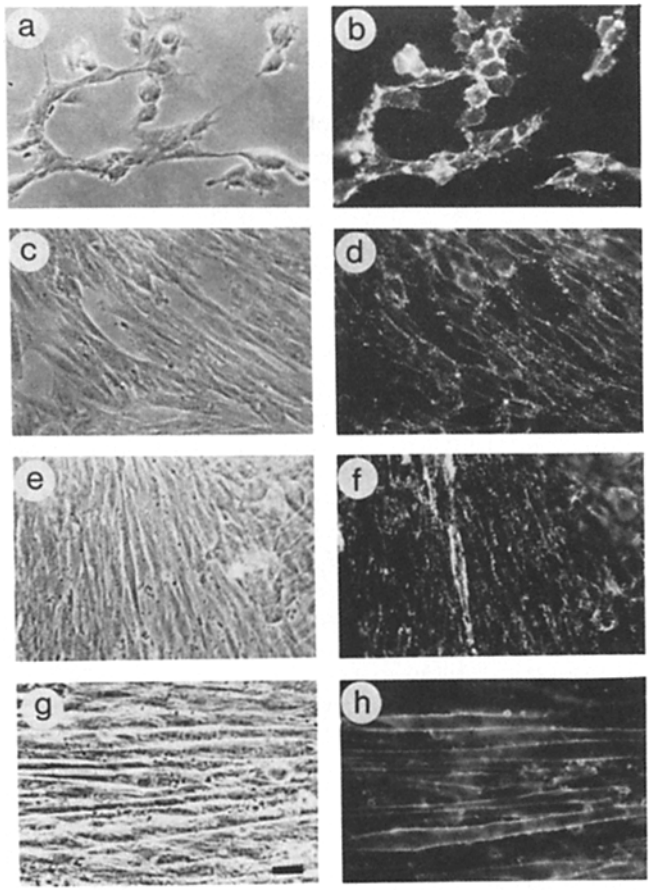

Figure 1. Expression of N-CAM in cell cultures of mouse G8-1 cells during myogenesis. Indirect immunofluorescence was used to show the distribution of N-CAM after different periods of time in culture. (*a*, *c*, *e*, and *g*) Phase-contrast views of cells; (*b*, *d*, *f*, and *h*) the same cells stained with anti-N-CAM. Samples were examined after different times of culture. These were (*a*) 1 d, (*c*) 3 d, (*e*) 6 d, and (*h*) 10 d. Bar, 100 μm.

Fig. 1, *a*, *c*, *e*, and *g* shows the morphological development of G8-1 cells after various periods of time in culture and Fig. 1, *b*, *d*, *f*, and *h* shows the same cells stained with anti-N-CAM. These cells start as mononucleate cells that divide rapidly and after alignment begin fusing to form myotubes after 5 d in culture. Fusion continues beyond this time and by day 10 the myotubes increase in size and start contracting. Like all other rodent muscle cell lines previously analyzed, such as C2 cells, BC3H1 cells (7), L6 cells (46), and 10T½ clones (Moore, S. E., and F. S. Walsh, unpublished observations), the G8-1 cells expressed N-CAM. This was shown by immunofluorescence using the mouse N-CAM-specific mAb H28 that reacts with a backbone polypeptide determinant of mouse N-CAM (15). No reactivity with the cells was observed when mAb H28 was omitted from the staining reaction or a control mAb was substituted for mAb H28. In addition, similar staining patterns were found with the polyclonal rabbit anti-N-CAM antibody. Indirect immunofluorescence analysis of N-CAM expression during myogenesis in G8-1 cultures revealed the following. At the earliest time point (24 h) after plating, when all cells in the culture are mononucleate, all cells were positive for N-CAM (Fig. 1, *a* and *b*). This shows that N-CAM is constitutively expressed

by cycling myoblasts in culture, and that myoblasts do not have to commit to become myocytes before N-CAM is expressed. This expression of N-CAM remained stable at the next time point immediately before the onset of fusion (Fig. 1, *c* and *d*) and it was not until the third time point, when fusion was starting to occur, that qualitative changes in N-CAM staining intensity became apparent (Fig. 1, *e* and *f*). As soon as small myotubes began to appear in the G8-1 cultures they could be clearly identified from background myoblasts on the basis of a much enhanced fluorescence staining intensity. Thus, myotubes appeared to express more N-CAM per unit area than myoblasts. This distinction became more pronounced as the myotubes became larger and more differentiated (Fig. 1, *g* and *h*). The immunostaining was distributed over the entire myotube's surface with no observable areas of specialization or local concentration. The residual myoblasts in the differentiated cultures still expressed N-CAM but less than myotubes. These results show that in this model system all muscle cells express N-CAM at the cell surface but that myotubes exhibit an apparent enhanced expression. This profile of expression of N-CAM in G8-1 cells was not peculiar to these cells, as we found similar results with C2 cells (data not shown).

