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Serine/Threonine/Tyrosine Phosphorylation of the LHX3 LIM-Homeodomain Transcription Factor

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Abstract LHX3 is a LIM homeodomain transcription factor with essential roles in pituitary and motor neuron development in mammals. Patients with mutations in the *LHX3* gene have combined pituitary hormone deficiency and other symptoms. In this study, we show that the LHX3 protein can be modified post-translationally by phosphorylation. LHX3 can serve as a substrate for protein kinase C and casein kinase II. Overexpression of these kinases reduces the transcriptional capacity of LHX3 to activate target genes. Following exposure of LHX3 to cellular kinases, mass spectrometry was used to map the phosphorylation of five amino acid residues within the human LHX3a isoform. Two phosphorylated residues (threonine 63 and serine 71) lie within the first LIM domain of the protein. Three other modified amino acids (tyrosine 227, serine 234, and serine 238) are located in the carboxyl terminus. Targeted replacement of these amino acids with non-modifiable residues significantly reduced the ability of LHX3 to activate both synthetic and pituitary hormone reporter genes. However, the amino acid replacements did not significantly affect the capability of LHX3 to interact with the NLI, PIT1, and MRG1 partner proteins, or its ability to bind to a high affinity DNA site. In conclusion, we have identified unique amino acids within LHX3 that are important for its transcriptional activity and are phosphorylated. J. Cell. Biochem. 94: 67–80, 2005.

Key words: pituitary; hormone; PKC; casein kinase

The LIM homeodomain (LIM-HD) family of proteins is comprised of multifunctional transcription factors with cell-, tissue-, and organ-specific roles in development [reviewed in Retaux and Bachy, 2002]. In mammals, there are at least thirteen LIM-HD genes encoding regulatory proteins featuring two amino terminal LIM domains and a centrally located HD. The LIM domains are zinc-coordinated structures that confer multiple functions, including mediating protein–protein interactions, and the HD is used to interact with DNA. The actions of LIM-HD proteins are modulated by their interactions with partner proteins [reviewed in Bach, 2000]. These partner proteins include other members of the LIM-HD family, NLI/LDB/CLIM, MRG1, SLB, the PIT1 pituitary transcription factor, and RLIM, a ubiquitin protein ligase that modulates interactions of LIM-HD proteins with cofactors [e.g., Bach et al., 1995, 1997; Glenn and Maurer, 1999; Bach, 2000; Howard and Maurer, 2000; Ostendorff et al., 2002].

The LHX3 (LIM3/P-Lim) LIM-HD transcription factor is required for both pituitary gland organogenesis and motor neuron specification in the developing nervous system [Sheng et al., 1996 and reviewed in Savage et al., 2003; Sheng et al., 1997; Sharma et al., 1998]. Disruption of the mouse *Lhx3* gene blocks anterior pituitary development at an early stage: the homozygous null animals die around the time of birth and lack the gonadotrope, thyrotrope, somatotrope, and lactotrope hormone-secreting pituitary cell

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types [Sheng et al., 1996, 1997]. In addition to early roles in pituitary development, LHX3 appears to directly influence the transcription of some pituitary trophic hormone and transcription factor genes, including the *alpha-glycoprotein subunit (αGSU)*, *prolactin (PRL)*, *thyroid-stimulating hormone beta (TSHβ)*, and *PIT1* genes [Bach et al., 1995; Sloop et al., 1999, 2001a]. Heritable mutations in the human *LHX3* gene that cause changes predicted to disable LHX3 protein functions are associated with complex diseases in patients featuring losses of pituitary hormones and other symptoms [Netchine et al., 2000; Howard and Maurer, 2001; Sloop et al., 2001b]. Mammalian *Lhx3* genes, such as the human *LHX3* gene, produce at least two mRNA transcripts that can be translated into three protein isoforms, LHX3a, LHX3b, and M2-LHX3 [Sloop et al., 1999, 2001a]. These LHX3 proteins possess different capacities to bind DNA and activate pituitary hormone gene promoters [Sloop et al., 1999, 2001a].

LHX3 appears to activate target genes by several mechanisms, involving both DNA binding-dependent actions and transcriptional activation mediated by a major carboxyl terminus activation domain as well as activation functions in other parts of the protein, including the LIM domains [Sloop et al., 2001a]. In experiments studying pituitary hormone promoters, LHX3 has been demonstrated to function both alone and in synergy with other transcription factors such as PIT1 [Bach et al., 1995; Bach et al., 1997; Sloop et al., 1999, 2001a and reviewed in Savage et al., 2003]. In the developing nervous system, LHX3 acts in multiprotein complexes that include other LIM-HD proteins, helix-loop-helix proteins, and the LIM-interacting partner protein NLI [Thaler et al., 2002]. The association of LHX3 with the nuclear matrix, its affinity for AT-rich DNA sequences, and its ability to alter local DNA topology also suggest that LHX3 may exert some of its actions through architectural roles to promote the activities of other transcription factors [Parker et al., 2000; Bridwell et al., 2001].

Little is known about the involvement of post-translational modifications such as phosphorylation in the regulation of most neuroendocrine transcription factors. Phosphorylation of proteins can induce allosteric conformational changes [Barford and Johnson, 1989] and electrostatic repulsive effects [Hurley et al., 1990] can result from phosphorylation at multiple sites. Phosphorylation of transcription factors can modulate their activity in several ways [reviewed in Hunter and Karin, 1992]. Phosphorylation can sequester factors within an intracellular compartment thereby controlling access to DNA target sequences. For example, phosphorylation precedes nuclear localization of the STAT and SMAD transcription factors [Levy and Darnell, 2002; Shi and Massague, 2003]. Further, phosphorylation can affect DNA binding abilities, either positively or negatively. For example, in response to signals, such as phorbol esters and adenosine 3′,5′-monophosphate (cAMP), phosphorylation at two sites may alter the DNA binding properties of the pituitary transcription factor, PIT1 [Kapiloff et al., 1991]. More recent studies, however, suggest that direct phosphorylation of these residues in PIT1 may not be required for PIT1-mediated hormone responses [Fischberg et al., 1994; Okimura et al., 1994] and that regulatory signaling pathways may largely impact PIT1 interactions with cofactor proteins [Xu et al., 1998]. Phosphorylation also has been shown to modulate the ability of transcription factors to interact with protein partners, thereby regulating transcriptional activities. For example, phosphorylation of the SF1 nuclear receptor has been proposed to modulate its recruitment of transcriptional cofactors [Hammer et al., 1999]. Each of these mechanisms may work concurrently in the regulation of transcription factor function.

