Impairment of SHOX nuclear localization as a cause for Léri-Weill syndrome

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Accepted 12 February 2004
Journal of Cell Science 117, 3041-3048 Published by The Company of Biologists 2004
doi:10.1242/jcs.01152

Summary

We report the characterization of the nuclear localization signal (NLS) of the short stature homeobox gene *SHOX*. Mutations within the *SHOX* gene cause Léri-Weill dyschondrosteosis (LWD) and Langer mesomelic dysplasia (LD) as well as idiopathic short stature (ISS). Furthermore, haploinsufficiency of *SHOX* has also been implicated in Turner syndrome. SHOX has been shown to be a cell-type-specific transcriptional activator that localizes to the nucleus. The SHOX protein contains a central homeodomain that together with its transactivation domain regulates the transcription of its target sequences within the nucleus. The sequences for its nuclear localization have not been identified yet. Experimental characterization of SHOX-NLS by deletion mapping identified a non-classic type basic signal, AKCRK, in the recognition helix of the homeodomain. Fusion of this stretch of five amino acids to a cytoplasmic reporter protein resulted in its nuclear translocation. Functional analysis of a missense mutation R173C (C517T) affecting the identified SHOX-NLS in two families with LWS and LD showed that the mutated SHOX protein is unable to enter the nucleus. Conversely, we can demonstrate that insertion of the identified signal adjacent to the mutant site can restore its nuclear translocation. These results establish impairment of nuclear localization as a mechanistic basis for *SHOX*-related diseases.

Key words: Nuclear localization signal (NLS), Short stature homeobox gene (*SHOX*), Léri-Weill dyschondrosteosis (LWS), Red fluorescent protein (RFP)

Introduction

The nuclear membrane divides the cell into nuclear and cytoplasmic compartments. Across this membrane, a continuous and regulated transport of molecules is required. Proteins with predominantly nuclear functions are translocated to the nucleus after their translation in the cytoplasm in a highly organized way. This translocation occurs via the nuclear pore complex, consisting of nucleoporins and is mediated by an active and selective mechanism controlled by saturable transport receptors/carriers. The carrier proteins (kaps) are members of the Importin β/Karyopherin β family, which is divided into importins and exportins. Some commonly found importins are importin α, importin β and transportin1, kap123 and kap121. Exportin1 (also called Crm1 or Xpo1) was the first export carrier identified. Other related exportins are exportin 4, Tap-1/Mex67 and calreticulin. The karyopherins recognize the corresponding cis-acting transport signals, known as nuclear localization signals (NLSs) on the proteins to be imported or nuclear export signals on the proteins to be exported (Mattaj and Englmeier, 1998; Macara, 2001). The best characterized nuclear import signals are the monopartite and the bipartite signals. PKKKRKV of the simian virus 40 large T-antigen represents a classic type monopartite, and KRPAATKKAGQAKKKK of the cellular nucleoplasmin protein a bipartite signal (Kalderon et al., 1984; Robbins et al., 1991). Many import sequences are recognized by a heterodimeric receptor composed of importin α and β. Importin α works as an adaptor between the protein carrying a NLS and importin β which is recognized by the nuclear pore complex. The entire heterotrimer is then translocated into the nucleus in an energy-dependent manner catalyzed by the Ran-GTPase system. Besides these classic nuclear localization signals, a variety of nonclassic signals have also been recognized in many viral and cellular proteins including homeodomain proteins such as PDX1 and Nkx2.2 (Hessabi et al., 1999; Hessabi et al., 2000; Moede et al., 1999; Parker et al., 2000; Wang et al., 2002; Lischka et al., 2003). Impairment of the nuclear localization of proteins caused by either mutations in their NLS or the disruption of the transportation machinery itself has emerged as a mechanism leading to disease phenotypes such as Holt-Oram syndrome, tricho-rhino-phalangeal syndrome (TRPS) and Saethre-Chotzen syndrome (Ghouzzi et al., 2000; Fan et al., 2002; Kaiser et al., 2004).

The *SHOX* gene encodes a cell-type-specific transcriptional activator that has been demonstrated to reside in the nucleus of a variety of different cell types (Rao et al., 2001). The heterozygous loss of *SHOX* function by deletions or other mutations has been shown to cause Léri-Weill dyschondrosteosis while its homozygous loss results in Langer mesomelic dysplasia (Rao et al., 1997; Belin et al., 1998; Shears et al., 1998). In addition, *SHOX* haploinsufficiency has also been demonstrated in patients with idiopathic short stature and Turner syndrome (Clement-Jones et al., 2000; Schiller et al., 2000; Rappold et al., 2002). Impairment of the SHOX nuclear translocation caused by mutations within its NLS is expected to interfere with its inherent function and therefore may provide a molecular explanation for the phenotype, e.g. in Léri-Weill patients.

Since SHOX does not contain a classic NLS, we have carried out deletion mapping of *SHOX* cDNA and were able to narrow down the localization signal to a stretch of five amino acids within the recognition helix of the homeodomain. A missense mutation R173C (C517T) within this NLS occurring in patients with Léri-Weill dyschondrosteosis and Langer mesomelic dysplasia was analyzed in detail. We can show that this mutated SHOX protein does not translocate to the nucleus. These data elucidate the disruption of the SHOX-NLS as a cause for Léri-Weill dyschondrosteosis and Langer mesomelic dysplasia.

