Comparative Proteomes of the Proliferating C<sub>2</sub>C<sub>12</sub> Myoblasts and Fully Differentiated Myotubes Reveal the Complexity of the Skeletal Muscle Differentiation Program*§

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When cultured in low serum-containing growth medium, the mouse C<sub>2</sub>C<sub>12</sub> cells exit cell cycle and undergo a well-defined program of differentiation that culminates in the formation of myosin heavy chain-positive bona fide multinucleated muscle cells. To gain an understanding into this process, we compared total, membrane- and nuclear-enriched proteins, and phospho-proteins from the proliferating C<sub>2</sub>C<sub>12</sub> cells and the fully differentiated myotubes by the combined methods of two-dimensional PAGE, quantitative PDQuest image analysis, and MS. Quantification of more than 2,000 proteins from C<sub>2</sub>C<sub>12</sub> myoblasts and myotubes revealed that a vast majority of the abundant proteins appear to be related to the essential, housekeeping and structural functions, and their steady state levels remain relatively constant. In contrast, 75 proteins were highly regulated during the phenotypic conversion of rapidly dividing C<sub>2</sub>C<sub>12</sub> myoblasts into fully differentiated, multi-nucleated, post-mitotic myotubes. We found that differential accumulation of 26 phospho-proteins also occurred during conversion of C<sub>2</sub>C<sub>12</sub> myoblasts into myotubes. We identified the differentially expressed proteins by MALDI-TOF-MS and LC-ESI-quadrupole ion trap MS/MS. We demonstrate that more than 100 proteins, some shown to be associated with muscle differentiation for the first time, that regulate inter-and intracellular signaling, cell shape, proliferation, apoptosis, and gene expression impinge on the mechanism of skeletal muscle differentiation. Molecular & Cellular Proteomics 3:1065–1082, 2004.

The de novo myogenesis from mesoderm-derived committed muscle precursor cells has been studied in the embryos of mouse, chicken, frog, and zebra fish, and in a number of cell and tissue culture models of muscle differentiation. As a result of these studies, key anatomic, genetic, and molecular aspects of this multi-step process have been elucidated (1–3). The final step of myogenesis in vivo entails that the proliferating myoblasts withdraw from cell cycle, elicit a muscle-specific gene expression program and fuse to become multinucleated myotubes.

The induction of muscle-specific genes during myogenic differentiation is regulated by basic helix-loop-helix (bHLH) transcription factors such as MyoD, Myf-5, myogenin, and MRF4. The known muscle-specific regulatory factors (MRFs) exhibit distinct but somewhat overlapping spatio-temporal patterns of expression during development of the skeletal muscle. For example, Myf5 is the first of the myogenic bHLH factors to be expressed in the developing embryo followed by MyoD and myogenin. The expression of myogenin and MRF4 occurs later, and apparently the two MRFs directly control transcription of muscle-specific genes prior to the formation of multinucleated myotubes (4, 5). In contrast, MyoD and Myf-5 are not only expressed in the proliferating myoblasts but may also be needed in an early step of myogenesis (i.e. muscle cell fate specification). Varying degrees of defects in muscle development are caused by loss-of-function mutations in the individual MRF genes (6, 7). Consistent with their unique roles, the combined mutations in two MRFs in mice (e.g. MyoD –/–; MRF4 –/–) result in more severe defects in muscle differentiation than those seen in either MyoD –/– or MRF4 –/– mice (2). Additionally, MEF2 and regulators of cell cycle such as p21<sub>cip1</sub>, p27, and p57<sub>kip2</sub> are also coordinately regulated during muscle differentiation (1–3).

Mouse C<sub>2</sub>C<sub>12</sub> cells have been used extensively to study the process of myogenic differentiation in culture. The application

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The abbreviations used are: bHLH, basic helix-loop-helix; MRF, muscle-specific regulatory factor; 2D, two-dimensional; QIT, quadrupole ion trap; GM, growth medium; DMEM, Dulbecco’s modified Eagle’s medium; DM, differentiation medium; MHC, myosin heavy chain; RIPA, radio-immunoprecipitation assay; TBST, TBS-Tween 20; LIMK1, LIM kinase 1; PKA, protein kinase A; MKK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; HSP, heat shock protein; CRE, cAMP-responsive element; TIF, transcription intermediary factor; HAT, histone acetyl transferase; FGF, fibroblast growth factor.
of DNA microarray technology to the differentiating C2C12 cells has led to the identification of numerous differentially expressed messenger RNAs (8, 9). Shen et al. (9) reported that the differentially expressed genes of C2C12 cells grown in low serum medium represented regulators of cell cycle (e.g. cyclin D1, p27Kip1, PP2A, and Rb), apoptosis such as DAD1, BAK, Caspase 11, and glycogen synthase kinase-3β, and muscle-specific genes (e.g. MyoD, myogenin, dystroglycan, troponin c, and creatine kinase). These authors showed that expression of Cyclin D1 was readily detected in the proliferating myoblasts while p21WAF1/Cip1 expression increased only when <40% of cells were fused into multinucleated myotubes. In a related study, assessment of global gene expression in the differentiating C2C12 cells up to the stage of myogenin induction showed that <1,500 genes, which could be classified into 12 coordinate regulated groups of genes, were significantly altered (8). Similar to what was demonstrated by Shen et al. (9), Dalgado and colleagues found that numerous cell cycle signaling-, apoptosis-, cell architecture-, and transcriptional control-specific genes were significantly altered during early phase of myogenic differentiation (8).

Although the genomics-based analyses of myogenesis in C2C12 cells have been highly instructive, we believe that the molecular mechanisms by which proliferating myoblasts leave cell cycle, initiate a program of myogenic gene expression, and become fused into multinucleated myotubes cannot be fully understood from the analysis of the transcriptome alone. This is because the signal transduction pathways mediating the phenotypic conversion of myoblasts into myotubes utilize proteins and the analysis of transcriptome informs us little about the dynamic changes in the rates of translation of various mRNAs or about proteins produced by translation of alternatively spliced mRNAs. Therefore, it is desirable to complement the global gene expression analyses with studies examining the proteomes of C2C12 cells undergoing myogenesis in vitro. With a goal to compare the proteomes of the C2C12 cells undergoing differentiation, we analyzed the total cellular, membrane-, and nuclear-enriched proteins from proliferating myoblasts and fully differentiated myocytes by two-dimensional (2D)-PAGE. The differentially regulated protein spots were identified by PDQuest image analysis of the silver nitrate-stained 2D gels followed by MALDI-TOF-MS and LC-ESI-quadrupole ion trap (QIT)-MS/MS. Furthermore, because the status of phosphorylation, a key modification of proteins that regulates numerous signaling cascades, cannot be discerned from analyses of the protein abundance, we also compared the phospho-proteomes of proliferating C2C12 cells and myotubes by using the Pro-Q® Diamond phosphoprotein gel staining. We demonstrate that in addition to many well-known proteins involved with myogenesis, the expression of a number of new proteins capable of regulating inter- and intracellular signaling, cell cycle and apoptosis, cell shape, and transcription is also altered during skeletal muscle differentiation.

EXPERIMENTAL MATERIALS AND METHODS

Cell Culture—C2C12 cells were bought from American Type Culture Collection (ATCC-CRL 1772; Bethesda, MD). Cells were cultured in growth medium (GM; Dulbecco’s modified Eagle’s medium [DMEM] containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin) in a humidified incubator at 37 °C with 5% CO2. Cells cultivated in GM were subcultured after they became 70–80% confluent, and the cell passage number was not allowed to exceed 10. To induce differentiation, nearly confluent C2C12 cells were incubated in DMEM containing 2% heat-inactivated horse serum (differentiation medium; DM) for varying lengths of time. The fraction of cells converted into myotubes was assessed by light microscopy of unstained cells or after staining with a monoclonal myosin heavy chain (MHC)-specific primary antibody. The primary antibody was diluted 1:1 with 1% BSA/PBS-Tween 20 followed by secondary reaction with a goat anti-mouse IgG conjugated with FITC. The detailed methods for staining of C2C12 cells with antibody and detection of FITC fluorescence have been outlined previously (10).

