FEBS Letters 574 (2004) 138–144 FEBS 28762

Comparative analysis of phosphoprotein-enriched myocyte proteomes reveals widespread alterations during differentiation

Lawrence G. Puente[a,b], Jean-François Carrière[b], John F. Kelly[c], Lynn A. Megeney[a,b,d,*]

[a] *Ottawa Health Research Institute, Molecular Medicine Program, Ottawa Hospital, 501 Smyth Road, Ottawa, Ont., Canada K1H 8L6*
[b] *Ontario Genomics Innovation Centre, Ottawa Hospital, 501 Smyth Road, Ottawa, Ont., Canada K1H 8L6*
[c] *Institute for Biological Sciences, National Research Council, Ottawa, Ont., Canada K1A 0R6*
[d] *Department of Cellular and Molecular Medicine and Centre for Neuromuscular Disease, Faculty of Medicine, University of Ottawa, Ottawa, Ont., Canada K1H 8M5*

Received 15 June 2004; revised 14 July 2004; accepted 3 August 2004

Available online 21 August 2004

Edited by Lukas Huber

Abstract The differentiation of skeletal muscle has been associated with altered phosphorylation status of individual proteins. However, a global analysis of protein phosphorylation during myogenesis has yet to be undertaken. Here, we report the identification of over 130 putative phosphoproteins from murine C2C12 muscle cells. Cell extracts were fractionated on phosphoprotein enrichment columns and the resulting proteins were detected by two-dimensional gel electrophoresis and silver stain, and identified by liquid chromatography coupled to electrospray tandem mass spectrometry. The early differentiation of C2C12 myoblasts was found to be accompanied by changes in the phosphorylation or expression of numerous proteins including cytoskeletal, heat shock and signaling proteins, the pp32 family of nuclear phosphoproteins, several disease-associated gene products and other characterized and uncharacterized proteins.

Keywords: Proteomics; Phosphoproteome; Myogenesis

1. Introduction

Skeletal myogenesis has served as a useful model for the study of cellular differentiation. Myogenic precursor cells undergo a distinctive differentiation program characterized by exit from the cell cycle and cell–cell fusion to form multinucleate myotubes. This process has been extensively studied at the transcriptional level [1–3]. Transcriptional control of muscle specification and development is exerted by the myogenic regulatory factors (MRFs) MyoD, Myf5, Myogenin and MRF4 [4], and by myocyte enhancer factor-2 (MEF-2) [5]. Expression of MyoD or Myf5 promotes assumption of myoblast characteristics, whereas differentiation of myoblasts to myotubes is linked to the expression of myogenin and MRF4 [4].

In addition to the transcriptional paradigm, several lines of evidence indicate that kinase-dependent signaling events also regulate myogenesis. For example, the serine/threonine kinase p38α/SAPK2 is activated upon induction of differentiation and p38α inhibitors block skeletal muscle differentiation [6–9]. p38γ/SAPK3 (ERK6) expression is associated with and appears to promote muscle differentiation [10,11]. Akt activity has been implicated in myogenesis [12,13]. Inhibition of the protein tyrosine kinase Src blocks myotube formation, suggesting that tyrosine phosphorylation is required for complete muscle differentiation [14].

An obvious question arising from these observations is the identity of proteins that are phosphorylated during skeletal myogenesis. MEF2A and MEF2C can be phosphorylated by p38 and this potentiates their pro-myogenic activity [15,16]. Recently, SAPK2/p38 was shown to phosphorylate MRF4 [17]. Although MEF2 and MRFs are essential for myogenesis, they are unlikely to be the only targets of phosphorylation. However, no large-scale analysis of protein phosphorylation in skeletal myogenesis has been conducted as yet.

In the present study, we utilized both phosphoprotein binding columns and anti-phosphotyrosine immunoprecipitation to prepare phosphoprotein-enriched samples for proteomic analysis by comparative two-dimensional gel electrophoresis (2DGE) and LC-MS/MS. These methods revealed a large set of known and putative phosphoproteins that are expressed during myoblast growth and early myogenesis. Together, these results indicate that the differentiation process in the skeletal muscle lineage may be regulated by an extensive alteration of the endogenous phosphoproteome.

2. Materials and methods

2.1. Cells and antibodies

C2C12 cells were obtained from ATCC and maintained as previously described [18]. Differentiation was induced by replacing growth medium (DMEM, 10% FBS) with differentiation medium (DMEM, 2% horse serum). Anti-phosphotyrosine antibody PY100 was purchased from Cell Signalling Technology (Beverly, MA).

