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Neural cell adhesion molecule (NCAM) marks adult myogenic cells committed to differentiation

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Abstract

Although recent advances in broad-scale gene expression analysis have dramatically increased our knowledge of the repertoire of mRNAs present in multiple cell types, it has become increasingly clear that examination of the expression, localization, and associations of the encoded proteins will be critical for determining their functional significance. In particular, many signaling receptors, transducers, and effectors have been proposed to act in higher-order complexes associated with physically distinct areas of the plasma membrane. Adult muscle stem cells (satellite cells) must, upon injury, respond appropriately to a wide range of extracellular stimuli: the role of such signaling scaffolds is therefore a potentially important area of inquiry. To address this question, we first isolated detergent-resistant membrane fractions from primary satellite cells, then analyzed their component proteins using liquid chromatography-tandem mass spectrometry.

Transmembrane and juxtamembrane components of adhesion-mediated signaling pathways made up the largest group of identified proteins; in particular, neural cell adhesion molecule (NCAM), a multifunctional cell-surface protein that has previously been associated with muscle regeneration, was significant. Immunohistochemical analysis revealed that not only is NCAM localized to discrete areas of the plasma membrane, it is also a very early marker of commitment to terminal differentiation. Using flow cytometry, we have sorted physically homogeneous myogenic cultures into proliferating and differentiating fractions based solely upon NCAM expression.

Keywords

satellite cell; adult myogenesis; myogenic differentiation; neural cell adhesion molecule; muscle regeneration; membrane raft

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INTRODUCTION

Skeletal muscle is a terminally differentiated tissue consisting of ordered arrays of multinucleated, contractile myofibers. In vertebrates, skeletal muscle is formed during fetal and postnatal development by differentiation of previously specified myoblasts, which irreversibly exit the cell cycle and become committed myocytes. This transition is accompanied by changes in gene expression, growth factor responsiveness and structural protein production (reviewed in [1]). Upon differentiation myocytes subsequently fuse with each other or with existing myotubes to produce contractile syncytial myofibers [2].

In adult vertebrates myogenesis is believed to be primarily carried out by satellite cells, the somatic stem cells responsible for *in vivo* maintenance and regeneration of skeletal muscle tissue [3, 4]. These cells, which comprise a very small (1-6%) fraction of total muscle-associated nuclei, are defined anatomically by their position between the basement membrane and the sarcolemma of differentiated muscle fibers [3, 5, 6]. In response to injury, otherwise mitotically quiescent satellite cells become activated and proliferate extensively. The resulting population of adult myoblasts will then transit to the site of injury and differentiate into myocytes to replace the damaged myofibers, either by fusion with each other to form new muscle fibers or by fusing into existing post-mitotic muscle fibers [7, 8]. While the satellite cell compartment is repopulated following completion of a cycle of acute regeneration, it remains unclear what the exact cellular source(s) of these new quiescent cells may be: evidence exists for satellite cell self-renewal, either by asymmetric division [9] or stochastic events [10], as well as possible contributions from muscle-associated mesenchymal stem cell populations [9, 11].

The extracellular milieu encountered by newly-activated satellite cells requires that they detect and respond appropriately to a diverse array of rapidly changing stimuli. In addition to the damaged host muscle, local signaling sources would include coincidentally damaged connective tissue, vasculature and nervous tissue, as well as infiltrating cells of the immune system [3]. Local extracellular signals would also be expected to vary with time after the initial injury. Thus, critical roles have been demonstrated for many soluble factors and matrix/adhesion molecules in the muscle tissue during satellite cell-mediated muscle repair [12-14], and there is a significant amount of ongoing investigation into the signaling pathways that function in satellite cells during regeneration.

An area that has not yet been addressed with respect to satellite cell signaling is the possible involvement of higher-order signaling complexes, such as those that have been proposed to assemble in membrane 'rafts'. Membrane rafts, also known as lipid rafts, are small (10-200 nm), cholesterol and sphingolipid enriched membranes that function to compartmentalize cellular processes [15, 16]. These regions of the plasma membrane play important roles in intracellular protein transport, membrane fusion and transcytosis; they have also been proposed to act as platforms for assembly of signaling complexes, cell surface antigens and adhesion molecules. Cellular events commonly associated with membrane raft complexes include cell activation, polarization and signaling [17, 18]. In other adult stem cells (i.e., hematopoietic stem cells) membrane rafts are critical for cell cycle regulation and survival [19, 20], however very little is known about signaling pathways mediated by membrane rafts in satellite cells.

In this study, we attempted to isolate and characterize plasma membrane proteins expressed by primary mouse satellite cells, with the goal of prospectively identifying additional signaling pathways that may impinge upon satellite cell activity. Using liquid chromatography-tandem mass spectrometry, we identified classes of transmembrane and membrane-associated proteins present in freshly isolated murine satellite cells and enriched

in detergent-resistant membrane domains. While surprisingly few of the expected transmembrane receptors were detected above the reliability threshold, multiple proteins associated with adhesion-mediated signaling were identified. Several have not previously been connected with myogenesis, although many have; a significant subset have also been reported to act via membrane raft complexes. One such protein, neural cell adhesion molecule (NCAM), was found to be present and enriched in the detergent-resistant membrane fraction, and was selected for further study.

