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IDENTIFICATION OF SERINES 201 AND 209 AS SITES OF PAX3 PHOSPHORYLATION AND THE ALTERED PHOSPHORYLATION STATUS OF PAX3-FOXO1 DURING EARLY MYOGENIC DIFFERENTIATION

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Abstract

Pax3, a member of the paired class homeodomain family of transcription factors, is essential for early skeletal muscle development and is key in the development of the childhood solid muscle tumor alveolar rhabdomyosarcoma (ARMS). ARMS is primarily characterized by a t(2;13) (q35;q14) chromosomal translocation, which fuses the 5'-coding sequences of Pax3 with the 3'-coding sequence of the forkhead transcription factor FOXO1 generating the oncogenic fusion protein Pax3-FOXO1. We previously demonstrated that Pax3 and Pax3-FOXO1 are phosphorylated by the protein kinase CK2 at serine 205 in proliferating primary myoblasts and that this phosphorylation event is rapidly lost from Pax3, but not Pax3-FOXO1 upon the induction of differentiation. However, reports suggested that additional sites of phosphorylation might be present on Pax3. In this report we use in vitro and in vivo analyses to identify serines 201 and 209 as additional sites of phosphorylation and along with serine 205 are the only sites of phosphorylation on Pax3. We provide solid evidence supporting the role of the protein kinase GSK3 β as phosphorylating Pax3 at serine 201. Using phospho-specific antibodies we demonstrate a changing pattern of phosphorylation at serines 201, 205, and 209 throughout early myogenic differentiation and that this pattern of phosphorylation is different for Pax3-FOXO1 in primary myoblasts and in several ARMS cell lines. Taken together, our results allow us to propose a molecular model to describe the changing pattern of phosphorylation for Pax3 and the altered phosphorylation for Pax3-FOXO1 during early myogenic differentiation.

Keywords

Pax3; Pax3-FOXO1; alveolar rhabdomyosarcoma; myogenesis; phosphorylation

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INTRODUCTION

Pax3, a member of the paired class homeodomain family of transcription factors, is important for early skeletal muscle development where it is required for the formation of muscles of the trunk and for the delamination and migration of myogenic progenitor cells to the limb buds (Williams and Ordahl, 1994, Buckingham and Relaix, 2007). In addition to its role in myogenesis, Pax3 is also involved in the formation of the childhood solid muscle tumor alveolar rhabdomyosarcoma (ARMS). ARMS is primarily characterized by the t(2;13)(p35;p14) chromosomal translocation, which results in the fusion of the 5'-sequences of Pax3 to the 3'-sequences of a member of the forkhead family of transcription factors, FOXO1, generating the oncogenic fusion protein Pax3-FOXO1. Pax3-FOXO1 retains the DNA binding and protein-protein interaction domains of Pax3. However, the Pax3 transcriptional activation domain is replaced by the bisected FOXO1 DNA binding domain and the more potent FOXO1 transcriptional activation domain (Shapiro et al., 1993, Galili et al., 1993).

Several reports have demonstrated that the biological activities of Pax3-FOXO1 are altered when compared to Pax3. First, Pax3-FOXO1 mRNA and protein levels are increased in ARMS cell lines when compared to Pax3 (Davis and Barr, 1997). Second, Pax3-FOXO1 is a more potent transcriptional activator than Pax3 even though it has a reduced DNA binding activity (Fredericks et al., 1995). Third, Pax3-FOXO1 is unresponsive to the regulatory effects of the Pax3 co-repressor hDaxx despite a demonstrated interaction (Hollenbach et al., 1999). Fourth, Pax3-FOXO1 has a greater post-translational stability than Pax3 during myogenic differentiation (Miller and Hollenbach, 2007). Finally, Pax3-FOXO1 is able to regulate genes that are not normally regulated by wild-type Pax3 (Epstein et al., 1998). As a consequence of these altered activities, Pax3-FOXO1 is believed to be a key contributor to the development of ARMS.

Due to the importance of Pax3 in early muscle development and the essential role that Pax3-FOXO1 plays in the development of ARMS, it is critical to understand the basic molecular mechanisms by which both proteins are regulated. One potential mechanism is phosphorylation, which has been widely studied due to its various roles in transcription factor regulation (Hunter and Karin, 1992). Several reports suggest that phosphorylation is important for Pax3 and Pax3-FOXO1 activities by contributing to their protein stability (Miller and Hollenbach, 2007), DNA binding and transcriptional activity (Amstutz et al., 2008), and Pax3-FOXO1-dependent cellular proliferation (Zeng et al., 2010). The latter activity is dependent on the protein kinase GSK3 β , a ubiquitously expressed serine-threonine protein kinase important for the regulation of numerous biological events (Doble and Woodgett, 2003) including myogenic differentiation (Brack et al., 2008, van der Velden et al., 2008).

Consistent with the role of phosphorylation in regulating Pax3 and Pax3-FOXO1 biological activities, we demonstrated that the protein kinase CK2 (formerly known as casein kinase II) phosphorylates Pax3 and Pax3-FOXO1 at serine 205 in proliferating primary myoblasts. However, unlike Pax3, whose phosphorylation at this site is rapidly lost upon the induction of differentiation, phosphorylation at serine 205 aberrantly persists on Pax3-FOXO1 throughout early myogenic differentiation (Dietz et al., 2009, Miller et al., 2008). To date, serine 205 is the only identified site of phosphorylation on Pax3 and Pax3-FOXO1. However, several reports suggest that additional sites of phosphorylation may be present near this site (Amstutz et al., 2008, Miller et al., 2008). In the present report we identify serines 201 and 209 as additional sites of phosphorylation on Pax3 in vitro and in vivo. We provide evidence supporting the role of GSK3 β in phosphorylating serine 201 and by using phospho-specific antibodies we demonstrate a changing pattern of phosphorylation at all

three sites on Pax3 during early myogenic differentiation. Further, we demonstrate that Pax3-FOXO1 has an altered pattern of phosphorylation relative to wild type Pax3 in primary myoblasts and ARMS cell lines. These results allow us to propose a model to describe a molecular mechanism for the changing status of phosphorylation on Pax3 in early myogenic differentiation and for how another altered biological characteristic of Pax3-FOXO1 may contribute to the development of ARMS.