To further characterize the apparent differences in N-CAM levels between myoblasts and myotubes a quantitative dot blot ELISA assay for N-CAM was developed to measure the binding of mAb H28 to G8-1 cell extracts adsorbed to nitrocellulose. In addition, at each time point in culture, two independent indices of myogenesis were studied, namely CK activity and AchR levels. These parameters serve as internal indices for fusion and myotube formation in the cell cultures, as it has been found that with fusion both CK and AchR levels rise in muscle cultures in parallel (25). Graphs of N-CAM immunoreactivity versus protein concentration in G8-1 and C2 cells at different times of culture were drawn to determine the linear region of the curve (not shown). N-CAM relative abundance was calculated and an attempt was then made to correlate the increase in N-CAM in these cultures with CK and AchR levels. Fig. 2 shows a comparison of N-CAM levels with those of CK and AchR in G8-1 and C2 cells during differentiation. CK and AchR levels are low in G8-1 and C2 myoblasts, but, as shown previously (25), concomitant with the appearance of myotubes the level of these markers increases in parallel and continues to do so at each of the further time points analyzed. N-CAM levels do not increase with the same kinetics as CK and AchR. Fig. 2 *a* shows the rise in N-CAM levels in G8-1 and Fig. 2 *b* shows the levels in C2 cells. Both these cell lines, which fuse with different kinetics, behave in the same way. N-CAM levels are low but above background levels at the first time point. The N-CAM levels rise rapidly with time in culture and the main phase of increase precedes CK and AchR increases. This was found for both G8-1 and C2 cells. Thus, the rise in N-CAM levels is not linked to the coordinate activation of CK and AchR.

N-CAM shares some properties with the AchR in muscle with respect to its pattern of expression and location in muscle in vivo and in its response to denervation and paralysis (6, 26, 27, 32). One feature of the expression of the AchR in vitro is that it can be regulated by membrane activity (22) and hormones (35). We therefore wished to determine whether these factors could influence N-CAM levels. We grew G8-1 and C2 cells for 4 d in 0.5 μg/ml tetrodotoxin to alter muscle membrane activity or hormone treated with 0.89 μg/ml T4. In three independent experiments we were unable to show any statistically significant change in N-CAM levels with the tetrodotoxin or T4 treatment, suggesting that in addition to showing different kinetics of accumulation to AchR, N-CAM does not appear to be modulated by some factors that regulate AchR.

N-CAM Isoform Changes during Myogenesis

Immunoblotting was used to analyze the changes in N-CAM protein forms during myogenesis. Fig. 3, *a* and *b* shows the result of immunostaining of cell extracts of G8-1 and C2 cells

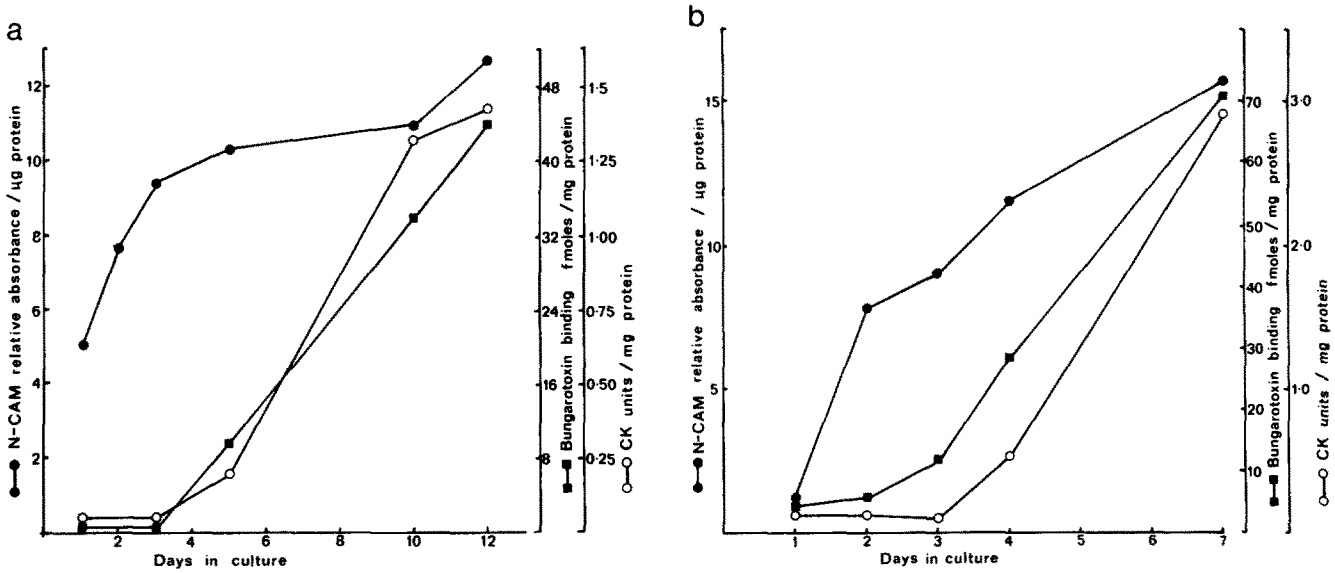

Figure 2. Comparison of the kinetics of accumulation of N-CAM in cultures of G8-1 (*a*) and C2 (*b*) cells with CK and AchR. Graph shows the rate of N-CAM (*solid circles*), CK (*open circles*), or AchR (*squares*) accumulation with time of culture.