When examined in heterogeneous populations of adult myoblasts and myocytes, we found NCAM expression to be coincident with the earliest detectable markers of commitment to differentiation. In order to unequivocally differentiate between proliferation-competent myoblasts and committed myocytes, it is common to assay for expression of differentiation-associated proteins such as myogenin, p21, or muscle structural proteins. However, all of these proteins are cytoplasmic or nuclear, and it would be desirable to determine cell status by assaying for a cell-surface epitope, allowing analysis of living unfixed cells. To date, no such marker has been reported. Here we show that by indirect immunohistochemistry, NCAM labels only nonproliferating cells that, based on their expression of differentiation markers including myogenin and muscle creatine kinase have committed to differentiation. We also use NCAM expression to separate a heterogeneous population of adult myoblasts into proliferating and differentiated fractions, as confirmed by expression of either proliferation or pro-myogenesis proteins. This molecular tool therefore represents a novel, non-terminal assay for the early identification and sorting of committed myocytes from heterogeneous populations derived from primary mouse satellite cells.

MATERIALS AND METHODS

Primary Satellite cell isolation and culture

Mouse satellite cells were isolated and cultured as described previously [21]. Briefly, muscle was dissected from the hind limbs of adult female mice (B6D2F1; Jackson labs) between 80 and 130 d of age. Muscles from both legs were minced and digested in 400 U/ml collagenase type I (Worthington) diluted in Ham's F-12 medium (Invitrogen). The resulting cells were collected and pre-plated on gelatin-coated (0.66%) petri dishes in growth medium [Ham's F-12 (Gibco), 15% horse serum (Equitech) and penicillin/streptomycin (Gibco) supplemented with 0.5 nM rhFGF-2.] After 24 hrs, non-adherent cells (consisting primarily of myoblasts and residual red blood cells) were collected and replated on new gelatin-coated plates in growth medium supplemented with FGF-2; the original plates with the adherent cells (primarily fibroblasts) were discarded. After 72 hours total, the myoblasts had become adherent; they were washed and taken up with warmed PBS then replated as appropriate: cells to be expanded for an additional 2 days were replated on new gelatin-coated plates in growth medium supplemented with FGF2, and cells to be used for immunofluorescent staining were plated on glass coverslips coated with a thin layer of gelatin in growth or differentiation media as specified. Differentiation medium consisted of F-12 K (Gibco), 2% horse serum and penicillin/streptomycin.

Detergent resistant membrane fractionation

Primary satellite cells were isolated and cultured as above for 6 days in growth medium supplemented with FGF-2. The resulting subconfluent cells were washed with PBS, dissociated using collagenase, and harvested by centrifugation. The cell pellet was resuspended in 1 ml MNE (25 mM MES pH 6.5, 150 mM NaCl, 5 mM EDTA) containing 0.2% (v/v) Triton X-100 and 1x Roche Complete Protease Inhibitors. Cells were lysed with 10 strokes of a chilled Dounce homogenizer on ice then mixed with an equal volume (1 ml) of 80% (w/v) sucrose in MNE and placed into the bottom of an ultracentrifuge tube. Cell

lysates were overlaid with 2 ml of 30% (w/v) sucrose followed by 1 ml of 5% (w/v) sucrose in MNE and ultracentrifuged at 38,800 rpm in a Sorvall TH-660 rotor for 18 hours at 4°C. Fractions of 550 µl were collected from top to bottom and designated 1-9. Fraction(s) containing visible raft material were noted and processed for further analysis.

Liquid chromatography-tandem mass spectrometry, sample preparation and data acquisition

The protein concentration of each fraction was measured using a Pierce BCA assay. Protein samples (40 µg) in 80% acetone were incubated at -20°C for 2 hours then centrifuged for 20 minutes at 13,000rpm at 4°C. Acetone was removed and the pellet was dried. Protein pellets were re-suspended in 20 µL of buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris-HCl pH 8.0. Cysteines were reduced and alkylated with 10 mM DTT (100 mM stock in Tris-HCl pH 8.0), and 40 mM IAA (200 mM stock in 100mM Tris-HCl pH 8.0) respectively, using 1 hour incubations at RT for each. Remaining IAA was neutralized with an addition of DTT to 30mM. Urea concentrations for each sample were adjusted to 1 M with 100 mM Tris-HCl pH 8.0. Digestion was performed with 8.0 µL of a 0.2 µg/mL solution of sequencing grade-modified trypsin (Promega, Madison WI) in 50mM acetic acid to get a 1:25(w/w) ratio of trypsin to sample protein. The pH was adjusted to neutrality with 100mM Tris pH 8.0, and the mix was then incubated at 37°C for 12 hours. Tryptic digests were lyophilized, and reconstituted in 30 µL 0.1% formic acid in water. Samples were analyzed on an LTQ ProteomeX linear ion trap LC-MS/MS instrument (Thermo Fisher, San Jose, CA). Sample was loaded onto a C18 packed nanospray column and eluted using an acetonitrile gradient (0-90% in 120 min). LC separation was performed using fused silica nanospray needles (360 mm outer diameter, 150 mm inner diameter; Polymicro Technologies), packed with “Magic C18” (100Å, 5 mm particles; Michrom Bioresources) in 100% methanol. Samples were analyzed in the data-dependent positive acquisition mode. Following each full scan (400–2000 *m/z*), a data-dependent MS/MS scan for the three most intense parent ions was acquired. The nanospray column was held at ion sprays of 3.1 kV and a flow rate of 100 nL/min.