Extraction of Proteins—Cell monolayers (~106 cells/15-cm diameter Petri dish) were washed twice with 10 ml of 0.35 M ice-cold sucrose, scraped in 4 ml of 0.35 M ice-cold sucrose, and collected by centrifugation (4,000 rpm for 5 min at 4 °C). Whole-cell proteins were extracted in radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, and 100-fold diluted fresh cocktails of phosphatase inhibitor I (microcystin LR, cantharidin, and (-)-p-bromotetramisole; catalog no. p2850, Sigma-Aldrich, St. Louis, MO), phosphatase inhibitor II [sodium vanadate, sodium molybdate, sodium tartrate, and imidazole; catalog no. p5726, Sigma-Aldrich], and 40 μl of protease inhibitor mixture (catalog no. 1697498, Roche, Indianapolis, IN). Proteins were precipitated in acetone (final concentration of 80%) at −20 °C overnight, pelleted by centrifugation (14,000 rpm for 20 min at 4 °C), and pellets were air-dried. The protein pellet obtained from cells harvested from a single 15-cm diameter dish was taken up in 200 μl of rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS]. The protein solution in rehydration buffer was supplemented with immobilized pH gradient buffer (8 μl/ml), 1 μl (0.5%) of bromphenol blue, and DTT (10 mg/ml) and kept for 1 h at room temperature. These samples were centrifuged (14,000 rpm for 20 min at 25 °C), and supernatant containing the proteins in complete solution was used for 2D-PAGE.

We extracted crude membrane fraction according to the published protocol (11). Cells were suspended in HES buffer [20 mM HEPES, 1 mM EDTA at pH 7.4, and freshly added mixture of protease and phosphatase inhibitors] and broken by freezing and thawing (~80 °C for 30 min) and 30 passes in a Dounce homogenizer (clearance of 0.1016–0.1524 mm). Unlysed cells and nuclei were removed from the cell homo-
genate by centrifugation (900 × g for 10 min at 4 °C). The crude plasma membranes were recovered as a pellet by centrifuging the post-nuclear supernatant at 100,000 × g for 45 min at 4 °C. Membrane proteins were extracted in RIPA buffer, precipitated by acetone as above and dissolved in re-hydration buffer (12).

Cells harvested from 15-cm diameter dishes as above were used to isolate nuclei according to the previously reported protocol (13). Cells were sequentially washed three times in 3 ml of ice-cold isolation buffer [3.75 mM Tris-Cl pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA, 1% thiodiglycol, 20 mM KCl] containing protease and phosphatase inhibitors, and centrifuged (900 × g for 5 min at 2 °C) after each wash. The supernatant containing the membrane and cytoplasm was removed after each spin. The crude nuclear pellets were then taken up in 3 ml of ice-cold Triton X-100 lysis buffer [isolation buffer] and homogenized. The mixture was centrifuged at 900g 10 min in 3 ml of solution containing 6M urea, 1.5M Tris-HCl, 0.5M EDTA, 1% SDS, 2% (v/v) glycerol, 30% (v/v) acetic acid, and 1% w/v thiodiglycol. The supernatant containing the membrane and cytoplasm was removed, and the remaining pellet was homogenized again, and centrifuged as above. This digestion and separation was repeated five times. The supernatant was concentrated by centrifugation in a Centricon-10 filter (Amicon, Beverly, MA). Nuclei were resuspended in 0.5% Triton X-100 containing protease inhibitors, and centrifuged (900g 10 min) in 3 ml of isolation buffer (14). The supernatant was saved for analysis of nuclear proteins, and the pellet was again resuspended and centrifuged. This digestion and separation was repeated three times by stepwise resuspension and centrifugation. The supernatant containing the membrane and cytoplasm was removed after each spin. The crude nuclear pellets were then taken up in 3 ml of ice-cold Triton X-100 lysis buffer [isolation buffer] containing protease and phosphatase inhibitors, and centrifuged (900 × g for 5 min at 2 °C) after each wash. The supernatant containing the membrane and cytoplasm was removed after each spin. The crude nuclear pellets were then taken up in 3 ml of ice-cold Triton X-100 lysis buffer [isolation buffer] containing protease and phosphatase inhibitors, and centrifuged (900 × g for 5 min at 2 °C). Finally, nuclear proteins were extracted in 500 µl of RIPA buffer, precipitated in 80% acetone at −20 °C overnight, and made soluble in 100 µl of re-hydration buffer. Concentration of proteins from total cell, membrane, or nuclear extracts was determined by a modified Bradford assay kit (14) as described by the manufacturer (Pierce, Rockford, IL).

2D-PAGE—One hundred-microgram aliquots of whole-cell, membrane, or nuclear proteins, taken up in 360 µl of re-hydration buffer, were electrofocused in Immobiline™ DryStrips (180 × 3 × 0.5 mm, pH 3–10/4–7 linear) with the IPGphor (Amersham Pharmacia Biotech, Piscataway, NJ) (15). The IPG strip was rehydrated for 12 h and subjected to sequential IEF at 100 V for 200 V-h, at 500 V for 500 V-h, at 1,000 V for 1,000 V-h, and at 8,000 V for 80,000 V-h. The platform temperature was maintained at 20 °C, and 50-µA current was passed per strip. The IPG strips were equilibrated to reduce the disulfide bonds in a tray containing 3 ml of equilibrating solution per strip [6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT] with gentle rocking for 10 min. The protein SH groups were blocked by rocking each strip for 10 min in 3 ml of solution containing 6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 2.5% (w/v) iodoacetamide.

Solubilization of proteins, equilibration of first-dimension strips, and electrophoresis in the second dimension were done in the Protean Dodeca Cell (Bio-Rad, Hercules, CA) apparatus capable of running 12 gels simultaneously. After equilibration, according to the published method (16), the IPG strips were transferred onto vertical 10% SDS-PAGE slab gels (1,800 × 1,800 × 1 mm), using 1% melted agarose as stacking gel. One microliter of the molecular mass marker (Amersham Rainbow marker RPN 800) mixed with 4 µl of running buffer was loaded on 2 mm² filter paper, which was placed on the acidic end of the IPG strip (17). Electrophoresis was carried out for 400 min at a steady voltage of 200 V (12).

Staining of 2D-PAGE and Image Analysis—Proteins separated by 2D gels were visualized after staining with Mann’s modified silver staining method, which is compatible with trypsin digestion and MALDI-TOF-MS (18, 19). The staining of 2D gels for phospho-proteins was done according to the instructions provided by the manufacturer (Molecular Probes, Eugene, OR). Gels were fixed in 250 ml of 50% methanol and 10% TCA overnight. The fixed gels were then sequentially washed with 250 ml of distilled water for 15 min, incubated with 250 ml of Pro-Q® Diamond phospho-protein stain for 3 h in the dark and destained with 20% ACN, 50 mM sodium acetate (pH 4) for 3 h. After scanning the images of the gels for phospho-proteins, the gels were silver stained for visualization of the spots for MS analyses. The stained 2D gels were scanned (Hewlett Packard 4470c scanner) and saved as TIFF files using Adobe Photoshop software. The images of the scanned 2D gels were analyzed by PDQuest (version 6.2.1) software (Bio-Rad) (20). To identify valid spots, PDQuest spot detection software was used with appropriate selection of the faintest and the smallest spots and a large representative section of the image containing spots, streaks, and background gradations to make corrections for noise filter. Absorbance of individual protein spots from the replicate gel images was combined to make “master gels” representing proteins from C2C12 cells at different stages of differentiation. The differentially regulated protein spots were identified by quantitative comparisons of master gels. The reproducibility of PDQuest-based quantification of 2D gel images was ensured by three complementary approaches. First, at least three independent sets of synchronized cultures of C2C12 cells were grown in GM or DM to extract myoblast and myotube-specific proteins. All Petri dishes were individually assessed for cell morphology, density of culture, and myotube formation (in DM) on a daily basis to select two to three uniform replicates for each condition of growth. Second, in the initial tests of reproducibility of 2D gels, protein extracts from individual replicate cultures were analyzed by 2D-PAGE. Subsequently, extracts from cells harvested from three to four Petri dishes incubated under identical conditions were mixed and subject to 2D-PAGE to “normalize” dish-to-dish variability. Finally, regardless of the source of the protein extract, either from a single dish or from multiple dishes grown under identical conditions, all samples were run on three to four replicate gels in the Protein Dodeca Cell (Bio-Rad). Unpaired Student’s t test was used to determine if the averages of the myoblast or myotube-specific samples were significantly different using the Microsoft Excel®. Protein spots of interest were subject to MALDI-TOF-MS or LC-ESI-MS/MS (21).