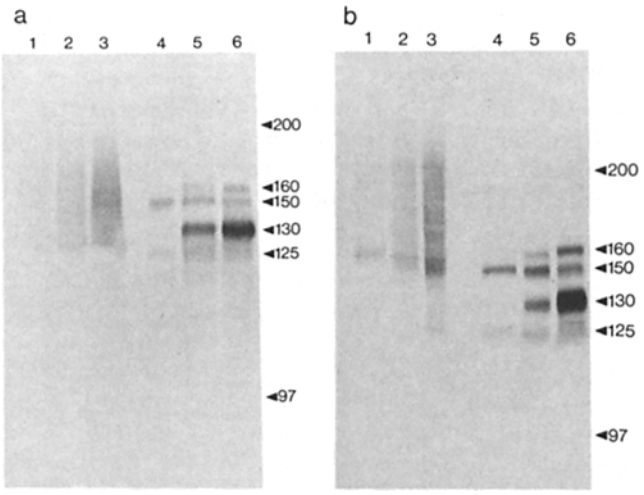

Figure 3. Immunoblot analysis of N-CAM in cell cultures of G8-1 (*a*) and C2 (*b*) cells. Three time points were chosen for each cell line, namely prefusion (lanes *1* and *4*), midfusion (lanes *2* and *5*), and postfusion (lanes *3* and *6*). Samples were harvested from culture dishes and extracted with NP-40 and 50 μg of untreated (lanes *1–3*) or neuraminidase-treated sample (lanes *4–6*) added to the gel. After electrophoresis protein was transferred to nitrocellulose and reacted with anti-N-CAM. Molecular mass markers are shown to the right of each blot and are given in kilodaltons.

during myogenesis. 50 μg of NP-40–extracted cellular protein from each time point was added per gel track and stained with rabbit anti-N-CAM. As in previous studies there were low levels of N-CAM in myoblasts (Fig. 3, *a* and *b*, lanes *1*), but a faint band of ~150 kD could be seen. As time progressed the amount of this band increased, as did a diffuse band extending up to ~220 kD. As previously shown (7), there was no observable shift in the spread of this band with time in culture. Neuraminidase treatment was used to generate desialo N-CAM that can be resolved into discrete bands on polyacrylamide gels. Fig. 3, *a* and *b*, lanes *4–6*, shows the results for G8-1 and C2 cells. For C2 cells these data can be compared with those reported previously (see Fig. 3, lanes *11* and *12* in reference 7). Fig. 3 *b*, lane *6*, shows four protein bands of 120, 125, 145, and 155 kD present in a ratio of 1:9:2:2. On long exposures a minor band of 180–190 kD was sometimes found in cultures, but the significance of this is not clear at present. The major band of 125 kD corresponds to the previously described 125-kD band and the band of 145 kD corresponds to the previously described 140-kD band (7).

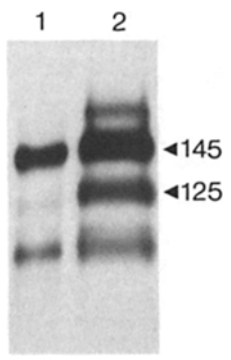

Figure 4. Biosynthetic labeling of N-CAM in G8-1 myoblasts and myotubes. G8-1 myoblasts or myotubes were metabolically labeled with [^{35}S]-methionine, the cells solubilized with NP-40, and N-CAM–reactive polypeptides isolated. Samples were then treated with neuraminidase and separated on polyacrylamide gels. Lanes *1* and *2* show [^{35}S]methionine-labeled immunoprecipitates from (lane *1*) myoblasts and (lane *2*) myotubes. Molecular mass markers are given in kilodaltons.

A similar banding pattern was found in cultures on G8-1 myotubes (Fig. 3 *a*, lane *6*). The first myoblast time point mainly shows the 145-kD band in both G8-1 and C2 cells (Fig. 3, *a* and *b*, lane *4*) with small amounts of a 120-kD band. The levels of these two bands do not change appreciably in culture. However, there is a characteristic increase during myogenesis in the 125- and 155-kD bands during the second (Fig. 3, *a* and *b*, lane *5*) and third (Fig. 3, *a* and *b*, lane *6*) time points. Thus, myogenesis in G8-1 and C2 cells is accompanied by the appearance of 125- and 155-kD bands. Little change was found in the level of the 145- and 120-kD bands. Similar results were found if we used mAb H28 to study N-CAM protein forms.