Database searching and protein identification

The National Center for Biotechnology Information (NCBI; <ftp.ncbi.nih.gov/blast/>) non-redundant protein database (as of June 2007) was used for querying all data. The FASTA database utilities and indexer of the BioWorks Rev 3.3, software was used to create a mouse database (keywords *Mus musculus*, and “mouse”) from the NCBI non-redundant database and to index it for trypsin cleavage with cysteine modified by carboxyamidomethylation (+57Da). Search parameters were set to include oxidation of methionine (+16) as a variable modification. Protein hits were filtered with the following criteria: peptide probability less than 0.001, XCorr values greater than 1.5, 2.0 and 2.5 for +1, +2, and +3 charged ions, respectively.

Immunohistochemistry

Primary satellite cells were amplified in culture under growth conditions for 4 days then plated on gelatin-coated glass coverslips. In time course studies cells were then cultured for 0, 12, 24, 48 or 72 h in differentiation medium; BrdU was added to a final concentration of 0.01 mM for 4 hrs before fixation and staining. Cells were fixed in 4% paraformaldehyde for 10 min at RT and blocked in 10% goat serum containing 1% NP-40. If anti-syndecan-4 was to be used an additional block in 10% Blokhen (Aves) was used. To detect BrdU incorporation, fixed cells were treated with 4 M hydrochloric acid for 10 min at RT. Primary antibodies used were rat anti-NCAM (Chemicon) at 1:100, chicken anti-syndecan-4 at 1:1500 [21], mouse anti-caveolin-3 (mAb 26, BD Biosciences) at 1:200, mouse anti-BrdU (Roche) at 1:20, and mouse anti-myogenin (F5D; Developmental Studies Hybridoma Bank)

neat. For GM1 detection Cholera Toxin Subunit B conjugated to Alexa 488 (Invitrogen) was used at 1:1000. Secondary antibodies conjugated to Alexa 488 or Alexa 594 (Invitrogen) were used at 1:500. Subtracting background, secondary only fluorescence, normalized all images (Slidebook, Intelligent Imaging Innovations).

For analysis of NCAM expression kinetics primary cells were plated onto coverslips in triplicate. For each time point, cells from five randomized 20x fields were counted. Total cell number was determined by DAPI-stained nuclei (or by brightfield images when BrdU was used). Error bars represent the standard error of the mean for $n=3$.

Scanning electron microscopy

Correlative SEM was performed essentially as has been described [22]. Briefly, primary satellite cells grown on a gridded, gelatin-coated coverslips were fixed in 4% paraformaldehyde before imaging by immunofluorescence and noting the location of individual cells on the grid. Samples were then post-fixed in 2.5% glutaraldehyde, dehydrated in ethanol, critical point dried, and coated with a thin film of evaporated platinum. The previously identified cells were then identified and viewed on a Hitachi S4700 field emission SEM at the University of Missouri Electron Microscopy core facility.

Fluorescence-activated cell sorting analysis

MM14 myoblasts [23, 24] were amplified in culture under growth conditions, which for this satellite cell-derived line includes addition of supplementary FGF-2 every 12 hours to prevent commitment to terminal differentiation during the G1 phase of the cell cycle. To produce a heterogeneous population of proliferating and recently-committed cells, cultures that had previously been supplemented with FGF-2 were not given additional FGF-2 for 12 hrs, leading to depletion of the available FGF2 and subsequent commitment of a fraction of the cells to differentiation. Cells were taken up with 0.05% collagenase and blocked in 10% normal goat serum for 45 min at 4°C. NCAM staining was performed using rat anti-NCAM (Chemicon) at 1:100 for 45 min at 4°C followed by secondary staining with anti-rat Alexa 488 (Invitrogen) at 1:500 for 30 min at 4°C. Cells were then sorted on a BD FACS DiVa' Vantage (Becton Dickinson, San Jose, CA) at the University of Missouri Cell and Immunobiology core facility; collection gates P1, P2 and P3 were defined as described in the text.

Western blotting

Cells from NCAM⁺ and NCAM⁻ sorted populations were lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholic acid, 1 mM NaF, 1 mM sodium orthovanadate, 1 mg/ml Pepstatin A and 1x Roche Complete Protease Inhibitors) and protein concentration was determined using a Pierce BCA assay. Equal amounts (20 µg) of cell lysate were loaded in alternating lanes on an Invitrogen Bis-Tris 4-12% gradient gel and blotted onto PVDF. Each of the four blots were probed then stripped and reprobed with at least two antibodies, one predicted to be found in the NCAM⁺ lysate and another predicted in the NCAM⁻ lysate. Primary antibodies were: mouse anti-caveolin 3 (BD Transduction Laboratories) at 1:5000, goat anti-creatine kinase-M (Santa Cruz) at 1:200, mouse anti-myogenin (F5D; Developmental Studies Hybridoma Bank) at 1:50, rat anti-NCAM (Chemicon) at 1:200, rabbit anti-PCNA (Abcam) at 1:200, rabbit anti-phospho-histone H3 (Ser 10) (Santa Cruz) at 1:200, rabbit anti-phospho-GSK3β (inhibitory Ser 9, Cell Signaling) at 1:500. Primary antibody binding was detected with secondary antibodies conjugated to horseradish peroxidase (Pierce) and detection of the signal was obtained using enhanced chemiluminescence (Pierce). Total protein loading per lane was confirmed by staining two lanes from the same blot with Coomassie Brilliant blue G-250 (Fisher Scientific).