MATERIALS AND METHODS

Cell culture conditions and stable transduction of mouse primary myoblasts

Mouse primary myoblasts were isolated from 2 – 4 day old C57/Bl6 mice and cultured in proliferation media as previously described (Miller and Hollenbach, 2007, Rando and Blau, 1997). To induce differentiation, the proliferation media was removed, the cells were washed twice with PBS, and the media was replaced with 10ml of differentiation media, as previously described (Miller et al., 2008, Miller and Hollenbach, 2007). All cells were grown on collagen-coated dishes (Becton Dickinson Labware, Bedford, MA), were passage-matched to prevent possible differences due to different passage conditions, were not used past passage nine, and were not allowed to grow past approximately 80% confluency to maintain the cells in an undifferentiated state.

Mouse primary myoblasts stably expressing Pax3-FOXO1, FLAG epitope-tagged Pax3 (FLAG-Pax3), or a FLAG-Pax3 construct in which Serines 201, 205, and 209 were mutated to phosphoincompetent alanines (FLAG-Pax3[3A]), were generated by a modification of retroviral transduction using an MSCV-IRES-Puromycin construct, which contains the gene for puromycin resistance. Cells were transduced, selected, cultured and expanded as previously described (Dietz et al., 2009). Human ARMS Rh30 cells were purchased from the American Type Culture collection (Manassas, VA), and human ARMS Rh4 cells were a kind gift of Dr. Gerard Grosveld, St. Jude Children's Research Hospital (Memphis, TN), and were cultured as previously described (Dietz et al., 2009).

Creation of expression vector constructs

The GST fusion construct pGEX-5X-1-Pax3 was a kind gift from Dr. Gerard Grosveld, St. Jude Children's Research Hospital (Memphis, TN) and as received lacked the transcriptional activation domain. All phosphoincompetent pGEX-5X-1-Pax3 or point mutant constructs, in which the indicated serines were mutated to alanines, were created as previously described (Miller and Hollenbach, 2007) and as created contained the full-length protein. The wild-type and point mutant vectors were individually transformed into Rosetta(DE3)pLysS chemically competent bacteria (EMD Chemicals, Gibbstown, NJ), subsequently used for expression and purification (Miller et al., 2008), and the purified proteins were used without elution from the resin, as previously described. Protein expression and purity were confirmed by SDS-PAGE analysis with Coomassie blue staining and the relative protein concentrations on the resin were estimated by comparison to proteins of known concentration (data not shown).

In vitro kinase assay and two-dimensional phosphopeptide analysis

GST-Pax3 or the indicated GST-Pax3 point mutants were prepared as described above and 8µl of resin was subsequently used for in vitro kinase assays using proliferating mouse primary myoblast total cell extract as previously described (Dietz et al., 2009, Miller et al., 2008). Following the in vitro kinase assay, the radiolabeled protein band corresponding to phosphorylated Pax3 was extracted from the gel and submitted to two-dimensional phosphopeptide analysis as previously described (Miller et al., 2008, Boyle et al., 1991). Alternatively, non-radioactive ATP was used in the reaction and the resulting

phosphorylated protein was transferred to Immobilon-P membrane (Millipore, Bedford, MA) for Western blot analysis using the previously described anti-Pax3 (Lam et al., 1999) or anti-Pax3(p205) (Miller et al., 2008) antibodies, or the anti-Pax3(p201) and anti-Pax3(p209) antibodies described below.

Alternatively, GST-Pax3, GST-Pax3(S201A) or GST-Pax3(S205A) present on the resin (8µl of resin), were used for in vitro kinase assays using purified CK2 and GSK3β. The indicated proteins were “primed” by incubating with purified CK2 and non-radioactive ATP, as previously described (Dietz et al., 2009). The primed proteins were subsequently washed three times with 100µl of PBS and resuspended in a 30µl reaction containing GSK3β reaction buffer [40mM MOPS, pH 7.0; 1mM EDTA], 30µM ATP, and 50µCi of [γ -³²P]ATP (MP Biomedicals) prior to the addition of 5ng of purified GSK3β (Millipore, Bedford, MA). Following the addition of GSK3β the reaction mixture was incubated at 30°C for 1h with periodic gentle agitation, washed three times with 100µl of PBS, and separated by 10% SDS-PAGE. The resulting gels were dried and visualized by autoradiography. Alternatively, the in vitro kinase assays were performed using 84µM non-radioactive ATP with visualization by Western blot analysis using either the anti-Pax3, anti-Pax3(p201), or anti-Pax3(p205) antibodies.

[³²P]-Orthophosphate Metabolic Labeling

Mouse primary myoblasts containing FLAG-Pax3 or FLAG-Pax3(3A) were grown to 70%-80% confluency and were metabolically labeled with [³²P]-Orthophosphate or [³⁵S]-methionine (MP Biomedicals, Aurora, OH) as previously described (Miller et al., 2008). The metabolically labeled protein was immunoprecipitated with a FLAG-specific antibody, the immunoprecipitate was washed and separated by 10% SDS-PAGE, and the presence of radiolabeled FLAG-Pax3 or FLAG-Pax3(3A) visualized by autoradiography, as previously described (Miller et al., 2008).