2.2. Phosphoprotein enrichment

To obtain cell lysates enriched for phosphoproteins, Phosphoprotein Purification Kit 37101 was purchased from Qiagen (Mississauga, Ont.) and used in accordance with the manufacturer's instructions. Briefly, cells were washed, lysed using the supplied lysis buffer, and centrifuged to remove insoluble material. Protein lysate was loaded onto the supplied phosphoprotein binding columns, washed, and eluted. The eluate containing putative phosphoproteins was concentrated and desalted using the supplied ultrafiltration columns (10 kDa cut-off).

* Corresponding author. Fax: +1-613-737-8023.
E-mail address: lmegeney@ohri.ca (L.A. Megeney).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.08.019

2.3. Immunoprecipitation

Cells were washed twice with PBS and lysed by the addition of 1% TX-100 lysis buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 20 mM NaF, and 10 mM sodium pyrophosphate, PMSF, aprotinin, pepstatin, leupeptin, and sodium vanadate). Cells were incubated in lysis buffer for 20 min on ice, then scraped and collected into 1.5 ml tubes. Samples were further incubated for 10 min at 4 °C, then centrifuged for 5–10 min at $20\,000 \times g$. 20 μl of agarose-bound PY100 mAb was added to 0.8 mg of postnuclear protein lysate and incubated at 4 °C overnight. Pellets were washed three times in 1% TX-100 lysis buffer and resuspended in Laemmli reducing sample buffer.

2.4. Electrophoresis

For 2DGE, samples were diluted in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 1% DTT) and absorbed into 17 cm ReadyStrip™ IPG strips (Bio-Rad) following the manufacturer's directions. Isoelectric focusing was performed on a Protean IEF Cell (Bio-Rad) with the following program: 200 V for 1 h, 500 V for 1 h, 5000 V ramp for 5 h, and 5000 V for 80 000 VH. For separation by molecular weight, 10% SDS–PAGE gels or 8–16% gradient precast gels (Bio-Rad) were used in a Protean II electrophoresis cell (Bio-Rad).

2.5. Silver stain

Silver stain was performed as previously described [19].

2.6. In-gel digest

Selected protein spots were excised manually and in-gel digested with trypsin using the Investigator Progest automated digestor (Genomic Solutions, Ann Arbor, MI). Briefly, the automated digestion procedure was as follows. The gel pieces were destained with 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate [20], washed 3 times with water and shrunk with acetonitrile. The gel pieces were reswollen with 20 μL of 50 mM NH_4HCO_3 containing 20 ng/μL of sequencing grade trypsin (Promega, Madison WI). Peptides were first extracted with 5% acetic acid (50 μL) and then with 5% acetic acid in 50% methanol (50 μL). The extracts were pooled and evaporated to dryness. The samples were stored at −20 °C prior to analysis.

2.7. NanoLC-MS/MS analysis of in-gel digests

Samples were redissolved with 0.2% formic acid in 5% acetonitrile (10 μL) and analyzed by nanoLC-MS/MS using a CapLC liquid chromatograph (Waters) coupled with a Q-TOF Ultima hybrid quadrupole time-of-flight mass spectrometer (Waters). The samples were first injected onto a 0.3×5 mm C_{18} micro pre-column cartridge (Dionex/LC Packings) to remove salts and other soluble contaminants. The trap was then brought on-line with a 75 mm × 150 mm C_{18} NanoSeries column (Dionex/LC-Packings) and the peptides were separated by gradient elution (5–55% acetonitrile, 0.2% formic acid in 45 min). The mass spectrometer was set to operate in survey mode and MS/MS spectra were automatically acquired on doubly, triply, and quadruply charged ions.

The MS/MS fragment ion spectra were searched against the NCBI nr protein sequence database using the MASCOT database search engine (Matrix Science, London, UK). The lists of identified proteins were then critically evaluated by hand.

3. Results

3.1. Identification of proteins after phosphoprotein enrichment

Protein lysates were prepared from C2C12 cultures after 0 or 24 h in differentiation-inducing medium and phosphoprotein enrichment was performed as described. Proteins were separated by 2DGE and visualized by silver stain (Fig. 1). Spots were excised from replicate gels and peptides were extracted by in-gel digestion for analysis by HPLC-ESI-MS/MS. 272 silver stained gel spots were cut and 591 identifications representing 190 different proteins were made (many proteins were identified multiple times as they were present in more than one gel and/or in more than one spot per gel). 132 proteins that gave both quality MS/MS spectra and consistent silver-stain expression patterns are listed in Tables 1–4. Of these proteins, 73 have been described in the literature as phosphoproteins, 31 are uncharacterized or poorly characterized at the protein level, two are thought to be non-phosphorylated, and for 26 no information on phosphorylation status was found.