In-gel Trypsin Digestion—The individual protein spots from the 2D gels were excised with pipette tips, minced using a 0.5-ml pestle (Nalge Nunc, Rochester, NY), and destained in 1:1 (v/v) of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (22). Gel spots were dehydrated by sonication for
20 min in 100 μl of solution containing 50% (v/v) ACN and 100 mM ammonium bicarbonate until the gel turned opaque white. Twenty micrograms of lyophilized trypsin (883 pmol; Promega, Madison, WI) was reconstituted in 100 μl of 50 mM acetic acid to form the stock solution that was diluted [1:12 (v/v)] in 50 mM ammonium bicarbonate and incubated for 15 min at 37 °C. The gel fragments were dried by vacuum centrifugation for 30 min and incubated overnight with 50 μl of trypsin (16 ng/μl) at 37 °C.

The supernatant from trypsin digest was transferred to a siliconized microcentrifuge tube. Peptides from the gel pieces were sequentially extracted three times in 50 μl of extraction buffer [60% (v/v) ACN, 5% (v/v) TFA in water]. Each extraction involved 20 min of sonication, followed by centrifugation and removal of the supernatants. The original supernatant and the supernatants from three sequential extractions were combined and dried in a vacuum centrifuge for 3–4 h. The dried peptides were dissolved in 3 μl of 12.5 mg/ml of α-cyano-4-hydroxy-cinnamic acid in 60% (v/v) ACN in water and deposited on paraffin wax-coated stainless-steel MALDI plate (23). Alternatively, dried peptides were dissolved in 15 μl of 0.1% (v/v) TFA in water and taken up in siliconized microcentrifuge tube for LC-ESI-QIT-MS/MS.

MALDI-TOF-MS—Mass spectrometer analyses were performed using the PerSeptive Biosystems (Framingham, MA) MALDI-TOF Voyager DE™-RP BioSpectrometry™ Workstation operated in the delayed extraction and reflector mode for positive ion detection (24). The laser wavelength and the repetition rate were 337 nm and 3Hz, respectively. The parameters set were as follows: maximum accelerating voltage, 20,000 V; grid voltage, 57%; mirror voltage ratio, 1.08; guide wire, 0.07% and the extraction delay time of 150 ns. The masses were calibrated internally with the masses of two trypsin auto-digest products: fragment 108–115 ([M+H]⁺ = 842.509 Da) and fragment 58–77 ([M+H]⁺ = 2211.104 Da). The results were analyzed with Data Explorer software (Applied Biosystems). The protein identification was carried out using the PeptIdent search engine (us.expasy.org/tools/peptident.html), and the Swiss-Prot/TrEMBL databases were used for the protein search (25). The search parameters used were: pl range of ± 1.0, molecular mass range of ± 40%, mass tolerance of ± 100 ppm, one allowed missed cleavage, cysteine treated with iodoacetamide to form carbamidomethyl-cysteine and methionine as the oxidized form.

LC-ESI-QIT-MS/MS—To remove the residual gel and to desalt the peptides, the mixtures were purified with ZipTipC18 micro-columns (Millipore, Bedford, MA) and eluted into 3 μl of 50% ACN in water. To the eluate, 3 μl of 0.1% phosphotolurobutyric acid was added and the sample mixture was injected onto the column of LC-nanoESI-QIT MS on the LCQDeca instrument (ThermoFinnigan, San Jose, CA). PicoFrit™ columns (360-μm outer diameter, 75-μm inner diameter, 15-μm tip inner diameter) from New Objective (Woburn, MA) were used for LC. Samples were analyzed using a gradient program consisting of initial 5-min isocratic elution with 0% B, followed by linear gradient 0–70% B in 50 min, 5-min isocratic elution with 70% B and a linear gradient 70–0% B in 15 min (A = 0.1% formic acid and B = 90% ACN–10% water–0.1% formic acid). Thirty-minute column equilibration time was used between subsequent injections and the injector port was washed with 50 μl of 30% acetonitrile:30% isopropanol:40% water followed by 50 μl of water between injections to prevent carryover of samples. The peptides eluted at 400 nl/min were introduced online into the mass spectrometer and MS and MS/MS spectra were obtained in the data-dependent mode. The instrument cycled through acquisition of a full-scan MS spectrum, followed by five MS/MS scans of the most abundant ions from the MS scans obtained by collision with helium gas. For the MS/MS analysis the collision energy was set to 35% and the dynamic exclusion was 0.5 min.

The search engine Sequest (LCQDeca software package) was used to analyze the MS/MS data by searching the SwissProt mouse protein sequence database. The protein matches were considered valid if MS/MS data for multiple unique peptides were matched. For single peptide match, the identification was confirmed by manual examination using the following criteria: 1) Sequest cross-correlation (Xcorr) score ≥ 2.0 (doubly charged peptides)/≥ 3.5 (triply charged peptides); 2) a good-quality MS/MS spectrum with the difference in the observed and the theoretical masses of the product ions not more than 0.5 mass unit; 3) continuous stretch of the peptide sequence covered by either the y- or b-ion series; 4) intense y-ions corresponding to a proline residue (if proline was present in the sequence); 5) similar values of observed and theoretical pl and molecular mass of a protein (26).

Western Blot Analysis—C2C12 cells were washed twice with warm PBS and incubated for 30 min at 4 °C in RIPA buffer as described above. After removing the insoluble material by centrifugation (14,000 rpm for 5 min at 4 °C), protein concentrations were determined by Bio-Rad DC Protein Assay. Thirty-microgram aliquots of proteins were separated by SDS-PAGE and subject to Western blotting as detailed previously (27). Proteins were transferred onto 0.45-μm nitrocellulose membranes. Binding of nonspecific proteins to membranes was blocked by incubating these in the blocking buffer consisting of 5% non-fat milk in TBS plus 0.1% Tween 20 (TBST) for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with primary antibodies diluted in the blocking buffer. Dilution of various antibodies was done according to the recommendation of the manufacturers as follows: anti-MHC (MF20), 1:1,000, anti-cAMP-dependent protein kinase (PKA) α-1 and -I regulatory subunit, 1: 200; anti-PKA β-I regulatory subunit, 1:200; anti-actin, 1:200; anti-MyoD, 1:200; anti-MEF-2, 1: 200; anti-LIM kinase 1 (LIMK1), 1:200; anti-phospho serine/threonine protein kinase (pAkt), 1:1000; anti-phospho mitogen-activated protein kinase kinase (MKK) 3/6, 1:1000; anti-phospho P70(S6) kinase, 1:1000; anti-phospho extracellular signal-regulated kinase (pERK) 1/2, and 1:1,000.

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FIG. 1. A, detection of MHC in C2C12 cells grown either in GM (left) or DM for 72 h (right). A monoclonal antibody against MHC was used as primary antibody that was detected by FITC-conjugated secondary antibody as described in “Materials and Methods.” B, whole-cell lysates prepared from C2C12 cells cultured in GM or cultivated in DM for indicated number of hours were subjected to SDS-PAGE, transferred to membranes, and probed with antibodies for MyoD, MEF, MHC, and actin as detailed in the “Materials and Methods.” The antibody against actin did not discriminate between ubiquitous and muscle-specific actin.

**RESULTS**

**Morphology of C2C12 Cells in GM and DM**—When cultured in DMEM containing 10% fetal bovine serum, proliferating C2C12 cells grow as mononucleated flattened cells in a monolayer. When confluent cells were incubated in DMEM containing 2% horse serum for 48–72 h, the majority of C2C12 cells assumed elongated morphology and fused to become multinucleated myotubes or myocytes. Such morphological conversion of myoblasts into myotubes accompanies accumulation of muscle-specific proteins, as demonstrated by staining by MHC-specific antibody (Fig. 1A). Changes in the steady-state levels of muscle-specific transcription factors such as MyoD and MEF, and structural proteins (e.g. actin and MHC) could also be readily detected by Western blot analysis of proteins from C2C12 cells grown in DM for various durations (Fig. 1B).

**Proteome of Total Cell Proteins**—Total proteins were extracted from proliferating C2C12 myoblasts cultivated in GM and fully differentiated myotubes harvested from cultures incubated for 72 h in DM (80–90% multinucleated myotubes). Four aliquots of 100 μg of protein from each cell type were fractionated on replicate 2D gels (10%, 3–10 linear pH gradient) and images of silver-stained replicates were analyzed by PDQuest to assess their reproducibility. We discovered that <550 protein spots that were detected from either proliferating myoblasts or myotubes on replicate gels had 86–99% reproducibility; this range of reproducibility for replicated 2D patterns is similar to what has been reported previously (28). The representative silver-stained 2D images of total cell proteins from C2C12 myoblasts and myocytes fractionated on a 3–10 pH gradient and 10% gels are shown in Fig. 2, A and B, respectively. The PDQuest image analysis of the master images representing myoblasts and myotubes elucidated the putative differentiation-specific protein spots.

