NLS-dependent nuclear localization of p120^{ctn} is necessary to relieve Kaiso-mediated transcriptional repression

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Summary

The Armadillo catenin p120^{ctn} regulates cadherin adhesive strength at the plasma membrane and interacts with the novel BTB/POZ transcriptional repressor Kaiso in the nucleus. The dual localization of p120^{ctn} at cell-cell junctions and in the nucleus suggests that its nucleocytoplasmic trafficking is tightly regulated. Here we report on the identification of a specific and highly basic nuclear localization signal (NLS) in p120^{ctn}. The functionality of the NLS was validated by its ability to direct the nuclear localization of a heterologous β-galactosidase-GFP fusion protein. Mutating two key positively charged lysines to neutral alanines in the NLS of full-length p120^{ctn} inhibited both p120^{ctn} nuclear localization as well as the characteristic p120^{ctn}-induced branching phenotype that correlates with increased cell migration. However, while these findings and others suggested that nuclear localization of p120^{ctn} was crucial for the p120^{ctn}-induced branching phenotype, we found that forced nuclear localization of both wild-type and NLS-mutated p120^{ctn} did not induce branching. Recently, we also found that one role of p120^{ctn} was to regulate Kaiso-mediated transcriptional repression. However, it remained unclear whether p120^{ctn} sequestered Kaiso in the cytosol or directly inhibited Kaiso transcriptional activity in the nucleus. Using minimal promoter assays, we show here that the regulatory effect of p120^{ctn} on Kaiso transcriptional activity requires the nuclear translocation of p120^{ctn}. Therefore, an intact NLS in p120^{ctn} is requisite for its first identified regulatory role of the transcriptional repressor Kaiso.

Key words: p120^{ctn}, Kaiso, NLS, Nuclear import, Transcriptional repression

Introduction

The Armadillo catenins, β-, γ- and p120^{ctn}, are multifunctional proteins whose malfunction contributes to tumor progression by various means (Behrens, 1999; Van Aken et al., 2001). They are members of a larger family of Armadillo proteins that are characterized by the presence of an Armadillo domain consisting of ten or more tandem copies of a 42 amino-acid Armadillo repeat (Peifer et al., 1994). The importance of the Armadillo catenins in tumorigenesis was first credited to their involvement in the multiprotein cadherin-catenin complex, which forms the major cell-cell adhesion system in epithelial cells. Dysfunction of this complex, through defects in any of its components, correlates with the metastatic phenotype in ~50% of human carcinomas (Behrens, 1999; Yap, 1998). The key participant in this complex is the transmembrane glycoprotein and tumor suppressor E-cadherin that mediates cell-cell adhesion by the homophilic interactions of its extracellular domain. E-cadherin is then anchored to the underlying actin cytoskeleton by the classical catenin cofactors, β- and γ-catenin, that bind E-cadherin in a mutually exclusive manner (reviewed by Nollet et al., 1999). These classical catenins bind simultaneously to a highly conserved carboxy-terminal domain of E-cadherin and to the actin-binding protein α-catenin (reviewed by Behrens, 1999; Rimm et al., 1995). Like β- and γ- catenin, the nonclassical Armadillo catenin p120^{ctn} binds the cytoplasmic tail of E-cadherin via its Arm domain (Reynolds et al., 1994; Reynolds et al., 1992). However, β- and γ-catenin compete for binding to the distal conserved catenin-binding domain, and function by anchoring E-cadherin to the actin cytoskeleton, whereas p120^{ctn} binds E-cadherin at a distinct juxtamembrane domain (JMD) and does not interact with α-catenin (Daniel and Reynolds, 1995; Thoreson et al., 2000). Importantly, the JMD is implicated in E-cadherin stability (Ireton et al., 2002; Pettitt et al., 2003), modulating cadherin adhesive strength (Aono et al., 1999; Ohkubo and Ozawa, 1999; Thoreson et al., 2000; Yap et al., 1998) and regulating cytoskeletal dynamics (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000), and as such p120^{ctn} is speculated to be a key mediator of these effects. This hypothesis is supported by recent observations that p120^{ctn} modulates the activities of RhoA, Rac and Cdc42, which are key mediators of cytoskeletal organization (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000).

Originally identified as a Src kinase substrate (Reynolds et al., 1989), it was the structural similarity of p120^{ctn} to β-catenin that led to the discovery of p120^{ctn} as a novel catenin component of the cadherin complex (Daniel and Reynolds,

1995; Reynolds et al., 1994). In fact, p120*ctn* is now the prototype of a subfamily of Armadillo proteins (e.g. ARVCF, p0071, δ-catenin/NPRAP, and plakophilins 1 and 2) that are linked both to cell adhesion events at the plasma membrane and gene regulatory events in the nucleus (reviewed by Anastasiadis and Reynolds, 2000; Hatzfeld, 1997; Reynolds and Daniel, 1997). Another key distinction between p120*ctn* and the classical catenins is that p120*ctn* exists as multiple isoforms that compete for cadherin binding (Keirsebilck et al., 1998; Mo and Reynolds, 1996). These isoforms differ solely by their amino- and carboxy-termini and arise from the differential use of N-terminal ATG translational start sites and alternate splicing of three exons, A, B, and C (Keirsebilck et al., 1998). The functional significance of the p120*ctn* isoforms is currently unknown but it is thought that their divergent N- and C-termini confer functional diversity through specific protein-protein interactions (Anastasiadis and Reynolds, 2000). It is therefore noteworthy that the characteristic branching phenotype induced by overexpression of p120*ctn* (Grosheva et al., 2001; Reynolds et al., 1996) is attributed only to certain splice variants; p120*ctn* isoforms 1A, 2A and 3A induce branching whereas the shortest isoform, p120*ctn*-4A does not (Aho et al., 2002).