Many peptide and steroid hormonal signals control the development and activity of the anterior pituitary gland. It is, therefore, likely that the actions of pituitary transcription factors, such as LHX3, may be controlled in part by post-transcriptional modifications resulting from pathways activated by such hormones. In this study, we tested the hypothesis that LHX3 is a phosphoprotein. We show that LHX3 can serve as a substrate for protein kinase C (PKC) and casein kinase II (CKII) and that when these enzymes are active, LHX3 activation of target genes is inhibited. By mass spectrometry, we mapped five phosphorylated amino acid residues in the LIM domains and carboxyl terminus of LHX3. These amino acids are important for the ability of LHX3 to activate synthetic and pituitary hormone promoters, but not for interaction with the NLI, PIT1, and

MRG1 partner proteins, or binding to a consensus DNA site.

MATERIALS AND METHODS

DNA Plasmids and Site-Directed Mutagenesis

Vectors for the expression of myc epitope-tagged LHX3a in eukaryotic cells and for the expression of recombinant GST-LHX3a proteins in *E. coli* have been described [Bach et al., 1995; Meier et al., 1999; Sloop et al., 1999, 2001a; Parker et al., 2000]. Most studies were performed using human LHX3a cDNAs; in some cases, murine or porcine cDNAs also were used. Site-directed mutations causing amino acid substitutions in human LHX3a were generated using the QuikChange PCR protocol (Stratagene) with the hLHX3a-myc expression vector as a template and the following sense primers: 5′-tgcagcgactgccacgccccactggccgagcgc-3′ (T63A); 5′-gccgagcgctgcttcgcccgaggggagagcgtt-3′ (S71A); 5′-cagcgctgggggcagtttttccgcaacatgaag-3′ (Y227F); 5′-cgcaacatgaagcgcgcccgcggcggctccaag-3′ (S234A); and 5′-cgctcccgcggcggcgccaagtcggacaaggac-3′ (S238A). Expression vectors with double and triple mutations were generated by repeating the procedure using single mutant templates. All plasmids were confirmed by DNA sequencing (Biochemistry Biotechnology Facility, Indiana University School of Medicine). The rat *prolactin* enhancer/promoter reporter gene, mouse *TSHβ* promoter reporter gene, the 3 × LBC luciferase reporter gene, the rat PIT1 expression vector, and the mouse NLI expression vector have been described [Bach et al., 1995; Bridwell et al., 2001; Sloop et al., 2001b]. The mouse MRG1 plasmid [Han et al., 2001] was a generous gift from Dr. Yu-Chung Yang (Case Western Reserve University). The constitutively active bovine protein kinase C alpha (PKCα) and human alpha casein kinase II (CKII) catalytic subunit expression vectors [Heller-Harrison and Czech, 1991; James and Olson, 1992] were kind gifts from Dr. Stephen Konieczny (Purdue University, West Lafayette).

Cell Culture, Luciferase Assays, In Vivo Labeling

Human embryonic kidney (HEK) 293T cells were cultured and transfected as described previously [Parker et al., 2000]. Cells were transiently transfected using the CalPhos system (Clontech Laboratories, Inc.) or Lipofectamine (Invitrogen). Luciferase activity in cell extracts was measured 48–72 h after transfection as described [Parker et al., 2000]. Protein assays were performed by Bradford method (BioRad) and luciferase activity was normalized to protein concentration.

To label living cells with ^{32}P-phosphate, the growth medium of transfected cells was replaced with phosphate-free Dulbecco's Modified Eagle Medium (DMEM). After 1 h at 37°C, the media was replaced with phosphate-free DMEM supplemented with 1 mCi/ml ^{32}P-orthophosphate (New England Nuclear) and the cells were grown for an additional 4 h.

Immunoprecipitation

After radiolabeling, cells were rinsed twice with cold phosphate-buffered saline (PBS), scraped into cold PBS, and pelleted. Cells were washed in wash buffer (0.3% NP40, 0.3% NaCl, 0.3% Na deoxycholate, and 50 mM Tris-Cl, pH 7.4), vortexed, and sonicated for 15 s. The cell slurry was spun at 16,000*g* for 10 min at 4°C. The supernatant was precleared using 25 μl of protein G agarose beads (Pierce) for 30 min at room temperature. After removing the beads, LHX3-myc protein was precipitated from the supernatant using the mouse 9E10 anti-myc monoclonal antibody (obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa) and protein G beads. After washing, the beads were incubated at room temperature for 2 h on a rotary stirrer. The beads then were washed 4 times with wash buffer. The proteins were solubilized in SDS-loading dye, boiled, separated using 12% SDS–PAGE gels and dried onto Whatman 3MM paper. Assays were analyzed by exposure to BioMax MR film (Kodak) with intensifying screens at −80°C.

Phosphatase Treatment/Western Analysis

Forty-eight hours following transfection, cells were scraped into ice cold PBS, pelleted, and resuspended in 20 μl phosphatase buffer (Promega, Madison, WI). Cell suspensions were split into two fractions. One fraction was saved as a negative control. Two units of calf intestinal alkaline phosphatase (CIAP, Promega) was added to the second fraction and incubated at 30°C for 20 min. The fractions then were solubilized in SDS-loading buffer and proteins

separated by SDS–PAGE. Western analysis was performed using the mouse 9E10 anti-myc monoclonal antibody as previously described [Sloop et al., 2001a].

Two-Dimensional Protein Electrophoresis

Transfected cells were washed with cold PBS and collected by centrifugation. Cell pellets were solubilized in buffer containing 9.8 M urea, 4% CHAPS, 0.2% (w/v) Bio-Lytes (BioRad, pH 3–10), and 100 mM DTT. The protein sample then was actively rehydrated onto an 11 cm IPG ReadyStrip (BioRad), pH 3–10. Proteins were separated in the first dimension for 35,000 V h using a PROTEAN® IEF Cell (BioRad). After isoelectric focusing, the strips were equilibrated in DTT buffer (6 M urea, 2% SDS, 0.375 Tris-HCl, pH 8.8, 20% glycerol, 130 mM DTT) for 15 min followed by equilibration in iodoacetamide buffer (6 M urea, 2% SDS, 0.375 Tris-HCl, pH 8.8, 20% glycerol, 135 mM iodoacetamide) for 15 min. Proteins then were separated in the second dimension on 10% SDS–PAGE gels and transferred to PVDF membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH) at 4°C and 15 V for 12–16 h. Western analysis then was performed as described above.

In Vitro Kinase Assays

In vitro kinase assays were performed with commercially purchased enzymes according to manufacturer's instructions. One microgram of recombinant protein was incubated with either CKII (New England Biolabs) or rat brain PKCα (Calbiochem) in the presence of the appropriate kinase buffer, cold ATP, and [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham). Parallel positive controls were performed with casein or histone proteins (Calbiochem), as appropriate. Reactions were incubated at 30°C for 5 min and stopped by the addition of cold 25% TCA and bovine serum albumin. Precipitated proteins were neutralized with Tris-Cl, pH 8.0, solubilized in SDS-loading buffer, boiled, separated on 12% SDS–PAGE gels, and dried onto Whatman 3MM paper. Dried gels were exposed to BioMax MR film with intensifying screens at −80°C.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed as described [Haring et al., 1985]. Briefly, labeled proteins were separated by SDS–PAGE, transferred to PVDF membranes, stained with Coomassie Brilliant Blue, and rinsed in water. The LHX3 protein was excised and hydrolyzed in 5.7 M HCl at 110°C for 90 min. After several rounds of drying and resuspension in water, the sample was resuspended in water with phosphoamino acid standards and dried onto cellulose TLE plates without flouro indicators (VWR). Plates were pre-wetted with 5% acetic acid/0.5% pyridine running buffer, pH 3.5 before separation at 500 V for 45 min. The plates then were dried under a heat lamp and sprayed with 0.25% ninhydrin in acetone. Dried plates were exposed to BioMax MR film with intensifying screens at −80°C.

