A Point Mutation in the LIM Domain of Lhx3 Reduces Activation of the Glycoprotein Hormone α-Subunit Promoter*

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Lhx3, a member of the LIM homeodomain family of transcription factors, is required for development of the pituitary in mice. A recent report has described a point mutation in the human LHX3 gene that is associated with a combined pituitary hormone disorder. The mutation is predicted to lead to the replacement of a tyrosine residue with a cysteine in the second LIM domain of LHX3. We have characterized the effects of this point mutation (Y114C) when analyzed in the context of the mouse Lhx3 coding sequence. Mobility shift assays demonstrated that the Lhx3 Y114C mutant is capable of binding DNA, although a decrease in the formation of a specific complex was observed. Transfection assays using an expression vector for either full-length Lhx3 or a GAL4-Lhx3 LIM domain fusion provided evidence that the Lhx3 Y114C mutant has a decreased ability to stimulate transcription. In particular, a GAL4-Lhx3 Y114C LIM mutant was unable to support Ras responsiveness of a modified glycoprotein hormone α-subunit reporter gene. Protein interaction studies suggest that the Y114C mutation may modestly reduce binding to the POU transcription factor, Pit-1. Interestingly, the Y114C mutation essentially abrogated binding to the putative co-activator/adapter, selective LIM-binding protein. The findings provide insights into the mechanisms mediating transcriptional activation by Lhx3 and suggest that the observed phenotype of the human mutation probably involves reduced transcriptional activity of the mutant LHX3.

The Lhx1 family of transcription factors contain a homeodomain and two LIM domains each consisting of two zinc fingers (1–3). Lhx3 is expressed in the pituitary and a subset of neurons and is able to enhance the transcription of several pituitary hormone reporter genes in transient transfection assays (4–8). Lhx3 can synergize with the POU homeodomain transcription factor, Pit-1, to stimulate prolactin promoter activity (4, 9). Lhx3 also appears to play a role in permitting the promoter for the α-subunit of the glycoprotein hormones to respond to the mitogen-activated protein kinase signaling pathway (10).

Members of the LIM homeodomain family of transcription factors all contain a homeodomain which functions as the DNA-binding domain and two amino-terminal LIM domains (1, 2). The LIM domains each contain two zinc finger structures and appear to function as protein-protein interaction domains (11, 12). The LIM domains of Lhx3 have been shown to specifically bind several proteins. Lhx3 can bind to the pituitary-specific transcription factor, Pit-1 (4). Lhx3 has also been shown to interact with several co-activator/adapter proteins. Several laboratories have identified the widely expressed nuclear adapter protein nuclear LIM interactor (NLI, also designated LBD or CLIM) as an Lhx3 interacting protein (13–16). Recently, the LIM domains of Lhx3 have also been shown to bind the novel putative adapter, selective LIM-binding protein (SLB) (17). Unlike NLI, which binds to all nuclear LIM domains tested, SLB appears to bind to a subset of LIM domains with the highest affinity for Lhx3 and the closely related protein, Lhx4.

The developmental role of Lhx3 has been clearly demonstrated through the use of gene disruption studies in mice and Drosophila melanogaster (6, 18–20). In the mouse Lhx3 is required for pituitary development and for specification and pathway selection of certain motor neurons (6, 18, 19). Disruption of the mouse lhx3 and lhx4 genes have demonstrated the role of these LIM factors in pituitary gland development and in differentiation and proliferation of pituitary cell lineages (18, 19). There is also evidence that LHX3 plays an important role in pituitary hormone gene expression in humans. A recent report has described mutations in the human LHX3 gene in patients displaying a syndrome called combined pituitary hormone deficiency (21). This syndrome is characterized by the loss of all but one (adrenocorticotropin) of the five hormones produced in the anterior pituitary resulting in severe growth retardation. Two mutations which are associated with this syndrome in humans have been identified. One of the mutations is predicted to produce a truncated protein lacking the homeodomain. Thus, this protein would likely be inactive due to failure to bind DNA. Interestingly, the other mutation is a point mutation that is predicted to convert a tyrosine residue to cysteine in the first zinc finger structure of the second LIM domain. It is not clear how this mutation alters the activity of LHX3.