Antibodies and Western blot analysis

An antibody specific for phosphorylation of Pax3 at serine 201 (anti-Pax3[p201]) was produced by rabbit immunization using the following synthetic peptide: NH₂-CSERASAPQ(pS)DEG-CO₂ (GenScript Corporation, Piscataway, NJ). An antibody specific for phosphorylation of Pax3 at serine 209, (anti-Pax3[p209]), was independently produced by rabbit immunization using the following synthetic peptide: NH₂-DID(pS)EPDLPLKRC-CO₂ (GenScript Corporation, Piscataway, NJ). Both antibodies were affinity purified and their specificity confirmed by Western blot analysis. The Pax3-specific antibody and the anti-Pax3(p205) phospho-specific antibody were described previously (Lam et al., 1999, Miller et al., 2008). To remove minor background reactivity of the anti-Pax3(p209) antibody, the antibody was preincubated with 0.5µg/ml of the non-phospho 209-synthetic peptide in TBS-Tween with 3% dry milk for 1h. Following this pre-incubation period, standard Western blot analysis procedures were followed.

Total cell extracts from proliferating primary myoblasts, myoblasts that were induced to differentiate for a specific period of time, or the indicated ARMS cell lines were prepared as previously described (Dietz et al., 2009). A constant amount of total cell extract (50µg) was separated by 8% SDS-PAGE, proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), and the presence of Pax3, Pax3-FOXO1, or Pax3 phosphorylated at serines 201, 205, or 209 were detected using the affinity purified, Pax3 antibody (1:5000 dilution), the anti-Pax3(p201) antibody (1:1000 dilution), the anti-Pax3(p205) antibody (1:5000 dilution), or the anti-Pax3(p209) antibody (1:500 dilution), as previously described (Lam et al., 1999, Miller et al., 2008).

RESULTS

Pax3 is phosphorylated at serines 201 and 209 in vitro

We demonstrated previously that Pax3 is phosphorylated at serine 205 by the protein kinase CK2 in proliferating but not differentiating mouse primary myoblasts (Dietz et al., 2009, Miller et al., 2008). However, literature reports indicated that additional sites of phosphorylation might be present in this same region of Pax3 (Miller et al., 2008, Amstutz et al., 2008). Therefore, we individually mutated the serines present in this region to phospho-incompetent alanines (S180A, S187A, S193A, S197A, S201A, S209A, and S222A), and used these GST-Pax3 mutants in our in vitro kinase assay followed by two-dimensional phosphopeptide analysis. We observed the characteristic phospho-peptide pattern for wild-type Pax3 (Figure 1A). Similar to results previously observed for serine 205 (Miller et al., 2008), the individual mutation of either serine 201 or 209 resulted in the loss of a single radiolabeled peptide, a loss that was not observed for the remaining point mutants (Figure 1A), suggesting that Pax3 is phosphorylated at serines 201 and 209 in vitro.

A close examination of the biochemical characteristics of the tryptic peptide containing serines 201, 205, and 209 explains how mutation of two independent sites could result in the loss of the same radiolabeled species on the phosphopeptide map. In addition to containing serines 201, 205, and 209, this peptide contains six potential tryptic cleavage sites at its carboxyl-terminus, of which two are predicted to be favored (Figure 1B) (Boyle et al., 1991). The alternative use of these two preferred sites, combined with the potential for one, two, or three phosphorylation events would result in peptides with different charge and hydrophobicity characteristics. These differences would ultimately result in alternative migrations for the same peptide fragment dependent on the exact tryptic sites used and the number of phosphorylation events present. In addition to predicting the observed Pax3 phosphopeptide map, mobility prediction models (Boyle et al., 1991) determined that phosphopeptide #1, which is lost upon the mutation of serines 201, 205, or 209, could result from a triply phosphorylated phosphopeptide and would be lost upon mutation of any of these sites.

To further confirm that serines 201 and 209 are sites of Pax3 phosphorylation and to determine whether along with serine 205 these sites serve as the only sites of phosphorylation in vitro, we created a series of GST-Pax3 point mutants in which we individually mutated these serines (S201A, S205A, S209A), mutated these serines in combinations of two (S201:205A, S201:209A, S205:209A), or mutated all three serines (S201:205:209A) and used these proteins in our in vitro kinase assay. Mutation of serines 201 or 209, either individually or together resulted in no significant qualitative loss of radioactive incorporation relative to wild-type Pax3 (Figure 2A). Further, mutation of serine 205 in any combination resulted in an observable, but not complete loss of radioactive incorporation, indicating that sites of phosphorylation are still present on the protein. Only upon the mutation of all three serines do we observe a complete loss of radioactive incorporation, demonstrating that serines 201, 205, and 209 are the only sites of phosphorylation on Pax3 in vitro (Figure 2A).

To determine whether serines 201, 205, and 209 are the only sites of phosphorylation on Pax3 in vivo, we stably transduced proliferating primary myoblasts with a retroviral construct containing either FLAG-epitope tagged Pax3 (FLAG-Pax3) or a Pax3 mutant in which all three serines were mutated to the phosphoincompetent alanines (FLAG-Pax3[3A]). We metabolically labeled the cells with either [³²P]-orthophosphate or [³⁵S]-methionine, immunoprecipitated the proteins with a FLAG-specific antibody, and examined the incorporated radiolabel by SDS-PAGE analysis. Consistent with previous results (Dietz et al., 2009, Miller et al., 2008) we observed the specific incorporation of both radiolabels

into FLAG-Pax3, demonstrating it is expressed and phosphorylated (Figure 2B). However, despite its efficient expression and immunoprecipitation, we observe no detectable incorporation of [³²P]-orthophosphate into FLAG-Pax3(3A) (Figure 2B), indicating that the mutation of these sites prevents the phosphorylation of Pax3. Therefore, our results demonstrate that serines 201, 205, and 209 are the only sites of phosphorylation on Pax3 in vitro and in vivo.