Materials and Methods

Plasmid construction and site directed mutagenesis

Various *SHOX* deletion constructs in the vector pcDNA4/HisMax (Invitrogen) were generated by subcloning from full-length *SHOX* cDNA in pBluescript SK(+) (Stratagene) named as pBSK-*SHOX* (Rao et al., 2001). The homeobox fragment was generated by PCR using the primers HDFor/*Bam*HI+ HDRev/*Eco*RI and pBSK-SHOX as the template DNA. The double-digested and purified PCR product was cloned in an equivalently digested pcDNA4/HisMax.

For the fusion constructs, the red fluorescent protein/RedStar was first amplified by PCR using the primers FP/RedStar/*Xho*I+ RP/RedStar/*Xba*I and the plasmid p415Gal1RedStar (Knop et al., 2002) as the template DNA and this PCR product was cloned in pcDNA4/HisMax. The selected clone was called pcDNA4/HisMax/RedStar. RedStar is a mutant red fluorescent protein (RFP) and an improved version of drFP583 also termed DsRed cloned from corallimorpharian *Discosoma*. Wild-type drFP583 has several drawbacks, including inefficient folding of the proteins, slow maturation of the chromophore, and tetramerization even in dilute solutions. We have chosen to use RedStar as a reporter protein because it is a truly cytoplasmic protein unlike the conventionally used green fluorescent protein (GFP). Three RedStar fusion constructs containing either the homeodomain (aa 117-176), the first half of the homeodomain (aa 117-146) or the second half of the homeodomain (aa 147-176) fused to the N terminus of RedStar were generated by PCR-based cloning using the primer combinations HDFor/*Bam*HI+HDRev/*Xho*I, HDFor/*Bam*HI+HDMRev/*Xho*I and HDMFor/*Bam*HI+HDRev/*Xho*I, respectively. Additional fusion constructs with RedStar called HDLF (homeodomain last fragment, aa 161-176), 7AAs (aa 168-174), 6AAs (aa 169-174), 5AAsL (aa 169-173), 5AAsR (170-174) and 4AAs (aa 170-173) were generated using the corresponding forward and reverse oligos with *Bam*HI and *Xho*I sites, respectively. The oligos were annealed together by cooling slowly after denaturing at 95°C for 5 minutes. The annealed oligos were ligated to the N terminus of the RedStar.

Wild-type *SHOX* in pcDNA4/TO was subcloned from pBSK-*SHOX* and was named as pcDNA4/TO/*SHOX*. The *SHOX* mutant C517T was first generated in pGEX using the primer C517T Quick-Change with the Quick-Change Multi Site-directed Mutagenesis Kit (Stratagene) following the instructions from the manufacturer. The mutant was subcloned into pcDNA4/TO from pGEX-4T1 and was called pcDNA4/TO/*SHOX*/C517T.

Deletion of NLS from the wild-type *SHOX* and the insertion of NLS in the mutant *SHOX* C517T were done by PCR-based cloning using the primer combination SHOXHDFor*Xho*I+ DelNLSRev*Nsi*I and SHOXHDFor*Xho*I+ NLSMidRev*Nsi*I with pBSK-SHOX and pcDNA4/TO/SHOX/C517T as the template respectively. The *Xho*I-*Nsi*I fragments were first cloned in pBSK-*SHOX* and from here the *Xho*I-*Xba*I fragments were further subcloned in pcDNA4/TO/*SHOX*. The final clones were called pcDNA4/TO/*SHOX*/WT-NLS and pcDNA4/TO/*SHOX*/C517T+NLS, respectively.

Please refer to the primer chart (Table 1) for all the details concerning the primers.

Cell culture and transfections

Osteosarcoma cells U2Os-T-Rex (Rao et al., 2001) and human embryonic kidney cells HEK293 were grown on coverslips in Dulbecco's minimum essential medium (DMEM) with 10% fetal calf serum. Transient expression of different constructs was achieved by transfecting the cells using FuGENE reagent (Roche) following the manufacturer's instructions.

Interspecies heterokaryon assay

Interspecies heterokaryon assay was performed as previously described (Chestukhin et al., 2002). The stable cell line F6 expressing wild-type SHOX in a doxycyclin-inducible manner (Rao et al., 2001) was grown on coverslips to a confluency of around 40%. Twelve hours post induction, mouse fibroblast cells NIH-3T3 were plated along with F6 cells at the same confluency and the cells were allowed to grow overnight. After 24 hours of SHOX induction, cells were washed twice with serum-free DMEM and incubated for 1-2 hours in the same medium containing cycloheximide (Sigma) at a concentration of 100 μM to block the protein synthesis. Fusion of cells was obtained using 100 μl of 50% polyethylene glycol (PEG) (Sigma). The cells were washed twice with DMEM containing cycloheximide and were finally incubated in a medium containing 10% fetal calf serum in the presence of 100 μM cycloheximide. The cells were processed for immunohistochemistry as described below. The M9 domain of human hnRNP A1 protein (Michael et. al., 1995; Michael, 2000) fused to GFP was used as a positive control for the heterokaryon assay (data not shown).