When total cell proteins, regardless of whether extracted from myoblasts or myocytes were subject to IEF in a 3–10 pH gradient, distribution of these spots was nonrandom because most spots were clustered in a 4–7 pI range. Therefore in the subsequent experiments we fractionated total cell proteins from the two cell types on a 4–7 pH gradient in the first dimension. Isoelectric focusing of proteins in a 4–7 pH gradient resolved many more spots than those seen in a 3–10 pH gradient 2D gel; 653 and 433 protein spots were resolved from samples of proteins from myoblasts, in a 4–7 versus 3–10 pH gradients, respectively. Similarly, 558 protein spots were observed for myotube-specific proteins subject to IEF on 4–7 pH gradients in the first dimension, whereas about 300 proteins were resolved in a 3–10 pH gradient. A PDQuest-based quantification of the master patterns representing myoblast- and myotube-specific 2D gels revealed numerous pro-
tein spots that seemed to have variable expression in the two cell types as judged by their staining intensities (Figs. 2 and 3).

Proteome of Crude Membrane-enriched Fractions from the Proliferating and Differentiated C2C12 Cells—While extracts of whole-cell proteins subjected to IEF on either 3–10 or 4–7 pH gradients in the first dimension resolved numerous proteins, we were aware that these represented only a fraction of the most abundant proteins expressed in C2C12 cells, regardless of their state of differentiation. Therefore, we prepared subcellular fractions from crude plasma membranes and nuclei. Aliquots of membrane-enriched proteins from either C2C12 myoblasts or myotubes were fractionated on three to four replicate pH 4–7 and 10% 2D gels. We should stress here that the purpose of our subcellular fractionation was to enhance...
the relative abundance of a subset of proteins that may not be detectable in the whole-cell extracts. Thus, our "crude membrane-enriched" fraction is likely to contain additional proteins that are not bona-fide membrane proteins. Furthermore, as has been articulated in detail (29, 30), analysis of integral membrane proteins by 2D SDS-PAGE poses a number of challenges that include i) their lower solubility in the first dimension followed by 10% SDS-PAGE as detailed in "Materials and Methods." Arrows indicate protein spots that were identified by MS and bioinformatics. The detailed parameters of protein identification are outlined in Table I.

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Proteome of \( C_2C_{12} \) Cells Undergoing Myogenesis

\[\text{Relative Intensity}\]

A

B

C

\[\text{Relative Intensity}\]
per cell of some membrane proteins (e.g. receptors for hormones). However, these caveats notwithstanding, we achieved our main objective of analyzing many more proteins as a result of subcellular fractionation of C2C12 myoblasts and myotubes than would have been otherwise possible (31, 32).

Two features of the 2D gel patterns from membrane-enriched proteins shown in Fig. 4 can be readily appreciated visually. First, the overall patterns of membrane-enriched proteins analyzed by 2D gels are less complex compared with the patterns seen with total-cell proteins. Corroborating the visual impression, PDQuest analysis showed that 213 and 144 unique protein spots were detected in the master-images of myoblast- and myocyte-specific membrane protein 2D gels, respectively. Second, a large fraction of the membrane-enriched proteins generated a horizontal string of spots with heterogeneous pl that likely result from post-translational modifications of proteins. A comparative PDQuest image analysis of master gels representing crude membrane-enriched proteins from myoblasts and myotubes revealed that several proteins were differentially expressed (Fig. 4). We identified a number of these proteins by MALDI-TOF-MS and LC-ESI-QIT-MS/MS. As expected, although the majority of the proteins from crude membrane-enriched fractions were membrane proteins such as IGF-I receptor (spot 39) FGFR-4 (spot 40), jagged 2 (spots 46 and 47), and NADH-ubiquinone oxidoreductase subunit (spot 49), a number of nonmembrane proteins such as baculovirus IAP repeat-containing protein (spot 16), glucosamine-6-phosphate isomerase (spot 62), and LIM domain kinase 1 (spot 11) were also found in this fraction.

Proteome of Nuclear Proteins from C2C12 Myoblasts and Myotubes—To enhance detection of less-abundant proteins that may be differentially regulated during myogenesis in vitro, we also analyzed proteins from “crude nuclei-enriched” fractions from C2C12 myoblasts and myotubes. We obtained “nuclear-enriched” fractions according to Mirkovitch et al. (13), as modified to isolate nuclei from C2C12 cells (33). Extensive analysis of nuclear fractions obtained by this approach has revealed that although vast majority of the proteins detected from such preparations represent bona-fide nuclear proteins a large number of cytoplasmic and cytoskeletal proteins (e.g. vimentin cytokeratin and desmin) are also associated with nuclei (33). Therefore, the caveat articulated above with respect to “crude membrane-enriched” fractions should also be applied to the interpretation of our data on the proteome of the “crude nuclear fraction.” In addition to the problem of contamination of nuclei with non-nuclear components, resolution of nuclear proteins on a narrow 4–7 pH gradient 2D gel is incomplete because many nuclear proteins are highly basic.

Representative 2D images of nuclear proteins from myoblasts and myotubes are shown in Fig. 5. The PDQuest image analysis showed that 413 and 276 nuclei-enriched proteins could be consistently detected from cultures of myoblasts and myotubes, respectively. As we had observed for the membrane-enriched proteins earlier, the 2D patterns of nuclei-associated proteins are also significantly less complex than those of whole-cell proteins. A comparison of the staining intensities of common proteins by PDQuest analysis revealed that several proteins accumulated preferentially either in the nuclei of proliferating myoblasts or fully differentiated myotubes (Fig. 5).

Quantification of Differentially Expressed Proteins—To quantify potential gene products that were differentially regulated during conversion of C2C12 myoblasts into myocytes, we assessed the absorbance of individual protein spots from 2D gels representing total, and membrane-, and nuclear-enriched proteins from each cell type. The staining intensity of each spot was normalized against the sum total of intensities of all detectable spots in the 2D gel; this normalization maneuver of the PDQuest program is designed to correct for the minor differences in protein loading or staining intensity among replicate gels. The values of spot densities were obtained as arbitrary units ranging from $1 \times 10^9$ to $1 \times 10^{10}$ and were divided by a factor of $10^9$. To confirm normalization of spot intensities derived from the PDQuest analysis, we compared the staining intensities of seven protein spots, shown as standard spot protein (SSP) in Fig. 2. This analysis was also used to assess the intrinsic variability of the densitometry-based quantification of protein expression data.

Quantification of staining intensities of the individual protein spots from 2D gels, as examined by PDQuest-based image analysis, revealed that the vast majority of 2,139 total cell-, membrane-, and nuclei-associated protein spots were qualitatively similar regardless of whether they represented proliferating C2C12 cells or fully differentiated myotubes. Additionally, we observed that the relative staining intensities of numerous protein spots showed only low (10–20%) to moderate (20–50%) change associated with the state of cellular differentiation (31, 32). In contrast, a small subset of polypeptides that were abundant in the extracts of proliferating C2C12 cells such as a LIM domain-containing protein (spot 7), insulin-like growth factor I receptor (spot 39), jagged 2 (spots 46 and 47) were not seen in myotube-specific 2D gels, while...
other proteins such as MKK3 (spots 9) or a baculovirus IAP-repeat containing protein-4 (spot 16) were detected only in the 2D gels of the fully differentiated myotubes (3). As shown in Fig. 6, although the regulation of about 70 proteins fell between the “all or none” extremes their steady-state levels were significantly different between myoblasts and myotubes (p < 0.05 by unpaired Student’s t test). The extent of differential expression of such proteins ranged from as low as 1.32-fold enhancement noted for calsenilin (spot 36) to a 42-fold increase in the steady-state levels of the microtubule-associated, APC-binding protein EB1 (spot 17). We reasoned that these highly regulated proteins were mechanistically related to the process of myogenic differentiation of C2C12 cells and, therefore, systematically established their identities by peptide mass fingerprinting and amino acid sequencing as outlined below.