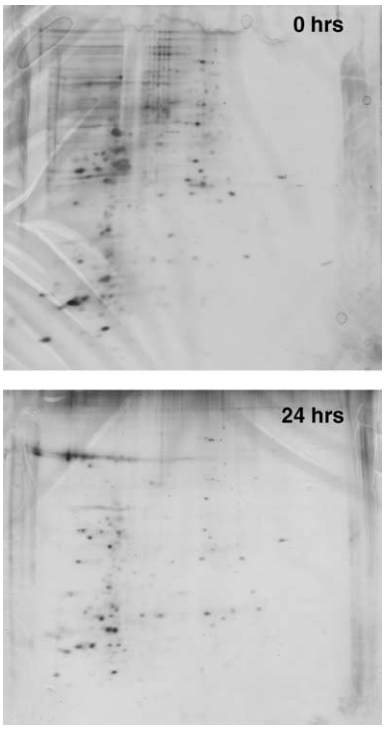

Fig. 1. Phosphoprotein content varies between growing and differentiating myoblasts. Protein was extracted from C2C12 cells after 0 or 24 h culture in differentiation medium and loaded onto phosphoprotein enrichment columns as described in Section 2. Column eluate was concentrated using 10 kDa cut-off ultrafiltration columns and subjected to 2DGE on a pH 3–10 isoelectric focusing gradient and an 8–16% polyacrylamide gel gradient. Proteins were visualized by silver stain.

Each protein was characterized as to whether its apparent abundance increased, decreased or remained unchanged between growth and differentiation conditions (Tables 1–3). Since silver staining is known to be non-linear, only the strongest changes were considered significant; 68/82 (83%) of the proteins listed in Tables 2 and 3 were detected exclusively under growth or differentiation conditions, respectively. As expected, many phosphoproteins did not significantly change in abundance between 0 and 24 h of myogenesis (Table 1). A large number of proteins that were detected in proliferating cells displayed a loss in phosphorylation or expression at 24 h of differentiation (Table 2). Some such as nucleophosmin [21] and small glutamine-rich tetratricopeptide repeat containing protein [22] are known to be involved in cell cycle progression. Lamin C phosphorylation has been associated with mitosis [23]. BIN1 was detected in proliferating but not in differentiating myoblasts and is known to be a phosphoprotein associated with myogenesis [24].

3.2. Detection of a pSer site in PEA-15

Automated LC-MS/MS can identify large numbers of proteins with a high degree of confidence, but specific sites of phosphorylation are rarely detected since phosphopeptides do

Table 1
Proteins identified in both undifferentiated and differentiating C2C12

Heat shock and chaperone
Calreticulin
Heat shock protein 1β
Stress-induced phosphoprotein 1
Tumor rejection antigen gp96

Proteasome
Proteasome subunit, α type 2
Proteasome subunit, α type 4
Proteasome subunit, α type 5
Proteasome subunit, α type 6
Proteasome subunit, β type 3

Structural and mechanical
Myosin light chain alkali
Myosin regulatory light chain-like
Similar to tropomyosin 4
Tropomyosin α
Tropomyosin β
Tropomyosin β2
Tropomyosin 5
Tubulin, α2

Translation
Eukaryotic translation elongation factor 1 β2
Eukaryotic translation initiation factor 2, subunit 2
Ribosomal protein, large, P1
Ribosomal protein, large, P2

Other functions
Acidic leucine-rich nuclear phosphoprotein 32 family, member A
Barrier to autointegration factor 1
Cathepsin D
Hepatoma-derived growth factor
Nascent polypeptide-associated complex α
p32-RACK (complement component 1 q subcomponent binding protein)
ras-GTPase-activating protein SH3-domain binding protein
Tumor protein, translationally-controlled 1
Valosin containing protein

Unnamed gene products
Unnamed protein similar to septin 6

Table 2
Proteins exclusively or preferentially detected in undifferentiated C2C12

Cytoskeleton/regulatory
Caldesmon
Collapsin response mediator protein 2
Myosin regulatory light chain polypeptide 9
Nuclear distribution gene E homolog 1

Cytoskeleton/structural
Tubulin, α6
Putative β-actin
Actin-like
γ-Actin
Drebrin A
Villin 2

Heat shock and chaperone
DNA K-type molecular chaperone grp78
DNA K-type molecular chaperone hsc70

Metabolic
Pyruvate dehydrogenase, β polypeptide
Similar to α enolase

Nuclear and chromatin
Dosage compensation-related protein DPY30
Heterogeneous nuclear ribonucleoprotein U
Lamin C
Nucleophosmin 1
RAN binding protein 1
Similar to small nuclear ribonucleoprotein F

Proteasome
Proteasome subunit, β type 6
Proteasome subunit, β type 7
Proteasome subunit, α type 3

Translation
Eukaryotic translation elongation factor 2
Eukaryotic translation initiation factor 3 subunit 4
Eukaryotic translation initiation factor 3 subunit 5
Eukaryotic translation initiation factor 4e