Immunohistochemistry and microscopic analysis

For various *SHOX* deletion constructs in the vector pcDNA4/HisMax, cells were grown on coverslips and fixed in 3.7% paraformaldehyde/phosphate-buffered saline (PBS) for 15 minutes at room temperature after 48 hours of transfection. After blocking in PBS with 0.2% Tween 20 and 10% milk powder at room temperature for 1 hour, the coverslips were incubated at room temperature for 1 hour with mouse anti-His primary antibody (Sigma) diluted 1:200 in blocking reagent. The slides were washed five times with PBST (1× PBS with 0.2% Tween 20) followed by incubation at room temperature for 1 hour in a humidified dark chamber with FITC-conjugated goat anti-mouse secondary antibody (Sigma) diluted 1:500 in blocking solution. After washing three times with PBST, cells were counterstained with 0.2 μg/ml of bis benzimide Hoechst 33258 dye (Sigma) for 10 minutes at room temperature, washed again and mounted in DABCO. Cells transfected with RedStar fusion constructs were fixed and counterstained with Hoechst dye after 12-24 hours of transfection and analyzed under the microscope. Cells transfected with pcDNA4/TO constructs were induced after 4-6 hours of transfection using doxycyclin at a concentration of 4 μg/ml of culture medium and were immunostained after 36-48 hours of induction, as before, using rabbit anti-SHOX (Rao et al., 2001) and FITC-conjugated goat anti-rabbit antibodies (Sigma).

Slides were analyzed using a Carl Zeiss Axiophot microscope (Germany) at 40× magnification with the Isis3 software package and later all the results were confirmed by confocal microscopy.

Protein expression, subcellular fractionation and western blot analysis

U2Os-T-Rex cells were grown in 135 mm tissue culture dishes to a confluency of 30-40% and were transfected using FuGENE reagent. Transfected cells with pcDNA4/TO constructs were first induced with doxycyclin after 4-6 hours of transfection and harvested after 36-48 hours.

For subcellular fractionation, cells were centrifuged at 836 ***g*** for 5 minutes at 4°C and the pellet was washed with ice cold PBS twice,

Table 1. Primers used for cloning and site-directed mutagenesis

Name of the primers	Primer sequence (5′ to 3′)	Purpose
HDFor/*Bam*HI	A AAA GGA TCC CAG AGG CGC AGC CGC ACC	PC
HDRev/*Eco*RI	A AAA GAA TCC TTG TTT GCG GCA CTC	PC
FP/RedStar/*Xho*I	A AAA CTC GAG ATG AGT AGA TCT TCT	PC
RP/RedStar/*Xba*I	A AAA TCT AGA TTA CAA GAA CAA GTG	PC
HDRev/*Xho*I	A AAA CTC GAG TTG TTT GCG GCA CTC	PC
HDMRev/*Xho*I	A AAA CTC GAG CAT GAA GGC GTC GGG	PC
HDMFor/*Bam*HI	A AAA GGA TCC CGC GAG GAG CTC AGC	PC
HDLFFor/*Bam*HI	GA TCC GTG CAG GTT TGG TTC CAG AAC CGG AGA GCC AAG TGG CGC AAA CAA GAG C	OC
HDLFRev/*Xho*I	TC GAG CTC TTG TTT GCC CCA CTT GGC TCT CCG GTT CTG GAA CCA AAC CTG CAC G	OC
7AAsFor/*Bam*HI	GA TCC CGG AGA GCC AAG TGG CGC AAA C	OC
7AAsRev/*Xho*I	TC GAG TTT GCG CCA CTT GGC TCT CCG G	OC
6AAsFor/*Bam*HI	GA TCC AGA GCC AAG TGG CGC AAA C	OC
6AAsRev/*Xho*I	TC GAG TTT GCG CCA CTT GGC TCT G	OC
5AAsLFor/*Bam*HI	GA TCC AGA GCC AAG TGG CGC C	OC
5AAsLRev/*Xho*I	TC GAG GCG CCA CTT GGC TCT G	OC
5AAsRFor/*Bam*HI	GA TCC GCC AAG TGG CGC AAA C	OC
5AAsRRev/*Xho*I	TC GAG TTT GCG CCA CTT GGC G	OC
4AAsFor/*Bam*HI	GA TCC GCC AAG TGG CGC C	OC
4AAsRev/*Xho*I	TC GAG GCG CCA CTT GGC G	OC
C517TQuickChange	P-GAT TCT CTT GTT TGC AGC ACT TGG CTC TCC GG	SDM
SHOXHDFor*Xho*I	A AAA CTC GAG CGA CTC TTC GAC GAG ACC	PC
DelNLSRev*Nsi*I	A AAA ATG CAT TCT CCG GTT CTG GAA CCA AAC	PC
NLSMidRev*Nsi*I	A AAA ATG CAT TTT GCG GCA CTT GGC CTG ATT CTC TTG TTT	PC

PC, PCR based cloning; OC, cloning by oligo annealing; SDM, site-directed mutagenesis.
Restriction sites introduced are underlined.

resuspended in 300 μl of hypotonic buffer A (10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.05% NP40, 1 mM DTT and 0.1 mM EDTA; protease inhibitors from Roche) and after vortexing were incubated on ice for 20 minutes. The lysed cells were centrifuged at 5000 rpm for 5 minutes at 4°C and the supernatant was taken as the cytoplasmic extract. The remaining pellet was washed with ice cold PBS, resuspended in 200 μl of hypotonic buffer B (hypotonic buffer A with 0.4 M KCl) and incubated on ice for 40 minutes followed by centrifugation at 2324 ***g***. The supernatant contained the nuclear extract and the pellet the cell debris.