In the present studies we have explored how this point mutation in the second LIM domain alters the functional properties of Lhx3. To study this issue, we have prepared the corresponding mutation in the mouse Lhx3 coding sequence. We have analyzed the ability of the mutant transcription factor to bind DNA, participate in transcriptional activation, and interact with other transcription factors and co-activator/adapter proteins.

MATERIALS AND METHODS

Cell Culture, DNA Constructs, and Transfections—GH4c1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with...
15% equine serum and 2.5% fetal bovine serum. Human embryonic kidney 293 cells (HEK 293) and the gonadotropin-derived αT-3 cell line (22) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Luciferase reporter genes containing 0.6 kilobase pairs of 5’-flanking region of the rat prolactin gene (23) or 5 copies of a radiolabeled probe corresponding to an Lhx3-binding element (designated the pituitary glycoprotein basal element from the human LHX3) were generated by polymerase chain reaction using standard protocols. The products were all confirmed by automated DNA sequencing. Cells were transfected with a total of 1 µg of DNA and 5 µl of LipofectAMINE (Life Technologies, Inc.) in 35-mm well plates, or 0.4 µg of DNA and 2 µl of LipofectAMINE in 22-mm well plates using a protocol provided by the supplier.

Preparation of Cell Extracts—For immunoblotting or immunoprecipitations, αT-3 or 293 cells were scraped from the culture dishes in 100 mM sodium phosphate, pH 7.8. The cells were pelleted in a microcentrifuge and resuspended in the same buffer but with 0.1% Nonidet P-40. The cells were disrupted by cycles of freeze thaw using dry ice/ethanol and 37 °C water baths. After centrifugation at 10,000 × g for 5 min at 4 °C, the supernatant was saved as a whole cell extract. For preparation of cell extracts for luciferase assays and for some mobility shift studies, cell monolayers were rocked for 15 min in Passive Lysis Buffer (Promega). Cell debris was removed by transferring the extract to microcentrifuge tubes and centrifuging for 2 min.

Mobility Shift Assay for Protein/DNA Interaction—Cell extracts were made from transfected 293 cells as described above for immunoprecipitations. A duplex DNA probe was synthesized containing the sequence, ATATCGGCTTACGTTAATTAAATGCCT, which corresponds to the pituitary glycoprotein hormone basal element (26). This DNA element contains a LIM factor-binding site (7). Binding reactions contained 10,000 cpm of 32P-labeled DNA probe, varying amounts of cell extract, 1 µg of sheared salmon sperm DNA, 10 µg of bovine serum albumin, 10 mM Tris, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol in a total volume of 25 µl. In some reactions 0.5 µl of preimmune serum or antiserum to Lhx3 was included. Reactions were incubated for 20 min at room temperature and then analyzed on nondenaturing, 4% polyacrylamide gels. The gels were then dried and exposed to x-ray film.

Antiserum, Immunoprecipitations, and Immunoblotting—The antiserum to SLB has been described previously (17). Monoclonal antibody to the αT-3 epitope was obtained from the Berlex Antibody Company. The antiserum to Lhx3 was produced by immunizing rabbits with a fusion protein containing glutathione S-transferase fused to residues 266 to 400 of mouse Lhx3 (GST-Lhx3266–400). This antigen represents the carboxy-terminal domain of Lhx3 facilitating the use of the results of the antiserum to detect Lhx3 proteins with point mutations in the zinc finger of the Lhx3 sequence, it is likely the findings also apply to human LHX3 as the second LIM domain of human LHX3 is identical to the second LIM domain of mouse Lhx3. The Y114C mutation is adjacent to histidine 115, one of four residues predicted to coordinate a zinc atom in the first zinc finger of the second LIM domain (2, 28, 29). It seemed possible that the introduction of a cysteine residue which potentially could coordinate with the zinc atom might distort the zinc finger structure. Therefore we created a point mutation which replaced tyrosine 114 with an alanine residue (Y114A) which should not coordinate a zinc atom. We also prepared a mutant Lhx3 in which histidine 115 was replaced with alanine (H115A) presumably disrupting the zinc finger structure. The ability of the wild type and mutant Lhx3 proteins was then tested for DNA binding, transcriptional activity, and co-factor binding.