Increasing evidence now indicates that many Armadillo repeat proteins (e.g. APC, β-catenin, plakoglobin, plakophilins 1 and 2) are dual localization proteins that shuttle between the nucleus and the cytoplasm or plasma membrane (Chen et al., 2002; Fagotto et al., 1998; Korinek et al., 1997; Mertens et al., 2001; Morin et al., 1997; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000; Wiechens and Fagotto, 2001; Zhang et al., 2000). These observations are even more interesting in light of the fact that the major nuclear import receptor, importin-α, is composed almost entirely of Armadillo repeats (Conti et al., 1998; Gorlich, 1998). This raises the exciting possibility that Armadillo repeat proteins possess intrinsic nuclear import ability. Consistent with this idea, we and others have detected nuclear p120*ctn*, particularly in cells of tumor lineage or in cells lacking functional cadherins (our unpublished data) (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). Interestingly, the nuclear localization of p120*ctn* appears to be an isoform-specific phenomenon; all isoforms capable of inducing branching (e.g. p120*ctn*-1A, -2A, -3A) apparently localize to nuclei, whereas those incapable of inducing branching (e.g. p120*ctn*-4A) localize to the cytosol and at sites of cell-cell contact (Aho et al., 2002). However, while the nuclear localization of p120*ctn* appears coincident with the branching phenotype, it is not clear whether the nuclear trafficking of p120*ctn* is a cause or consequence of this cellular phenotype.

Accumulating research further implicates the cell-adhesion Armadillo catenins in tumorigenesis through their interaction with, and regulation of, various transcription factors (Behrens, 1999; Gottardi and Gumbiner, 2001). Hence, in addition to their direct role in cell-cell adhesion, the Armadillo catenins also regulate gene expression. This is best exemplified by the classical β-catenin, which, as the downstream effector of the Wnt signaling pathway, translocates to the nucleus and binds to the Lef/TCF transcription factor (reviewed by Gumbiner, 1995; Peifer, 1995). Within the nucleus, the β-catenin/TCF heterodimer then activates tumorigenesis-associated genes such as *cyclinD1*, *c-myc*, *matrilysin*, *Id2* and *ITF-2* (Crawford et al., 1999; He et al., 1998; Kolligs et al., 2002; Rockman et al., 2001; Shtutman et al., 1999). Our identification of the novel BTB/POZ transcriptional repressor Kaiso as a p120*ctn*-specific binding partner (Daniel and Reynolds, 1999) thus raised the exciting possibility that p120*ctn*, like β-catenin, regulates gene expression. Indeed, we have found that p120*ctn* inhibits both the DNA-binding ability of Kaiso in vitro (Daniel et al., 2002), and Kaiso-mediated transcriptional repression of target genes via the sequence-specific Kaiso consensus site (our unpublished data).

Despite the unveiling of a nuclear role for p120*ctn* in modulating Kaiso transcriptional activity and the observation that p120*ctn* is capable of nucleocytoplasmic shuttling, the exact mechanisms of p120*ctn* nuclear import and export remain unknown. Two putative p120*ctn* nuclear localization signals (NLS) have been described (Aho et al., 2002; Anastasiadis et al., 2000; Roczniak-Ferguson and Reynolds, 2003) but neither has been directly or empirically tested for their ability to target a heterologous protein to the nucleus, the classical test for NLS functionality (Kalderon et al., 1984). To date, just one p120*ctn* nuclear export signal (NES) has been characterized but this NES is present only in isoforms encoding exon B (van Hengel et al., 1999). This suggests that alternative NESs may facilitate the nuclear export of p120*ctn* splice variants lacking exon B. Consistent with this idea, some isoforms of p120*ctn* lacking exon B are sensitive to leptomycin B (LMB), a specific inhibitor of CRM-1-mediated nuclear export (our unpublished data) (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). These results suggest that p120*ctn* is capable of nucleocytoplasmic shuttling in a CRM-I-dependent manner. Although these data collectively highlight the nucleocytoplasmic shuttling abilities of p120*ctn*, a definitive nuclear localization signal(s) for p120*ctn* and the mechanism of p120*ctn* nuclear import remains to be elucidated.