Protein amounts were estimated using the BCA Protein Estimation kit (Pierce). 20 μg of protein fractions were separated on a 12% polyacrylamide gel and blotted on a Hybond P membrane (Amersham Biosciences). After blocking, membranes were incubated with rabbit anti-SHOX primary antibody at a dilution of 1:3000 (Rao et al., 2001). As cytoplasmic and nuclear controls, mouse anti-M2PK (DF4) (pyruvate kinase isozyme M2) (Schebo) (Tani et al., 1988) and mouse anti-C23/nucleolin (Santa Cruz) (Lischew et al., 1981) were used at a dilution of 1:1000 and 1:300, respectively. After washing, the respective HRP-conjugated goat anti-rabbit and goat anti-mouse (Sigma) IgG, at a dilution of 1:10,000, was used as the secondary antibody. Detection of the bound antibody was performed using the ECL Detection Kit (Amersham Biosciences).

Results

The SHOX homeodomain contains a nuclear localization signal

We had previously described that SHOX acts as a transcriptional activator (Rao et al., 2001). Since predictions using the PSORT II server (http://psort.nibb.ac.jp) did not reveal a classic nuclear localization signal within the SHOX protein, we have experimentally analyzed nuclear translocation using several deletion constructs along with the full length *SHOX* (Fig. 1A, construct 1). Initially four deletion constructs of the protein were established. These deletion constructs covering amino acids 1-84 (N-terminal ATG-*Eco*RI fragment), 85-216 (middle *Eco*RI-*Pst*I fragment), 217-292 (C-terminal *Pst*I-TGA fragment) and the *SHOX* homeodomain from amino acids 117-176 (Fig. 1A, constructs 2-5). After transient transfections into U2Os cells, we analyzed the subcellular localization of these constructs after 36-48 hours of transfection by indirect immunofluorescence using anti-His antibodies. Only constructs 1, 3 and 5, all encompassing the homeodomain were found to be located in the nucleus indicating that the homeodomain of the protein contains the signal for proper nuclear localization (Fig. 1B).

A stretch of five amino acids within the recognition helix of the SHOX homeodomain functions as a nonclassic nuclear translocation signal

Since the SHOX protein is potentially diffusible and detection of small portions of the full length protein or the homeodomain in the nucleus may be the result of passive diffusion and/or retention, we generated reporter constructs of the SHOX homeodomain fused N-terminally to RedStar. The homeodomain fusion protein was detected only in the nucleus showing a discrete and non-diffusible signal while the N terminus of SHOX fused to RedStar (negative control) was truly cytoplasmic, excluding the possibility of diffusion or retention for the full length protein. To narrow down the nuclear localization signal, we further dissected the SHOX homeodomain into two halves from amino acids 117-146 and 147-176 and fused them N-terminally to the reporter protein RedStar (Fig. 2A, constructs 4, 5). The N-terminal amino acids 1-84 of the SHOX protein and the complete homeodomain (amino acids 117-176) were fused to RedStar as negative and positive controls, respectively (Fig. 2A, constructs 2, 3). After transient transfections of fusion cDNAs into U2Os cells, we

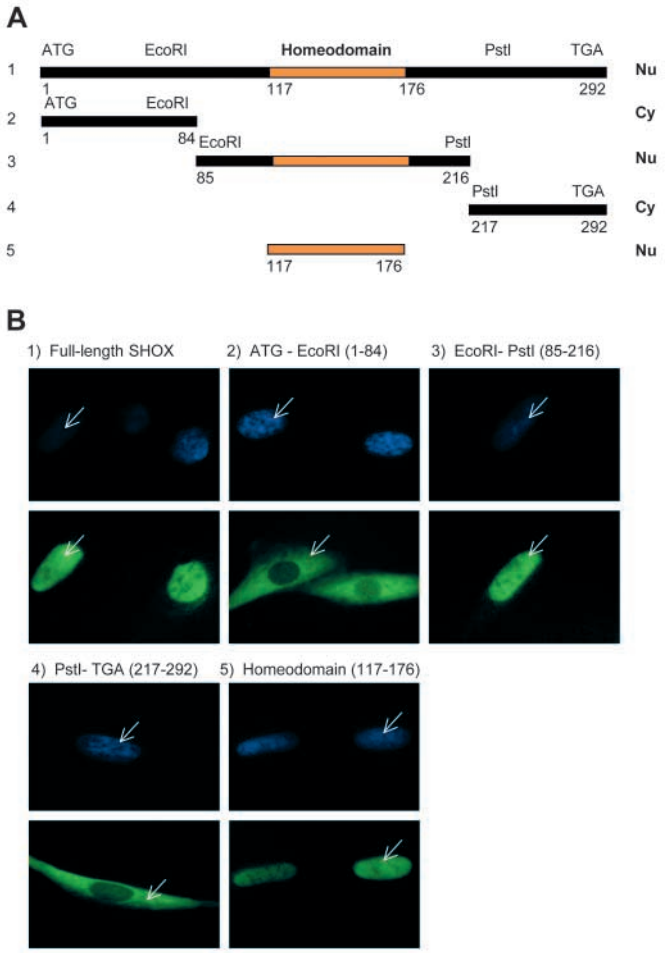

Fig. 1. Deletion constructs of SHOX cDNA and their subcellular localization. (A) Schematic representation of deletion constructs generated in pcDNA4/HisMax and their subcellular localization. Nu, nuclear; Cy, cytoplasmic. (B) Fluorescent images of immunostained slides. The first and third rows of images show the cell nuclei stained with Hoechst dye (blue) and the second and fourth rows of images show green staining for the SHOX deletion constructs immunostained with anti-His (green) antibodies. Note that all the constructs containing the homeodomain are localized in the nucleus. Arrows indicate subcellular localization of the SHOX deletion constructs (green) and the nuclei (blue) in corresponding cells.