Mutant Lhx3 Is Capable of Binding DNA—A point mutation might reduce the ability of a transcription factor to stimulate gene expression through any of several mechanisms. One of the most straightforward mechanisms would involve a decrease in DNA binding activity. Although the Y114C mutation is outside of the homeodomain of Lhx3, there is evidence that the LIM domain of Lhx3 may inhibit the DNA binding activity of the homeodomain (4, 9, 30). To test for DNA binding activity, HEK 293 cells were transiently transfected with expression vectors for wild type and mutant Lhx3 and then cell extracts prepared for binding experiments. The cell extracts were incubated with a radiolabeled probe corresponding to an Lhx3-binding element designated the pituitary glycoprotein basal element from the gonadotropin α-subunit promoter (7, 26). Bound complexes were separated from free probe by electrophoresis on nondenaturing polyacrylamide gels (Fig. 1). Extracts prepared from the HEK 293 cells transfected with the empty expression vector formed several weak complexes which were insensitive to addition of an antiserum to Lhx3. Transfection of the vector for wild type Lhx3 resulted in the formation of two major complexes. Both complexes were apparently disrupted by antiserum to Lhx3 but not by preimmune serum. The more slowly migrating complex, C2, has a similar migration to a complex from nontransfected cells. However, the Lhx3-directed, C2 complex can be distinguished from the endogenous complex (designated C1NS) by the disrupting effects of an Lhx3 antiserum. Formation of two Lhx3-directed complexes is consistent with the observation that the related LIM homeodomain protein, Lhx2, also forms two complexes on DNA fragments containing this sequence (7). As this DNA element contains an imperfect palindromic sequence, it was possible that the slower migrating, C2 complex contain Lhx3 dimer. Analysis of extracts from

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LIM Point Mutation Reduces Transcription

Recently it has been shown that a point mutation which results in replacement of a tyrosine residue with a cysteine residue in the LIM domain of the human LHX3 transcription factor is associated with combined pituitary hormone deficiency (21). It seems likely that this point mutation alters the ability of LHX3 to modulate a transcriptional event important for the development of pituitary cell lineages (18, 19). In addition, the point mutation may alter the ability of LHX3 to stimulate pituitary hormone gene expression (4–10). To examine the effects of this point mutation on LHX3 activity, we created the corresponding mutation (Y114C) in the mouse Lhx3 coding sequence. Although we have studied this mutation in the context of the mouse Lhx3 sequence, it is likely the possibility that the introduction of a cysteine residue which potentially could coordinate with the zinc atom might disrupt or distort the zinc finger structure. Therefore we created a point mutation which replaced tyrosine 114 with an alanine residue (Y114A) which should not coordinate a zinc atom. We also prepared a mutant Lhx3 in which histidine 115 was replaced with alanine (H115A) presumably disrupting the zinc finger structure. The ability of the wild type and mutant Lhx3 proteins was then tested for DNA binding, transcriptional activity, and co-factor binding.

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cells transfected with vectors for the mutant Lhx3 proteins demonstrated that the mutant proteins can also bind to the DNA element. Expression vectors for all of the Lhx3 mutants led to formation of a C1 complex which was disrupted by the Lhx3 antisera and was comparable in magnitude to the C1 complex for the wild type protein. The H115A mutation which presumably disrupts the zinc finger appeared to produce more C1 complex than was obtained with the wild type protein, consistent with other observations that the LIM domain can inhibit DNA binding (4, 9, 30). Analysis of the formation of the C2 complex by the mutants is somewhat complicated due to substantial variation in the amount of the NS complex in the various cell extracts. However, as indicated above, the C2 complex from cells transfected with wild type Lhx3 is disrupted by the Lhx3 antisera. None of the Lhx3 mutants produced a C2 complex which was sensitive to the Lhx3 antisera. A horseradish peroxidase-labeled anti-rabbit secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins.