To facilitate subsequent analyses of Pax3 phosphorylation at serines 201 and 209, we raised antibodies that recognize Pax3 when phosphorylated only at serine 201 [anti-Pax3(p201)] or only at serine 209 [anti-Pax3(p209)]. To confirm the specificity of these antibodies we first qualitatively determined whether the antibodies recognize phosphorylated Pax3. GST-Pax3 was phosphorylated with primary myoblast total cell extracts using our in vitro kinase assay and subsequently used in Western blot analyses using the anti-Pax3(p201) or anti-Pax3(p209) antibodies. As a control we also utilized the previously described anti-Pax3(p205) phosphospecific antibody (Miller et al., 2008). Despite the presence of equal amounts of protein, we observed reactivity of all three phosphospecific antibodies only with the phosphorylated form of Pax3 (Figure 3A).

To further confirm the specificity of the phosphospecific antibodies, we performed a Western blot analysis with each individual phospho-specific antibody on GST-Pax3, GST-Pax3(S201A), GST-Pax3(S205A), or GST-Pax3(S209A) phosphorylated using our in vitro kinase assay and primary myoblast total cell extracts. As previously described, the anti-Pax3(p205) antibody showed a strong reactivity only when serine 205 was phosphorylatable (Figure 3B) (Miller et al., 2008). In a similar manner, the anti-Pax3(p209) antibody reacted very efficiently with the protein when serine 209 was phosphorylatable, but not when serine 209 was mutated to the phosphoincompetent alanine (Figure 3B). Finally, the anti-Pax3(p201) antibody demonstrated a strong reactivity with wild type Pax3 and the GST-Pax3(S209A) mutant. However, we also observed a loss of reactivity with anti-Pax3(p201) when serine 205 could not be phosphorylated (Figure 3B), a result consistent with the primacy of phosphorylation at serine 205 (Miller et al., 2008) and suggesting that phosphorylation at serine 201 is dependent on phosphorylation at serine 205. Therefore, combined with subsequent results, which demonstrate that the anti-Pax3(p201) antibody reacts with Pax3 only when phosphorylated at serine 201 (Figure 4C), these results confirm the specificity of the anti-Pax3(p201) and anti-Pax3(p209) antibodies.

Phosphorylation of serine 201 by GSK3 β

At present we found no evidence to indicate the identity of the kinase responsible for phosphorylating Pax3 at serine 209. In contrast, two pieces of evidence strongly support the role of GSK3 β in phosphorylating serine 201. First, GSK3 β is unique in that it requires a priming phosphorylation event at the n+4 residue (where “n” is the site of phosphorylation by GSK3 β) (Frame and Cohen, 2001). Consistent with this fact our data indicates that phosphorylation at serine 201 is dependent on phosphorylation at serine 205 (the n+4 residue) (Figure 3B). Second, a visual examination of the primary amino acid sequence surrounding serine 201 demonstrates that when Pax3 is phosphorylated at serine 205, serine 201 is present in the context of a nearly perfect GSK3 β recognition sequence (Figure 4A).

Therefore, to test whether GSK3 β is capable of phosphorylating Pax3 following a “priming” phosphorylation event at serine 205, we “primed” wild-type GST-Pax3 by incubating it with *non-radiolabeled* ATP in the presence or absence of purified CK2, which phosphorylates Pax3 at serine 205 (Dietz et al., 2009). An independent parallel reaction using purified CK2 and radiolabeled ATP demonstrated the efficient phosphorylation of GST-Pax3 by CK2 (Figure 4B, lane 1). After extensive washing we incubated the “primed” proteins with *radiolabeled* ATP in the presence or absence of purified GSK3 β . We observed minimal

GSK3 β -dependent incorporation of radiolabel on CK2-primed Pax3 in the absence of purified GSK3 β or on Pax3 that had not been primed by CK2 (Figure 4B, lanes 2 and 3). Only when Pax3 had been primed by CK2 did we observe a substantial increase in GSK3 β -dependent radiolabel incorporation on Pax3 (Figure 4B, lane 4). This result is not due to the artifactual phosphorylation of GST itself, since neither CK2 nor GSK3 β are capable of phosphorylating GST [data not shown and (Dietz et al., 2009)]. Therefore, these results demonstrate that purified GSK3 β phosphorylates Pax3 when Pax3 is phosphorylated at serine 205 by CK2.

In order to demonstrate that the observed GSK3 β -dependent phosphorylation occurs on serine 201 and that this event requires priming by CK2 phosphorylation at serine 205, we used GST-Pax3 that had been phosphorylated using *non-radiolabeled* ATP by purified CK2 alone, purified GSK3 β alone, or CK2 followed by GSK3 β and the presence of phosphorylation at serine 201 or serine 205 was detected by Western blot analysis using our phosphospecific antibodies. Consistent with our previous work (Dietz et al., 2009) we observed phosphorylation of serine 205 only in the presence of CK2 (Figure 4C, lanes 1 and 3, bottom panel). Further, in the absence of priming by CK2, GSK3 β alone was unable to phosphorylate Pax3 at serine 201 (Figure 4C, lane 2, middle panel). We observed efficient phosphorylation of serine 201 only when GST-Pax3 had been phosphorylated at serine 205 by CK2 (Figure 4C, lane 3, middle panel). Taken together, these results demonstrate that purified GSK3 β can phosphorylate Pax3 at serine 201 only after phosphorylation of serine 205 by CK2. In addition, the inability of the anti-Pax3(p201) antibody to react with wild-type GST-Pax3 even in the presence of efficient phosphorylation of serine 205 (Figure 4C, lane 1, compare middle and bottom panels) further supports the specificity of the anti-Pax3(p201) antibody.