Using an in vivo nuclear localization assay (Sorg and Stamminger, 1999) and site-directed mutagenesis, we have identified an NLS in p120*ctn* that is necessary for its nuclear localization in HeLa and NIH 3T3 cells. Minimal point mutations of key basic residues within the NLS abolished its functionality. In agreement with previous reports (Anastasiadis et al., 2000), expression of the cytosol-restricted p120*ctn* NLS-mutant did not induce the characteristic p120*ctn*-overexpression branching phenotype that correlates with increased cell motility (Grosheva et al., 2001; Reynolds et al., 1996). We also show definitively that the branching phenotype is not triggered by the nuclear localization of p120*ctn*. Furthermore, in contrast to wild-type p120*ctn*, this p120*ctn* NLS-mutant failed to inhibit Kaiso-mediated transcriptional repression, indicating that the p120*ctn* inhibitory effect occurs in the nucleus and is not due to sequestration of Kaiso in the cytosol. Our work suggests an NLS-dependent nuclear role for p120*ctn* in modulating Kaiso-mediated transcriptional repression.

Materials and Methods

Cells, tissue culture and drug treatments

HeLa (human cervical carcinoma) and NIH 3T3 (mouse fibroblast) cell lines were used in this study. Both cell lines were grown at 37°C, 5% CO_2 in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.5 μg/ml). Leptomycin B (Calbiochem) treatment was performed for 18 hours at 50 nM.

Immunofluorescence

Twenty-four hours post-transfection, the transfected cells were washed once in PBS, fixed for 10 minutes in 4% paraformaldehyde/PBS on ice, and permeabilized for 5 minutes at RT with 0.2% Triton-X-100/PBS. Nonspecific antibody binding was blocked with 3% nonfat milk-PBS for 10 minutes at RT, then aspirated. Antibodies were diluted in 3% milk-PBS. Cells were incubated with anti-pp120*ctn* antibody (Transduction Labs, San Jose, CA) at 2 μg/ml for 30 minutes at RT, then washed three times for 5 minutes each in PBS. Coverslips were briefly immersed in 3% milk-PBS, aspirated, then incubated for 30 minutes at RT with Alexafluor 594-conjugated donkey anti-mouse IgG (Molecular Probes, Eugene, OR) at a dilution of 1/400 in 3% milk-PBS. The cells were finally washed three times with PBS, 5 minutes per wash, and mounted onto glass slides with Aqua Polymount (Polysciences, Warrington, PA) and imaged using a Zeiss Axiovert fluorescent microscope (Carl Zeiss, Thornwood, NY).

Plasmid constructs

To generate p120*ctn* deletion mutants, the various cDNAs were amplified by PCR using p120*ctn* isoform 1A (murine full-length) as a template, and subcloned into the pHM829 vector. This mammalian expression vector was specifically designed for expression of the protein of interest (e.g. p120*ctn*) simultaneously fused to β-galactosidase (β-gal) at its N-terminus and green fluorescent protein (GFP) at its C-terminus (Sorg and Stamminger, 1999). Each PCR primer was designed to incorporate a 5′ *Sac*II site and a 3′ *Xba*I site (underlined) into the PCR product. The PCR primers utilized for each p120*ctn* deletion construct are as follows: p120*ctn*-1 (bp 1-543), AM-1 5′-GCATATCCGCGGATGGACGACTCAGAGGTG-3′ and AM-2 5′-GCATATTCTAGAATCACGGCCCAAGGTCTG-3′; p120*ctn*-2 (bp 524-1070), AM-3 5′-GCATATCCGCGGCAGACCTTGGGCCGT-GAT-3′ and AM-4 5′-GCATATTCTAGACATTCCCTTTCGCAAA-CT-3′; p120*ctn*-3 (bp 1053-1616), AM-5 5′-GCATATCCGCGGAG-TTTGCGAAAGGGAATG-3′ and AM-6 5′-GCATATTCTAGATG-AGCTTACGTTCCGAAG-3′; p120*ctn*-4 (bp 1599-2171), AM-7 5′-GCATATCCGCGGCTTCGGAACGTAAGCTCA-3′ and AM-8 5′-GCATATTCTAGAAGCAGCTTTTACTACTCG-3′. The p120*ctn*-5 (bp 2154-2729) primers AM-13 5′-GCATATGCTAGCCGAGTAGT-AAAAGCTGCT-3′ and AM-11 5′-GCATATCACGTGCTTCTGCA-TCAAGGGTGC-3′ incorporated *Nhe*I and *Pml*I sites (underlined). All oligonucleotides were synthesized by the Central Facility of the Institute for Molecular Biology and Biotechnology (MOBIX), McMaster University. To evaluate NLS functionality, double-stranded oligonucleotides encoding the putative NLS were flanked with *Sac*II and *Xba*I sites, and ligated into pHM829. All ligations were performed using T4 DNA Ligase (NEB, Beverly, MA) according to the manufacturer's instructions.

Site-directed mutagenesis

NLS point mutations using pBluescript-mp120*ctn*-1A as template were generated using the Quickchange XL Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) according to the manufacturer's protocols. The mutated p120*ctn* cDNAs were then subcloned into the pEGFP-C1 plasmid (Clontech, Palo Alto, CA) and these constructs gave rise to a panel of p120*ctn* deletion mutants fused to eGFP at their amino terminus. Heterologous fusion of p120*ctn* to the SV40 Large T Antigen (LTag) NLS or the HIV-1 Rev NES was performed using pEGFP-C1-mp120*ctn*-1A as template and subcloned into the pRC/RSV and pEGFP-C1 plasmids. The nucleotide sequence of each construct was verified by automated fluorescence sequencing at MOBIX, McMaster University.