Fig. 1. DNA binding activity of wild type and mutant Lhx3. A, extracts from 293 cells transfected with the indicated expression vectors were incubated with a 32P-labeled DNA probe containing the pituitary glycoprotein hormone basal element, a LIM factor binding site (7) from the mouse glycoprotein hormone α-subunit promoter. Protein-DNA complexes were resolved on a nondenaturing polyacrylamide gel, the gel dried and exposed to x-ray film. B, the same cell extracts used for the mobility shift in panel A were assayed for protein expression. The extracts were resolved by denaturing polyacrylamide gel electrophoresis, transferred to a membrane, and then incubated with a 1:10,000 dilution of Lhx3 antisera. A horseradish peroxidase-labeled anti-rabbit secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins.

could possibly participate in transcriptional activation. Therefore, their activity was further characterized.

LIM Point Mutations Decrease Transcriptional Activity of Lhx3—Previous studies have shown that Lhx3 can synergize with the pituitary specific transcription factor, Pit-1, to activate the prolactin promoter in heterologous cells (4, 9, 17). We used this assay to explore the transcriptional activity of the Lhx3 LIM domain mutants in HEK 293 cells (Fig. 2). As previously reported (4, 9), wild type Lhx3 weakly activated the prolactin promoter alone and showed strongly synergistic activation in the presence of Pit-1 (Fig. 2A). All three of the mutant Lhx3 proteins were found to synergize with Pit-1, although reporter activity was reduced somewhat as compared with wild type Lhx3. Immunoblot analysis demonstrated that the wild type and mutant Lhx3 proteins were expressed at approximately the same levels (Fig. 2B). These findings suggest that replacement of tyrosine 114 of Lhx3 with either cysteine or alanine modestly reduces the ability of Lhx3 to act in concert with Pit-1 to synergistically activate transcription.
increased basal reporter gene activity and supported a substantial response to transfection of the activated Ras. In contrast, GAL4 fused to any of the three mutant Lhx3 proteins did not support Ras responsiveness. Immunoblot analysis demonstrates that all the GAL4 fusion proteins are expressed at approximately the same levels (Fig. 3B). Thus, this assay using a glycoprotein hormone α-subunit reporter gene demonstrates that NLI can interact with a number of different LIM factor-binding sites. Previous studies have demonstrated that for Lmx1, a different LIM homolog of NLI, with the LIM homeodomain transcription factor, both LIM domains contribute to high affinity binding to NLI (16). In the case of Lhx3, the Y114C and Y114A mutations of Lhx3 greatly reduce the ability of the transcription factor to mediate transcriptional responses to the Ras/mitogen-activated protein kinase pathway.

Point Mutations in the LIM domain of Lhx3 Greatly Reduce Interaction with the Putative Co-activator, SLB—The Y114C Lhx3 mutation occurs in a domain that is known to be important for binding of several proteins including other transcription factors such as Pit-1 (4) as well as several putative co-activator proteins including NLI, MRG1, and SLB (10, 13, 14, 16, 17). The possible effects of the point mutations on interactions with the putative co-activators seemed particularly interesting. Of the three potential co-activators which might play a role in mediating transcriptional responses to Lhx3, NLI has been studied the most extensively. Several studies have demonstrated that NLI can interact with a number of different LIM factors and there is evidence that NLI may play a role in mediating transcriptional responses (13–16, 31). Perhaps the most compelling evidence for a role for NLI as a LIM factor mediating transcriptional responses to the Ras/mitogen-activated protein kinase pathway.