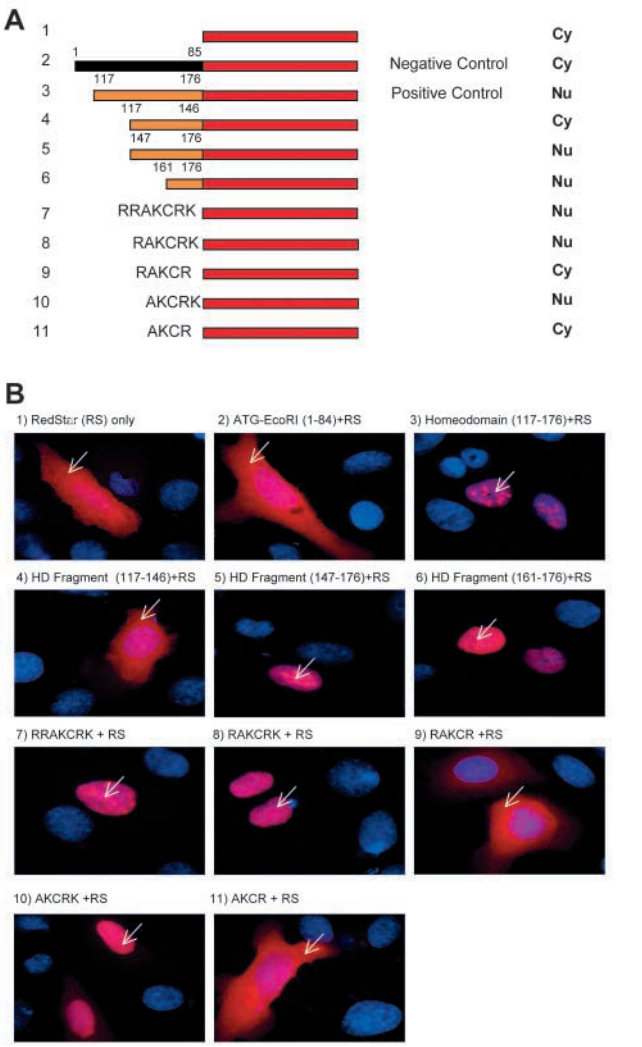

Fig. 2. Deletion fusion constructs and their subcellular translocation. (A) Schematic representation of the deletion fusion constructs of the SHOX homeodomain with RedStar and their subcellular localization. Nu, nuclear; Cy, cytoplasmic. (B) Fluorescent images of cells transfected with the RedStar fusion constructs. All constructs containing the five amino acids AKCRK are localized in the nucleus (blue, Hoechst staining). Arrows indicate the subcellular localization of the RedStar deletion fusion constructs. Red/pink staining is for red fluorescent protein (RFP).

found the C-terminal half of the homeodomain to be located in the nucleus whereas the N-terminal portion resided in the cytoplasm, indicating the presence of a signal in the second half of the homeodomain. Further analysis demonstrated that the last 16 amino acids from 161 to 176 were sufficient for nuclear translocation (Fig. 2A, construct 6). This peptide contains a seven amino acids motif, RRAKCRK from 168 to 174, which is basic in nature and represents a good candidate for a putative NLS. To confirm this hypothesis and define the minimal peptide capable of conferring nuclear translocation, we established RedStar fusion constructs containing various parts of this peptide (Fig. 2A, constructs 7-11). All fusion constructs encompassing the five amino acids AKCRK (aa 170-174 of SHOX/aa 54-58 of the homeodomain) localized in the nucleus indicating that these five amino acids are required and sufficient to promote nuclear import of the protein (Fig. 2B). Similar results were also obtained using HEK293 cells (data not shown).

To confirm that the identified signal is really the NLS for SHOX, we deleted these five amino acids from the wild-type protein (Fig. 3A). By immunofluorescence, this mutant SHOX was found to reside in the cytoplasm (Fig. 3B) confirming the amino acids AKCRK as the NLS for SHOX.

The SHOX-NLS mutant R173C in LWD and LD patients is deficient in nuclear transport but can be restored by insertion of the wild-type NLS

After having defined the nuclear localization signal for SHOX, we searched the human *SHOX* mutation database (www.shox.uni-hd.de) (Niesler et al., 2002) for short stature