Transient transfections

For each transfection, cells were plated onto coverslips in a 6-well dish at least 12 hours before transfection and incubated overnight (37°C, 5% CO_2), to achieve an approximate confluency of 60-70% at the time of transfection. All transfections were performed using the XGen-500 reagent (MBI Fermentas, Bethesda, MD) or SuperFect (Qiagen, Mississauga, Ontario). For each construct, 2 μg of plasmid DNA were diluted in Opti-MEM serum-free medium (Gibco, Carlsbad, CA). Six microliters of XGen-500 or SuperFect reagent was added to the DNA-media solution and the mixture gently vortexed. The mixture was incubated without agitation at RT for 15-20 minutes to allow reagent/DNA complex formation, before its addition to the cells in supplemented DMEM. Unless otherwise noted, the cells were incubated with the DNA complexes for 3 hours at 37°C, 5% CO_2, after which time the transfection mixture was aspirated and cells washed with 1× Phosphate buffered saline (PBS, pH 7.4). The cells were then incubated for 24 hours in supplemented DMEM medium before fixation in 4% paraformaldehyde/PBS (pH 7.0) for 10 minutes on ice.

X-gal staining of cells

Twenty-four hours post-transfection, cells were stained for β-galactosidase activity. Cells were washed once with 1× PBS (pH 7.4), followed by fixation with either 4% paraformaldehyde-PBS alone or methanol at –20°C for 7 minutes. After fixation, cells were washed twice with 1× PBS and incubated with X-gal reagent (1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 5 mM potassium ferricyanate, 5 mM potassium ferrocyanate, 2 mM $MgCl_2$, in PBS pH 7.4) for 2-16 hours (37°C, 5% CO_2), as specified. After incubation with X-gal, cells were washed once in 1× PBS, once in dH_2O, and mounted on slides using Aqua Poly/Mount anti-fade solution (Polysciences). The cells were then imaged by phase contrast microscopy using a Zeiss Axiovert 200 microscope.

Microscopy

For eGFP or GFP imaging, samples were prepared and fixed as described above before the coverslips were mounted onto slides using Aqua Poly/Mount anti-fade solution (Polysciences). The coverslips were then sealed with clear nail polish (toluene and formaldehyde free) and visualized using epi-fluorescence microscopy on the Zeiss Axiovert 200.

Artificial promoter assays

Twenty-four hours before transfection, 90% confluent cells were washed twice with 5 ml of PBS and trypsinized for 2 minutes. Cells were counted on a hemacytometer and seeded at 2×10^5 cells per well in 6-well dishes. All transfections were performed using SuperFect transfection reagent (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. We used 800 ng of reporter construct DNA (either pGL3 Control or 4× KBS-pGL3 Control) and 200 ng of effector DNA (Kaiso-pcDNA3, p120-1A-pRc/RSV, p120NLSmut-pRc/RSV, or backbone vector alone). Cells were incubated with the transfection mix at 37°C in 5% CO_2 for 3 hours, washed twice with 2 ml PBS, and incubated for 24 hours at 37°C in 5% CO_2 with fresh supplemented DMEM before luciferase assay analysis. Each well was treated with 350 μl of Passive Lysis Buffer (PLB) (Promega, Madison, Wisconsin) and the plates kept at room temperature for 20 minutes with vigorous shaking. Lysates were resuspended by pipetting, after which 20 μl of lysate from each well were assayed for luciferase activity on a Lumat LB 9501 Berthold Luminometer (Fisher Scientific, Toronto, Ontario). Each experiment was performed in triplicate and all data are representative of the mean of three independent trials.

Immunoprecipitation and immunoblot analysis

Cells were washed twice with 5 ml of PBS followed by incubation on ice with lysis buffer containing 0.5% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM EDTA for 5 minutes. Cells were harvested from the plates, and transferred to an 1.5 ml tube. The lysate was centrifuged at 16,000 ***g*** for 5 minutes at 4°C and the supernatant transferred to a new tube. Lysates were quantified by Bradford assay and equal amounts of total protein were used for immunoprecipitation with the murine-specific anti-p120*ctn* mAb 8D11 (Wu et al., 1998). The immunocomplexes were then subjected to SDS-PAGE as previously described (Daniel and Reynolds, 1999).

After SDS-PAGE, proteins were transferred from the gel to a nitrocellulose membrane using the Hoeffer semi-dry transfer apparatus (Amersham/Pharmacia, San Francisco, CA). The membrane was then briefly blocked at room temperature with 3% milk in TBS pH 7.4 before incubating at 4°C overnight with anti-p120*ctn* antibodies (8D11) used at 2 μg/ml in 3% milk-TBS. The primary antibodies were removed by washing with TBS and the membranes incubated for 2 hours at RT with peroxidase-conjugated donkey anti-mouse secondary antibody, diluted 1:40,000 in 3% milk-TBS. Membranes were finally rinsed five times with water, once with TBS pH 7.4 for 5 minutes, and processed using the enhanced chemiluminescence (ECL) system (Amersham/Pharmacia) according to the manufacturer's protocols.