Biosynthesis and Posttranslational Modifications of N-CAM in G8-1 Cells

The immunoblot study was unable to resolve the question of the relationship between the four desialo N-CAM bands shown above. To further address this question we analyzed aspects of N-CAM biosynthesis and processing. First, G8-1 cells at myoblast and myotube stages of growth were metabolically labeled with [^{35}S]methionine and afterwards N-CAM polypeptides immunoisolated from NP-40–extracted cells. Fig. 4 (lanes *1* and *2*) shows the N-CAM polypeptides found in immunoprecipitates of G8-1 myoblasts and myotubes after neuraminidase treatment. The profile observed is very similar to that found on the Western blots. Confluent myoblasts predominantly express a band of 145 kD with an additional less intense band at 120 kD. Myotubes also express these bands, and, in addition, bands at 125 and 155 kD. A phosphorylation study was carried out to further analyze the relationship between isoforms. Neuronal N-CAM polypeptides of 180 and 140 kD but not of 120 kD have been shown to be phosphorylated (12, 23, 34). We therefore metabolically labeled myoblasts and myotubes with [^{32}P]inorganic phosphate in the presence or absence of tunicamycin and then immunoisolated N-CAM polypeptides. Fig. 5 (lane *1*) shows that the major N-CAM isoform of 145 kD found in myoblasts incorporates [^{32}P]inorganic phosphate while the additional minor bands do not. This result suggests that the 145-kD N-CAM isoform is a transmembrane protein, as [^{32}P]inorganic phosphate is believed to be added to the cytoplasmic domains of membrane proteins (12). The 145-kD phosphor-

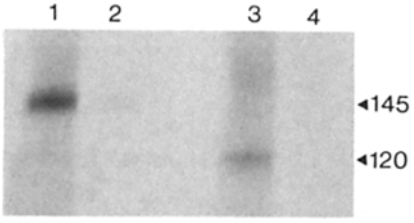

Figure 5. Phosphorylation of N-CAM polypeptides in G8-1 muscle cultures and the effect of tunicamycin. G8-1 myoblasts and myotubes were grown in the presence of [^{32}P]inorganic phosphate in the absence or presence of tunicamycin, the cells solubilized with NP-40, and N-CAM–reactive polypeptides isolated. Samples were then treated with neuraminidase and separated on polyacrylamide gels. Lanes *1–4* show [^{32}P]inorganic phosphate samples from (lane *1*) myoblasts or (lane *2*) myotubes. Lane *3* shows tunicamycin-treated samples from myoblasts or (lane *4*) myotubes. Molecular mass markers are given in kilodaltons.

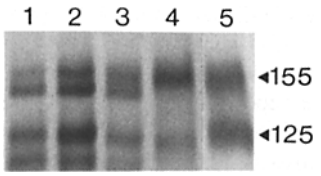

Figure 6. Pulse-chase analysis of N-CAM in G8-1 cells and effect of phospholipase C on release of labeled proteins. G8-1 myotube cells were pulse-labeled for 20 min with [^{35}S]methionine and chased with unlabeled media for various periods of time. Samples were then solubilized with NP-40 and N-CAM–reactive polypeptides isolated. Lane *1* shows N-CAM polypeptides after a 20-min pulse and lanes *2–4* after 10 (lane 2), 30 (lane *3*), and 45 min (lane *4*) of chase. Lane *5* shows the effect of phospholipase C on release of N-CAM proteins from myotubes. G8-1 cells were labeled with [^{35}S]methionine and the culture treated with phospholipase C for 1 h at 37°C. The supernatant was collected, N-CAM polypeptides immunoisolated, and samples were neuraminidase treated. Molecular mass markers are given in kilodaltons.

ylated N-CAM isoform also appears to be down regulated with fusion, as only very low levels are found in myotubes (Fig. 5, lane *2*). Tunicamycin treatment (Fig. 5, lanes *3* and *4*) of ^{32}P-labeled cultures showed that the phosphoprotein band of 145 kD in myoblasts shifts down in molecular mass to 120–125 kD, suggesting that the label is incorporated on amino acids rather than carbohydrate. As this 120–125-kD phosphorylated band is only found in myoblasts and is not present in myotubes, the major desialo N-CAM transcript in myoblasts is probably a transmembrane isoform that can be phosphorylated and is down regulated with fusion. It is, however, not possible to rule out that this apparent down regulation could also be a consequence of differential phosphorylation. The presence of a transmembrane N-CAM isoform in myoblasts is also consistent with other studies that show that myoblasts express a 6.7-kb mRNA species (7, 10) that contains the coding potential to generate a transmembrane isoform (10). In an attempt to further analyze the biosynthesis of N-CAM isoforms pulse-chase experiments were carried out. Cultures of G8-1 myotubes were pulsed with [^{35}S]methionine for 20 min and then chased for up to 90 min. Fig. 6 shows the four main N-CAM bands found in [^{35}S]methionine-labeled myotubes. After a chase of 30 min the bands of 120 and 145 kD appear to decrease in intensity. By 45 min these bands have disappeared and the 120-kD band appears to be chased into the 125-kD band and the 145-kD band is chased into the 155-kD band (Fig. 6). It is likely that the major desialo isoforms of myotubes are of 125 and 155 kD and that the bands of 120 and 145 kD are processing intermediates. This result also shows that the myoblast 145 kD isoform co-migrates with an additional intermediate destined to become a 155-kD isoform. Further proof that the 125- and 155-kD isoforms are expressed at the cell surface came from experiments that showed they could be released from the cell surface by phospholipase C. The brain N-CAM isoform of 120 kD, but not the 140- or 180-kD isoforms, has recently been shown to be nontransmembrane and bound to the membrane via linkage through phosphatidylinositol (18). G8-1 myoblasts and myotubes in culture were labeled with [^{35}S]methionine and cells treated with phospholipase C. Released N-CAM was then immunoisolated. Under the con-