To begin to explore possible mechanisms mediating the decreased transcriptional activity of mutant Lhx3, we analyzed the binding of several factors to Lhx3. To directly assess the ability of SLB and NLI to bind Lhx3 we performed in vitro binding studies. Radiolabeled wild type or mutant Lhx3 were incubated with immobilized proteins consisting of GST fused to the LIM interacting domains of NLI or SLB, and the bound proteins were analyzed by denaturing gel electrophoresis (Fig. 4A). Wild type Lhx3 bound strongly to GST-NLI and GST-SLB. The Y114A and Y114C mutants bound GST-NLI quite well (PhosphorImager analysis of several experiments indicated 60% or greater as compared with wild type binding). However, the Y114A and Y114C mutants bound GST-SLB only marginally better than GST alone (PhosphorImager analysis indicated about 1–2% of wild type binding). The H115A Lhx3 mutant which disrupts the zinc finger did not bind appreciably to either GST-SLB or GST-NLI in this assay. We also analyzed binding of Lhx3 to Pit-1. For these binding experiments, Pit-1 containing a FLAG epitope at the amino terminus was produced in baculovirus. The epitope-tagged Pit-1 was then immobilized on resin containing a monoclonal antibody to the FLAG epitope. Radiolabeled wild type or mutant Lhx3 was incubated with the immobilized FLAG-Pit-1, and the bound proteins were analyzed by denaturing gel electrophoresis (Fig. 4B). Consistent with previous reports (4), a substantial amount of wild type Lhx3 was bound to interact with Pit-1. All three mutant Lhx3 proteins also bound Pit-1 at about 50 to 60% of wild type binding. As Bach et al. (4) demonstrated that each of the two LIM domains of Lhx3 can individually strongly interact with Pit-1, it is perhaps not surprising that a point mutation in one of the two LIM domains reduces binding to Pit-1 by only about 50%. Overall, the in vitro binding data provide evidence that the Lhx3 Y114C and Y114A mutations which greatly reduce transcriptional responses to Ras, essentially abrogate binding to SLB, with more modest effects on binding to NLI and Pit-1. We also used a mammalian version of the two-hybrid assay to further test for the apparent interaction of Lhx3 with NLI and SLB in intact cells (Fig. 5). HEK 293 cells were transfected with a GAL4-dependent reporter gene and expression vectors for a GAL4-Lhx3 LIM domain fusion and a fusion of NLI or SLB to the strong transcriptional activation domain of VP16. As expected, transfection of NLI-VP16 or SLB-VP16 with the wild type GAL4-Lhx3 LIM fusion resulted in strong stimulation of reporter gene activity suggesting substantial interaction of wild type Lhx3 with both NLI and SLB. Similar to the in vitro binding studies, the Y114C and Y114A mutations of Lhx3 modestly reduced the apparent interaction with NLI, but abrogated apparent binding to SLB. Disruption of the zinc finger structure by replacement of histidine 115 with alanine, abrogates apparent binding to both NLI and SLB. The mammalian two-hybrid assay offers further evidence confirming that replacement of tyrosine 114 of Lhx3 with either cysteine or alanine disrupts in vivo interaction with SLB with rather modest effects on binding to NLI.

Previous studies have demonstrated that for Lmx1, a different LIM homeodomain transcription factor, both LIM domains contribute to high affinity binding to NLI (16). In the case of
Lhx3 were prepared by cell-free translation in the presence of [35S]methionine and then incubated with GST, GST-NLI, GST-SLB, or GST-SLB1213–1265 (GST-SLB) fusion proteins immobilized on agarose beads. The agarose beads were washed and the eluted proteins analyzed by denaturing gel electrophoresis. The gel was dried and exposed to x-ray film. B, the experiment was performed as in panel A except Lhx3 proteins were incubated with FLAG epitope-tagged Pit-1 immobilized on agarose beads.

FIG. 4. Analysis of the interaction of NLI, SLB, and Pit-1 with wild type and mutant Lhx3 in vitro. A, radiolabeled wild type and mutant Lhx3 were prepared by a reporter construct containing five copies of a GAL4-binding site upstream of the minimal thymidine kinase luciferase reporter and expression vectors for the GAL4 DNA-binding domain alone (−) or the GAL4 DNA-binding domain fused to either wild type or mutant Lhx3 LIM domain as indicated. The cells were also transfected with an empty expression vector control or an expression vector for VP16 fused to either residues 295–375 of NLI (NLI-VP16) or residues 1213–1265 of SLB (SLB-VP16). The cells also received an expression vector for β-galactosidase driven by a cytomegalovirus promoter as an internal standard. The amount of expression vector was kept constant for all transfections by the inclusion of empty expression vector. Values were corrected for β-galactosidase activity and are the average ± S.E. of three independent transfections. B, a separate experiment 293 cells were transfected with the expression vectors for the GAL4 DNA-binding domain fusions used in panel A. Whole cell extracts were then prepared and proteins resolved by denaturing polyacrylamide gel electrophoresis, transferred to a membrane, and then incubated with a 1:10,000 dilution of the GAL4 monoclonal antibody. A horseradish peroxidase-labeled anti-mouse secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins.