Results and Discussion

p120*ctn* has a conserved, monopartite NLS within its Armadillo domain

In tumor-derived cells and cells lacking functional E-cadherin, p120*ctn* displays an increased nuclear localization (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). Moreover, the interaction of p120*ctn* with the transcriptional repressor Kaiso (Daniel and Reynolds, 1999), and p120*ctn*'s inhibition of Kaiso-mediated transcriptional repression of target genes (our unpublished data) clearly indicates a nuclear role for this novel Armadillo catenin. Because the molecular weight of p120*ctn* (~120 kDa) exceeds the upper limit for passive diffusion through the nuclear pore (~50 kDa), it has been postulated that p120*ctn* might possess one or more nuclear localization signals that facilitate its active nuclear import. The canonical NLS-dependent nuclear import pathway is mediated by the importin-α/β heterodimer (reviewed by Gorlich, 1998). The importin-α subunit binds directly to the NLS of the import substrate, whereas importin-β bridges the trimeric complex to nuclear pore complex (NPC) proteins. Once in the nucleus, the importin-β subunit interacts with the GTPase Ran and dissociates from the importin-α/β heterodimer, thereby releasing the import cargo in the nucleoplasm. Typically, proteins imported via this pathway contain a highly basic NLS that is either monopartite (e.g. SV40 large T antigen, SV40-LTAg) or bipartite (e.g. nucleoplasmin) in nature (Gorlich, 1998).

Visual inspection of the p120*ctn* amino acid sequence revealed a highly basic region between Armadillo repeats 6 and 7 with the potential to serve as an NLS. Interestingly, this region encoded by amino acids 622-629 is also thought to be crucial for p120*ctn*-mediated inhibition of the RhoA GTPase (Anastasiadis et al., 2000). In that study, however, amino acids 622-629 were not tested for NLS functionality, which is classically defined as the ability of a short amino acid sequence to facilitate

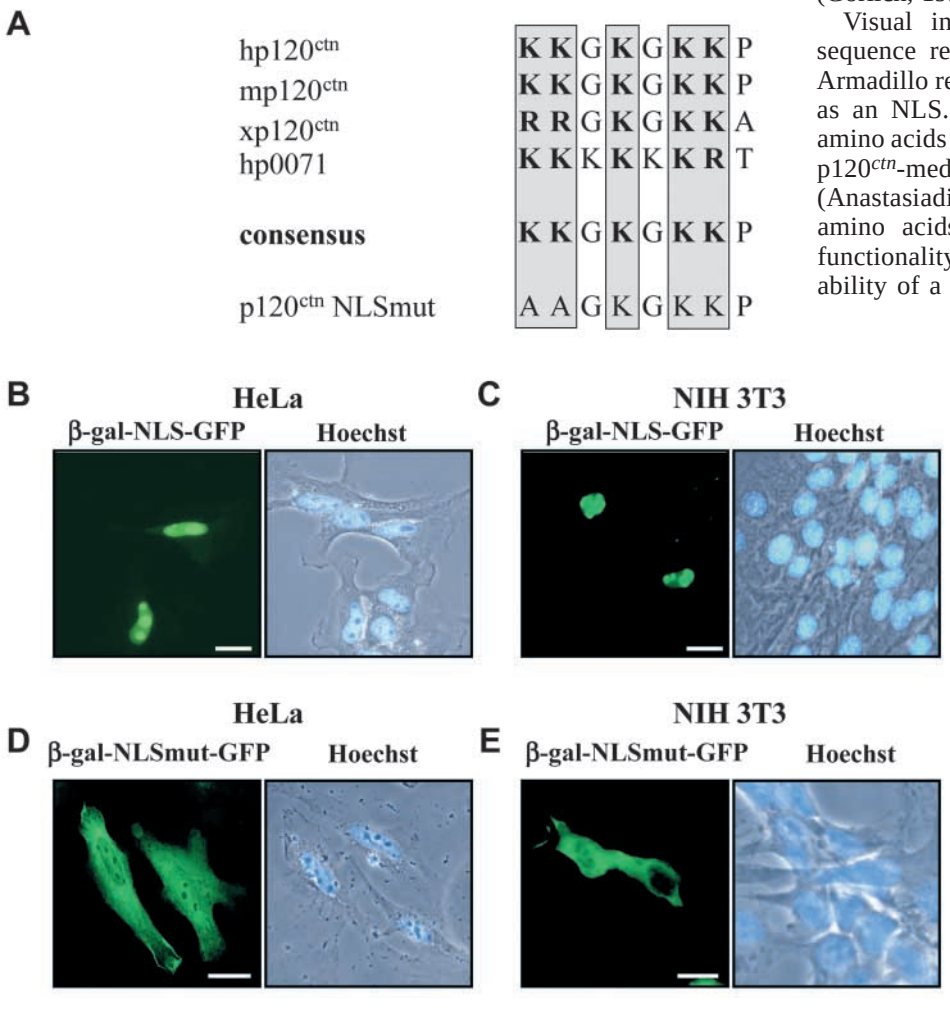

Fig. 1. Identification of an NLS in the p120*ctn* Armadillo domain. (A) A cross-species comparison of the p120*ctn* amino acid sequence revealed the presence of a highly conserved putative NLS. Key positively charged residues are boxed. p120*ctn*-NLSmut denotes the point mutations generated for NLS functionality experiments in D and E. (B,C) Direct fusion of the putative p120*ctn* NLS (a.a. 622-629, KKGKGKKP) to β-galactosidase and GFP directs the heterologous fusion protein to the nuclei of HeLa and NIH 3T3 cells, respectively (β-gal-NLS-GFP). (D,E) The functionality of the putative NLS was validated by the failure of a β-gal-NLSmut fusion protein to localize to the nuclei of HeLa and NIH 3T3 cells, respectively. This mutant possessed a double point mutation within the p120*ctn* NLS as indicated. The images shown are representative of at least 100 cells scored for each construct. Bars, 20 μm.