RESULTS AND DISCUSSION

Detergent-resistant membrane domain proteins of primary satellite cells

The light buoyant density and other physical properties of membrane raft domains facilitate their isolation based on insolubility in cold nonionic detergents [25]. To determine what proteins are partitioned into the detergent-resistant membrane domains (DRMs), we used cold Triton X-100 insolubility and flotation over a discontinuous sucrose gradient to isolate them from cultures amplified from primary murine satellite cells. We then used liquid chromatography-tandem mass spectrometry to identify the extracted proteins and construct a representative list of DRM-associated proteins of satellite cells. Table 1 includes 72 of the approximately 250 proteins identified. Many of them are present in or associated with membrane rafts of other cell types [18, 26], and/or have been previously described in muscle. Overall, satellite cell DRMs contained primarily signaling intermediates, adhesion and cytoskeletal proteins (Table 1).

While detergent-resistant membrane extraction has historically been used as a benchmark for membrane raft association [15, 25, 27], we note that more recent work would suggest that detergent-resistance can be a subjective approach because of its dependence on type and concentration of detergent, temperature and cell type [16, 27]. In spite of this caveat, it remains a straightforward and effective means of isolating individual membrane raft proteins and complexes [16] subject to later verification. Using this method, we identified many probable satellite cell raft proteins, several of which also form multicomponent signaling complexes with one another, suggesting the potential for functional organization within the DRMs.

Integrins $\beta 1$ (fibronectin receptor β) and $\alpha 6$ were reliably represented in the DRM fractions (Table 1); microarray and RT-PCR analysis of primary satellite cells suggest that integrins $\alpha 5$ (fibronectin receptor α), $\alpha 3$ and $\alpha 4$ and are expressed by satellite cells as well (Cornelison and Olwin, in prep.) Integrins are ubiquitous transmembrane adhesion proteins known to be influenced by plasma membrane lipids; the functional activation of integrins can be linked to membrane raft localization [28, 29]. The lack of inherent catalytic activity requires integrins to be associated with other molecules for signaling activity. Both fibronectin-binding integrins and syndecan-4, a marker of satellite cells, are actively localized to membrane rafts and cooperate for the formation of vinculin-containing focal adhesions in vascular endothelial cells [30-32]. Detection of integrins in satellite cell DRMs raises the possibility that similar mechanisms of raft-based, adhesion-mediated signaling may apply in this cell type.

The ferlins are a family of proteins important for maintenance and repair of muscle membranes [33], particularly in the process of cellular fusion to form syncytial myofibers. In muscle, dysferlin interacts with other raft-localized proteins such as caveolin-3 and annexins for correct plasma membrane trafficking and repair, respectively [34-36]. Mutations in either dysferlin or caveolin-3 cause specific, non-Duchenne's type muscular dystrophies [33, 37-39]. We detected myoferlin, dysferlin, and annexins A1, A2 and A5 with confidence in satellite cell DRMs (Table 1); while ferlins have been extensively characterized in myoblasts, this is the first indication that they are both expressed by primary adult satellite cells and potentially localized to membrane raft microdomains in this cell population. Further analysis of their expression and activity in these cells will provide useful information in disease models as well as nonpathogenic myogenesis.

We also detected multiple signaling molecules that, while not inserted into the plasma membrane, are known to be tethered to juxtamembrane cytoskeletal elements, particularly in membrane microdomains. Examples of these include multiple small G-proteins such as R-

ras and Cdc42, the tyrosine kinases fyn and yes, and α - and β -catenin (Table 1). These data suggest that tightly-associated signaling complexes can be co-isolated in the DRM fractions used in this analysis. β -catenin in particular is a component of the canonical Wnt signaling pathway (reviewed in [40]) and acts as a transcriptional activator in synergy with Tcf/Lef family proteins (reviewed in [41]). Wnt signaling in satellite cells has been shown to act in initial specification and promotes both growth and differentiation [42-44]. Intriguingly, Wnt signaling through Frizzled has also recently been implicated in satellite cell transdifferentiation *in vitro* and *in vivo* [45]. Inactive β -catenin is localized at the plasma membrane by associations with the intracellular domains of cadherins, which act to regulate their activity [46]. Caveolin-1 expression inhibits Wnt/ β -catenin /Lef-1 signaling by recruiting β -catenin to caveolae membrane microdomains [47], and it has been shown that inhibition of membrane raft structure by methyl- β -cyclodextrin affects myogenesis downstream of Wnt signaling [48, 49]. Thus, our isolation and identification of both α - and β -catenin further supports the hypothesis that DRM-based complexes may be key regulators of satellite cell activity.