Signaling and other functions
Calcium-regulated heat stable protein 24 kD
COP9 signalosome subunit 4
Craniofacial development protein 1
High mobility group protein 1
Phosphoprotein enriched in astrocytes 15
Proliferation-associated 2G4
SH3 domain protein 3
SH3 domain protein 9 (BIN1)
Small glutamine-rich tetratricopeptide repeat containing protein
Transcription elongation factor B (SIII) polypeptide 2
Ubiquitin conjugating enzyme 3b
Vacuolar protein sorting 4b

Unnamed gene products
RIKEN 2410002K23 (similar to HSP75)
RIKEN 2610024N24 (similar to RANBP3b)
RIKEN G430041M01 (similar to transformer-2 α)
Unnamed protein similar to tumor protein D52
Unnamed protein similar to eTIF-3 subunit 1
Unnamed protein similar to gp96
Unnamed protein similar to tumor protein D52-like 2
Unnamed protein similar to CG7519-PA
Unnamed protein similar to caldesmon
Unnamed protein 5031425D22Rik
Unnamed protein similar to putative transcription intermediary factor

not ionize well in positive ion mode and tend to lose their phosphate moiety. However, site-specific phosphorylation of one protein was readily detected. Phosphoprotein enriched in astrocytes 15 (PEA-15) is a DED-domain protein with known phosphorylation sites at Ser104 and Ser116 [25]. Although no peptides covering Ser104 were seen, two out of three mass spectra covering the Ser116 region indicate the presence of phosphoserine at the 116 site (Fig. 2). Ser116 is a CaMKII substrate and is required for the anti-apoptotic activity of PEA-15 [25].

3.3. Proteins exhibiting altered gel mobility

Proteins whose electrophoretic mobility changed between growing and differentiating cells (Fig. 3 and Table 4) were identified by matching silver stain patterns to protein identities. Such observations directly demonstrate a physical alteration to the protein such as post-translational modification or protein cleavage. The Niemann–Pick type C2 protein was observed to exhibit two forms of different isoelectric point (p*I*) in undifferentiated myoblasts but three p*I* forms during differentiation. Lamin A was seen to have three p*I* forms, one of which was only detected in undifferentiated cells. Three mobility forms of SET were detected in undifferentiated C2C12 and in one experiment a unique mobility form of SET was seen in differentiating cells. Two SET-interacting proteins were also detected: acidic leucine-rich nuclear phosphoprotein 32 family member A (Table 1) and prothymosin-α (detected sporadically under each condition, not shown in tables).

Table 3
Proteins preferentially or exclusively detected in differentiating C2C12

Cytoskeleton/regulatory
Cortactin
Cytoskeleton/structural
α-Actin
Tubulin α
Tubulin β2
Tubulin α3
Tubulin β
Heat shock and chaperone
α-B crystallin
HSP 27
Metabolic
Triosephosphate isomerase
Translation
Eukaryotic translation initiation factor 3 subunit 3
Similar to eukaryotic translation initiation factor 3 subunit 1
Signaling and other functions
14-3-3β
14-3-3ε
Autophagy Apg3p/Aut1p-like
Cathepsin Z
Divalent cation tolerant protein CUTA
Epithelial protein lost in neoplasm
Heterogeneous nuclear ribonucleoprotein C
Methyl-CpG binding domain protein 3
Prosaposin
Protein kinase C and casein kinase substrate in neurons 2
SAPK3/ERK6/p38γ
Similar to Sid3177p
Similar to TATA box binding protein associated factor
Sorting nexin 9
Telomerase binding protein p23
U2af1 protein
unc-33-like phosphoprotein (CRMP-4)
Unnamed gene products
RIKEN 2610019P18 (similar to trinucleotide repeat containing 5)
RIKEN 2310039E09 (similar to polymerase I and transcript release factor)
RIKEN 2310047C17 (similar to periaxin)
Unnamed protein similar to RAN-BP3A

Table 4
Proteins exhibiting changes in electrophoretic mobility during early myogenesis

Cytoskeleton/regulatory
Moesin
Stathmin
Cytoskeleton/structural
Tubulin β5
Vimentin
Heat shock and chaperone
Heat shock protein 1α
Nuclear
Acidic nuclear phosphoprotein 32 family member B
Acidic nuclear phosphoprotein 32 family member E
Chromobox homolog 1
Lamin A
Nucleolin
Transcription factor BTF3
Signaling and other functions
14-3-3ζ
Niemann–Pick type C2
PDGF-associated protein
Protein disulfide isomerase precursor
SET
Suppression of tumorigenicity 13
Unnamed gene products
RIKEN 2610029G23
Unnamed protein product similar to eTIF2 subunit 1α

The nascent polypeptide-associated complex (NAC) is a heterodimer of NACα (Table 1) and the basic transcription factor 3, splice isoform b (BTF3b) (Table 4) [26]. Although a splice isoform of NACα termed skNAC is thought to be a muscle-specific transcription factor [27], the NACα peptides detected here were from the C-terminal region common to both isoforms and the 2DGE spot position was closer to that expected for the ubiquitous isoform. BTF3b appeared as a single spot in growing cells, but as a pair of spots with different p*I* in differentiating cells.