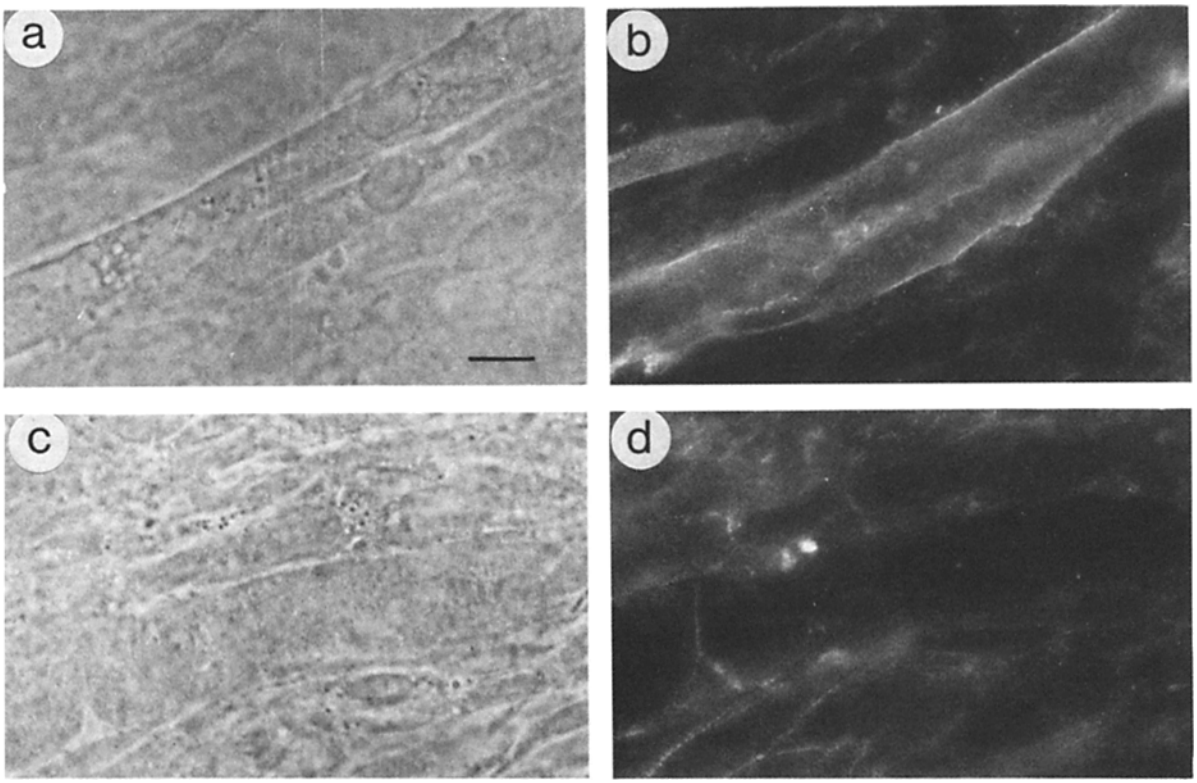

Figure 7. Effect of phosphatidylinositol-specific phospholipase C on cell surface N-CAM. Indirect immunofluorescence was used to analyze the expression of N-CAM in the absence and presence of the enzyme. (*a* and *c*) Phase-contrast views focused to highlight the myotubes that sit above the cell monolayer; (*b* and *d*) the same cells stained with anti–N-CAM in the absence (*b*) or presence (*d*) of phosphatidylinositol-specific phospholipase C. Bar, 50 μm.

ditions of assay, cells were not detached from the culture dish by enzyme treatment. Fig. 6 (lane *5*) shows that two N-CAM polypeptides of 125 and 155 kD are released from the cell membrane by phospholipase C in myotubes. No N-CAM polypeptides were detected in media after similarly labeled cells were incubated in the absence of phospholipase C. These data show that two individual N-CAM isoforms are specifically released from the myotube membrane by phospholipase C with no evidence of bands of 120 and 145 kD, suggesting that these intermediates in myotubes are not exposed at the cell surface, and, as shown above, are likely to be cytoplasmic processing intermediates.