NCAM and membrane raft marker expression in proliferating and differentiating cultures

Neural cell adhesion molecule (NCAM) was detected in satellite cell DRMs with high confidence, based on the large number of peptides retrieved, percent coverage and cross correlation value (Table 1). NCAM is an immunoglobulin superfamily adhesion molecule; its expression on human satellite cells was first observed in monoclonal antibody studies [53] while later work demonstrated that, in human [54] and rat [55], NCAM is expressed on quiescent satellite cells and is maintained during proliferation and differentiation, through the formation of regenerated myofibers. It is also detected on the widely-used myoblast cell line C2C12 [56] where its expression is associated with the early stages of differentiation, concurrent with expression of T-type Ca^{2+} channels and inward rectifier K^{+} channels [57]. However, these studies and others [58] also established that NCAM is *not* expressed by quiescent mouse satellite cells, and that in the mouse its expression on satellite cells is heterogeneous during regeneration *in vivo* [59, 60]. In spite of significant efforts to assign a function to NCAM in satellite cell physiology, its molecular role is still unclear.

NCAM has previously been found to localize and act in membrane rafts in C2C12 myoblasts as well as neuronal cells and various NCAM transfected cell lines [50-52]. To investigate the localization of NCAM in relation to known membrane raft markers in primary satellite cells, satellite cell cultures amplified for 96 hours after harvest from the mouse were either fixed (as a proliferating sample) or grown for an additional 48 hours under differentiating conditions then fixed. All samples were labeled with anti-NCAM and co-stained with either fluorescently labeled cholera toxin subunit B (CTB), anti-caveolin-3, or anti-syndecan-4 (Figure 1). CTB binds to ganglioside M1 (GM_1), a glycosphingolipid considered to be a specific marker of membrane rafts [27, 62]. Caveolin-3, or M-caveolin, is a muscle-specific isoform of caveolin that localizes to caveolae, a subset of membrane rafts, in the plasma membrane (reviewed in [63]). Syndecan-4, which has also been shown to associate with membrane rafts [32], is used as a marker of primary satellite cells [64].

Figure 1 A and B illustrate that GM_1 and NCAM appear to identify distinct subpopulations of satellite cells: GM_1 is prevalent in cells cultured under growth conditions that express little or no NCAM, while NCAM predominates in cultures that have been stimulated to differentiate, primarily on morphologically distinct, differentiating myotubes that are also expressing caveolin-3 (Figure 1D and 1C). This coexpression is particularly intriguing in light of recent studies indicating caveolin-3 appears to mark terminally differentiated myotubes [65].

Scanning electron microscopy of NCAM stained primary myocytes

We next analyzed the surface architecture of NCAM-positive primary adult myocytes using scanning electron microscopy (SEM). Primary satellite cell cultures were induced to differentiate for 12 hours then fixed and immunostained for NCAM. The cells were imaged under fluorescence optics and their location on the coverslip recorded; the coverslips were then processed for SEM imaging. Figure 2 shows one myocyte sequentially imaged by immunofluorescence for NCAM and SEM; NCAM staining is punctate and overlaps with small structures at the plasma membrane (Figure 2C and 2D). Note that in figures 2B and 2D, the viewing plane is focused at the surface of the cell near the nucleus, thus NCAM staining on other portions of the cell is out of focus.

We speculate that NCAM localization to these domains may explain why NCAM staining is observed in comparatively large areas across the plasma membrane despite the extremely small size of membrane rafts. The identity and function of these structures remains to be determined.

Time course of NCAM expression in differentiating myotubes

Based on the expression of NCAM in proliferating and differentiating cultures we went on to test the potential for NCAM to be an early extracellular marker of commitment to myogenic differentiation. To test this we examined NCAM expression on primary mouse satellite cell cultures at fixed times after they were switched to differentiation medium. To measure proliferation the cells were incubated in BrdU for 4 hr prior to fixation and staining. We found that NCAM labels an expanding subpopulation of syndecan-4 positive cells over time in differentiation medium (Figure 3A). Prior to induction of differentiation, 10.7% of all cells were NCAM positive; 48 hours later almost all syndecan-4 positive cells were also NCAM positive, with NCAM expression on 97.9% of cells (Figure 3, Figure 4A). At 0 hours BrdU incorporation was 50.2%; after 24 hours in differentiation media BrdU incorporation was 1.1 % indicating a general exit from the cell cycle coincident with increasing incidence of NCAM expression (Figure 3B). Concurrent with NCAM expression, myogenin expression also increased throughout differentiation (Figure 3C and 4B). By 48 hours all NCAM positive cells were also myogenin positive. These results suggest NCAM is a proximal marker of commitment to myogenic differentiation.

FACS sorting of NCAM⁺ and NCAM⁻ cells

To test the utility and specificity of NCAM as an extracellular marker of differentiation, we separated a heterogeneous population of proliferating and recently-committed differentiating MM14 myoblasts by flow cytometry into two populations based on their NCAM immunoreactivity. MM14s are a satellite cell-derived cell line with morphology and gene expression very similar to primary satellite cells [23, 66]. They are dependent on FGF2 stimulation during the G1 phase of the cell cycle to prevent commitment to terminal differentiation [24]. The cells were amplified in culture under growth conditions then deprived of additional FGF2 for 12 hours before staining and sorting (Fig. 5A). Forward scatter and side scatter, measures of the size and granularity of cells, respectively, (Fig. 5B, left) were used to gate a physically homogenous population of small, mononucleated cells. Thus, sorted cells were either proliferating, or at such an early stage in myogenic differentiation that they had not begun to elongate and were thus morphologically indistinguishable from proliferative myoblasts. Figure 5B (right) shows the histogram of NCAM immunoreactivity for cells within the P1 gate; two clear peaks can be observed, with a small region of overlap between. Collection gates P2 and P3 were set to exclude this region of overlap, and NCAM⁻ and NCAM⁺ cells were sorted and collected for further analysis.