Solid Phase Kinase Reactions

Solid phase kinase reaction protocols were modified from the method of Hibi et al. [Hibi et al., 1993]. Briefly, 2×10^7 293T cells were scraped into cold PBS and resuspended in 150 μl lysis/kinase buffer (25 mM HEPES, 0.3 M NaCl, 0.2 mM EDTA, 0.1% Triton-X 100, and Sigma phosphatase inhibitor cocktails I and II at 1:1,000). Cell lysates then were incubated at 30°C for 30 min with GST-human LHX3a protein (attached to glutathione-agarose beads, Sigma), [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham), and cold ATP. The beads were then washed with five times with lysis/kinase buffer. The LHX3 protein was solubilized in SDS-loading buffer, boiled, separated using 12% SDS–PAGE gels, and dried onto Whatman 3MM paper before exposure to BioMax MR film with intensifying screens at −80°C or to a Storm phosphorimager screen.

Mass Spectrometry

On-line capillary liquid chromatography electrospray tandem ion trap mass spectrometry (LC-MS/MS) was performed with the Biochemistry Biotechnology Facility, Indiana University School of Medicine. Methods were modified from a previously described procedure [Haque et al., 2001]. After solid-phase kinase reactions using cold ATP, LHX3 protein was excised from gels and dried under acetonitrile in a vacuum centrifuge. Gel samples then were digested with trypsin, extracted with an acetonitrile:water (50:50) solution containing 5% trifluoroacetic acid, sonicated, and soluble material separated by centrifugation. Samples then were dried, washed, and redried. Peptides from trypsin digests were analyzed by LC-MS/MS using an Applied Biosystems 140D solvent delivery sys-

tem. Samples were injected directly into silica capillaries packed with C18 resin (300 micron internal diameter) and eluted directly into a Finnigan LCQ mass spectrometer via an electrospray ionization source. Nitrogen was used as the sheath gas at 35 pounds/square inch, and no auxiliary gas was used. Electrospray ionization was conducted with a spray voltage of 4.8 kV, a capillary voltage of 26 V, and a capillary temperature of 200°C. Spectra were scanned over an m/z range of 200–2,000. Base peak ions were trapped using the quadrupole ion trap and analyzed further with a high-resolution scan performed at an isolation width of 3 m/z and with collision-induced dissociation scans at a collision energy of 40.0. Sequences of all eluted peptides were confirmed on the collision-induced dissociation scans using the Protein Analysis Work Sheet program (PAWS, ProteoMetrics/Genomic Solutions). Ion currents at m/z ratios corresponding to the peptides fragments were integrated over the entire peak using Finnigan Bioworks software (Thermo Electron Corporation).

Protein–Protein Interaction Assays

Labeled NLI, PIT1, and MRG1 proteins were synthesized in vitro using TNT rabbit reticulocyte lysate reagents (Promega) and ^{35}S-methionine. Protein–protein interaction assays using labeled NLI, PIT1, or MRG1 proteins and wild type and mutant GST-LHX3 fusion proteins were performed as described previously [Sloop et al., 2001b]. Assays were analyzed by exposure to BioMax MR film with intensifying screens at −80°C.

Electrophoretic Mobility Shift Assays (EMSA)

EMSA were performed using protein extracts of 293T cells transfected with LHX3a expression vectors and radiolabeled oligonucleotide probes containing the LHX3 binding consensus (LBC) site [Bridwell et al., 2001] as described [Parker et al., 2000]. Results were visualized by autoradiography using BioMax MR X-ray film or a Storm phosphorimager (Amersham Biosciences).

RESULTS

Phosphorylation of LHX3

To examine whether LHX3 could be modified by phosphorylation, we first transfected heterologous 293T cells with an expression vector encoding myc epitope-tagged LHX3 or empty vector as a control. The cells were then cultured in the presence of ^{32}P-orthophosphate. After labeling, LHX3 was immunoprecipitated using an anti-myc antibody. Immunoprecipitated proteins were separated by electrophoresis and radiolabeled proteins were visualized by autoradiography. A ^{32}P-labeled protein migrating with an apparent mass consistent with the predicted mass of LHX3 was observed in the LHX3-expressing cells but not the controls (Fig. 1A). Next, protein extracts of cells expressing myc-LHX3 were incubated in the presence or absence of calf intestinal alkaline phosphatase, followed by Western analysis using an anti-myc monoclonal antibody. The detected complexes migrated more rapidly in the phosphatase-treated samples, consistent with the removal of phosphate groups from LHX3 (Fig. 1B). In similar experiments, proteins from cells expressing myc-LHX3 were separated by two-dimensional gel electrophoresis followed by

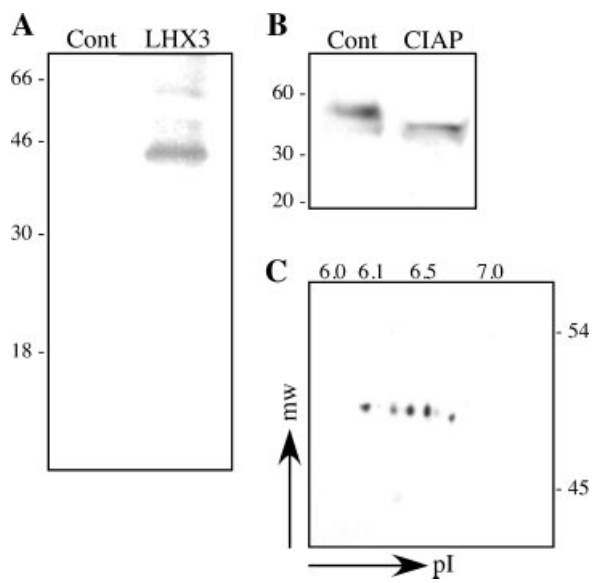

Fig. 1. Phosphorylation of LHX3. **A**: In vivo labeling of LHX3. HEK 293T cells were transfected with myc-LHX3 and then incubated with ^{32}P orthophosphate. LHX3 was immunoprecipitated and washed complexes were visualized by autoradiography. Cont = immunoprecipitation from mock transfected cells as a negative control. **B**: Whole cell lysates from 293T cells expressing myc-tagged LHX3 were incubated in the presence or absence (Cont) of calf intestinal alkaline phosphatase (CIAP). LHX3 was then detected by Western analysis using an anti-myc monoclonal antibody. **C**: Proteins from 293T cells expressing myc-LHX3 were separated by 2D gel electrophoresis followed by Western analysis using an anti-myc antibody. A ''charge train'' with at least six myc-LHX3 species is observed.