Phosphatidylinositol-specific Phospholipase C Specifically Releases N-CAM from Myotubes

We showed above that phospholipase C specifically released N-CAM bands of 125 and 155 kD from myotube cultures. To determine whether this release of N-CAM was specific to myotubes, as may be expected from the size of the released N-CAM proteins, we carried out an indirect immunofluorescence analysis of myotube cultures for N-CAM in the absence and presence of phosphatidylinositol-specific phospholipase C. Fig. 7, *a* and *b* shows the staining profile of a C2 myotube culture reacted with N-CAM antibody and is very similar to the profile shown in Fig. 1; both myoblasts and myotubes are strongly stained. Myotube cultures that had been treated with phosphatidylinositol-specific phospholipase C gave a dramatically different result (Fig. 7, *c* and *d*). Here we found that N-CAM immunoreactivity was removed from the myotube membrane while the staining intensity on myoblasts was little changed. Taken together with the results shown above, we conclude that the 125- and 155-kD N-CAM isoforms are linked to the membrane via phosphatidylinositol linkage and that this occurs specifically in myotubes but not in myoblasts.

Northern Blot Analysis of N-CAM in G8-1 Cells

Fig. 8 shows a Northern blot analysis of poly(A)$^+$ RNA from G8-1 cells at four different stages of development in culture probed with the mouse brain N-CAM probe pM1.3 (16). G8-1 myoblasts express two N-CAM hybridizing bands only of 6.7 and 5.2 kb and at no timepoint did we find only the 6.7-kb band. It is not until the second time point that the 2.9-kb band is seen. The level of the 2.9-kb band continues to rise through the third and fourth time point and is the major N-CAM mRNA at the fourth time point. Myogenesis in G8-1 cells is also accompanied by the down regulation of the 6.7-kb band. Thus, myogenesis is accompanied by activation of the 2.9-kb band, a down regulation of the 6.7-kb band, and a relative increase in the 5.2-kb band. There is also a slight but reproducible increase in apparent size of the 5.2-kb band during myogenesis. Whether this means there is heterogeneity in the 5.2-kb band due to alternative splicing remains to be determined.

To quantitate accurately the relative changes in the various N-CAM mRNA species, Northern blot autoradiographs were scanned after hybridization with the pM1.3 probe. In addition, the blot was stripped and rehybridized with a cDNA probe to GPDH (24) (Fig. 8 *b*). The GPDH mRNA does not change during myogenesis (13) and can therefore be used as an internal standard to quantitate N-CAM mRNAs. CK levels in the cultures were also measured to allow the kinetics of fusion to be followed. Fig. 9 shows the changes in various N-CAM mRNAs after normalizing to GPDH levels. The broad changes shown in Fig. 7 are reproduced here. Myogenesis, as indexed by increases in CK, is accompanied by a down regulation of the 6.7-kb band, an up regulation of the 2.9-kb band, and an increase in the 5.2-kb band. Interestingly, the temporal expression of the 2.9-kb band follows the rise in CK, whereas the 5.2-kb band does not. The rise in the 5.2-kb band does, however, parallel the profile of total N-CAM protein accumulation in the culture.

Analysis of mRNA from C2 cells gave essentially the same results as those found with the G8-1 cells with respect to the molecular size of mRNAs and their alteration with differentiation in culture (data not shown). This is in contrast to a previous study using total RNA where no hybridizing bands were detected in C2 myoblasts (7).

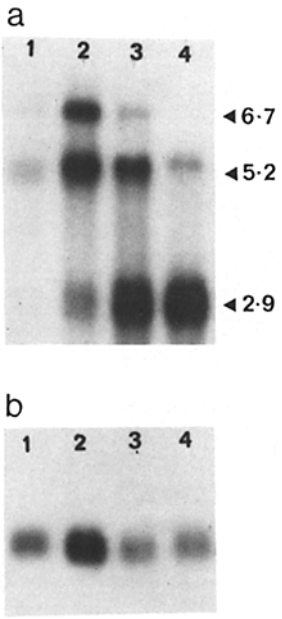

Figure 8. Northern blot analysis of N-CAM mRNA during myogenesis of G8-1 cells. G8-1 cells were grown for (lane *1*) 2, (lane *2*) 3, (lane *3*) 6 , and (lane *4*) 10 d of culture, harvested, and mRNA extracted. mRNA samples were processed for Northern blot analysis and hybridized to the N-CAM cDNA probe pM1.3. Filters were autoradiographed at −70°C in the presence of intensifying screens. (*b*) The Northern blot from *a* was stripped of hybridized N-CAM probe and rehybridized with a GPDH probe recognizing a transcript of invariant abundance during myogenesis to allow accurate relative quantitation of total poly (A)$^+$ RNA per gel track.