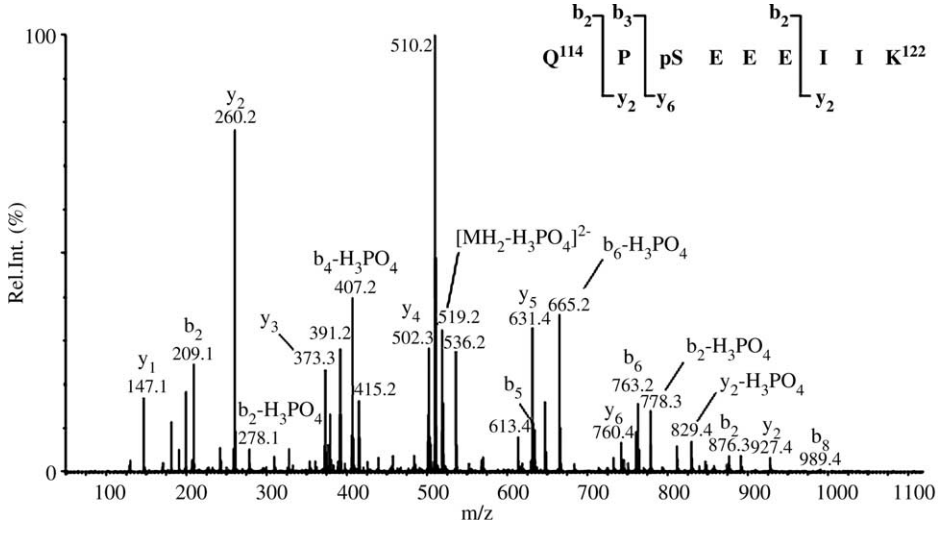

Fig. 2. NanoLC-MS/MS analysis of a tryptic phosphopeptide from PEA-15. Product ion spectrum of the doubly protonated ion at *m/z* 568.2. The most informative fragment ions are indicated in the MS/MS spectrum. The sequence of the peptide is shown in the inset. The N-terminal glutamine has been transformed into a pyroglutamic acid.

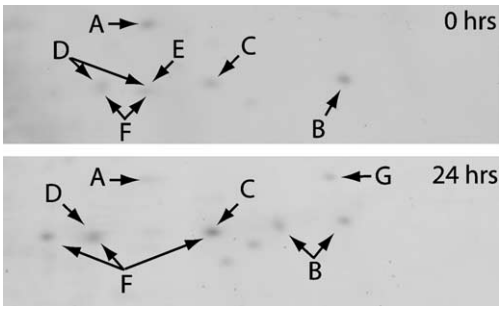

Fig. 3. Close-up view of two-dimensional gels shows proteins exhibiting altered isoelectric points and/or gel mobility between 0 and 24 h of myoblast differentiation. Silver-stained protein spots were identified by LC-MS/MS. (A) Calcium-regulated heat-stable protein; (B) transcription factor BTF3; (C) Cofilin; (D) Stathmin 1; (E) SH3 domain protein 3; (F) Niemann–Pick C2; and (G) α-B-crystallin. Altered mobility is evident relative to spots (A) and (C) which are invariant.

3.4. Anti-phosphotyrosine immunoprecipitation

In addition to the phospho-column purification approach, we also investigated the utility of phospho-antibody immunoprecipitations (IPs) as a strategy for the isolation/enrichment of phosphoproteins. C2C12 cells were grown, then switched to differentiation-inducing medium and cultured for varying amounts of time. Tyrosine-phosphorylated proteins were immunoprecipitated, separated by one-dimensional SDS–PAGE, and detected by silver stain (Fig. 4). Bands exhibiting strong changes in intensity were excised, digested and identified by nanoLC-ESI-MS/MS. The effectiveness of this method was reduced due to a background signal of abundant proteins. However, several non-background proteins were observed. Tyrosine phosphorylation of cortactin was detected at 24 h of differentiation. Phosphorylated cortactin was also detected by affinity column enrichment (Table 3) and by PY20 anti-phosphotyrosine IP (not shown). Several components of the Arp2/3 complex were detected. Both Arp3 and subunit 2 (p34-Arc) appeared at 24 h of differentiation. Arp2 was detected at all time points but only weakly at time 0. The actin-binding phosphoprotein drebrin was detected in the affinity column-based screen solely in undifferentiated cells (Table 2) but was also detected during differentiation in the PY100 IP (Fig. 2). Interestingly, drebrin appears at a slightly higher MW in the phosphotyrosine IP at time 0 (Fig. 4), suggesting that a multiply phosphorylated form of drebrin may be present during growth that becomes less phosphorylated upon differentiation.