To confirm that phosphorylation at serine 201 is the only site of phosphorylation by GSK3 β on Pax3, we used GST-Pax3, GST-Pax3(S201A), or GST-Pax3(S205A), primed the proteins using purified CK2 and *non-radiolabeled* ATP, and after extensive washing incubated the primed proteins with *radiolabeled* ATP in the absence or presence of purified GSK3 β . Consistent with the above results, we observed efficient incorporation of GSK3 β -dependent radiolabel only when GST-Pax3 has been primed by CK2 (Figure 4D, lanes 1 and 2). This increase in radiolabel does not result from the presence of residual CK2 since we observed minimal radiolabel incorporation either in the absence of GSK3 β or on the GST-Pax3(S201A) point mutant, which can only be phosphorylated at serine 205 in this assay (Figure 4D, lanes 1 and 3). Further, the inability of GST-Pax3 to be radioactively phosphorylated by GSK3 β when the required priming site is absent [GST-Pax3(S205A)] or the site of GSK3 β phosphorylation is absent [GST-Pax3(S201A)] demonstrates that serine 201 is the only site of phosphorylation by purified GSK3 β on Pax3.

Finally, we wanted to determine whether GSK3 β is the kinase present in primary myoblast total cell extracts responsible for phosphorylating Pax3 at serine 201. We pre-incubated extracts with increasing concentrations of the commonly used GSK3 β inhibitor lithium chloride (LiCl) or the highly GSK3 β -specific inhibitor AR-A014418 (Bhat et al., 2003) for 30 minutes prior to the addition of unlabeled ATP and GST-Pax3. The resulting phosphorylated proteins were then analyzed by Western blot analysis using our anti-Pax3, anti-Pax3(p201), or anti-Pax3(p205) antibodies. We observed a nearly complete loss of phosphorylation on serine 201 with no apparent affect on phosphorylation at serine 205 using LiCl or with AR-A014418 (Figure 4E) at concentrations commonly used in similar experiments (Bain et al., 2007, Bhat et al., 2003). Unfortunately, we were unable to determine if GSK3 β is the responsible kinase *in vivo* since the inhibitors resulted in primary myoblast cell death within several hours and primary myoblasts were intractable to transfection with siRNA (<5% efficiency), as determined using fluorescently labeled

controls and a variety of transfection conditions. Regardless, our results provide strong evidence that GSK3 β is the kinase present in total cell extracts that phosphorylates Pax3 at serine 201 after phosphorylation by CK2 at serine 205.

Pax3 and Pax3-FOXO1 phosphorylation during early myogenic differentiation

To confirm serines 201 and 209 are sites of Pax3 phosphorylation *in vivo* and to determine how this phosphorylation changes throughout early myogenic differentiation, we performed a Western blot analysis on total cell extracts from proliferating primary myoblasts, which are known to express endogenous Pax3, and primary myoblasts induced to differentiate for up to eight hours, during which we observe stable Pax3 expression (Miller and Hollenbach, 2007). We qualitatively determined the presence of Pax3 using the general Pax3 antibody and phosphorylation at serines 201 or 209 with our phospho-specific antibodies. In addition, we analyzed phosphorylation at serine 205 as a positive control and to provide a complete picture of phosphorylation during early differentiation. The general Pax3 antibody reacted with two distinctly migrating species with apparent molecular weights of 56kD and 66kD in proliferating myoblasts of which only the apparent 66kD species is phosphorylated at serine 205. Upon the induction of differentiation, we observed a complete loss of the 66kD species with a corresponding loss of phosphorylation at serine 205 (Figure 5), all consistent with previous results (Dietz et al., 2009, Miller et al., 2008).

In contrast, we observed minimal reactivity of the anti-Pax3(p209) antibody with either species of Pax3 in proliferating myoblasts (Figure 5) with low levels of phosphorylation being detected on the 56kD species with longer exposures times (data not shown). However, within fifteen minutes of the induction of differentiation, we observed a substantial increase in the phosphorylation at serine 209 on the apparent 56kD form, which persisted throughout the first two hours of differentiation followed by a gradual decrease and complete loss by eight hours of differentiation (Figure 5). Finally, we observed reactivity of the anti-Pax3(p201) antibody with the apparent 56kD form of Pax3 in proliferating primary myoblasts, which persisted throughout early myogenic differentiation. These results confirm that in addition to phosphorylation at serine 205, Pax3 is also phosphorylated at serines 201 and 209 and a distinct pattern of phosphorylation of Pax3 at these sites exists throughout early myogenic differentiation.

Pax3-FOXO1, the oncogenic fusion protein present in ARMS, retains key regulatory regions of Pax3 including serines 201, 205, and 209. Therefore, we were interested in determining whether serines 201 and 209 are phosphorylated on Pax3-FOXO1 during early myogenic differentiation. We used primary myoblasts stably transduced with Pax3-FOXO1 and induced differentiation for up to eight hours. We observed no distinguishable morphological changes between uninfected primary myoblasts and primary myoblasts stably transduced with Pax3-FOXO1 during the first eight hours of differentiation (data not shown), consistent with previous reports (Miller and Hollenbach, 2007). We isolated total cell extracts at various time points during differentiation and performed a Western blot analysis on equal amounts of total cell extracts using our anti-Pax3 or the phospho-specific antibodies. The anti-Pax3 and the anti-Pax3(p205) antibodies reacted with a 97kD band corresponding to Pax3-FOXO1 throughout differentiation (Figure 6A), consistent with previous reports (Dietz et al., 2009). Similar to results seen with wild-type Pax3 (Figure 5), we observed phosphorylation at serine 201 throughout differentiation (Figure 6A). However, in direct contrast to results obtained with wild-type Pax3 (Figure 5), we observed no reactivity of the anti-Pax3(p209) antibody with Pax3-FOXO1 in either proliferating or differentiating primary myoblasts (Figure 6A). Therefore, these results demonstrate that phosphorylation of Pax3-FOXO1 at serines 205 and 209, but not serine 201, is altered during early myogenic differentiation relative to wild-type Pax3.