the nuclear translocation of a heterologous protein (Kalderon et al., 1984). We thought that this region may serve the dual purpose of regulating p120^{ctn} nuclear localization as well as the p120^{ctn} inhibitory effects on RhoA function. Moreover, the high conservation of this sequence, KKGKGKKP, in all p120^{ctn} isoforms, from diverse species such as *Homo sapiens*, *Mus musculus*, and *Xenopus laevis*, further underscored its potential to regulate p120^{ctn} nuclear import (Fig. 1A). In our NLS assay, this region (amino acids 622-629), when fused directly to β-gal-GFP, strongly targeted the β-gal-GFP fusion protein (β-gal-NLS-GFP) to 88% of HeLa and 92.5% of NIH 3T3 nuclei as detected by fluorescence microscopy (Fig. 1B,C). To confirm that the NLS had indeed targeted the entire heterologous fusion protein (i.e. β-gal-NLS-GFP) to the nucleus and that the carboxy-terminal GFP moiety had not been cleaved, transfected cells were stained with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and we detected β-gal enzymatic activity in the nucleus (data not shown). To test further the functionality of the p120^{ctn} NLS, we inactivated the NLS by mutating two positively charged lysine residues to neutral alanines (β-gal-NLSmut-GFP). As shown in Fig. 1D, this fusion protein was no longer predominantly nuclear but rather localized equally to the cytosol and nucleus in 93% of transfected HeLa cells. However, in NIH 3T3 cells (Fig. 1E), this β-gal-NLSmut-GFP fusion protein was predominantly excluded from the nucleus in 85.5% of transfected cells. The quantification of these results and subsequent NLS characterization experiments are summarized in Table 1. Our results definitively show that p120^{ctn} amino acids 622-629 (KKGKGKKP) represent a bona fide and functional monopartite NLS that is sufficient to direct the nuclear localization of heterologous proteins.

To validate our findings and identify any additional NLS activity in p120^{ctn}, we used a deletion mutagenesis approach in conjunction with an established in vivo assay for mapping functional NLS sequences (Sorg and Stamminger, 1999). We created five p120^{ctn} deletion mutants spanning the entire p120^{ctn} open reading frame (Fig. 2A). p120^{ctn} isoform 1A was used for these experiments because it lacks the NES present in exon B as identified by van Hengel et al. (van Hengel et al., 1999). Each mutant was fused N-terminally to β-galactosidase to add bulk and prevent passive diffusion into the nucleus, and C-terminally to GFP, which allows subcellular visualization (Sorg and Stamminger, 1999). The constructs were transfected into HeLa and NIH 3T3 cells, and the subcellular distribution of the fusion proteins were scored by fluorescence microscopy. Each construct was analyzed relative to a β-gal-GFP negative control, which remained cytosolic, and a GFP-SV40-LTAg-NLS-β-gal positive control that localized to the nucleus (Fig. 2B). Full-length p120^{ctn} fused to β-gal and GFP (p120^{ctn}-full) localized equally to the cytosol and nuclei of all transfected HeLa cells (Fig. 2C). Of the five initial p120^{ctn} deletion mutants, only one deletion mutant (p120^{ctn}-2, a.a. 176-357) showed any nuclear localization (Fig. 2D). However, this mutant also localized to cytosolic aggregates, suggesting that we were not observing a robust nuclear localization phenotype. Interestingly, this mutant contains a putative bipartite NLS (a.a. 306-320, RRTGTPSDPRRRLRS), previously suggested by Aho et al. (Aho et al., 2002) to be important for the nuclear import of p120^{ctn}. When this sequence was fused directly to β-gal-GFP, we observed negligible NLS activity and the resulting β-gal-GFP fusion protein localized predominantly to the cytosol in 84.5% of transfected HeLa cells (Fig. 2E). This mutant produced similar results in NIH 3T3 cells. We therefore concluded that amino acids 306-320 of p120^{ctn} are insufficient to serve as an NLS and are unlikely to regulate p120^{ctn} nuclear import.