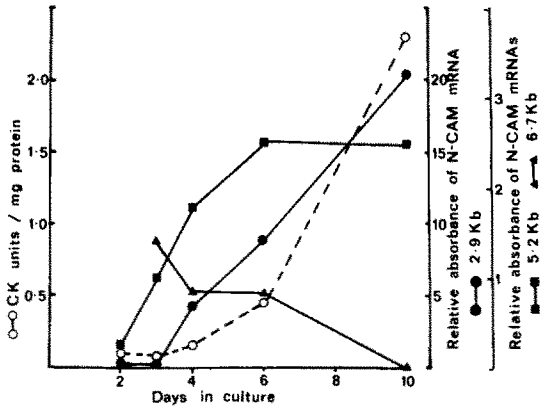

Figure 9. Quantitation of N-CAM mRNA bands during myogenesis of G8-1 cells. Autoradiographs of Northern blots were scanned on a densitometer after probing with N-CAM or GPDH probes. The N-CAM hybridizing bands at 6.7 (*triangles*), 5.2 (*squares*), and 2.9 kb (*solid circles*) were normalized against the GPDH band. The graph is plotted as relative N-CAM levels versus time in culture for each of the mRNA bands. These graphs are compared with CK levels (*open circles*) in the same cultures.

Discussion

A number of studies have now shown N-CAM to be expressed by skeletal muscle (6–9, 17, 26, 27, 30, 32, 33, 38, 43, 44, 46). During muscle development N-CAM is present at the cell membrane of growing myofibers, but as development proceeds it is down-regulated and adult myofibers do not express N-CAM (8, 26, 28). N-CAM is, however, still concentrated at the neuromuscular junction in adults (6, 26, 32). Several experimental or pathological states can cause N-CAM to be expressed at the sarcolemma. For instance, denervation or nerve crush cause rapid increases in N-CAM transcription in muscle (6, 27, 32), and similar effects are found by blocking muscle activity by tetrodotoxin (6) or botulinum toxin (27). Studies on experimental muscle regeneration (Moore, S. E., and F. S. Walsh, unpublished observations) or human disease (43) where regeneration is part of the pathology, show N-CAM–positive fibers indicating that N-CAM expression is a correlate of the program of muscle regeneration.

N-CAM protein expression has now been studied in muscle cell cultures (7, 8, 26, 30, 46), and one study has reported on N-CAM–related mRNA species in culture (7). One of the difficulties in comparing the above studies is that different cell types, both primary and cell lines, have been used; another is that different stages of development and different analytical methods have been used to analyze N-CAM expression. The most detailed study (7) has shown that there are clear changes in N-CAM polypeptides during myogenesis in vitro. Myoblasts predominantly express a 140-kD polypeptide, while myotubes predominantly express a 125-kD polypeptide. Analysis of Northern blots using a mouse N-CAM cDNA probe showed that there were three N-CAM–related bands of 6.7, 5.2, and 2.9 kb, and of these, the 6.7-kb band predominates in myoblasts, while the 5.2- and 2.9-kb bands predominate in myotubes. cDNA clones encoding human muscle N-CAM have recently been isolated by us (10, 45) and additional brain clones have been isolated by others (16, 19, 28, 29). We identified a cDNA clone that encodes a transmembrane N-CAM isoform and an alternatively spliced version of this that encoded nontransmembrane isoforms. The transmembrane isoform is encoded by the 6.7-kb mRNA (10), and these data plus the present studies strongly suggest that the 145-kD isoform is the protein product of the 6.7-kb mRNA. The 6.7-kb muscle transcript shares extensive sequence homology with the 140-kD brain N-CAM isoform and it remains to be determined by what mechanism arises the slight increase in size of this isoform versus the brain homologue. mRNA species of 5.2 and 4.3 kb in human muscle and 5.2 and 2.9 kb in G8-1 cells encode nontransmembrane isoforms, and, via alternative splicing (40), these transcripts contain a muscle-specific N-CAM sequence of 37 amino acids in their extracellular region (10). As the 5.2- and 2.9-kb mRNAs accumulate predominantly in myotubes it seems likely that the 125- and 155-kD isoforms are their protein products. It is, however, not yet possible to definitely state which mRNA encodes which protein.

In primary human cell cultures we showed (26) that N-CAM was present on myoblasts and co-distributed with other myoblast cell surface antigens. Similar conclusions have been made from analyses carried out on primary chicken and rat muscle cell cultures (8, 17). We show here that mouse muscle cell lines behave in a manner similar in most respects to the primary cultures. All mononucleate cycling myoblasts were found to be N-CAM positive. One of the striking features of the expression of N-CAM in primary muscle cultures and cell lines is the increased intensity of staining in well differentiated cultures. This qualitative impression has been quantitated for the first time and we found almost a 4-fold increase in N-CAM levels in G8-1 cells and a 15-fold increase in C2 cells during myogenesis. These two cell lines grow differently in culture. G8-1 cells fuse with much slower kinetics and are not fully mature until ~12 d after plating, while C2 cells are maximally fused after only 7 d. The process of activation of N-CAM is, however, the same in the two cell lines in that the main rise in N-CAM levels precedes fusion. This was found by comparing N-CAM with two other independent markers of myogenesis, namely the cytoplasmic enzyme CK and cell membrane AchR. The AchR and N-CAM have a number of properties in common. These include accumulation, albeit topographically distinct, at the neuromuscular junction (6, 27, 32) and their coregulation on myofibers by innervation status (6, 9, 27). However, they differ in that N-CAM is present on cycling myoblasts (8, 17, 26), and we show here that the activation of N-CAM during in vitro myogenesis precedes AchR. It is not known which cells are accumulating greater amounts of N-CAM before fusion. Some possibilities are that either committed myoblasts whose numbers are likely to increase before fusion, or, alternatively, cell confluency may play a role such that as the number of cells in the culture increased there was an increase in N-CAM levels per cell. Further work will be required to distinguish between these or other possibilities. One additional difference between N-CAM and AchR is their ability to be regulated in culture by certain exogenously added agents such as tetrodotoxin (22) and thyroid hormones (35), but we were unable to show any changes in N-CAM levels with these agents.