Finally, to determine if the observed differences in phosphorylation of Pax3-FOXO1 are present on the endogenous protein, we examined two ARMS cell lines (Rh4 and Rh30) known to express endogenous Pax3-FOXO1 (Khan et al., 1998, Hazelton et al., 1987, Petak et al., 2000). We isolated total cell extracts from the ARMS cells and performed a qualitative Western blot analysis using either the anti-Pax3 or phospho-specific antibodies. Similar to the results observed in primary myoblasts (Figure 6A), we observed reactivity of the anti-Pax3(p201) and anti-Pax3(p205) antibodies, but not with the anti-Pax3(p209) antibody (Figure 6B). Therefore, these results demonstrate that phosphorylation of Pax3-FOXO1 differs from that of wild-type Pax3 in primary myoblasts and ARMS cell lines.

DISCUSSION

Pax3 plays an essential role in early skeletal muscle development and is key in the formation of the childhood solid muscle tumor ARMS, where the most prevalent genetic mutation, a t(2;13)(p35;p14) chromosomal translocation, results in the formation of the oncogenic fusion protein Pax3-FOXO1. Recent evidence indicated that phosphorylation contributes to the biological regulation of Pax3 and Pax3-FOXO1 (Amstutz et al., 2008, Miller and Hollenbach, 2007). Consistent with this fact we demonstrated that Pax3 and Pax3-FOXO1 are phosphorylated at serine 205 in primary myoblasts and in ARMS tumor cell lines (Dietz et al., 2009, Miller et al., 2008). However, previous results suggested that serine 205 is not the only site of phosphorylation on Pax3 (Miller et al., 2008, Amstutz et al., 2008). In this report we identify serines 201 and 209 as additional sites of phosphorylation on Pax3 in vitro and in vivo and determine the pattern of phosphorylation at all three sites throughout early myogenic differentiation. Further, we provide solid evidence supporting the identification of GSK3 β as the kinase responsible for phosphorylating Pax3 at serine 201 and that this phosphorylation event requires a prior phosphorylation at serine 205. Finally, we demonstrate that the pattern of phosphorylation on Pax3-FOXO1 differs from that of wild type Pax3 in primary myoblasts and ARMS cell lines.

Based on the results presented here and combined with our previously published work, we propose the following model to describe Pax3 phosphorylation throughout early myogenic differentiation (Figure 7A). In this model, Pax3 is initially phosphorylated by CK2 at serine 205 in proliferating cells (Dietz et al., 2009, Miller et al., 2008), which primes the protein for two, apparently independent events. First, we propose that phosphorylation at serine 205 promotes an as of yet unidentified post-translational modification to give rise to the 66kD species, the only form of Pax3 phosphorylated at serine 205. Alternatively, phosphorylation of serine 205 promotes the subsequent phosphorylation of serine 201 by GSK3 β . However, because we do not observe a single species of Pax3 dually phosphorylated at both serines 201 and 205 in vivo (Figure 5), we propose that phosphorylation at serine 201 promotes the rapid dephosphorylation at serine 205. Upon the induction of differentiation, phosphorylation of serine 201 persists accompanied by a rapid increase in phosphorylation at serine 209, a state that persists on Pax3 for the first four hours of differentiation (Figure 5). Finally, by 8 hours of differentiation, phosphorylation at serine 209 is lost, phosphorylation at serine 201 persists (Figure 5), and Pax3 protein is degraded with a half-life of approximately four hours (Miller and Hollenbach, 2007).

A closer examination of our results supports our proposed model in which the initial phosphorylation of serine 205 can promote two alternative pathways. In proliferating cells Pax3 is almost always present as two distinctly migrating species with apparent molecular weights of 56kD and 66kD. Of these two species only the 66kD form is phosphorylated at serine 205 (Figure 5), which we previously theorized resulted from an altered electrophoretic mobility due to phosphorylation at this site (Miller et al., 2008). However, in light of the results presented in this report, it is difficult to rationalize how phosphorylation

at serine 205 could produce an altered electrophoretic mobility when phosphorylation at serines 201 and/or 209 does not promote a similar mobility shift (Figure 5). Therefore, although still possible that the apparent shift may result from phosphorylation at serine 205, it is more likely that the 66kD form of Pax3 results from the presence of an as of yet unidentified post-translational modification.

In addition to a potential post-translational modification, our data firmly support the presence of a pathway in which phosphorylation at serine 205 promotes the GSK3 β -dependent phosphorylation of serine 201 with a subsequent dephosphorylation of serine 205. Serine 201 is present within the context of a nearly perfect GSK3 β consensus sequence when Pax3 is phosphorylated at serine 205 (Figure 4A). Consistent with the dependence of GSK3 β activity on a priming phosphorylation event, GSK3 β phosphorylates serine 201 only when Pax3 has been phosphorylated at serine 205 by CK2 (Figures 4A – D). Further, the presence of the commonly used GSK3 β inhibitor LiCl and the highly specific GSK3 β inhibitor AR-A014418 both prevent the phosphorylation of serine 201 by primary myoblast total cell extracts (Figure 4E). Although inhibitor toxicity to primary myoblasts and intractability of the myoblasts to the introduction of siRNA constructs prevented in vivo confirmation (data not shown), we feel that our results provide solid evidence to support the idea that the GSK3 β -dependent phosphorylation of serine 201 can only occur following an initial priming event at serine 205. This conclusion would suggest that we would expect to observe a species of Pax3 phosphorylated at both serines 201 and 205. However, our results clearly demonstrate that Pax3 is only phosphorylated at serine 201 or 205 but not both simultaneously (Figure 5). Therefore, the only logical explanation to describe this observation is that phosphorylation at serine 201 promotes the dephosphorylation of Pax3 at serine 205.