Intriguingly, none of our remaining four deletion mutants localized to cell nuclei, including p120^{ctn}-4, which contained our putative NLS (a.a. 622-629). This could be attributed to the presence of either a dominant novel NES that overshadowed our identified NLS, or perhaps a protein-folding event that rendered our NLS inaccessible to the nuclear import machinery. To determine whether any of the deletion mutants possessed potential NLS activity that was out-competed by a strong NES, we transfected NIH 3T3 cells with each p120^{ctn} deletion mutant and compared the localization of the fusion proteins following treatment with the CRM-1 nuclear export inhibitor LMB (Fig. 3). As previously shown, wild-type, full-length p120^{ctn} (p120^{ctn}-WT) was LMB sensitive in our assay (Fig. 3A). However, none of the p120^{ctn} deletion mutants used for NLS analyses (Fig. 2D) displayed LMB sensitivity (Fig. 3B). This suggests that the LMB insensitivity of these p120^{ctn} deletion mutants is due to the absence of CRM-1-type NES in these deletion mutants. Interestingly, our p120^{ctn}-4 mutant containing the basic monopartite NLS also failed to display

Table 1. Quantitative scoring of p120^{ctn} deletion mutant subcellular localization

Construct	Cell line	± LMB	% Nuclear	% Cytosolic	% Nuclear and cytosolic
β-gal-NLS-GFP	HeLa	–	88.0±9.9	0	12.0±9.9
	NIH 3T3	–	92.5±2.1	0	7.5±2.1
β-gal-NLSmut-GFP	HeLa	–	1.0±1.4	6.0±1.4	93.0±1.4
	NIH 3T3	–	0	85.5±3.5	14.5±3.5
p120^{ctn}-full	HeLa	–	0	0	100.0±0
p120^{ctn}-1	NIH 3T3	–	0	94.5±2.1	5.5±2.1
		+	0	90.5±2.1	9.5±2.1
p120^{ctn}-2	NIH 3T3	–	0	23.5±19.0	76.5±19.0
		+	-	32.0±13.4	68.0±13.4
p120^{ctn}-3	NIH 3T3	–	0	90.0±5.7	10.0±5.7
		+	0	91.5±2.1	8.5±2.1
p120^{ctn}-4	NIH 3T3	–	0	97.0±4.2	3.0±4.2
		+	0	98.0±0	2.0±0
p120^{ctn}-5	NIH 3T3	–	0	95.0±7.0	5.0±7.0
		+	0	98.0±1.4	2.0±1.4
β-gal-p120^{ctn} (306-320)-GFP	HeLa	–	0	84.5±4.9	15.5±4.9
p120^{ctn}-WT	HeLa	–	2.0±4.9	2.5±4.9	95.5±4.9
	NIH 3T3	–	0	67.5±6.4	32.5±6.4
	NIH 3T3	+	0	11.6±10.7	88.3±10.7
p120^{ctn}-NLSmut	HeLa	–	0	70.6±16.8	29.4±16.8
	NIH 3T3	–	0	92.0±5.6	8.0±5.6
	NIH 3T3	+	0	36.3±11.1	63.7±11.1

Each p120^{ctn} deletion mutant was scored blindly as either predominantly nuclear, predominantly cytosolic, or predominantly nuclear and cytosolic. Each value represents at least two scoring experiments and is expressed as the mean of these experiments±s.d.

LMB sensitivity and did not localize to the nuclear compartment. This suggests that the predominant cytosolic localization of this mutant, despite the presence of a functional NLS, may be due to an alternate nonclassical NES or that the conformational structure of this particular mutant prevents access of the NLS by the nuclear import machinery. The first possibility is plausible, considering that a putative NES exists within this region (a.a. 554-567) that resembles the loose consensus of the Rev/Rex type NES as defined by Bogerd et al. (Bogerd et al., 1996). Furthermore, a recent study suggested that p120^{ctn} Arm repeat 8, contained within our p120^{ctn}-4 mutant, may regulate the nuclear export of p120^{ctn} (Roczniak-Ferguson and Reynolds, 2003). Although this region of p120^{ctn} may serve to modulate the nuclear export of p120^{ctn} isoforms lacking the NES encoded by exon B (van Hengel et al., 1999), we found that a p120^{ctn}-4 mutant lacking Arm repeat 8 still localized predominantly to the cytosol in our assay (data not shown). This discrepancy may be explained by the fact that our study examined p120^{ctn} nucleocytoplasmic shuttling in the context of a β-gal-GFP fusion. Thus, the inability of the p120^{ctn}-4 mutant to localize to the nucleus may be due to a folding event of the β-gal-GFP fusion that masked the identified NLS and rendered it inaccessible to nuclear import factors.