Phospho-proteomes of C2C12 Myoblasts and Myocytes— To determine changes in protein phosphorylations in C2C12 undergoing myogenesis, we stained the 2D gels of whole-cell protein extract with the Pro-Q® Diamond phospho-protein gel stain. The representative gels of whole-cell proteins of myoblasts and myocytes fractionated in 4–7 pH range and stained for phospho-proteins are shown in the Fig. 7. PDQuest-based comparisons of the gels stained for phospho-proteins showed that 108 and 122 putative phospho-protein spots were resolved in the myoblasts and myotubes, respectively. Thus, only about 15–20% spots of the total cellular proteins stained by silver nitrate were stained by Pro-Q® Diamond stain, regardless of whether cells were actively proliferating or fully post-mitotic. A PDQuest-based image analysis was done to quantify the staining intensities of the putative phospho-proteins and is represented in the Fig. 8. The absorbance in arbitrary units in a given spot was normalized, and all the absorbance values were divided by a factor of 10^4. The targeted phospho-protein spots were identified by MALDI-TOF-MS (Table I). Although the specificity of the Pro-Q® Diamond for staining phospho-proteins has been rigorously established (34–36), we wish to inject a note of caution in the interpretation of these data. The identification of a spot, stained with Pro-Q® Diamond, as a phospho-protein by MS may be seriously biased if an abundant protein (which may or may not be a phospho-protein) co-migrates with this particular spot. Therefore, the MS-mediated identification of a spot as a phospho-protein must be independently validated by other methods (i.e. by precursor ion scanning for phosphate ion or neutral loss by MS technique). We have not independently verified whether every spot assigned to the “phospho-proteome” of C2C12 cells, as judged by Pro-Q® Diamond staining, is indeed a bona-fide phospho-protein. However, it is worth noting that out of 26 differentially regulated “phospho-proteins,” at least half (spots 25, 83, 84, 89, 91, 92, 95, 96, 97, 100, 101, 104, and 105) are known phospho-proteins as evident from the published literature. In addition, we have obtained sequencing information on three additional spots by LC-ESI-QIT-MS/MS to confirm their phospho-protein nature (R. Raghow, unpublished observation). In light of this technical concern, the assignment of the remaining polypeptides as phospho-proteins may only be considered tentative.

MALDI-TOF-MS and LC-ESI-QIT-MS/MS Identification of Proteins— Table I represents a list of proteins extracted from
2D gels (Figs. 2–6) of C2C12 myoblasts and myocytes and identified by MALDI-TOF-MS and/or LC-ESI-QIT-MS/MS. Although majority of the proteins extracted from 2D gels were identified by MALDI-TOF-MS, the identity of some proteins was also corroborated by peptide sequencing using LC-ESI-QIT-MS/MS. Thus, heat shock protein β-90 (HSP-90β) was identified by MALDI-TOF-MS initially; the identity of HSP-90β was confirmed by amino acid sequence analysis of its tryptic peptides by LC-ESI-QIT-MS/MS. The combined sequence coverage by MALDI-TOF and LC-ESI-QIT MS unequivocally established the identity of HSP-90β. The list of 106 proteins and phospho-proteins identified by 2D-PAGE and MS and bioinformatics methods is presented in Table I. We have attempted to organize the differentially expressed proteins into functional categories, fully realizing that such a classification is somewhat arbitrary because a number of proteins could be assigned to more than one functional category.

Validation of Selected Proteomics Data by Western Blotting—The list of putatively regulated proteins depicted in Table I is a snapshot of proteins from proliferating myoblasts versus quiescent, multinucleated myotubes. Therefore, we assessed temporal changes in expression of a subset of proteins C2C12 myoblasts incubated in the DM for 6, 12, 24, 48, 72, 96, and 120 h. Cellular proteins were then fractionated by denaturing SDS-PAGE and analyzed by Western blotting. In addition to proteins known to be associated with skeletal muscle differentiation such as MyoD, MEF, MHC, and actin (Fig. 1B), we analyzed changed in the levels of LIMK1, PKA, AKT, p70S6 kinase, and various members of the MAP kinase family in C2C12 cells undergoing myogenesis in DM (N. Tannu, data not shown). The α-I, α-II, and β-I subunits of PKA were expressed constitutively in C2C12 cells regardless of their growth in GM or DM. The α-II subunit of PKA showed a steady decrease in expression up to 48 h, after which it showed enhanced accumulation up to the 120 h time point. The expression of LIMK1 was steady in the initial 12 h in DM but declined progressively as myoblasts exited cell cycle and became differentiated into bona fide multi-nucleated myotubes (72–120 h). The overall pattern of sequential expression of LIMK1 is consistent with the PDQuest and MS data. The phosphorylated form of Akt (pAkt) also showed steady expression up to 48 h after serum withdrawal but was up-regulated in C2C12 cells incubated in DM for 72–120 h. The apparent discrepancy in the expression Akt2 as assessed from PDQuest-based analysis and Western blot analysis may be due to failure of our antibody to distinguish between Akt1

![Fig. 8. Quantification of the phospho-proteins detected in myoblasts (black bars) and myocytes (white bars). The data were obtained by PDQuest analysis of phospho-proteins detected by Pro-Q® Diamond staining of 2D gels representing whole-cell proteins from myoblasts (black bars) and myocytes (white bars). The relative intensities of the phospho-proteins in myoblasts and myocytes are the normalized values in arbitrary units in a given spot as outlined in the “Materials and Methods.”](image-url)
## Proteome of C<sub>2</sub>C<sub>12</sub> Cells Undergoing Myogenesis

### TABLE I

The proteins identified by MALDI-TOF-MS and LC-ESI-QIT-MS/MS from C<sub>2</sub>C<sub>12</sub> myoblasts and myocytes

The major parameters of protein identification and mode of their regulation in response to myogenic differentiation is indicated by ↑ ↓. The precise quantification of the steady levels of a particular protein may be seen in the Figs. 6 and 8. An asterisk (\*) indicates proteins that were identified by LC-ESI-QIT MS/MS sequencing.