FIG. 5. Analysis of the interaction of wild type and mutant Lhx3 with NLI and SLB in HEK 293 cells. A, HEK 293 cells were transfected with a reporter construct containing five copies of a GAL4-binding site upstream of the minimal thymidine kinase luciferase reporter and expression vectors for the GAL4 DNA-binding domain alone (−) or the GAL4 DNA-binding domain fused to either wild type or mutant Lhx3 LIM domain as indicated. The cells were also transfected with an empty expression vector control or an expression vector for VP16 fused to either residues 295–375 of NLI (NLI-VP16) or residues 1213–1265 of SLB (SLB-VP16). The cells also received an expression vector for β-galactosidase driven by a cytomegalovirus promoter as an internal standard. The amount of expression vector was kept constant for all transfections by the inclusion of empty expression vector. Values were corrected for β-galactosidase activity and are the average ± S.E. of three independent transfections. B, αT3-1 cells were transfected with a luciferase reporter gene containing the −507 to −205 region of the mouse glycoprotein hormone α-subunit gene in which the LIM factor-binding site was replaced with a GAL4-binding site (10). The cells also received expression vectors for the GAL4 DNA-binding domain alone (−) or the GAL4 DNA-binding domain fusion to either both LIM domains (LIM1,2), the first LIM domain (LIM1), or the second LIM domain (LIM2) of Lhx3 as indicated. The cells were also transfected with an empty expression vector control or an expression vector for VP16 fused to either residues 295–375 of NLI (NLI-VP16) or residues 1213–1265 of SLB (SLB-VP16). The cells also received an expression vector for β-galactosidase driven by a cytomegalovirus promoter as an internal standard. The amount of expression vector was kept constant for all transfections by the inclusion of empty expression vector. Values were corrected for β-galactosidase activity and are the average ± S.E. of three independent transfections. C, αT3-1 cells were transfected with the expression vectors for the GAL4 DNA-binding domain alone (−) or the GAL4 DNA-binding domain fusion to either both LIM domains (LIM1,2), the first LIM domain (LIM1), or the second LIM domain (LIM2) of Lhx3 as indicated. The cells were transfected with an empty expression vector control or an expression vector for constitutively active Ras and all cells also received an expression vector for β-galactosidase driven by a cytomegalovirus promoter as an internal standard. The amount of expression vector was kept constant for all transfections by the inclusion of empty expression vector. Values were corrected for β-galactosidase activity and are the average ± S.E. of three independent transfections. A separate experiment αT3-1 cells were transfected with the expression vectors for the GAL4 DNA-binding domain fusions used in panels A and B. Whole cell extracts were then prepared and proteins resolved by denaturing polyacrylamide gel electrophoresis, transferred to a membrane, and then incubated with a 1:10,000 dilution of the GAL4 monoclonal antibody. A horseradish peroxidase-labeled anti-mouse secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins.

FIG. 6. Comparison of the ability of individual LIM domains of Lhx3 to bind to NLI and SLB and to support Ras responsiveness. A, 293 cells were transfected with a reporter construct containing five copies of a GAL4-binding site upstream of the minimal thymidine kinase luciferase reporter and expression vectors for the GAL4 DNA-binding domain alone or the GAL4 DNA-binding domain fused to either both LIM domains (LIM1,2), the first LIM domain (LIM1), or the second LIM domain (LIM2) of Lhx3 as indicated. The cells were also transfected with an empty expression vector control or an expression vector for VP16 fused to either residues 295–375 of NLI (NLI-VP16) or residues 1213–1265 of SLB (SLB-VP16). The cells also received an expression vector for β-galactosidase driven by a cytomegalovirus promoter as an internal standard. The amount of expression vector was kept constant for all transfections by the inclusion of empty expression vector. Values were corrected for β-galactosidase activity and are the average ± S.E. of three independent transfections. B, αT3-1 cells were transfected with a luciferase reporter gene containing the −507 to −205 region of the mouse glycoprotein hormone α-subunit gene in which the LIM factor-binding site was replaced with a GAL4-binding site (10). The cells also received expression vectors for the GAL4 DNA-binding domain alone (−) or the GAL4 DNA-binding domain fusion to either both LIM domains (LIM1,2), the first LIM domain (LIM1), or the second LIM domain (LIM2) of Lhx3 as indicated. The cells were transfected with an empty expression vector control or an expression vector for constitutively active Ras and all cells also received an expression vector for β-galactosidase driven by a cytomegalovirus promoter as an internal standard. The amount of expression vector was kept constant for all transfections by the inclusion of empty expression vector. Values were corrected for β-galactosidase activity and are the average ± S.E. of three independent transfections. C, αT3-1 cells were transfected with the expression vectors for the GAL4 DNA-binding domain alone (−) or the GAL4 DNA-binding domain fusion to either both LIM domains (LIM1,2), the first LIM domain (LIM1), or the second LIM domain (LIM2) of Lhx3 as indicated. The cells were transfected with an empty expression vector control or an expression vector for constitutively active Ras and all cells also received an expression vector for β-galactosidase driven by a cytomegalovirus promoter as an internal standard. The amount of expression vector was kept constant for all transfections by the inclusion of empty expression vector. Values were corrected for β-galactosidase activity and are the average ± S.E. of three independent transfections. A separate experiment αT3-1 cells were transfected with the expression vectors for the GAL4 DNA-binding domain fusions used in panels A and B. Whole cell extracts were then prepared and proteins resolved by denaturing polyacrylamide gel electrophoresis, transferred to a membrane, and then incubated with a 1:10,000 dilution of the GAL4 monoclonal antibody. A horseradish peroxidase-labeled anti-mouse secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins.
binding to the Lhx3 constructs and the ability of the constructs to support a Ras response when directed to the appropriate context in the glycoprotein hormone α-subunit gene.