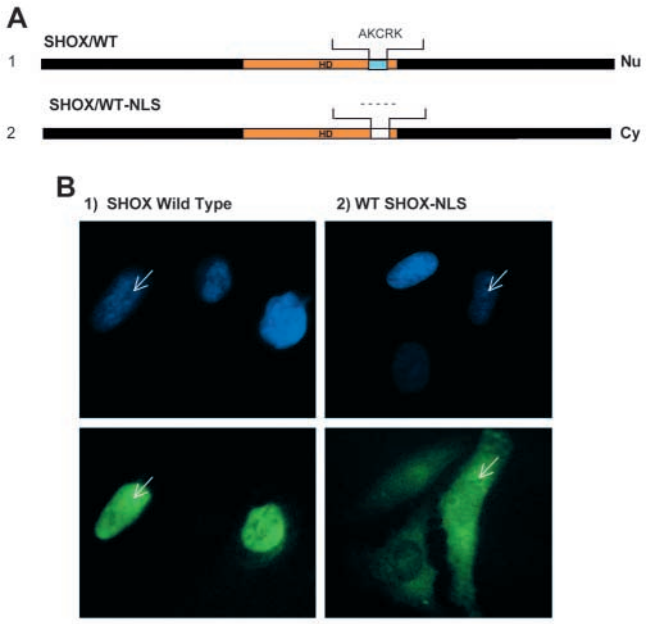

Fig. 3. Analysis of the wild-type SHOX and deletion construct SHOX-NLS and the respective subcellular localization. (A) Schematic representation of the wild-type SHOX and the deletion mutant SHOX-NLS where the NLS has been deleted from the wild-type SHOX. Nu, nuclear; Cy, cytoplasmic. (B) Fluorescent images of immunostained cells. The two upper panels show the cell nuclei stained with Hoechst dye (blue) and the two lower panel shows SHOX immunostaining with rabbit anti-SHOX antibodies (green). The constructs in pcDNA4/TO were overexpressed in U2Os cells. By contrast to the wild-type SHOX, which was found in the nucleus, deletion of the nuclear localization signal from the wild-type SHOX resulted in its cytoplasmic localization. Arrows indicate subcellular localization of the SHOX constructs (green) and the nuclei (blue) in corresponding cells.

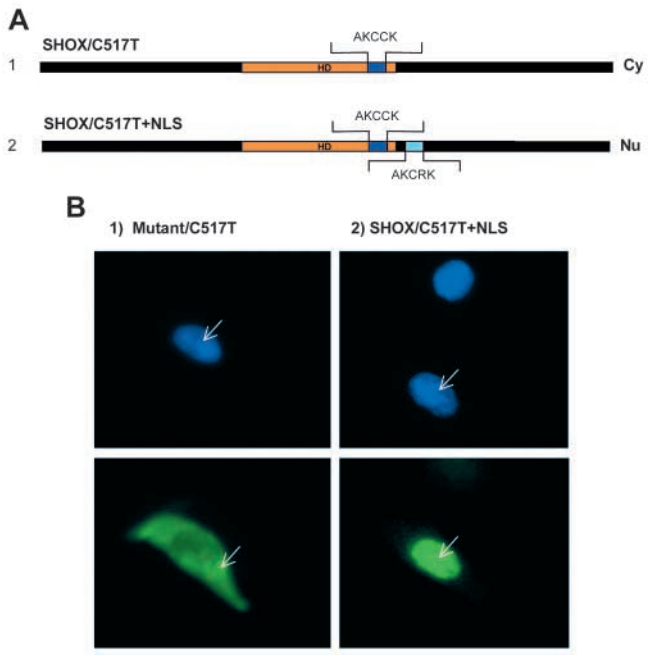

Fig. 4. Analysis of the mutant SHOX R173C (C517T) with the insertion construct SHOX/C517T+NLS and the respective subcellular localization. (A) Schematic representation of the missense mutant C517T with the mutation in the NLS, and the insertion mutant SHOX/C517T+NLS where the NLS has been inserted in the mutant SHOX C517T. Nu, nuclear; Cy, cytoplasmic. (B) Fluorescent images of immunostained cells. The two upper panels show the cell nuclei stained with Hoechst dye (blue) and the two lower panels show SHOX immunostaining with rabbit anti-SHOX antibodies (green) in corresponding cells. The constructs in pcDNA4/TO were overexpressed in U2Os cells. The missense mutation is within the nuclear localization signal resulting in its cytoplasmic localization (arrows in the two left panels). Insertion of the nuclear localization signal near the mutated site restored its nuclear localization activity (arrows in the two right panels).

patients with missense mutations in the identified NLS. Two families, one French (Huber et al., 2001) with Léri-Weill syndrome and a Spanish family with Langer mesomelic dysplasia were described with a missense mutation (R173C) (Shears et al., 2002). To investigate the subcellular location of this mutation, a *SHOX* construct harboring this point mutation was generated by site-directed mutagenesis and analyzed by immunofluorescence. By contrast to the wild-type SHOX, the SHOX construct with the point mutation R173C was retained in the cytoplasm (Fig. 4). Since the identified mutation resides within the recognition helix, we have also carried out EMSA experiments to examine the DNA-binding properties of this mutant. These experiments revealed that the SHOX mutant had a dramatic reduction in DNA-binding capacity as compared to the wild-type SHOX protein and hence would not trans-activate its targets (unpublished results). When we re-inserted the wild-type NLS adjacent to the mutated amino acid, the nuclear translocation capacity of the mutant SHOX protein was restored (Fig. 4).

To confirm the immunofluorescence results, we performed western blot analysis using subcellular fractions prepared from U2Os-T-Rex cells transiently transfected with wild-type and mutant SHOX constructs (Fig. 5). As controls for proper cytoplasmic and nuclear separation, we used pyruvate kinase isozyme M2 (M2PK) as a cytoplasmic and C23/nucleolin as a nuclear marker. Complementing the immunofluorescence results, wild-type and mutated SHOX with inserted NLS was found to be localized mainly in the nucleus whereas the missense mutant R173C and wild-type SHOX missing the AKCRK motif were detected in the cytoplasm.