| Spot no. | Protein                                      | Accession no. | Parameters | MALDI-MS/MS-ESI-QIT-MS/MS* | Regulation |
|----------|----------------------------------------------|---------------|------------|-----------------------------|------------|
|          | Cytoskeletal proteins                        |               |            |                             |            |
| 1        | Actin, cytoplasmic 1 (β)                     | P02570        | 41736      | 5.29                        | 19         | 52.4  |
| 2        | Actin, cytoplasmic 2 (γ)                     | P02571        | 41736      | 5.31                        | 9          | 34.2  |
|          | Actin, skeletal muscle 2 (α)                 | Q61276        | 41693      | 5.21                        | 7          | 26.9  |
|          | *Actin, skeletal muscle 2 (α)                | P53482        | 41976      | 5.23                        | 53.6*      |       |
| 2        | Actin, cytoplasmic 2 (γ)                     | P02571        | 41736      | 5.31                        | 5          | 17.1  |
| 3        | *F-actin capping protein α-2 subunit         | P47754        | 32967      | 5.57                        | 71*        |       |
| 4        | *Myosin heavy chain, fast skeletal muscle    | P02565        | 222816     | 5.68                        | 0.7*       |       |
| 5        | *Myosin light chain 1, skeletal muscle       | P05977        | 20594      | 4.98                        | 85.6*      |       |
| 6        | Tropomyosin β chain                          | P58774        | 32836      | 4.66                        | 6          | 21.1  |
| 7        | *Actin, skeletal muscle 2 (α)                | P53482        | 41976      | 5.23                        | 53.6*      |       |
| 8        | Calponin, acidic isoform (Calponin 3)        | Q15417        | 36414      | 5.69                        | 4          | 23.7  |
| 9        | Myosin light chain 1, atria/fetal isofrom   | P09541        | 21027      | 4.97                        | 8          | 47.4  |
|          | Signaling proteins                           |               |            |                             |            |
| 10       | Dual specificity MAP kinase kinase 3 (MKK3)  | P46734        | 39318      | 6.2                         | 6          | 22    |
| 11       | LIM domain kinase 1 (LIMK1)                  | Q13981        | 45387      | 4.96                        | 5          | 22.6  |
| 12       | Serine/threonine protein kinase Akt-2        | Q60989        | 56709      | 5.68                        | 4          | 9.3   |
| 13       | Serstrin 1 (p53-regulated protein PA26)       | P58006        | 56632      | 5.64                        | 4          | 16.3  |
| 14       | Bacularoviral IAP repeat-containing protein 4 | Q60989        | 56709      | 5.68                        | 4          | 9.3   |
| 15       | LIM domain-containing protein 1               | Q9BQ09        | UD         | UD                          | 14         | 4.1   |
| 16       | LIM and SH3 domain protein 1 (Lsp-1)         | Q61739        | 29994      | 6.61                        | 8          | 31.6  |
| 17       | *75-kDa glucose-regulated protein            | P38647        | 73528      | 5.91                        | 47*        |       |
| 18       | *Growth arrest-specific protein 1 (GAS 1)    | Q01721        | 35694      | 5.68                        | 3.1*       |       |
| 19       | *Follistatin-related protein 1               | Q62356        | 32461      | 5.41                        | 4          | 13.9  |
| 20       | Serine/threonine protein phosphatase 2A      | P13353        | 35608      | 5.3                         | 4          | 26.5  |
|          | Protein processing/folding                   |               |            |                             |            |
| 21       | Protein disulfide isomerase                  | P09103        | 57143      | 4.75                        | 21         | 33.7  |
| 22       | 78-kDa glucose-regulated protein             | P20029        | 72422      | 5.01                        | 32         | 47.3  |
| 23       | *78-kDa glucose-regulated protein            | P20029        | 72422      | 5.01                        | 48.5*      |       |
| 24       | *78-kDa glucose-regulated protein            | P20029        | 72422      | 5.01                        | 34.2*      |       |
| 25       | 60-kDa heat shock protein, mitochondrial     | P19226        | 60955      | 5.35                        | 9          | 23.4  |
| 26       | *Heat shock protein HSP90-β                  | P11499        | 56976      | 5.8                         | 26.3/50.4* |       |
| 27       | Endoplasmin                                  | P08113        | 92475      | 4.72                        | 30         | 36.4  |
| 28       | *Ubiquitin carboxyl-terminal hydrolase       | Q9WUP7        | 37616      | 5.24                        | 3.3*       |       |
|          | Membrane-associated proteins                 |               |            |                             |            |
| 29       | *Prefoldin subunit 2                         | O70591        | 16533      | 6                           | 8.3*       |       |
| 30       | *Phosducin-like protein (PHLP)               | Q9DBX2        | 34406      | 4.79                        | 3.4*       |       |
| 31       | Peptidyl-prolyl cis-trans isomerase E        | Q9Q2H3        | 33161      | 5.4                         | 4          | 21.1  |
| 32       | Chain 1: Plasminogen activator inhibitor     | P22777        | 42848      | 6.03                        | 6          | 19.8  |
| 33       | 26S Proteasome regulatory subunit            | Q03927        | 49164      | 5.87                        | 6          | 14.8  |
|          | Annexin I                                   | P04083        | 38583      | 6.6                         | 8          | 32.5  |
| 34       | Annexin IV                                  | P55260        | 35743      | 5.32                        | 5          | 17.9  |
| 35       | Annexin IV                                  | P97429        | 35858      | 5.43                        | 5          | 32.8  |
| 36       | Calsenilin (DRE-antagonist modulator)        | Q9QXT8        | 29430      | 5.46                        | 5          | 20.7  |
| 37       | Chain 1: Frizzled 4                          | Q9QYE5        | 132050     | 5.38                        | 10         | 19.3  |
| 38       | Ephrin type-B receptor 6 precursor           | Q9QYE5        | 132050     | 5.38                        | 8          | 24.3  |
| 39       | Insulin-like growth factor I receptor        | Q9QYE5        | 132050     | 5.38                        | 8          | 24.3  |
| 40       | Activin β-2                                 | P11835        | 82996      | 5.86                        | 4          | 5.6   |
| 41       | *Integrin α-V precursor                     | P43406        | 115277     | 5.46                        | 1.4*       |       |
| 42       | Chain 1: JAGGED 2                            | Q9QYE5        | 132050     | 5.38                        | 10         | 19.3  |
| 43       | Chain 1: JAGGED 2                            | Q9QYE5        | 132050     | 5.38                        | 8          | 24.3  |
| Spot no. | Proportion | Parameters | MALDI-MS/LC-ESI-QIT-MS/MS* Regulation |
|---------|------------|------------|--------------------------------------|
| 48 | Peripheral plasma membrane protein CASK | O70589 | 104710 | 6.16 | 13 | 15.5 |
| 49 | NADH-ubiquinone oxidoreductase 49-kDa subunit | P17694 | 49174 | 5.95 | 6 | 14 |
| 50 | Guanine nucleotide-binding protein β subunit 4 | P29387 | 37354 | 5.59 | 2.9* |
| 51 | Guanine nucleotide-binding protein G(l)/G(S)/G(T) β subunit 3 | Q61011 | 37240 | 5.41 | 4 | 8.8 |
| 52 | Guanine nucleotide-binding protein β subunit like protein 12.3 (receptor of activated protein kinase C1) | P25388 | 35076 | 7.6 | 4 | 10.7 |
| 53 | Granulocyte-macrophage colony-stimulating factor (GM-CSF) | Q00941 | 38775 | 8.02 | 5 | 12.8 |
| 54 | Myocyte-specific enhancer factor 2A (MEF2A) | Q60929 | 53724 | 6.43 | 3* |
| 55 | Ribonucleoprotein F (RNP F) | Q9Z2X1 | 45730 | 5.31 | 6.3 |
| 56 | TBP-associated factor (TAF) 28kD | Q99JX1 | 23333 | 5.16 | 4 | 19.9 |
| 57 | Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) | P09867 | 31665 | 5.07 | 4 | 19.6 |
| 58 | 40S ribosomal protein SA (34/67-kDa laminin receptor) | P14206 | 32719 | 4.74 | 23.7* |
| 59 | Vacuolar ATP synthase subunit E | P50518 | 26588 | 9.2 | 5.3* |
| 60 | *Secreted protein acidic and rich in cysteine (SPARC) | P07214 | 34450 | 4.77 | 14* |
| 61 | Zinc finger MYND domain-containing protein 10 | Q99ML0 | 50632 | 6.1 | 10.5 |
| 62 | Glucosamine-6-phosphate isomerase (glucosamine-6-phosphate deaminase) (Oscillin) | P48967 | 50406 | 6.38 | 4 | 12.1 |
| 63 | ATP synthase β chain, mitochondrial | P56480 | 50146 | 5.98 | 4 | 20.2 |
| 64 | Chain 1: Calreticulin | P14211 | 46000 | 4.33 | 6 | 21.8 |
| 65 | Adenylate kinase isozyme 5 | Q9Y6K8 | 22100 | 5.38 | 7 | 15.9 |
| 66 | NADPH-dependent carbonyl reductase 3 | O75828 | 30700 | 5.82 | 4 | 21 |
| 67 | Immunoglobulin-binding protein 1 | P78318 | 39220 | 5.26 | 5 | 22.4 |
| 68 | Immunoglobulin-binding protein 1 | P78318 | 39220 | 5.26 | 4 | 19.8 |
| 69 | Chain 1AATP synthase β chain | P56480 | 51749 | 4.99 | 17 | 47.2 |
| 70 | Aldo-keto reductase family 1 member C13 | Q8VC28 | 37057 | 6.67 | 5 | 27.6 |
| 71 | Guanine deaminase | Q9R111 | 51013 | 5.36 | 4 | 12.3 |
| 72 | Chain 1: protein C21ORF63 homolog | P58659 | 44037 | 5.98 | 4 | 20.2 |
| 73 | Protein-glutamine glutamyl transferase E | O08958 | 32550 | 6 | 21.1 |
| 74 | Heat shock protein HSP90-β (tumor-specific transplantation 84-kDa antigen) | P11499T | 83.19 | 4.97 | 16 | 20 |
| 75 | Vimentin | Q20152 | 53.55 | 5.06 | 8 | 15.9 |
| 76 | A disintegrin and metalloproteinase domain-containing protein 17 | Q9Z08F8-2 | 73.88 | 5.75 | 6 | 10.8 |
| 77 | Zinc finger protein 305 | Q08189 | 24973 | 5.54 | 7 | 24.7 |
| 78 | Quinone oxidoreductase-like 1 (OQH-1) (cystatin homolog) | O43309 | 70.22 | 6.28 | 5 | 8.3 |
| 79 | Annexin A5 (Annexin V) (Lipocortin V) | Q95825 | 38.66 | 5.49 | 5 | 24.1 |
| 80 | Annexin A5 (Annexin V) (Lipocortin V) | P48036 | 35.75 | 4.83 | 4 | 18.2 |
| 81 | NIF3-like protein 1 | Q98080 | 38.82 | 5.91 | 9 | 27 |
| 82 | Transcription factor E2-α | P15806 | 30.23 | 6.25 | 4 | 11.7 |
| 83 | CDK-activating kinase assembly factor MAT1 (CDK7/cyclin H assembly factor) (p36) (p35) | Q51949 | 35.84 | 5.64 | 4 | 8.4 |
| 84 | Mitogen-activated protein kinase 4 (MAP kinase isomorph p63) | P31152 | 62.62 | 6.05 | 4 | 8.3 |
| 85 | Lamin A | P48678 | 74.21 | 6.54 | 8 | 12.3 |
Proteome of C2C12 Cells Undergoing Myogenesis

and Akt2 (N. Tannu, data not shown). The pattern of p38 MAP kinase expression did not change during growth in DM. The levels of pMKK3/6 were dynamically regulated. The steady-state levels of MKK 3/6 and phosphorylated MKK7 went up when C2C12 cells were transferred to DM for 72 h. The p70S6 kinase was abundantly expressed in proliferating C2C12 myoblasts but dramatically declined after the cultures were transferred to DM for 12 h or longer.