Western analysis using an anti-myc antibody. A series of at least six reactive proteins was observed in the region of the gel corresponding to the predicted relative molecular mass and isoelectric point of myc-LHX3 (Fig. 1C). Such a "charge train" would be predicted if LHX3 proteins were present as a population reflecting multiple post-translational modifications, as has been observed in other factors [Deppmann et al., 2003]. Overall, these data are consistent with phosphorylation of LHX3.

Modification of LHX3 by Protein Kinase C and Casein Kinase II

The amino acid sequence of LHX3 contains several predicted recognition sites for protein kinases including PKC and CKII. To test whether the LHX3 protein could be a substrate for PKC, recombinant LHX3 proteins (as fusions with glutathione-S-transferase [GST]) were incubated with PKCα enzyme in the presence of [γ-^{32}P]ATP. The full-length LHX3 protein was phosphorylated by PKCα and a truncated LHX3 protein lacking the amino terminal LIM domains (LHX3 ΔLIM) was weakly labeled (Fig. 2A). By contrast, GST was not a substrate for PKCα (Fig. 2A). Phosphoamino acid analysis of the phosphorylated LHX3 indicated that in these reactions LHX3 was predominantly modified on serine residues with perhaps some phosphothreonine (Fig. 2B). The influence of PKC signaling on the transcriptional capacity of LHX3 was then tested by overexpressing a constitutively active form of PKCα in cells that were cotransfected with an LHX3 expression vector and LHX3-responsive reporter genes (Fig. 2C,D). First, a synthetic reporter gene containing three copies of a high-affinity LHX3 binding consensus site cloned next to a minimal promoter (3 × LBC luciferase) was tested. This reporter gene provides a valuable reagent for these studies because it is a simple LHX3-dependent reporter gene that does not contain complex response elements that might be influenced by signaling pathways via LHX3-independent mechanisms [Bridwell et al., 2001]. In experiments using 3 × LBC luciferase, coexpression of constitutively active PKCα inhibited transcriptional activation by LHX3 (Fig. 2C). Similarly, the synergistic transcriptional activation of a *prolactin* enhancer/promoter reporter gene by LHX3 and PIT1 was significantly reduced by constitutive PKC activity (Fig. 2D).

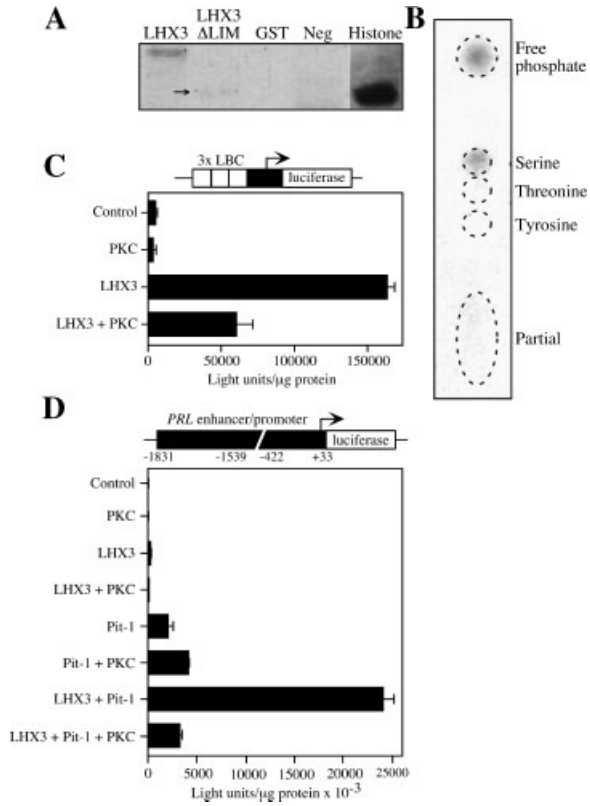

Fig. 2. Phosphorylation of LHX3 by PKC. **A**: In vitro phosphorylation of recombinant LHX3 proteins by PKCα. LHX3 = GST-full-length LHX3, LHX3 ΔLIM = GST-ΔLIM LHX3 (lacking the LIM domains; arrow indicates weak labeling), GST = GST alone as a negative control, Neg = control LHX3 reaction lacking enzyme, Histone = positive control. **B**: Phosphoamino acid analysis of ^{32}P-labeled full-length LHX3 protein following incubation with PKCα. Hydrolyzed proteins were separated by electrophoresis through a cellulose chromatography plate. The migration positions of added phosphoamino acid standards are indicated by the dashed circles. Partial = position of partially digested reactants. **C**: Activation of a synthetic luciferase reporter gene containing three LHX3 binding consensus (LBC) sites next to a minimal promoter (3 × LBC luciferase) [Bridwell et al., 2001] is reduced by PKCα. 293T cells were transiently transfected with 3 × LBC luciferase, expression vectors for LHX3, and a constitutively active form of PKCα. Promoter activity was assayed by measurement of luciferase activity after 48 h. Activities are mean [light units/10 s/μg total protein] of triplicate assays ± SEM. A representative experiment of at least three experiments is depicted. Negative controls (Control) received equivalent amounts of empty vector. **D**: Activation of a *prolactin (PRL)* luciferase reporter gene is reduced by PKCα.

Recombinant LHX3 and LHX3 ΔLIM proteins were also readily phosphorylated by CKII in in vitro kinase reactions (Fig. 3A). As for PKC, phosphoamino acid analysis indicated that CKII actions resulted in the modification of mostly serine side chains within the LHX3 protein (Fig. 3B). Further, overexpression of the

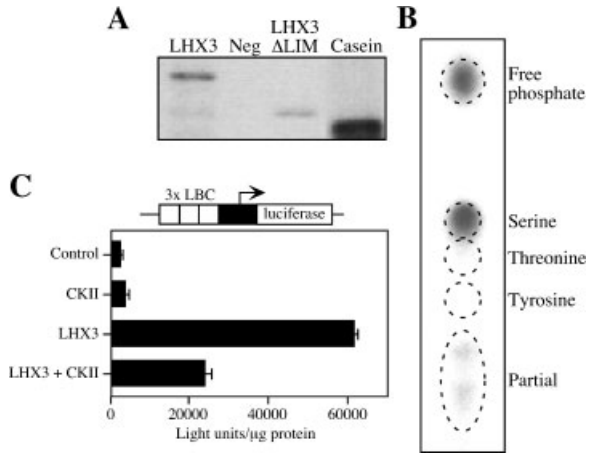

Fig. 3. Phosphorylation of LHX3 by casein kinase II. **A**: In vitro phosphorylation of recombinant LHX3 proteins by CKII. LHX3 = GST-full-length LHX3, Neg = control LHX3 reaction lacking enzyme, LHX3 ΔLIM = GST-ΔLIM LHX3, Casein = posi-positive control. **B**: Phosphoamino acid analysis of ^{32}P-labeled LHX3 protein following incubation with CKII. The migration positions of added phosphoamino acid standards are indicated by the dashed circles. Partial = position of partially digested reactants. **C**: Activation of 3 × LBC luciferase is reduced by CKII. 293T cells were transiently transfected with 3 × LBC luciferase, expression vectors for LHX3, and the catalytic α-subunit of CKII. Promoter activity was assayed by measurement of luciferase activity after 48 h. Activities are mean [light units/10 s/μg total protein] of triplicate assays ± SEM. A representative experiment of at least three experiments is depicted. Negative controls (Control) received equivalent amounts of empty vector.