Upon the induction of differentiation, we observe the loss of phosphorylation at serine 205 concomitant with the loss of the 66kD species, by an as of yet unidentified mechanisms. We also observe the continued phosphorylation of serine 201 with a significant increase in phosphorylation at serine 209. The absence of phosphorylation at serine 205, the priming event, during differentiation would suggest that phosphorylation of serine 201 is not a de novo phosphorylation during differentiation but instead is a “long-lived” event that persists from proliferation. Consistent with this fact, we previously demonstrated that Pax3 is a very stable protein during the first 8 – 10 hours of differentiation (Miller and Hollenbach, 2007). From this fact it is evident that Pax3 protein would be stable and persist from proliferation into minimally the first eight hours of differentiation, the time frame used in the assays described in the present work. It directly follows that in the absence of any dephosphorylation, Pax3 phosphorylation events in proliferating cells would also persist throughout early differentiation. Therefore, phosphorylation of serine 201 in proliferating cells, where it is phosphorylated with the help of the required priming event, would persist throughout the earliest hours of differentiation.

It is interesting to note that the loss of phosphorylation at serine 209 between 4 – 8 hours of differentiation (Figure 5) coincides with the decrease in Pax3 expression between 8 – 12 hours of differentiation (Miller and Hollenbach, 2007), a time frame consistent with the experimentally determined Pax3 half-life of four hours during later differentiation (Miller and Hollenbach, 2007). Since the only phosphorylation event present after 8 hours of differentiation is serine 201, it would suggest that the presence of this single event is sufficient to target Pax3 for degradation, potentially through the ubiquitin-proteasome pathway (Boutet et al., 2007). Experiments are presently underway to examine the role of phosphorylation at serine 201 in Pax3 degradation.

Our results also allow us to propose a molecular model to describe how the phosphorylation of Pax3-FOXO1 is altered relative to wild-type Pax3 during early myogenic differentiation and in ARMS cell lines (Figure 7B). In this model CK2 phosphorylates Pax3-FOXO1 at serine 205 (Dietz et al., 2009) followed by a GSK3 β -dependent phosphorylation at serine 201. However, unlike wild-type Pax3, we do not observe any detectable electromobility shift of Pax3-FOXO1 coinciding with phosphorylation at serine 205 (Figure 6), suggesting that the fusion protein seems not to undergo a similar post-translational modification. Further, Pax3-FOXO1 seems to maintain a phosphorylation status that is prominent only in proliferating cells since we observe the dual phosphorylation of the fusion protein at both serines 201 and 205, but not serine 209, throughout early differentiation (Figure 6).

Previous reports indicated that the presence of Pax3-FOXO1 is sufficient to inhibit myogenic differentiation (Miller and Hollenbach, 2007, Epstein et al., 1995). Therefore, our results demonstrating an altered phosphorylation status for Pax3-FOXO1 under differentiating conditions raises the question of whether the altered phosphorylation status *results* from the fusion protein being present in a non-differentiating, proliferative environment or whether the altered phosphorylation status of Pax3-FOXO1 *causes* the inability of the cells to terminally differentiate. Several lines of evidence indicate that the latter situation is the case. We previously demonstrated that when induced to differentiate, primary myoblasts stably expressing Pax3-FOXO1 were unable to *terminally* differentiate to form multi-nucleated myotubes. However, these cells had entered the differentiation program, were capable of expressing the late myogenic marker myosin heavy chain (Miller and Hollenbach, 2007), and were no longer proliferating (data not shown). This fact demonstrates that under the conditions of the experiment used in the present work the cells stably expressing Pax3-FOXO1 are in fact in a non-proliferative state and have entered the differentiation program. Therefore, since the fusion protein is in a differentiating and not proliferative environment, these results support the idea that the aberrant phosphorylation status of Pax3-FOXO1 maintains the fusion protein in a phosphorylation state prominent only in proliferating cells, thereby promoting the aberrant expression of genes that subsequently inhibit terminal differentiation to contribute to the development of ARMS.

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ABBREVIATIONS

ARMS	alveolar rhabdomyosarcoma
ATP	adenosine triphosphate
CK2	casein kinase 2
GSK3β	Glycogen Synthase Kinase 3 beta
GST	Glutathione S-Transferase
LiCl	Lithium Chloride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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A. **B.** **Figure 1.**

Two-dimensional phosphopeptide analysis of wild type Pax3 and Pax3 point mutants. (A) Bacterially expressed and purified GST-Pax3 or GST-Pax3 point mutants in which individual serines were mutated to non-phosphorylatable alanines (S180A, S187A, S193A, S197A, S201A, S209A, and S222A) were phosphorylated in vitro and subsequently analyzed by two-dimensional phosphopeptide analysis, as described in Materials and Methods. The arrow and the dotted circle indicate the phosphopeptide no longer present upon the mutation of either serine 201 or serine 209. (B) Schematic of the primary amino acid sequence of the tryptic peptide containing serines 201, 205, and 209. The location of serines 201, 205, and 209 are labeled and indicated by the asterisks. The arrows indicate the major sites of trypsin cleavage (Boyle et al., 1991).

Figure 2.

Serines 201, 205, and 209 are the only sites of Pax3 phosphorylation in vitro and in vivo. (A) Bacterially expressed and purified wild type GST-Pax3, GST-Pax3 single point mutants (S201A, S205A, and S209A), GST-Pax3 double point mutants (S201:205A, S201:209A, and S205:209A), and a Pax3 triple point mutant (S201:205:209A) were used in our in vitro kinase assay (see Materials and Methods) and visualized by autoradiography (top panel). A Coomassie stained gel demonstrates the presence of equal amounts of protein in the assay (bottom panel). The mobility of wild type Pax3 is indicated by the arrow on the left, the arrow on the right indicates the mobility of the point mutants, and the asterisk indicates commonly observed degradation products (Dietz et al., 2009, Miller et al., 2008). (B) Proliferating mouse primary myoblasts stably transduced with an amino-terminal FLAG epitope tagged wild type Pax3 (FLAG-Pax3) or the FLAG-Pax3 mutant in which serines 201, 205, and 209 are mutated to phosphoincompetent alanines (FLAG-Pax3[3A]) were metabolically labeled with either [³⁵S]-Methionine or [³²P]-orthophosphate. The proteins were immunoprecipitated from total cell extracts using an anti-FLAG antibody, the resulting

immunoprecipitates were separated by 10% SDS-PAGE, and the radiolabeled species were detected by autoradiography.