To determine the commitment status of the collected cell fractions we performed Western blots of lysates from the sorted cells with markers of proliferation and differentiation, sequentially (Fig. 5C). Lysates were assayed for protein concentration and 20 µg of total protein was run out in each lane. Phosphorylated histone H3 and PCNA, both markers of cycling cells, were detectable only in the NCAM^{-ve} fraction, consistent with actively proliferating, non-differentiating myoblasts. In contrast, the myogenic differentiation markers phospho-GSK3β, muscle creatine kinase (MCK), myogenin (myoG) and caveolin-3 (cav-3), as well as NCAM itself, were detected only in lysates from the NCAM^{+ve} fraction. We therefore conclude that NCAM is a marker of committed adult myocytes, which also has the technical advantage of providing a facile method for separating myoblasts from myocytes in a heterogeneous cell population.

Conclusions and future directions

Since the initial observation of NCAM expression on rat primary satellite cells, the role of NCAM during myogenesis has been elusive [12, 55]. In muscle, NCAM exists in multiple isoforms, which arise from alternative splicing of a single gene [67, 68]. NCAM is also subject to several forms of post-translational modification: polysialylation of residues in the immunoglobulin domains is commonly found during development and has recently been shown to increase membrane repulsion and to enhance directional migration in specific cell types [69, 70]. Conversely, O-linked glycosylation at the muscle specific domain (MSD) increases myoblast fusion [71]. *In vitro* overexpression of NCAM isoforms containing the MSD increases myoblast fusion, while the overexpression of NCAM isoforms lacking this domain has no effect [72-74]. Surprisingly, NCAM null mice display no gross defects in skeletal muscle during development, and primary myoblasts from these mice fuse at a similar rate as wild type *in vitro* [75]. Together, these data imply that the increased fusion noted in NCAM overexpression studies may be the result of increased overall adhesion, and that other adhesion molecules are capable of compensating for the loss of NCAM [12]. To this, the current study adds the suggestion that association of NCAM with complexes of adhesion and signaling molecules in specific, discrete physical microdomains and/or membrane structures could constitute an important aspect of NCAM regulation and activity. It also extends previous work on the heterogeneous expression of NCAM on activated mouse satellite cells by associating acquisition of NCAM expression with a change in cellular commitment status. Interestingly, we observe from the Western blots that while all three splice isoforms of NCAM (180kD, 140 kD, and 120 kD) appear to be present in the sorted cell lysates, the 120 kD form that has previously been associated with differentiation and enhanced fusion in cultured myocytes appears to be the most abundant.

This DRM proteomic study represents the first extraction and identification of putative membrane raft components from primary satellite cells. We find that satellite cell DRMs not only contain a multitude of established membrane raft-associated proteins and muscle specific proteins, but also show a strong potential to act as organizing scaffolds for higher-order, functional signaling complexes. Specific membrane raft components including GM₁, caveolin-3 and NCAM are dynamically expressed during differentiation of satellite cells, suggesting that these cells may represent a useful new model system for the study of membrane raft dynamics. The specific factors isolated in this screen also point to intriguing new avenues of inquiry in satellite cell physiology.

Our analysis of NCAM expression in heterogeneous cultures of primary myoblasts and the satellite cell line MM14 indicates that it may be a proximal marker of the commitment to myogenic differentiation. Flow cytometry on the basis of cell-surface NCAM detection is therefore a promising new molecular tool, that will allow live cell sorting of heterogeneous primary cell populations into discrete populations for either biochemical analysis or, potentially, downstream clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Immunolocalization of membrane raft markers and NCAM in primary satellite cells
Satellite cell-derived cultures were induced to proliferate in culture for 4 days then either fixed (**A**, **C**, **E**) or placed in differentiation media for an additional 48 hrs (**B**, **D**, **F**) before fixing and staining. In proliferative cultures most cells did not express NCAM (red) (**A**, **C**, **E**). NCAM staining is observed in most cells after culture in differentiation media (**B**, **D**, **F**). CTB (cholera toxin subunit B) labels GM₁ (green) on cells in proliferative cultures and appears restricted to round cells adjacent to myotubes in differentiating cultures (**A**, **B**). Cav-3 (green) has the opposite expression pattern, appearing predominately on myotubes (**C**, **D**). Yellow indicates overlap between green and red staining. Both proliferating and differentiating cells express the satellite cell marker syndecan-4 (green) (**E**, **F**). Nuclei were stained with DAPI (blue) to identify all cells present. Bar = 100 μ m.