Western blotting and biosynthesis studies were used to study N-CAM proteins during myogenesis. The Western blots showed an extremely polydisperse pattern for N-CAM, and it is not until after neuraminidase treatment that specific bands are observed. The banding pattern found after neuraminidase treatment was identical for G8-1 and C2 cells. Myoblasts expressed a band of 145 kD and small amounts of a 120-kD protein. These stayed effectively constant throughout the culture period but bands of 125 and 155 kD appeared. In myotube cultures the major band present was 125 kD (65% of total). Thus, at the protein level muscle development in culture is accompanied by the activation of 125- and 155-kD isoforms. The major problem with the Western blot analysis is that it shows the total cell-associated N-CAM only, and does not show which isoforms are expressed at the cell surface or whether one band is a precursor of another. To resolve this question, biosynthetic experiments were carried out after [^{32}P]inorganic phosphate or [^{35}S]methionine labeling. The major N-CAM protein present in myoblasts is of 145 kD and is likely to be transmembrane since it can be phosphorylated and myoblasts specifically express a 6.7-kb mRNA that encodes a transmembrane isoform (10). This isoform is down-regulated with fusion and does not appear in myotubes. Myotubes express two isoforms of 125 and 155 kD that are bound to the plasma membrane via phosphatidylinositol linkage and are therefore unlikely to be transmembrane. The

data from immunoprecipitates of [^{32}P]inorganic phosphate–labeled samples showed clearly that the 145-kD transmembrane isoform was down-regulated with fusion, yet by Western analysis and biosynthesis a strong band appeared at 145 kD in myotubes. Pulse-chase experiments allowed us to conclude that this band in myotubes and an additional band of 120 kD were precursors of 155- and 125-kD isoforms. It is likely that the 145- and 120-kD intermediates are not expressed at the cell surface as they could not be released from the cell membrane by phospholipase C, whereas the mature products were. Thus, myotubes are the first cell type to be identified that have two distinct N-CAM isoforms that are phosphatidylinositol linked. The only other N-CAM isoform to be identified that is phosphatidylinositol linked is the 120-kD isoform expressed by glial cells in culture (18). However, glial cells also synthesize an N-CAM isoform of 140-kD that is transmembrane. We showed that phospholipase C action could completely clear the myotube membrane of N-CAM, whereas in glial cells it is likely that the 140-kD isoform would remain. It has been suggested (18) that the linkage of N-CAM to the plasma membrane via phosphatidylinositol could provide a mechanism for rapid release of cell–cell bonds. Whether this mechanism plays a role in the formation and innervation of skeletal muscle remains to be determined.

In addition to the changes found in N-CAM levels and protein forms, Northern blot analysis showed changes in the expression of N-CAM mRNAs. The results of this study differed in two ways from the results of Covault et al. (7). First, the earliest time point studied in culture showed the presence of 6.7- and 5.2-kb N-CAM hybridizing bands, and, second, the 6.7-kb band was totally down-regulated in late myotube cultures. The common feature between the two studies is that a 2.9-kb mRNA species is up-regulated during myogenesis. Interestingly, this RNA species is activated with the same kinetics as CK, whereas the total cell-associated N-CAM immunoreactivity increases with different kinetics. We also found that the 6.7-kb band was completely down-regulated with fusion, suggesting that this is a myoblast-specific transcript.

A detailed picture is now available of N-CAM expression in skeletal muscle using a combination of whole animal and culture models, and a variety of biochemical assays. Although a number of roles have been suggested for N-CAM's participation in muscle functions, such as fusion, synaptogenesis, and histogenesis, there is little in the way of compelling evidence to support these hypotheses. There is no doubt that as a result of detailed molecular studies N-CAM seems to be an ideal candidate to play an important role in muscle because of its location and the qualitative and quantitative changes found in its expression. In addition, through alternative splicing, we have shown (10) that there are specific sequences expressed in the extracellular region of N-CAM isoforms found in myotubes identifying one additional mechanism for generating structural diversity in this molecule. However a variety of assay systems will have to be tested and more specific antibodies generated before conclusions can be made. Initial attempts to verify the proposed role of N-CAM in nerve–muscle interactions (33) have not been positive (3) and further studies are clearly required.

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