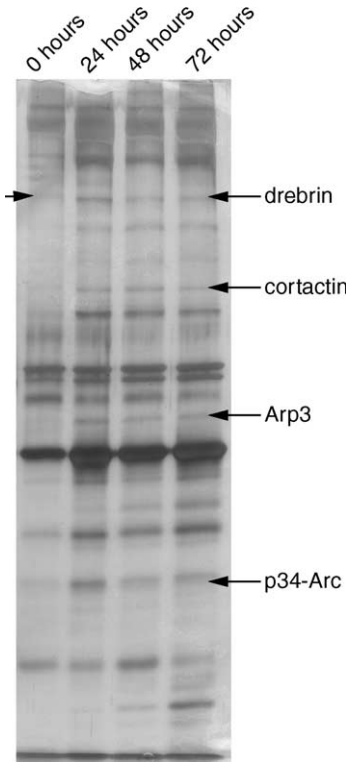

Fig. 4. Phosphotyrosine profiling during C2C12 myoblast differentiation. C2C12 cells were cultured in differentiation medium for 0, 24, 48 or 72 h. Tyrosine phosphorylated proteins were immunoprecipitated at the indicated time points from C2C12 protein lysates. Proteins were separated by SDS–PAGE on an 8% gel and silver stained. Bands of interest were identified by LC-MS/MS.

4. Discussion

4.1. Phosphoprotein enrichment

All large-scale analyses of phosphoprotein regulation rely on selective enrichment of phosphoproteins and recently, several strategies to accomplish this have been presented. Immunoprecipitation is effective for enrichment of tyrosine-phosphorylated proteins. Covalent modification of phosphorylated serine and threonine residues by β-elimination/Michael addition may be used to add biotin for affinity capture or to create novel proteolysis sites for later detection [28–30]. Immobilized metal affinity chromatography (IMAC) can be employed to retain negatively charged peptides after masking non-phosphate negatively charged residues by methylation [31]. The affinity columns used in the present study offer the advantage of not requiring covalent modification of the sample; this reduces sample processing time, circumvents issues of insufficient blocking or excessive labeling, and avoids creating covalent adducts that can interfere with the interpretation of mass spectra. Similar advantages were noted by Metodiev et al. [32] when preparing samples for MALDI analysis. The detection of a protein following any enrichment process is not on its own sufficient evidence to prove the presence of a phosphorylated residue. In some cases, phosphorylation sites are immediately evident (Fig. 2) but in most cases further steps such as phosphopeptide enrichment are required. In this respect, the IMAC strategy has been successfully applied to the large-scale phosphorylation-site-specific analysis of phospho-peptides [31], which was not examined here. Clearly, the use of multiple phosphoprotein enrichment techniques in series may be advantageous [33].

Comparative analysis of two-dimensional gels is a common technique for comparing protein expression between two biological conditions. In the present study, silver staining was sufficient to identify a large number of changes in protein abundance and/or gel mobility after phosphoprotein enrichment. In future studies, difference gel electrophoresis (DIGE) could be used to identify additional smaller alterations in protein abundance [34].

4.2. Implications of findings for the biology of skeletal myogenesis

A large number of proteins whose post-translational modification and/or expression changed during the first 24 h of myogenesis were identified. Several patterns were observed that may have biological implications.

Cytoskeleton. Myogenesis is characterized by a substantial change in cell morphology. The present data indicate that the known cytoskeleton-regulating factors, moesin, drebrin, caldesmon, DRP-2, nuclear distribution gene E homolog 1, Arp2/3, and cortactin, are expressed in differentiating muscle and suggest that these proteins participate in myogenesis in a phosphorylation-regulated manner. Tyrosine phosphorylation of cortactin (Fig. 4 and Table 3) is thought to be mediated by Src [35], suggesting that Src may be active during myogenesis. This finding supports recent evidence that pTyr signaling plays a role in myogenesis [14].

Genetic diseases. Two proteins linked to human genetic disease, Niemann–Pick type C2 (NPC2) and lamin A, were observed to be expressed in myoblasts and to exhibit altered electrophoretic mobility during myogenesis (Table 4). Mutations in the cholesterol-binding glycoprotein NPC2 cause a fatal syndrome whose symptoms can include sudden loss of muscle tone. Mutations in Lamin A have been associated with a number of muscular dystrophy syndromes [36].