catalytic α-subunit of CKII inhibited activation of the 3 × LBC luciferase reporter gene by LHX3 (Fig. 3C). We conclude that LHX3 can serve as a substrate for PKC and CKII and that signaling pathways mediated by these enzymes likely impart repressive signals upon LHX3-mediated transcription.

Modification of Serine, Threonine, and Tyrosine Residues in LHX3

To directly map residues within the LHX3 protein that can be modified by phosphorylation, recombinant human GST-LHX3a (hLHX3a) bound to glutathione-agarose beads was incubated with human 293T cell protein extracts as a source of kinase enzymes. Following the kinase reaction, GST-LHX3 was eluted, separated by SDS–PAGE, and analyzed by on-line capillary liquid chromatography electrospray tandem ion trap mass spectrometry (LC-MS/MS). Mass and sequence data indicated that hLHX3a can be phosphorylated on at least five residues, including serine, threonine, and tyrosine side chains (Figs. 4 and 5). Within the amino-terminal LIM domains, threonine 63 (T63) and serine 71 (S71) of LIM1 were phosphorylated (Fig. 4A). Threonine 63 is not well conserved in LHX3/LIM3 family proteins (Fig. 4B). By contrast, S71 lies within consensus sites for CKII and protein kinase A (PKA) in human LHX3a and this serine is conserved in vertebrate LHX3/LHX4/LIM3 proteins (Fig. 4B). The LHX3 carboxyl terminus contains both *trans*-activation and *trans*-repression domains [Parker et al., 2000; Sloop et al., 2001a]. Within the carboxyl terminal region following the HD, three additional sites of phosphorylation were mapped by mass spectrometry: tyrosine 227 (Y227), serine 234 (S234), and serine 238 (S238) (Fig. 5A,B). Tyrosine 227 is found in all LHX3/LHX4/LIM3 proteins, including *Drosophila* LIM3 (Fig. 5C). Serine 234 is conserved in vertebrate members of this family, except the pig (Fig. 5C). Serine 238 is predicted to be the reactive residue of consensus sites for CKII and PKA and is retained in all vertebrate LHX3/LHX4/LIM3 proteins (Fig. 5C).

Function of Modified Amino Acids in Gene Activation by LHX3

To examine the role of the five identified phosphorylation sites in human LHX3a, site-directed mutagenesis was used to substitute alanines for serines/threonines, and tyrosine was replaced by phenylalanine. Substitutions were made as single alterations (T63A, S71A, Y227F, S234A, S238A) and also in combinations of the LIM domain substitutions (T63A + S71A) and of the carboxyl terminal substitutions (Y227F + S234A; Y227F + S238A; S234A + S238A; and Y227F + S234A + S238A). The capacities of the altered LHX3 proteins were then compared to wild type LHX3 in transfection assays using 3 × LBC luciferase and *TSHβ* luciferase reporter genes (Fig. 6). In these experiments, substitution of any one of the modified amino acid groups, either alone or in any tested combination, reduced the ability of LHX3 to activate either of these promoters (Fig. 6A,B). The properties of the altered LHX3 proteins were similar for both the synthetic 3 × LBC luciferase promoter and the *TSHβ* pituitary hormone gene promoter, with the T63A + S71A protein retaining the most activity and Y227F + S238A possessing the least (Fig. 6A,B). We conclude that the phosphorylated amino acids are important for the transcriptional properties of LHX3.