Figure 3.

Creation of anti-Pax3(p201) and anti-Pax3(p209) phospho-specific antibodies. (A) Bacterially expressed and purified wild-type GST-Pax3 was used as purified (left lanes) or was phosphorylated using our in vitro kinase assay and non-radioactive ATP (right lanes). The proteins were used for Western blot analysis using the anti-Pax3(p201) or anti-Pax3(p209) antibodies. We also performed a Western blot analysis using our previously described anti-Pax3(p205) antibody (Miller et al., 2008) for completeness of analysis. A Coomassie stained gel demonstrates the presence of equal amounts of protein in the assay (top panel). (B) Bacterially expressed GST-Pax3 and the single GST-Pax3 point mutants (S201A, S205A, or S209A) were used in independent in vitro kinase assays using $[\gamma^{32}\text{P}]\text{-ATP}$ to confirm phosphorylation or with non-radioactive ATP. The non-radioactively labeled proteins were subsequently used for Western blot analysis using the phospho-specific antibodies. A Coomassie stained gel demonstrates the presence of equal amounts of protein in the assay (top panel). The mobility of wild type Pax3 is indicated by the arrow on the left, the arrow on the right indicates the mobility of the point mutants, and the asterisk indicates commonly observed degradation products (Dietz et al., 2009, Miller et al., 2008).

Figure 4.

GSK3 β phosphorylates Pax3 at Ser201 following a priming event at Ser205 by CK2. (A) Amino acid sequence of the GSK3 β consensus sequence and the region surrounding Ser201 on Pax3. (B) Bacterially expressed and purified GST-Pax3 was incubated in the presence (+) or absence (-) of CK2 and non-radioactive ATP. After extensive washing the “primed” protein was incubated in the presence (+) or absence (-) of GSK3 β and [γ^{32} P]-ATP. To confirm the initial phosphorylation of Pax3 by CK2, Pax3 was incubated with purified CK2 and [γ^{32} P]-ATP. The phosphorylated proteins were separated by 8% SDS-PAGE and visualized by Coomassie staining (bottom panel) or autoradiography (top panel). (C) Bacterially expressed and purified GST-Pax3 was incubated in the presence (+) or absence (-) of purified CK2 and non-radioactive ATP. After extensive washing the “primed” proteins were incubated in the presence (+) or absence (-) of GSK3 β and non-radioactive ATP. The phosphorylated proteins were separated by 8% SDS-PAGE and visualized by Coomassie staining (top panel) or Western blot analysis (bottom panels) using the anti-Pax3(p201) or the anti-Pax3(p205) phosphospecific antibodies. (D) Bacterially expressed and purified GST-Pax3, GST-Pax3(S201A), or GST-Pax3(S205A) were incubated with purified CK2 and non-radioactive ATP. After extensive washing the “primed” proteins were incubated in the presence (+) or absence (-) of GSK3 β and [γ^{32} P]-ATP. The phosphorylated proteins were separated by 8% SDS-PAGE and visualized by Coomassie staining (bottom panel) or autoradiography (top panel). The mobility of wild type Pax3 is indicated by the arrow on the left, the arrow on the right indicates the mobility of the point mutants, and the asterisk indicates commonly observed degradation products (Dietz et al., 2009, Miller et al., 2008). (E) Bacterially expressed and purified GST-Pax3 was phosphorylated using mouse primary myoblast total cell extracts that had previously been incubated with increasing concentrations of the commonly used GSK3 β inhibitor, LiCl, and the highly specific GSK3 β inhibitor, AR-A014418, as described in Materials and Methods. A Western blot analysis was performed using a Pax3-specific antibody to confirm the presence of equal amounts of protein (top panels), and the phospho-specific anti-Pax3(p201) (middle panels) and anti-Pax3(p205) (bottom panels) antibodies to determine the phosphorylation status at these two sites.

Figure 5.

Analysis of the phosphorylation status of endogenous Pax3 throughout early myogenic differentiation. Proliferating primary myoblasts were induced to differentiate, for 0 – 8 hours. At the indicated time points total cell extracts were made and a standard Western blot analysis was performed on 50 µg of total cell extract using the anti-Pax3 antibody (to demonstrate a qualitative presence of Pax3), or the indicated phospho-specific antibodies.

Figure 6.

Pax3-FOXO1 has an altered phosphorylation status relative to wild type Pax3. (A) Mouse primary myoblasts stably transduced with Pax3-FOXO1 were induced to differentiate for 0 – 8 hours as described in Materials and Methods. At the indicated time points total cell extracts were made and a standard Western blot analysis was performed on 50 μ g of total cell extract using the anti-Pax3 antibody (to demonstrate a qualitative presence of Pax3-FOXO1), or the indicated phospho-specific antibodies. (B) Total cell extracts were made from the ARMS cell lines Rh4 and Rh30 and a standard Western blot analysis was performed on 50 μ g of total cell extract using the anti-Pax3 antibody (to demonstrate a qualitative presence of Pax3-FOXO1), or the indicated phospho-specific antibodies.

Figure 7A

Figure 7B

Figure 7.

Schematic of a model for the phosphorylation of (A) Pax3 and (B) Pax3-FOXO1 throughout early myogenic differentiation.