SET complex and chromatin remodeling. The SET complex is a recently described protein complex that appears to influence transcription, nucleosome assembly, DNA replication, and apoptosis [37,38]. Three proteins detected in the present study, SET, LANP (Anp32a) and prothymosin-α, are members of this complex. Therefore, the SET complex may regulate myogenesis through one or more of its activities. SET and LANP are also found in the INHAT (inhibitor of acetyltransferases) complex [39,40]. Interestingly, it is well established that the histone deacetylases HDAC4 and HDAC5 repress skeletal myogenesis and that differentiation requires loss of this repressive activity [41–43]. Thus, it is very plausible that INHAT activity might also regulate myogenesis. Methyl-CpG binding domain protein 3 (MBD3), (Table 3) has also been implicated in transcriptional repression and chromosomal remodeling [44] and may be regulated by phosphorylation [45].

Regulators of apoptosis. PEA-15 [25], α-B-crystallin [46–48], and prosaposin [49,50] are thought to be anti-apoptotic. Crystallin has previously been associated with myogenesis [46–48]. Interestingly, ectopic expression of PEA-15 inhibits serum-withdrawal induced apoptosis [51] and C2C12 myoblasts respond to serum-withdrawal by entering differentiation. Previous work done in our group [18] revealed that transient caspase activation is in fact required for muscle differentiation. Therefore, the anti-apoptotic proteins observed in the present study may modulate the effects of transient caspase activity.

Similarity to neuronal differentiation. Stathmin-like 2, Anp32e, Anp32a (LANP), CRMP-2, CRMP-4, and nuclear distribution gene E homolog 1 have previously been associated with neuronal development. Here, we find that these proteins are also expressed in muscle and exhibit altered phosphorylation and/or expression during myogenesis. Therefore, muscle and nerve may share some common mechanisms for differentiation. Future studies of additional tissue types will further distinguish developmental proteins that are lineage specific from those that are common to all cells or to specific subsets of cells.

4.3. Conclusion

The phosphoprotein affinity column enrichment strategy described herein coupled to 2DGE-HPLC-ESI-MS/MS was effective in revealing a large number of proteins that exhibit altered post-translational modification and expression during myogenesis. These results offer multiple implications for the biology of myogenesis and present several avenues for future investigations.

Acknowledgements: The authors thank Luc Tessier and Tammy-Lynn Tremblay for technical assistance, and Pasan Fernando for useful discussions. This work was supported by Genome Canada and by a Canadian Institutes of Health Research (CIHR) Grant to L.A.M. L.A.M. is a CIHR new investigator.

References

[1] Bergstrom, D.A., Penn, B.H., Strand, A., Perry, R.L., Rudnicki, M.A. and Tapscott, S.J. (2002) Mol. Cell 9, 587–600.
[2] Parker, M.H., Seale, P. and Rudnicki, M.A. (2003) Nat. Rev. Genet. 4, 497–507.
[3] Charge, S.B. and Rudnicki, M.A. (2004) Physiol. Rev. 84, 209–238.
[4] Pownall, M.E., Gustafsson, M.K. and Emerson Jr., C.P. (2002) Annu. Rev. Cell. Dev. Biol. 18, 747–783.
[5] Molkentin, J.D., Black, B.L., Martin, J.F. and Olson, E.N. (1995) Cell 83, 1125–1136.
[6] Cuenda, A. and Cohen, P. (1999) J. Biol. Chem. 274, 4341–4346.
[7] Zetser, A., Gredinger, E. and Bengal, E. (1999) J. Biol. Chem. 274, 5193–5200.
[8] Li, Y., Jiang, B., Ensign, W.Y., Vogt, P.K. and Han, J. (2000) Cell. Signal. 12, 751–757.
[9] Wu, Z. et al. (2000) Mol. Cell. Biol. 20, 3951–3964.
[10] Lechner, C., Zahalka, M.A., Giot, J.F., Moller, N.P. and Ullrich, A. (1996) Proc. Natl. Acad. Sci. USA 93, 4355–4359.
[11] Tortorella, L.L., Lin, C.B. and Pilch, P.F. (2003) Biochem. Biophys. Res. Commun. 306, 163–168.
[12] Jiang, B.H., Aoki, M., Zheng, J.Z., Li, J. and Vogt, P.K. (1999) Proc. Natl. Acad. Sci. USA 96, 2077–2081.
[13] Xu, Q. and Wu, Z. (2000) J. Biol. Chem. 275, 36750–36757.
[14] Lu, H., Shah, P., Ennis, D., Shinder, G., Sap, J., Le-Tien, H. and Fantus, I.G. (2002) J. Biol. Chem. 277, 46687–46695.
[15] Ornatsky, O.I. et al. (1999) Nucleic Acids Res. 27, 2646–2654.
[16] Yang, S.H., Galanis, A. and Sharrocks, A.D. (1999) Mol. Cell. Biol. 19, 4028–4038.
[17] Suelves, M., Lluis, F., Ruiz, V., Nebreda, A.R. and Munoz-Canoves, P. (2004) EMBO J. 23, 365–375.
[18] Fernando, P., Kelly, J.F., Balazsi, K., Slack, R.S. and Megeney, L.A. (2002) Proc. Natl. Acad. Sci. USA 99, 11025–11030.
[19] Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Anal. Chem. 68, 850–858.
[20] Gharahdaghi, F., Weinberg, C.R., Meagher, D.A., Imai, B.S. and Mische, S.M. (1999) Electrophoresis 20, 601–605.
[21] Tarapore, P., Okuda, M. and Fukasawa, K. (2002) Cell Cycle 1, 75–81.
[22] Winnefeld, M., Rommelaere, J. and Cziepluch, C. (2004) Exp. Cell. Res. 293, 43–57.
[23] Ward, G.E. and Kirschner, M.W. (1990) Cell 61, 561–577.
[24] Wechsler-Reya, R.J., Elliott, K.J. and Prendergast, G.C. (1998) Mol. Cell. Biol. 18, 566–575.
[25] Renault, F., Formstecher, E., Callebaut, I., Junier, M.P. and Chneiweiss, H. (2003) Biochem. Pharmacol. 66, 1581–1588.
[26] Wiedmann, B., Sakai, H., Davis, T.A. and Wiedmann, M. (1994) Nature 370, 434–440.
[27] Yotov, W.V. and St-Arnaud, R. (1996) Genes Dev. 10, 1763–1772.
[28] Girault, S., Chassaing, G., Blais, J.C., Brunot, A. and Bolbach, G. (1996) Anal. Chem. 68, 2122–2126.
[29] Adamczyk, M., Gebler, J.C. and Wu, J. (2001) Rapid Commun. Mass Spectrom. 15, 1481–1488.
[30] Knight, Z.A., Schilling, B., Row, R.H., Kenski, D.M., Gibson, B.W. and Shokat, K.M. (2003) Nat. Biotechnol. 21, 1047–1054.