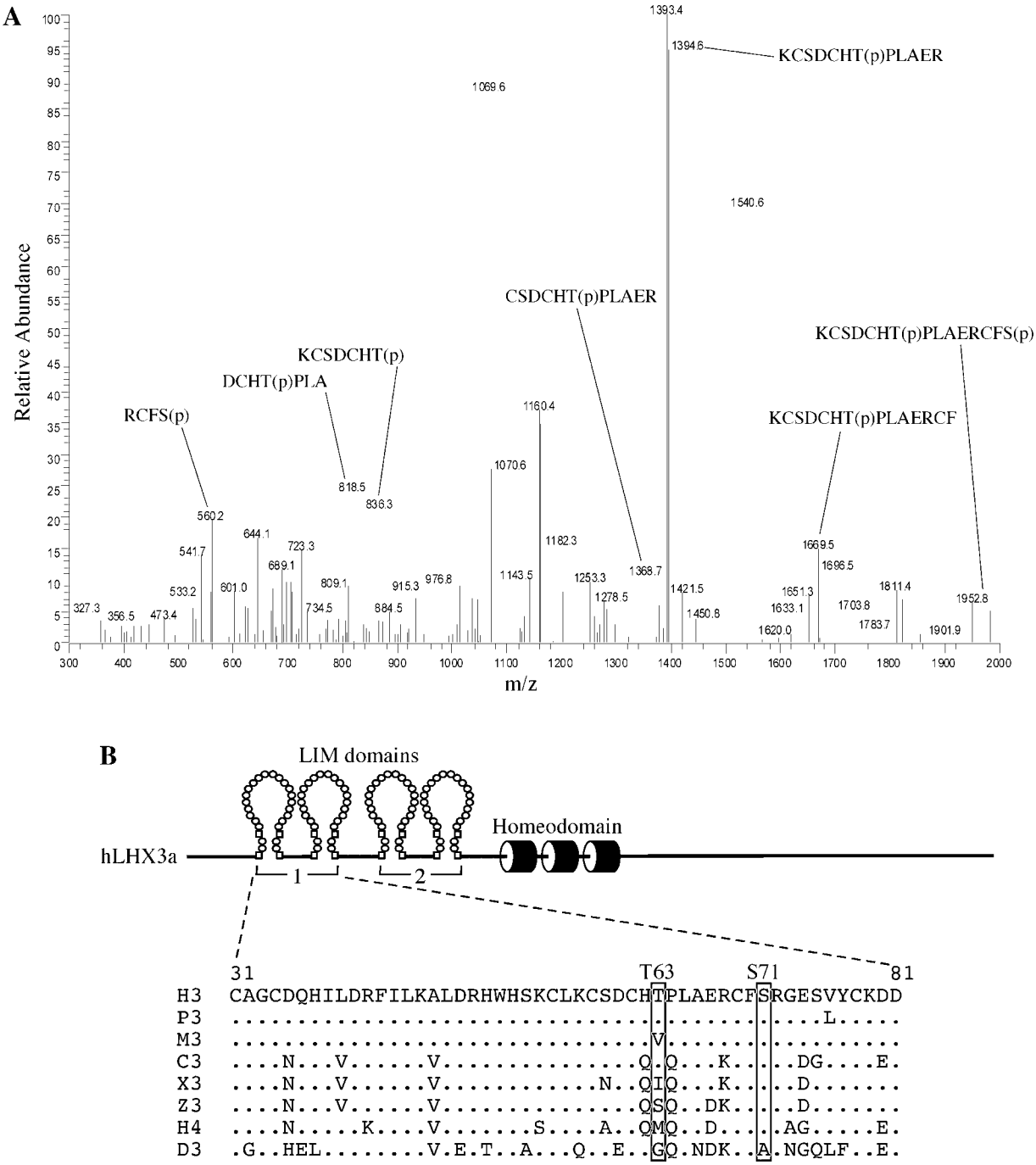

Fig. 4. Phosphorylation of amino acid residues within the LIM1 domain of human LHX3a. **A**: Recombinant GST-LHX3 protein was incubated with 293T cell extracts (as a source of protein kinases). Following washing, the protein was digested with trypsin. The resulting peptides were analyzed by on-line capillary liquid chromatography electrospray tandem ion trap mass spectrometry (LC-MS/MS) using a 300 micron id C18 capillary column and standard acetonitrile gradients. Amino acid sequence and position of phospho-groups were confirmed by collision-induced dissociation (CID). Representative data for the peptide CLKCSDCHT(p)PLAERCFS(p)R are shown. Examples of peptide fragments are labeled. **B.** Alignment of LHX3/LIM3 family amino acid sequences within the LIM1 region. Periods denote identity at that position. Comparison of human LHX3a (H3), porcine LHX3 (P3), mouse LHX3 (M3), chicken LIM3 (C3), *Xenopus laevis* LIM3 (X3), zebrafish LIM3 (Z3), human LHX4 (H4), and *Drosophila* LIM3 (D3). Residues threonine 63 and serine 71 are boxed. Residue numbers are for human LHX3a.

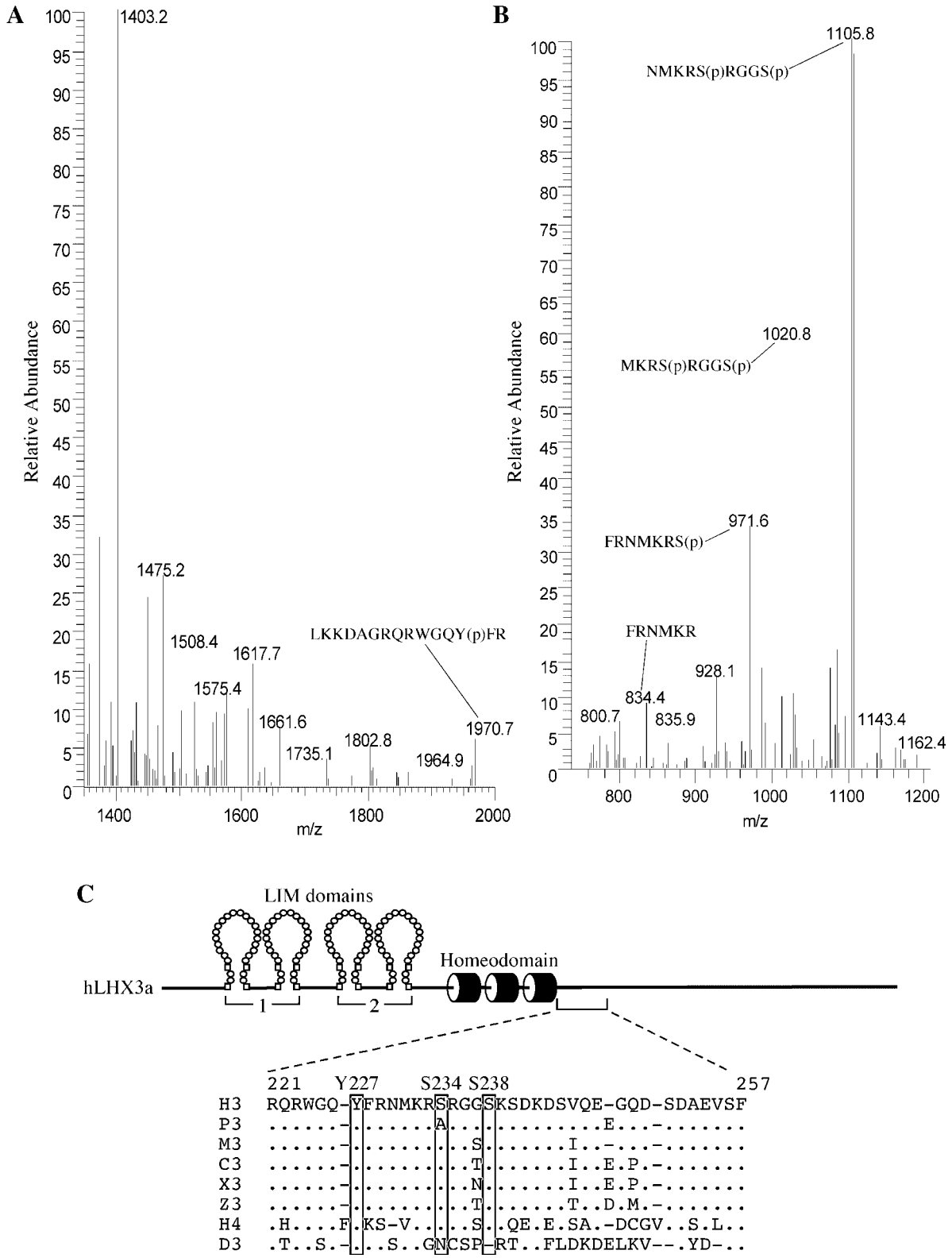

Fig. 5. Phosphorylation of amino acid residues in the carboxyl terminus of human LHX3a. **A, B**: Representative spectra showing analysis of the peptide RLKKDAGRQRWGQY(p)FRNMKRS-(p)RGGS(p)K by LC-MS/MS as described in Figure 4. Relevant peptide fragments are labeled. **C**: Alignment of LHX3/LIM3 family protein sequences within the carboxyl terminus. Periods denote identity at that position; dashes indicate gaps introduced to optimize alignment. Sequences are human LHX3a (H3), porcine LHX3 (P3), mouse LHX3 (M3), chicken LIM3 (C3), *Xenopus laevis* LIM3 (X3), zebrafish LIM3 (Z3), human LHX4 (H4), and *Drosophila* LIM3 (D3). Phosphorylated residues are labeled and boxed. Numbers are for human LHX3a.