DISCUSSION

We compared whole-cell, crude membrane-, and nuclear-enriched proteins from C2C12 myoblasts and myotubes by a noncandidate, large-scale method of proteomics that involved fractionation of proteins by 2D-PAGE, PDQuest image analysis of silver-stained gels, and identification of target proteins by MS. We should note, however, that assignment of some proteins as “membrane” or “nuclear” may be an artifact of our subcellular fractionation method. This caveat notwithstanding, such crude fractionation enabled us to quantify 20–30% more proteins than would have been otherwise possible from the analyses of the whole-cell proteins without subcellular fractionation (31, 32). PDQuest image analysis of the most abundant 2,139 proteins revealed that vast majority of these most likely represented gene products relegated to structural and/or housekeeping functions and apparently did not undergo major regulation. In contrast, expression of 75 polypeptides was consistently altered as mononucleated, proliferating C2C12 myoblast cells exited cell cycle and became MHC-positive, post-mitotic multi-nucleated myotubes. In addition, we identified 26 phospho-proteins that underwent differential expression during myogenic differentiation of C2C12 cells. Included among the differentially regulated proteins were mediators of inter- and intracellular signaling, cell shape, protein folding and stability, cell proliferation and apoptosis, and putative regulators of transcriptional and post-transcriptional modes of muscle-specific gene expression. We should note that although most of the differentially expressed proteins seen here are already known to be either directly or indirectly involved in myogenesis, a number of gene products (e.g. HSP90, transcription intermediary factor 1β (TIF1β) and IKB kinase α subunit) with unprecedented involvement in skeletal muscle differentiation were also uncovered by our experiments. Furthermore, although we found the proteomics-based approach to be valuable in generating new information, we were surprised that some well-known mediators of muscle differentiation such as myogenin and cell-cycle regulator p21 were not readily detected in our analysis. The most plausible explanation for this discrepancy is that a given spot on a 2D gel may consist of more than one protein and analysis of such spots by MALDI-TOF generates complex mixture of peptide fingerprints. Therefore, by choosing only the best-matched protein and ignoring additional less perfect peptide fingerprint matches, we are likely to miss minor proteins obscured by the more abundant ones.

Initiation of differentiation in C2C12 cells following their growth in DM leads to profound changes in the transduction of inter- and intra-cellular signals, locomotion, and cell shape that precede the fusion of myoblasts into multinucleated muscle cells and depends on the reorganization of their cytoskeleton and plasma membranes (37). Temporal changes in the steady-state levels of a number of key protein kinases known to regulate cell architecture, proliferation, and apoptosis seen in our study are consistent with this scenario. Coordinate regulation of p70S6K and pERK in cells incubated in DM suggests involvement of these kinases in the cell-cycle regulation and imposition of quiescence before induction of the myogenic differentiation program. Similarly, the levels of pAKT/PKB and p38 were also regulated during this process. The precise regulatory relationship among the various kinases remains to be determined. Furthermore, changes in the

| Spot no. | Protein                                      | Accession no. | Parameters | MALDI-MS/LC-ESI-QIT-MS/MS* | Regulation |
|---------|----------------------------------------------|---------------|------------|-----------------------------|------------|
| 93      | Lamin A                                      | P48678        | 74.21      | 6.54                        | 9          | 19.4       |
| 94      | TNF receptor-associated factor 5             | P70191        | 64.14      | 7.71                        | 7          | 12.4       |
| 95      | Mitogen-activated protein kinase kinase kinase 7 (MKK7) | Q62073        | 64.22      | 6.23                        | 6          | 9.7        |
| 96      | Ribosomal protein S6 kinase α 3 (pp90RSK2)   | P18654        | 83.69      | 6.41                        | 7          | 12.6       |
| 97      | Splice isoform 3 of Rho guanine nucleotide exchange factor 1 | Q61210–3 | 89.29 | 5.78 | 9 | 8.1 |
| 98      | Crooked neck-like protein 1                  | Q9CQC1        | 78.7       | 6.62                        | 4          | 7.3        |
| 99      | POU domain, class 5, transcription factor 1  | P20263        | 38.21      | 6.05                        | 4          | 11.4       |
| 100     | Protein kinase C-α binding protein           | Q62083        | 46.52      | 5.33                        | 5          | 9.4        |
| 101     | Ribosomal protein S6 kinase α 3              | P18654        | 83.69      | 6.41                        | 6          | 7.4        |
| 102     | Splice isoform 2 of tropomyosin α 3 chain    | P21107–2      | 29.02      | 4.75                        | 7          | 22.2       |
| 103     | Annexin V                                   | P48036        | 35.75      | 4.83                        | 4          | 16.3       |
| 104     | Inhibitor of nuclear factor α-B kinase α subunit | Q60680 | 66.8 | 7.23 | 5 | 12.7 |
| 105     | B-Raf proto-oncogene serine/threonine-protein kinase | P28028 | UD | UD | 4 | 8.2 |
| 106     | G2/mitotic-specific cyclin B1                | P24860        | 48.05      | 7.1                         | 4          | 8.6        |
steady-state levels of \( \alpha \)- and \( \beta \)-actin, calponin, CapZ, APC-EB1, and tropomyosin and myosin light chain during myogenesis in vitro underscore the role of cell shape changes during this process.

The C2C12 myoblasts accumulated the ubiquitous and muscle-specific actins and some proteins known to associate with actin. Tropomyosin binds to actin and in association with tropomyosin regulates calcium-dependent contraction of muscle. Anti-CapZ antibodies or expression of dominant-negative CapZ delay myofibril assembly and the formation of striations in the muscle cell (38). Phosphatidylinositol-4,5-bisphosphate dissociates CapZ from the barbed ends of actin filaments in vitro (39). Thus, the observed up-regulation of both CapZ and EB1 in the myotubes underscores a role of actin- and microtubule-based cytoskeleton network in differentiating muscle.

Cell-cell and cell-extracellular membrane interactions facilitate myotube formation and the enhanced levels of basement membrane proteoglycan agrin, the matricellular protein SPARC, and \( \alpha_\nu \) and \( \beta_\varepsilon \) integrin in the C2C12 myotubes are consistent with a role of extracellular matrix in myogenesis. Agrin facilitates clustering of acetylcholine receptors and acetylcholinesterase at neuromuscular junction (40). An enhanced expression of agrin in C2C12 myotubes reflects its intrinsic potential to form neuromuscular synapse. Three members of the calcium- and phospholipid-binding family of proteins, the annexins, were up-regulated in C2C12 myotubes. Annexin-I is known to promote membrane fusion and exocytosis, and elevated levels of annexin-I reflect its role in the fusion of C2C12 cells needed to form multinucleated myotubes.

The levels of two LIM domain-containing proteins and LIMK1 were significantly altered in C2C12 cells in DM. While the LIM- and SH3-domain containing protein (Lasp-1) preferentially accumulated in the myotubes, LIM-domain protein1 and the LIMK1 were both down-regulated. Lasp-1, a cAMP-dependent actin-binding protein (41, 42) may be causally associated with the changes in cell architecture. LIMK1 phosphorylates cofilin and abolishes its ability to de-polymerize actin. Apparently, LIMK1 initially rises in cells incubated in DM but declines progressively as myoblasts exit cell cycle and become myotubes (43, 44). Because the mature muscle cells do not express LIMK1, we propose that its steady decline during myogenic differentiation of C2C12 cells facilitates this process. Three members of the LIM-only proteins, FHL1, FHL2, and FHL3, are expressed in skeletal and heart muscle (45). Exogenous expression of FHL2 has been shown to enhance the rate of myotube formation in C2C12 cells (46). In light of these observations, we can only speculate whether changes in LIM-domains-containing protein 1 and LIMK1 are mechanistically related to the process of myogenic differentiation.