[31] Ficarro, S.B., McCleland, M.L., Stukenberg, P.T., Burke, D.J., Ross, M.M., Shabanowitz, J., Hunt, D.F. and White, F.M. (2002) Nat. Biotechnol. 20, 301–305.
[32] Metodiev, M., Timanova, A. and Stone, D.E. (2004) Proteomics 4, 1433–1438.
[33] Salomon, A.R. et al. (2003) Proc. Natl. Acad. Sci. USA 100, 443–448.
[34] Karp, N.A., Kreil, D.P. and Lilley, K.S. (2004) Proteomics 4, 1421–1432.
[35] Weed, S.A. and Parsons, J.T. (2001) Oncogene 20, 6418–6434.
[36] Genschel, J. and Schmidt, H.H. (2000) Hum. Mutat. 16, 451–459.
[37] Chakravarti, D. and Hong, R. (2003) Cell 112, 589–591.
[38] Lieberman, J. and Fan, Z. (2003) Curr. Opin. Immunol. 15, 553–559.
[39] Seo, S.B., McNamara, P., Heo, S., Turner, A., Lane, W.S. and Chakravarti, D. (2001) Cell 104, 119–130.
[40] Seo, S.B., Macfarlan, T., McNamara, P., Hong, R., Mukai, Y., Heo, S. and Chakravarti, D. (2002) J. Biol. Chem. 277, 14005–14010.
[41] Lu, J., McKinsey, T.A., Zhang, C.L. and Olson, E.N. (2000) Mol. Cell 6, 233–244.
[42] McKinsey, T.A., Zhang, C.L., Lu, J. and Olson, E.N. (2000) Nature 408, 106–111.
[43] McKinsey, T.A., Zhang, C.L. and Olson, E.N. (2001) Curr. Opin. Genet. Dev. 11, 497–504.
[44] Tyler, J.K. and Kadonaga, J.T. (1999) Cell 99, 443–446.
[45] Sakai, H., Urano, T., Ookata, K., Kim, M.H., Hirai, Y., Saito, M., Nojima, Y. and Ishikawa, F. (2002) J. Biol. Chem. 277, 48714–48723.
[46] Inaguma, Y., Goto, S., Shinohara, H., Hasegawa, K., Ohshima, K. and Kato, K. (1993) J. Biochem. (Tokyo) 114, 378–384.
[47] Kamradt, M.C., Chen, F., Sam, S. and Cryns, V.L. (2002) J. Biol. Chem. 277, 38731–38736.
[48] Morrison, L.E., Hoover, H.E., Thuerauf, D.J. and Glembotski, C.C. (2003) Circ. Res. 92, 203–211.
[49] Tsuboi, K., Hiraiwa, M. and O'Brien, J.S. (1998) Brain Res. Dev. Brain. Res. 110, 249–255.
[50] Misasi, R. et al. (2001) FASEB J. 15, 467–474.
[51] Condorelli, G. et al. (2002) J. Biol. Chem. 277, 11013–11018.