To address the question of whether the mutant SHOX was cytoplasmic because of impaired import or increased export of the SHOX protein, we carried out interspecies heterokaryon assays using the stable human osteosarcoma cell line F6 expressing SHOX and the mouse fibroblast cells NIH-3T3. Analysis of more than 100 heterokaryons in two different sets of experiments did not reveal a single mouse nucleus positive for SHOX staining, indicating that SHOX does not shuttle between the nucleus and the cytoplasm (Fig. 6).

Discussion

Homeodomain proteins are transcriptional regulators. Their functional specificities are dictated by several levels of regulation including nuclear-cytoplasmic transport (Abate-Shen, 2002). The pseudoautosomal gene *SHOX* encodes a cell-type specific transcription factor of the paired-like homeodomain proteins (Rao et al., 2001). SHOX is a clinically

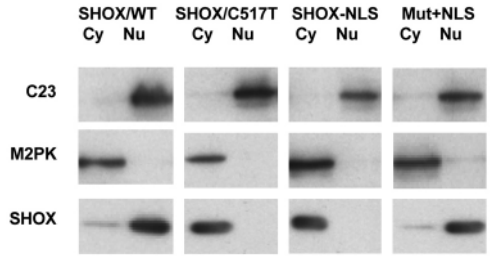

Fig. 5. Western blot analysis using cytoplasmic and nuclear extracts from U2Os cells transiently transfected with wild-type and mutant SHOX constructs. Immunofluorescence results using wild-type and mutant SHOX constructs were confirmed and complemented by western blots using cytoplasmic (Cy) and nuclear (Nu) extracts. Pyruvate kinase isozyme M2 (M2PK) at 57 kDa was used as a cytoplasmic marker and C23/nucleoloin at 116 kDa was used as a nuclear marker to detect the presence of SHOX (33 kDa) in both cytoplasmic or nuclear extracts.

important protein as its homozygous loss results in Langer mesomelic dysplasia while its haploinsufficiency has been shown to cause Léri-Weill dyschondrosteosis and is found in 2-3% of patients with idiopathic short stature. Additionally it has also been implicated in Turner syndrome (Rao et al., 1997; Belin et al., 1998; Shears et al., 1998; Clement-Jones et al., 2000; Schiller et al., 2000; Rappold et al., 2002; Binder et. al., 2003). Characterization of its functional domains is important for understanding the molecular mechanisms underlying its function. We have previously shown that the SHOX protein is translocated to the nucleus in a cell-type independent manner (Rao et al., 2001). In the light of these facts, we have investigated the molecular basis for the SHOX nuclear transport and identified the signal for its nuclear localization.

Since computational analysis of the SHOX sequence did not reveal any significant evidence for a classical NLS, we used several deletion constructs to identify the import signal within the homeodomain. Further analysis using expression constructs with different peptides fused to the RedStar, a red fluorescent reporter protein, identified five amino acids, AKCRK as the SHOX-NLS. This sequence is necessary and sufficient to promote nuclear translocation of the cytoplasmic reporter protein and its deletion from the full length wild-type SHOX resulted in its cytoplasmic localization. The motif AKCRK is basic in nature, a common feature shared by most of the NLSs identified. Two further basic amino acids, R168 and R169 reside next to the minimal stretch of five amino acids identified as the NLS. R169 is conserved in all the homeodomain proteins whereas the arginine at position 168 is conserved only in paired-like homeodomain proteins. We could experimentally show that they are not included in the minimal NLS, yet may be supporting the minimal signal by their basicity.

Interestingly, the identified SHOX-NLS resides within the helix III, also known as the recognition helix of the homeodomain involved in DNA binding. Database searches revealed that the recognition helix including the NLS motif exhibits extraordinary amino acid conservation among paired related homeodomain proteins. In particular, the amino acids A170 and R173 within the NLS are conserved throughout all paired-related homeodomain proteins (Fig. 7) (Banerjee-Basu and Baxevanis, 2001). Outside the family of homeodomain proteins, the identified NLS is not conserved. Missense mutations affecting this motif may therefore have structural or functional consequences. According to this idea, we could show that the mutation (R173C) drastically reduces the DNA-binding capacity of the SHOX protein in addition to its mis-localization. Also, the product of the closely related SHOX2 gene and its murine orthologue share the identical five amino acids motif, suggesting a comparable nuclear translocation mechanism.

Missense mutations within the *SHOX* gene have been identified to cause short stature, Léri-Weill and Langer syndrome. Searching the human *SHOX* mutation database (www.shox.uni-hd.de) (Niesler et al., 2002) revealed the existence of a missense mutation, R173C (C517T), within the identified NLS in a Léri-Weill and a Langer family from France and Spain, respectively (Huber et al., 2001; Shears et al., 2002). This mutation affects the highly conserved arginine residue at position 173. To investigate the functional consequences of this mutation, we have generated an expression construct carrying the observed R173C exchange. Immunofluorescence and western blot analysis of cells transfected with this construct clearly demonstrated that this mutation prohibits nuclear translocation of the SHOX protein. Transition of a positively charged amino acid arginine into a polar but uncharged amino acid cysteine is likely to disturb the charge-dependent recognition of the signal by the transportation machinery (Kalderon et al., 1984; Robbins et al., 1991; Mattaj and Englmeier, 1998; Macara, 2001). Restoration of nuclear transport of the mutant by insertion of the signal further proves that this signal is necessary and sufficient for the nuclear transport whereas the point mutation R173C is sufficient to