The steady-state levels of two signaling kinases, MKK3 and PKA, were reciprocally altered during in vitro conversion of C2C12 cells into myotubes. The MKK3, a dual-specificity MAP kinase, together with MKK4, MKK6, and MKK7, is an upstream activator of p38 MAP kinase involved in muscle differentiation (47). Expression of dominant-negative MKK3 severely inhibited the synthesis of MyoD, troponin T, p21 and p27, and myotube formation in C2C12 cells (48). Thus, preferential accumulation of MKK3 in the C2C12 myoblasts corroborates previous studies indicating that MAP kinase cascade plays a critical role in skeletal muscle differentiation.

Directed control of intracellular cAMP, PKA, phosphorylation of cAMP-responsive element (CRE) binding protein and transcription of CRE-driven promoters during myogenesis has also been documented (49). Thus, the reduced level of type II-\( \alpha \) regulatory subunit of PKA seen in C2C12 myoblasts is consistent with published data showing PKA to be a negative regulator of muscle differentiation.

We found that the serine/threonine protein kinase Akt2/PKB preferentially accumulated in C2C12 myotubes. A positive feedback loop between Akt2 and MyoD has been demonstrated previously. Akt2 promoter possesses multiple MyoD binding sites and is activated by MyoD; in turn, Akt2 facilitates the actions of MyoD and enhances muscle specific gene expression (50, 51). It was reported recently that Akt binds to HSP90 and if Akt-HSP90 complex formation was inhibited, it led to de-phosphorylation and inactivation of Akt (52). The de-phosphorylation of free Akt was catalyzed by phosphatase 2A (PP2A), a hetero-trimer composed of structural (A), phosphatase regulatory (B), and catalytic (C) subunits (53). The B subunit of PP2A, represented by \( \alpha \), \( \beta \), and \( \gamma \) isoforms, is abundant in muscle. Based on previously published observations (50–52), the reduced levels of HSP90 and a concomitant increase in PP2A protein seen here are likely to be mechanistically related to the process of muscle differentiation. We should also note that DNA microarray hybridization studies revealed that expression of mRNA-encoding PP2A was highly enhanced in C2C12 myoblasts (8).

Chromatin remodeling and regulation of transcription of muscle-specific genes are mechanistically related to the process of differentiation. The proliferating C2C12 cells reprogram their genome to induce muscle-specific gene expression prior to myotube formation. It is not surprising therefore that the steady-state levels of several nuclear proteins including Pax-7, MEF2A, TAF28, the transcription intermediary factor 1\( \beta \) (TIF1\( \beta \)), histone acetyl transferase (HAT), HnRNP A1, and RNP-F were altered in the differentiating C2C12 cells.

The Pax-3 and Pax-7 genes are expressed in the segmental plate and somites and play a rate role in the development of muscle (54). The muscles of Pax-7/–/– mice lacked satellite cells completely (55). Activated satellite cells expressing Pax-7 are involved in postnatal growth and repair of skeletal muscle fibers. Because expression of Pax-7 in fully differentiated muscle is negligible, the observed down-regulation of Pax-7 in C2C12 cells in DM is compatible with its putative role in the process of myogenesis.

The MADS-domain containing factor MEF2A is involved in muscle-specific and growth factor-related transcription (2, 3, 7). MEF2A forms homodimers and also associates with other
bHLH proteins that bind to the promoters/enhancers of muscle-specific genes to modulate their transcription. The MEF2 proteins also interact with HAT-containing co-activators such as p300/CBP, and some members of the MAP kinase family (56, 57). Altered regulation of TIF1β that binds to the heterochromatin protein 1 (HP1) has been shown to occur during the differentiation of F9 cells (58). Because treatment of C2C12 cells with a specific inhibitor of HAT or expression of dominant-negative CBP drastically reduced their ability to form myotubes (59), altered levels of MEF2A, TIF1β, and HAT seen here underscore a central role for chromatin remodeling in myogenesis.

A number of proteins known to regulate cell proliferation and apoptosis such as Gas1, BIRC4, follistatin-related protein, IGF-I, and FGF receptors were also altered in differentiating C2C12 cells. Both programmed cell death and myogenic differentiation share a need for dynamic assembly and disassembly of actin-based cytoskeleton. Caspases are the primary regulators of programmed cell death (60). BIRC4 inhibits caspase-3, -7, and -9 (61). The caspase3−/− mice elicited severe muscle deficit, and abolition of caspase-3 in C2C12 cells dramatically decreased myotube formation (62). The exact role of BIRC4 in promoting myogenesis in C2C12 remains to be established.

The IGF-I receptor was down-regulated in C2C12 myocytes. IGF-I apparently has time-dependent actions during myogenesis; initially it promotes C2C12 myoblast proliferation and inhibits cell differentiation while subsequently IGF-I induces cell-cycle exit and promotes differentiation. Regulated expression of myogenin in response to IGF-I is thought to mediate both early and late actions of this growth factor (63, 64).

We found that the receptor for FGF-4 was down-regulated during the differentiation of C2C12 myoblasts. FGFs stimulate myoblast proliferation and inhibit their differentiation into myotubes. Coordinated regulation of FGFs and their cognate receptors (FGFRs) in murine and chicken myoblasts undergoing differentiation has been reported (65–67). Enforced expression of FGFR1 increased myoblast proliferation and delayed differentiation, while expression of a truncated FGFR1 had the opposite effect (68). A seminal role of FGFR4 signaling in the regulation of Myf5, MyoD, and MHC genes and muscle differentiation was recently demonstrated (69).

Extracellular proteolysis is associated with myogenesis and uPA−/− mice have decreased ability for muscle regeneration (70). Thus, an increased accumulation of the plasmin activator inhibitor seen in C2C12 myocytes corroborates the central role of uPA in myogenesis.

**Notch 1** expression is associated with immature myoblasts, while cells expressing the Notch ligands, delta1 and serrate2, are more advanced in myogenesis. Interactions between Notch and its ligands are thought to sustain myoblasts in a proliferative state (71, 72). Because the levels of Jagged-2, a trans-membrane Notch ligand, declined in the multinucleated C2C12 myocytes, we posit that homothallic Notch1-Jagged signaling may modulate myotube formation.

Intracellular Ca2+ concentration regulates contraction-relaxation, cell motility, cell cycle progression, and apoptosis (73), and lowering of Ca2+ inhibits myogenic differentiation (74). Calreticulin (Crt), located in the endoplasmic reticulum, is thought to regulate Ca2+ homeostasis of muscle cells (75, 76). It was reported that although the Crt−/− embryonic stem cells differentiated into cardiac myocytes, they had severely reduced expression of ventricular myosin light chain 2. The accumulation of MEF2-C into the nuclei was also impaired in Crt−/− cardiac cells. A direct link between the observed up-regulation of Crt and myotube formation needs to be experimentally established.

The G-protein mediated GTP and GDP exchange is involved in regulating numerous physiological reactions. We noted that the levels of guanine deaminase that converts guanine to xanthine and ammonia, and also removes the guanine base from guanine containing metabolites, were significantly enhanced in C2C12 myotubes. Guanine deaminase may regulate the pools of intracellular GTP and guanylate, both as a housekeeping function and in response to differentiation signals.

DNA microarray-based analyses of about 12,000 genes revealed that ~12% transcripts were differentially regulated in differentiating C2C12 cells (8, 9). Our proteomics-based investigation has revealed that from more than 2,000 proteins analyzed here about 6% were differentially regulated. The apparent gap between the genomics and proteomics data may be reconciled if we consider the underlying differences between the two techniques. First, the analysis of gene expression by DNA microarrays represents an order of magnitude greater coverage of the genome, essentially 100%. Second, a greater sensitivity of the nucleic acid probes used for hybridization to DNA microarrays enables one to detect relatively small differences in the relative rates of steady-state accumulation of mRNAs. With these caveats notwithstanding, we conclude that the comparative analysis of protein expression from proliferating C2C12 cells and fully differentiated myotubes has yielded interesting and interpretable data that shed important light on the process of skeletal myogenesis.

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