We also analyzed the in vivo binding of Lhx3 to SLB using a co-immunoprecipitation assay (Fig. 7). Expression vectors encoding FLAG epitope-tagged wild type or mutant Lhx3 were transfected into HEK 293 cells with an expression vector encoding an AU1-tagged fragment of SLB that contains the LIM domain (17). We used this fragment of SLB as overexpression of the full-length protein appears to be toxic to many tissue culture cells (17). The FLAG antibody did not co-immunoprecipitate SLB in the absence of FLAG-tagged wild type and mutant Lhx3 as indicated. The total amount of expression vector was kept constant by the inclusion of empty expression vector. Whole cell extracts were prepared and then either directly electrophoresed on a denaturing gel (Input) or immunoprecipitated with a mouse anti-FLAG monoclonal antibody (Co-IP) and then resolved by denaturing gel electrophoresis. The separated proteins were transferred to a membrane and then incubated with a 1:5000 dilution of either Lhx3 antiserum (Input) or SLB antiserum (SLBCOOH). A horseradish peroxidase-labeled secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins.

We were particularly interested to examine the effects of the Y114C substitution on the interaction of Lhx3 with NLI. Several laboratories have shown that NLI can bind to a number of LIM homeodomain transcription factors including Lhx3 (13–15, 31). Genetic experiments have offered evidence that CHIP, the Drosophila homolog of NLI, plays a role in transcriptional responses to LIM homeodomain transcription factors (32–34). In view of the evidence supporting a functional role for the interaction of NLI with LIM transcription factors, it seemed quite possible that the Y114C mutation would disrupt the interaction of Lhx3 and NLI. Although we found that the Y114C mutation reduced the interaction of Lhx3 with NLI, the effects were rather modest, decreasing binding to about half of the binding achieved by wild type Lhx3. It seems somewhat unlikely that this modest change in binding to NLI accounts for the major change in the ability of Y114C Lhx3 to support Ras responsiveness of a glycoprotein α-subunit reporter gene. These findings suggest that although the binding of NLI to

![Co-immunoprecipitation of Lhx3 with SLB1213–1749](image)

**FIG. 7. Co-immunoprecipitation of Lhx3 with SLB1213–1749.** Cultured HEK 293 cells were transfected with expression vectors for SLB1213–1749 (SLBCOOH), Lhx3, or FLAG-tagged wild type and mutant Lhx3 as indicated. The total amount of expression vector was kept constant by the inclusion of empty expression vector. Whole cell extracts were prepared and then either directly electrophoresed on a denaturing gel (Input) or immunoprecipitated with a mouse anti-FLAG monoclonal antibody (Co-IP) and then resolved by denaturing gel electrophoresis. The separated proteins were transferred to a membrane and then incubated with a 1:5000 dilution of either Lhx3 antiserum (Input) or SLB antiserum (SLBCOOH). A horseradish peroxidase-labeled secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins.