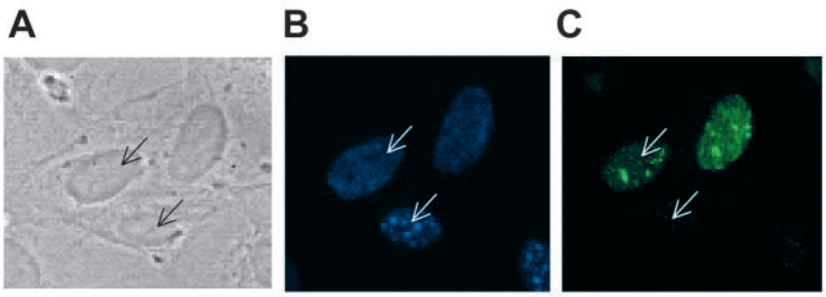

Fig. 6. Interspecies heterokaryon assay using the human osteosarcoma cell line F6, stably transfected with SHOX and the mouse fibroblast cell line NIH-3T3. After induction of SHOX expression, F6 cells were fused to NIH cells to make heterokaryons that were immunostained with anti-SHOX antibodies. (A) Phase contrast image of a representative heterokaryon with two larger F6 nuclei and one smaller NIH-3T3 nucleus (arrows). (B) Hoechst staining of the F6 and NIH-3T3 nuclei. Arrows indicate different nuclear staining for the F6 and NIH-3T3 nuclei that are shown in A. (C) Immunostaining using rabbit anti-SHOX antibodies (green). In more than 100 heterokaryons analyzed, not a single mouse nucleus showed positive staining for SHOX, indicating that once inside the nucleus, SHOX shows no shuttling between the nucleus and the cytoplasm. Arrows indicate positive SHOX staining only in F6, but not in NIH-3T3 nuclei.

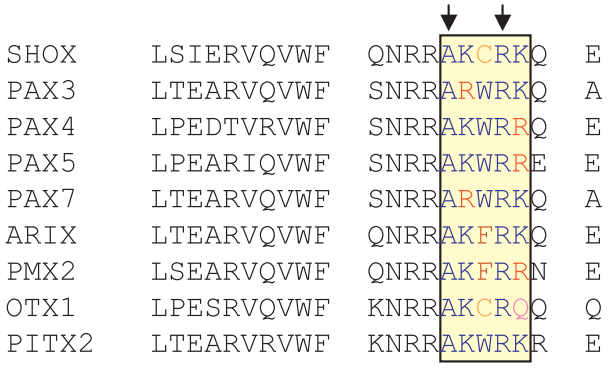

Fig. 7. Sequence alignment of the helix III of various paired-related homeodomain proteins. Amino acids from the characterized NLS for the SHOX protein are highlighted and aligned with other paired-related homeodomain proteins (boxed). Arrows at the top indicate the amino acids that are conserved in all the paired-related homeodomain proteins.

abolish this. The impairment of nuclear transport is therefore identified as the molecular basis for the observed phenotype in the patients with Léri-Weill and Langer syndrome.

Regulation of protein function by nucleo-cytoplasmic transport has recently emerged as a unique and somewhat overlooked mechanism for protein mis-regulation (Craig et al., 2002; Chestukhin et al., 2002). To check if the mutant SHOX protein was cytoplasmic as a result of impaired import or increased export, we carried out interspecies heterokaryon assays using the human osteosarcoma stable cell line F6, expressing wild-type SHOX and the mouse fibroblast cells, NIH-3T3. The assay revealed that the SHOX protein does not shuttle between the nucleus and the cytoplasm. Therefore the mis-localization of the SHOX mutant to the cytoplasm clearly results from its impaired import and not from its increased export. Several other disease phenotypes have also been attributed to the mis-localization of nuclear proteins, e.g. homeodomain proteins, helix-loop-helix or zinc finger transcription factors, nucleolar proteins and tumor suppressors (Marsh et al., 1998; Yang et al., 1999; Fan et al., 2002; Fabbro and Henderson, 2003; Kaiser et al., 2004). In addition, impairment of the NPC components and nuclear lamina has also been associated with the etiology of disease (Markiewicz et al., 2002).

In summary we have shown that mutations within, or deletion of, one copy of the NLS leads to consequences identical to SHOX haploinsufficiency, as only half of the protein is being transported to the nucleus resulting in the Léri-Weill phenotype. If both NLS copies are distorted, all of the SHOX protein remains in the cytoplasm leading to the clinically severe homozygous form of Langer mesomelic dysplasia. These results explain how single point mutations can lead to drastic forms of disease once an essential functional domain has been affected.

We thank Michael Knop from the European Molecular Biology Laboratory, Heidelberg, for providing p415-Gal1-RedStar and Dirk Görlich from the Center for Biochemistry and Molecular Biology, Heidelberg for providing the pQE80-M9-GFP clone. We also thank Jutta Finsterle from the Kirchoff Institute of Physics, University of Heidelberg, for help with the confocal microscope. This work was supported by the Deutsche Forschungsgemeineschaft and Eli Lilly and Company.

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