Figure 2. Scanning electron microscopy of an NCAM-stained primary myocyte SEM image (A) of a primary adult myocyte that had previously been imaged by indirect immunofluorescence using an antibody against NCAM (B). In higher magnification images, white arrows indicate small protrusions on the plasma membrane of the myocyte (C), while black arrows identify punctate NCAM staining that overlaps with these structures (D).

Figure 3. NCAM immunolocalization in differentiating satellite cells

Primary satellite cells were amplified in culture for 4 days then switched to differentiation medium for 0, 12, 24, 48 and 72 hrs. Immunostaining for NCAM reveals an increasing subpopulation of NCAM⁺ (red), syndecan-4⁺ (green) satellite cells (A). Overlap of red and green fluorescence appears yellow. BrdU was added to differentiating cells 4 hrs before fixing. Lack of detectable BrdU (green) incorporation suggests that all NCAM⁺ (red) cells have withdrawn from the cell cycle (B). Myogenin expression increases during differentiation; by 24 hrs most NCAM⁺ (red) cells are also myogenin⁺ (green) (C). Nuclei were stained with DAPI (blue) to identify all cells present. Bar = 100 μ m.

Figure 4. Kinetics of NCAM expression, cell cycle exit and myogenin expression in differentiating satellite cells

After serum withdrawal the percentage of NCAM positive cells increases from $10.7 \pm 3.5\%$ (mean $\pm$ SEM, $n=3$) at 0 hr to $96.4 \pm 0.59\%$ at 72 hr (A). Over the same period the percentage of cells incorporating BrdU decreased to zero and myogenin positive cells increased to 93.9% (B). The percentage of syndecan-4 positive cells stayed roughly the same over the 72 hr time course (B).

Figure 5. Isolation of NCAM positive cells by fluorescence activated cell sorting

Commitment of a subpopulation of proliferating MM14 myoblasts was induced by 12 hours of FGF2 depletion, then cells were sorted based on NCAM expression (A). Forward scatter (FSC) and side scatter (SSC) gating (B, left) and the parameters used to separate populations of NCAM⁺ and NCAM⁻ cells are displayed (B, right). Twenty micrograms of total protein from each sample were resolved by SDS-PAGE and transferred to PVDF, followed by Western blotting (C). Blots from left to right: phospho-histone 3 (pH3), phospho-serine 9 glycogen synthase kinase 3 β (pGSK3 β), muscle creatine kinase (MCK), proliferating cell nuclear antigen (PCNA), myogenin (MyoG), caveolin-3 (CAV-3), neural cell adhesion molecule (NCAM). One set of lanes from the blot was stained with Coomassie blue to confirm equivalent loading (D).

Table contains protein names, previously described raft association (“Y” refers to yes, the protein has been previously associated with rafts, “U” refers to unknown raft association, and “N/A” refers to not applicable), NCBI protein accession number and Gene ID, the number of unique tryptic peptides identified, composite protein p-value, cross-correlation score, and percent coverage. Individual peptide sequence, mass, and charge data are available in Supplemental Table 1. Prior raft associations were determined by searching the Harvester mouse database (<http://harvester.fzk.de/harvester/>) and combined searches in PubMed (“protein name” AND “raft”).

Table 1
Detergent-resistant membrane proteins identified by mass spectrometry

Name	Raft?	Protein Accession	Gene ID	#of unique peptides IDed	p-value (protein)	Xcorr (protein)	%Coverage
Adhesion, integrin associated							
Catenin alpha 1	Y	6753294	12385	1	2.85E-08	10.33	2.87
Catenin beta 1	Y	6671684	12387	1	4.66E-06	10.25	2.69
CD36 antigen	Y	729081	12491	5	1.82E-06	50.23	17.16
CD44 antigen isoformic	Y	85540468	12505	5	2.00E-10	50.41	13.97
CD47 antigen	Y	6754382	16423	2	2.59E-06	20.19	8.33
CD56/NCAM isoforms 120/140/180 *	Y	2181948	17967	13	4.80E-04	40.26	**19.6
CD9 antigen	Y	6680894	12527	1	5.42E-08	10.29	11.06
Galectin 1	Y	6678682	16852	1	3.20E-09	10.30	11.85
Integrin alpha 6	Y	408128	16403	3	1.88E-06	30.33	5.96
Integrin beta 1	Y	45504394	16412	2	7.60E-06	18.19	5.01
Integrin beta 4	Y	37747485	192897	2	1.83E-06	20.22	1.66
Myelin protein zero	Y	6678928	17528	9	2.04E-07	90.19	22.98
Myelin-associated glycoprotein	Y	3334470	17136	2	3.76E-05	20.28	7.00
Thy 1.2	Y	3980175	21838	1	5.72E-06	10.18	13.40
Cytoskeleton and associated							
14-3-3 gamma	U	9507245	22628	1	1.37E-13	10.28	11.70
14-3-3 zeta	Y	1841387	22631	2	9.69E-05	20.19	13.50
Actin, alpha 1 skeletal muscle	n/a	3182895	11459	2	1.29E-07	20.29	8.20
Actin, beta 1	n/a	1703156	11461	1	1.32E-04	8.16	2.90
Annexin A1	Y	124517663	16952	2	3.61E-04	20.17	7.80