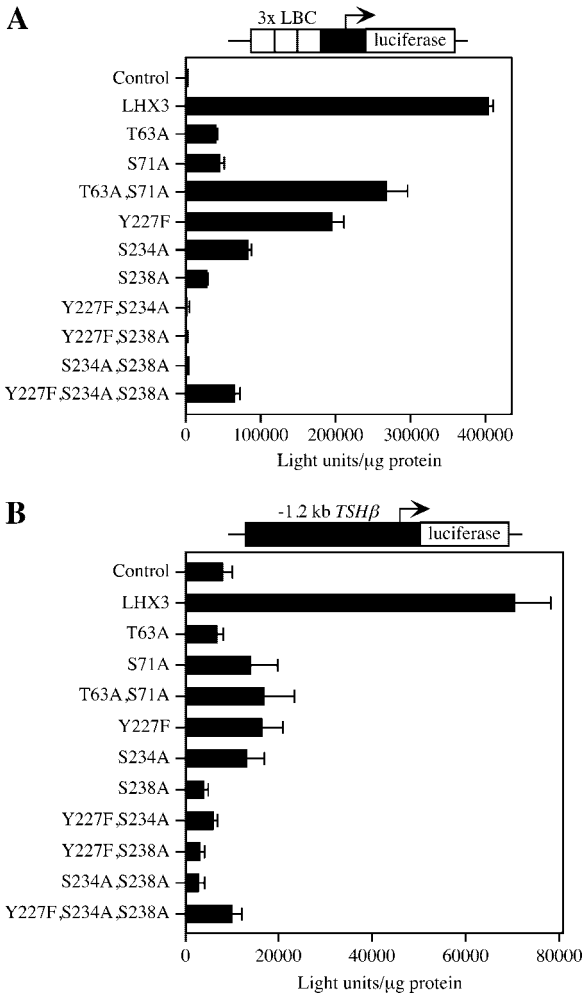

Fig. 6. The phosphorylated amino acids in LHX3 are critical for transcriptional activation. 293T cells were transiently transfected with 3 × LBC luciferase (**panel A**) or with a *TSHβ* luciferase promoter reporter gene (**panel B**) and expression vectors for the indicated wild type or mutated LHX3 proteins. Promoter activity was assayed by measurement of luciferase activity after 48 h. Activities are mean [light units/10 s/μg total protein] of triplicate assays ± SEM. A representative experiment of at least three experiments is depicted. Negative controls (Control) received equivalent amounts of empty vector.

The abilities of a transcription factor to activate or repress a target gene depend on multiple functional properties, including binding to its cognate DNA recognition element, interactions with regulatory partner proteins, interactions with coactivators and corepressors, and/or interactions with the components of the core transcriptional machinery. To determine whether the five identified amino acid residues were required for DNA binding, we performed EMSAs to compare the DNA binding properties of the LHX3 proteins carrying substitutions at these positions with wild type LHX3. In these assays, the Y227F, S234A, S238A, T63A + S71A, or Y227F + S234A + S238A substitutions did not affect the DNA binding ability of LHX3 (Fig. 7).

The LIM domains of LHX3 mediate interactions with regulatory and transcriptional protein partners. We therefore tested the protein–protein interaction properties of the LHX3 derivatives with substitutions of the phosphorylatable amino acid residues in the LIM structures (T63A, S71A, and T63A + S71A). Each of these proteins displayed wild type abilities to interact with the NLI, PIT1, and MRG1 proteins (Fig. 8). We conclude that the tested amino acid residues are not critical for DNA binding or interaction with these partner proteins. Im-

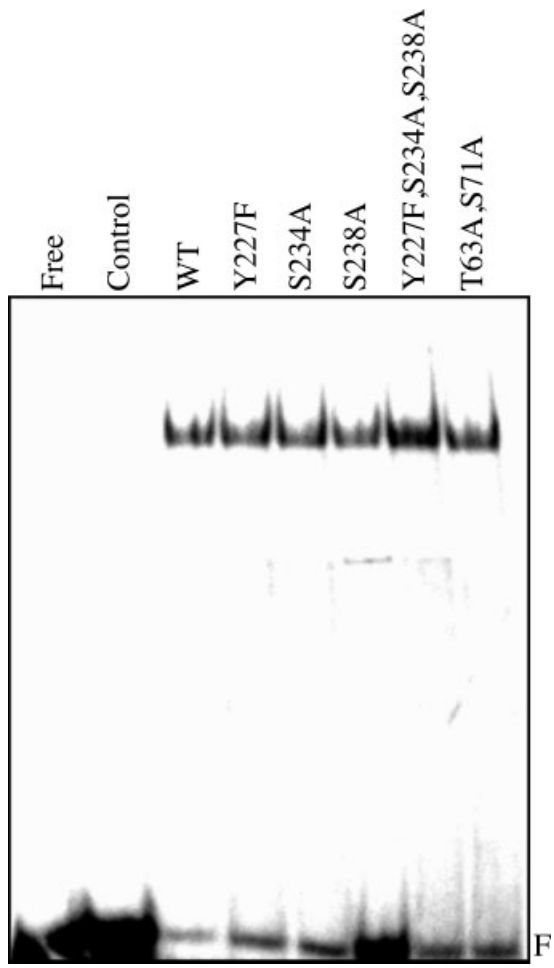

Fig. 7. The phosphorylated amino acids in LHX3 are not critical for DNA binding. 293T cells were transfected with the indicated wild type (WT) or mutated LHX3 expression vectors (or with vector alone as a negative control [Control]) and protein extracts were prepared. EMSAs then were performed using a radiolabeled LHX3 binding consensus site [Bridwell et al., 2001]. F = free probe.

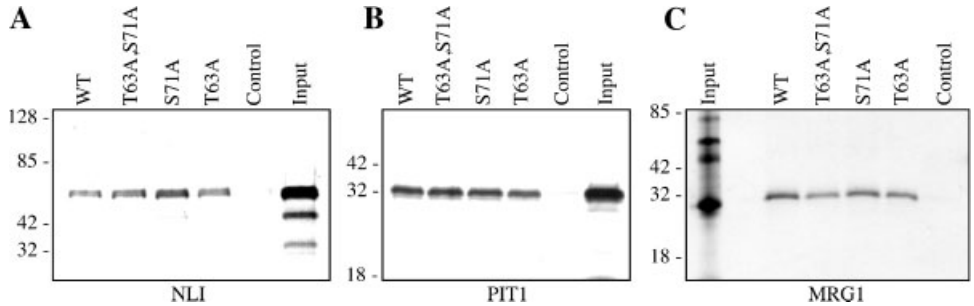

Fig. 8. Assay of the role of the phosphorylated amino acids in the LIM domains of LHX3 in protein–protein interaction with NLI, PIT1, and MRG1 binding. In vitro binding assays were used to measure the interaction of wild type and mutant LHX3 proteins with NLI (**A**), PIT1 (**B**), and MRG1 (**C**) proteins. Radiolabeled NLI, PIT1, or MRG1 proteins were generated by in vitro transcription/translation in the presence of [^{35}S]-methionine and incubated with the indicated LHX3 GST fusion proteins or with GST alone as a control. After washing, bound proteins were separated by electrophoresis and visualized by fluorography. The migration positions of molecular weight standards (in kilodaltons) are shown.

portantly, however, the observations that the alanine/phenylalanine-substituted forms of LHX3 retain wild type DNA binding and protein interaction properties suggest that the introduced residues do not significantly affect the structural integrity of the LHX3 protein and that the reductions in transcriptional activities shown in Figure 6 do indeed reflect the importance of these individual amino acids.