**DISCUSSION**

These studies provide insights into mechanisms mediating the effects of a specific point mutation in Lhx3 on pituitary hormone gene expression. The findings provide evidence that replacement of tyrosine 114 with a cysteine in the second LIM domain of Lhx3 reduces transcriptional activity. In one assay, the ability of the Lhx3 mutant to synergize with the pituitary specific factor, Pit-1, to activate the prolactin promoter was reduced. In another assay, the mutation substantially reduced the ability of Lhx3 to contribute to Ras responsiveness of the gonadotropin α-subunit promoter. Protein-protein interaction studies demonstrated that the Y114C LIM mutation essentially abrogates binding of Lhx3 to the putative co-activator/adapter protein, SLB. These changes in protein-protein interaction provide a possible mechanism mediating the reduced transcriptional activity. As Lhx3 has been shown to be capable of stimulating the promoters for a number of pituitary hormone genes (4), a decrease in the transcriptional activity of Lhx3 would presumably lead to decreased expression of several pituitary hormone genes leading to hormone deficiency. Lhx3 also has effects on the development of a number of pituitary cell lineages (18, 19). Thus, it is also possible that the decreased transcriptional activity of LHX3 may have effects on development of specific cells in the pituitary and the lack of these cells contributes to the hormone deficiency syndrome.

Although the Y114C Lhx3 mutant is clearly capable of binding to DNA, a qualitative difference in DNA binding by the wild type and mutant proteins was detected by mobility shift assay. Previous studies have provided evidence that the LIM domain may inhibit DNA binding activity as removal of the LIM domain enhances DNA binding (4, 9, 30). If the Y114C mutation acted to inhibit the function of the LIM domain, then it might be expected to enhance DNA binding activity, similar to the results obtained by deleting the LIM domain. Although the mobility shift studies did not reveal substantial increases in DNA binding activity of the mutant Lhx3, there was a difference in the nature of the complexes formed. The wild type protein produced two major complexes while the mutant protein yielded predominantly the faster migrating complex. This assay involved use of extracts from cells transfected with expression vectors for wild type or mutant Lhx3. Thus, the binding activity may represent Lhx3 plus other endogenous proteins. The nature of the components which make up the two complexes and the possible functional significance of the two complexes is not clear at this time and will require further study.

The studies of the interaction of the mutant Lhx3 with other proteins may provide insights into the mechanisms mediating the transcriptional activity of Lhx3. The Y114C mutation appeared to somewhat reduce the interaction of Lhx3 with Pit-1 and NLI. Although the effects of the mutation on the binding of Lhx3 to Pit-1 and NLI were rather modest, these effects may play a functional role. For instance, the reduced ability of the Lhx3 Y114C mutant to bind to Pit-1 was accompanied by a reduced ability of the mutant Lhx3 to synergize with Pit-1 to activate the prolactin promoter. One mechanism that might account for this observation would involve changes in cooperative DNA binding by Lhx3 and Pit-1. However, previous studies have failed to demonstrate cooperative DNA binding by Lhx3 and Pit-1 using a variety of different DNA-binding sites (4). Thus, it is perhaps more likely that the decreased ability of Lhx3 Y114C to bind to Pit-1 leads to decreases in the subsequent recruitment of co-activators or general transcription factors.
Lhx3 may be important, in at least some contexts the interaction probably is not sufficient for transcriptional activation. This conclusion would be consistent with previous studies demonstrating that forced recruitment of GAL4-NLI to the α-subunit reporter gene does not lead to transcriptional activation (10).

The most dramatic effect of the Y114C mutation on protein interactions involved essentially eliminating binding of Lhx3 to SLB. Interestingly, the Y114C mutation also blocked the ability of a GAL4-Lhx3 fusion to support Ras-stimulated activation of the glycoprotein hormone α-subunit promoter. This correlation is consistent with a possible involvement of SLB playing some role in mediating Ras responsiveness of specific promoters. Further studies will be required to determine if Ras activation leads to changes in the phosphorylation of Lhx3 or SLB or changes in the interaction of these factors with other transcription factors, co-activators, or adapter proteins.

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