Name	Raft?	Protein Accession	Gene ID	#of unique peptides IDed	p-value (protein)	Xcorr (protein)	%Coverage
Annexin A2	Y	6996913	12306	1	1.78E-14	20.29	11.20
Annexin A5	Y	6753060	11747	1	9.99E-04	10.26	9.40
CDC42	Y	74207606	12540	1	3.42E-05	10.23	6.80
Cofilin 1	Y	12861068	12631	1	5.59E-10	8.24	6.10
Cofilin 2, muscle	U	6671746	12632	1	6.23E-08	10.15	6.60
Erythrocyte protein band 4.1-like 2	U	29789052	13822	8	8.95E-10	70.27	11.90
Gnai3	Y	33563256	14679	1	1.81E-05	10.18	4.20
Gpr177/wntless homolog	U	12860275	68151	1	3.04E-09	10.23	4.10
Gprc5a	U	31126968	232431	1	1.56E-05	8.13	5.90
Gnb1	U	6680045	14688	1	1.97E-09	10.29	5.30
Gnb2	U	13937391	14693	3	1.24E-07	30.30	15.60
Keratin 71	n/a	9910294	56735	1	1.18E-06	8.23	2.30
Keratin, type II cytoskeletal 1	Y	4159806	16678	2	1.38E-06	20.23	3.70
MARCKS	Y	6678768	17118	2	2.26E-09	20.25	11.00
Myosin, light polypeptide 1	Y	29789016	17901	1	1.53E-08	10.21	10.60
Periaxin isoform 1	U	37674285	19153	38	3.23E-11	368.30	41.60
Periaxin isoform 2	U	9506999	19153	4	2.82E-10	40.34	41.20
Profilin 1	U	6755040	18643	2	4.01E-09	20.18	20.00
RAB14	Y	18390323	68365	1	9.02E-05	10.22	7.40
RAB15	Y	31559981	104886	2	1.33E-04	12.17	10.40
RAB1B	Y	21313162	76308	4	1.68E-07	46.27	31.30
RAB2	Y	10946940	59021	1	3.37E-09	10.20	7.50
RAB11B	Y	6679583	19326	2	2.75E-07	20.24	15.60
RAB21	Y	33859751	216344	1	2.81E-05	10.18	7.70
Ran	U	12846283	19384	1	9.09E-04	10.12	6.50
RAB338	Y	8394133	19338	1	4.17E-04	10.17	4.80
Rab5c	Y	13096181	19345	2	5.62E-06	20.28	23.20

Name	Raft?	Protein Accession	Gene ID	#of unique peptides IDed	p-value (protein)	Xcorr (protein)	%Coverage
Name	Raft?	Protein Accession	Gene ID	#of unique peptides IDed	p-value (protein)	Xcorr (protein)	%Coverage
RAB6B	Y	30424655	270192	1	4.17E-04	6.17	5.30
Rab7	Y	1050551	19349	3	1.02E-07	30.22	19.80
Ralb	Y	11612509	64143	2	4.24E-07	20.27	21.80
Stomatin	Y	12833038	66592	2	2.73E-07	20.23	12.50
Tubulin, alpha 1B	n/a	34740335	22143	6	1.32E-12	60.32	26.60
Tubulin, alpha 8	n/a	8394493	53857	9	7.35E-10	80.28	27.20
Tubulin, alpha 3	n/a	12963615	22152	3	3.41E-07	22.26	13.10
Tubulin, beta 6	Y	27754056	67951	6	4.82E-12	68.35	27.30
Tubulin, beta 5	n/a	74212109	22154	1	4.39E-11	10.34	4.00
Vasp	U	90110086	22323	1	2.45E-06	10.18	6.70
Vimentin	Y	26328885	22352	2	4.20E-08	20.23	18.50
Kinases and Phosphatases							
FGFR2	Y	2506801	14183	1	9.19E-04	10.13	2.30
Protein-tyrosine kinase fyn	Y	6679879	14360	3	4.75E-08	20.28	6.70
Phosphoglycerate kinase 1	N	70778976	18655	1	3.60E-06	10.16	4.30
Protein tyrosine kinase 7	U	30425042	71461	1	4.03E-04	10.13	2.70
Similar to adenylate kinase 1	U	94388048	633979	1	3.10E-04	10.13	2.70
Viral oncogene yes homolog	Y	6678617	22612	1	1.61E-05	10.18	4.10
Trapp	U	74188543	100683	1	2.26E-04	6.13	0.80
Other							
Basign isoform 2	Y	116014342	12215	3	3.13E-09	30.25	16.80
Baspl1/NAP-22	Y	45598372	70350	4	4.10E-11	50.29	47.80
Dysferlin	Y	149270772	26903	4	1.63E-07	40.23	3.20
Nradd	U	74227711	67169	1	1.27E-04	10.14	4.80
Myoferlin	Y	26327513	226101	1	3.76E-05	10.16	4.50
SNAP-23	Y	6678049	20619	1	3.43E-08	10.23	9.00

Name	Protein Accession	Gene ID	#of unique peptides IDed	p-value (protein)	Xcorr (protein)	%Coverage
Bin-1	6753050	30948	4	7.31E-08	40.28	12.40
Myadm	2463265	50918	1	3.17E-10	10.22	5.70

* values are averages of original isoform assignment values

** coverage calculated from NCAM isoform consensus sequence