DISCUSSION

In this study, we have demonstrated that the LHX3 LIM-HD transcription factor can be phosphorylated on at least five amino acids, including serine, threonine, and tyrosine residues. To our knowledge, this is the first report of phosphorylation of a member of the LIM-HD family of developmental regulatory proteins. The completion of a draft of the human genome led to predictions that the genome contains fewer genes than had been predicted [reviewed in Southan, 2004]. However, it is clear that the inherent regulatory capacity of the genome can be increased by the production of multiple mRNAs from single genes (via the use of alternate promoters and splicing), by the translation of multiple proteins from single mRNAs, by the post-translational modification of proteins, and by combinatorial interactions of proteins with regulatory partners. The human *LHX3* gene exhibits all of these features, producing two major mRNAs that encode three distinct proteins with different functional properties that can be modified by phosphorylation.

Phosphorylation of LHX3 may regulate its intracellular location, its stability, its DNA binding, and its interactions with partner proteins. Alternately, interactions with partner proteins may selectively modulate the accessibility of LHX3 as a substrate for regulatory protein kinases. In this study, we have shown that five amino acids can be modified by phosphorylation and that these positions are important to the transcriptional functions of the protein. However, alteration of these positions did not affect binding to a tested DNA consensus element or to the MRG1, NLI, and PIT1 partner proteins. Future experiments will be required to test interactions with additional DNA binding elements and partner proteins. The identified phosphorylated amino acids lie within the LIM domains and in the carboxyl terminus of the molecule just after the HD. The LIM domains of LHX3 reduce the DNA binding affinity of LHX3 but do not appear to affect the specificity of interaction with DNA [Bridwell et al., 2001]. The three phosphorylated amino acids in the carboxyl terminus of LHX3 may play complex roles in regulating both LHX3 location and activity because they lie within the B4 region that we have previously shown to contain both a potential repression function and one of the four LHX3 nuclear localization signals [Parker et al., 2000]. Further, phosphorylation may also modulate the intranuclear association of LHX3 with the nuclear matrix as has been shown for other factors such as p73 [Ben-Yehoyada et al., 2003].

The observation that multiple amino acids of LHX3, including serine, threonine, and tyrosine residues, can be phosphorylated suggests that modification of LHX3 may be a complex process, involving a hierarchy or specific sequence of combinatorial phosphorylation events. Such complex regulatory mechanisms have been well characterized in the basic region-leucine

zipper (bZIP) class of transcription factors that includes the FOS and JUN AP-1 proteins [Shaulian and Karin, 2001; Deppmann et al., 2003].

We have shown that LHX3 can serve as a substrate for the PKC and CKII enzymes and that the actions of these signaling enzymes inhibit the transcriptional actions of LHX3 on tested promoters. Future experiments will be required to delineate the specific target amino acids within LHX3 that are modified by these enzymes and the functional consequences of such phosphorylation in vivo. Under some circumstances, catalysis by PKC, CKII, or other signaling kinases may boost, rather than inhibit, LHX3 activity. Indeed, preliminary experiments suggest that protein kinase A (PKA)-dependent pathways increase LHX3-mediated transcription of specific pituitary target genes (GEP, BEW, SJR; unpublished data). In the pituitary, PKC and cAMP/PKA have been implicated in multiple processes, including mediating the complex second messenger effects of hypothalamic regulatory hormones in control of pituitary hormone gene transcription [e.g., Richards, 2001; Fowkes et al., 2002; Pickett et al., 2002; Malagon et al., 2003]. It will also be interesting to test the functions of altered forms of LHX3 in which the identified phosphorylatable amino acids are replaced by glutamate or aspartate residues to constitutively mimic the phosphorylated state; such approaches can often provide insights into the roles of specific protein phosphorylation events [e.g., Deng et al., 2004].

It is also interesting that LHX3 can be modified on a tyrosine residue (Y227 in human LHX3a) that is conserved in LHX3/LIM proteins, including the invertebrate *Drosophila* LIM3 protein. Although LIM3 may be expressed in the *Drosophila* ring gland, a hormone-secreting structure, invertebrates do not have a true pituitary gland, and the major roles of LIM3 in this organism appear to be in development of the nervous system [Thor et al., 1999]. In addition to roles in pituitary development, vertebrate LHX3 family proteins also serve essential functions in spinal cord neuron development [e.g., Sharma et al., 1998]. It is, therefore, intriguing to speculate that tyrosine phosphorylation, an important aspect of nervous system control [Purcell and Carew, 2003], may be a regulator of LHX3/LIM3 functions in the developing nervous system.

Two types of recessively acting mutations in the human *LHX3* gene are associated with a specific type of combined pituitary hormone deficiency disease (CPHD; [Netchine et al., 2000]). Similar to the *Lhx3* null mice, these patients display deficiencies in multiple anterior pituitary hormones [Netchine et al., 2000]. One mutation causes the substitution of a conserved tyrosine residue (Y111 in the LHX3a isoform LIM domains) with a cysteine; the other results in a truncated protein that likely lacks the HD. These mutations impair the ability of LHX3 to *trans*-activate specific pituitary gene promoters [Howard and Maurer, 2001; Sloop et al., 2001b]. In this study, we show that Y227 can be phosphorylated. To date, we have not seen evidence of Y111 phosphorylation in our experiments; however, it may be modified under specific conditions and the possibility remains that the substitution of a cysteine at this position prevents a phosphorylation event that is required for LHX3 function in humans. It will also be interesting to see whether mutations in the *LHX3* gene that cause substitutions of the five phosphorylated amino acids characterized here will be found in patients with loss of LHX3 function.

In conclusion, we have shown that the LHX3 LIM-HD protein is a phosphoprotein. This observation, therefore, extends the ways in which the actions of LHX3 can be regulated during neuroendocrine cell determination and differentiation. These studies are relevant to future investigations examining the role of LIM-HD factors, such as LHX3 and LHX4, in pituitary-associated diseases, such as combined pituitary hormone deficiency, precocious puberty, delayed puberty, and pituitary tumor formation. They are also more broadly relevant to future investigations of the role of post-translational modifications in the functions of other members of the LIM-HD family of gene regulatory proteins.

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