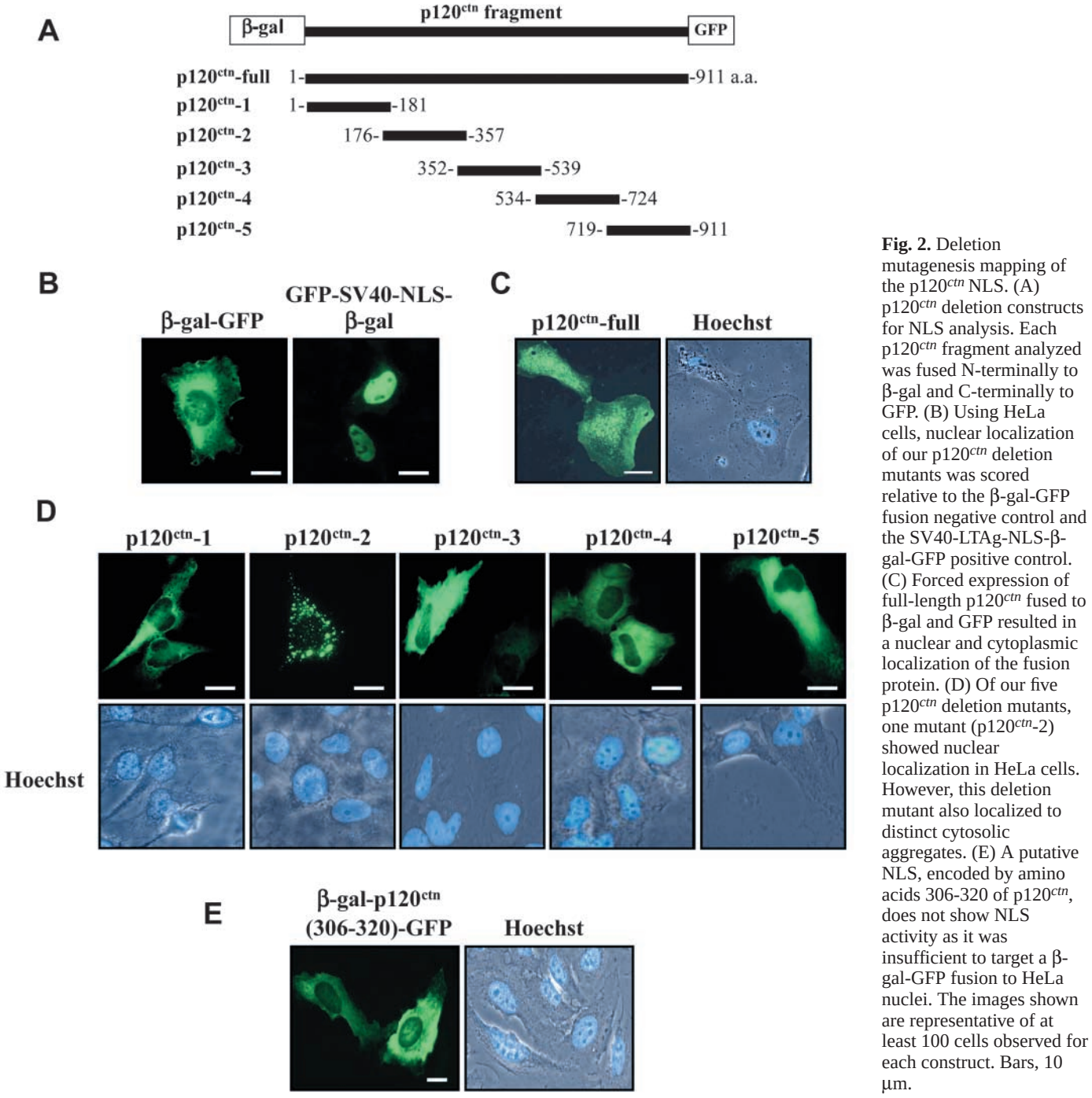

Fig. 2. Deletion mutagenesis mapping of the p120^{ctn} NLS. (A) p120^{ctn} deletion constructs for NLS analysis. Each p120^{ctn} fragment analyzed was fused N-terminally to β-gal and C-terminally to GFP. (B) Using HeLa cells, nuclear localization of our p120^{ctn} deletion mutants was scored relative to the β-gal-GFP fusion negative control and the SV40-LTAg-NLS-β-gal-GFP positive control. (C) Forced expression of full-length p120^{ctn} fused to β-gal and GFP resulted in a nuclear and cytoplasmic localization of the fusion protein. (D) Of our five p120^{ctn} deletion mutants, one mutant (p120^{ctn}-2) showed nuclear localization in HeLa cells. However, this deletion mutant also localized to distinct cytosolic aggregates. (E) A putative NLS, encoded by amino acids 306-320 of p120^{ctn}, does not show NLS activity as it was insufficient to target a β-gal-GFP fusion to HeLa nuclei. The images shown are representative of at least 100 cells observed for each construct. Bars, 10 μm.

NLS-regulated nuclear localization of p120*ctn*

To determine the necessity of our identified p120*ctn* NLS for the nuclear localization of full-length p120*ctn*, we generated an eGFP-p120*ctn* fusion construct that incorporated the NLS mutation (p120*ctn*-NLSmut) and assessed the consequences of its ectopic expression relative to wild-type p120*ctn* (p120*ctn*-WT) in NIH 3T3 and HeLa cells (Fig. 4). p120*ctn*-WT localized predominantly to the nucleus and cytosol in the majority of HeLa cells (95.5% of transfected cells), whereas the localization of this wild-type GFP-p120*ctn* fusion protein was mainly cytosolic in NIH 3T3 cells (67.5% of transfected cells) but also localized equally to the nucleus and cytosol in 32.5% of transfected cells. Consistent with amino acids 622-628 serving as a bona fide NLS, expression of the full-length p120*ctn*-NLSmut localized primarily in the cytosol of NIH 3T3 cells (92%) and HeLa cells (70.6%) (see Table 1 and Fig. 4A-ii and 4B-ii, respectively). This confirmed that the identified monopartite NLS (a.a. 622-629) was integral for the nuclear localization of p120*ctn* in these cells. However, the possibility remained that our p120*ctn* NLS-mutant was trafficking to the nucleus by an alternate mechanism, independent of or in conjunction with our identified NLS. To test this hypothesis, we sought to determine whether our p120*ctn* NLS-mutant was LMB-sensitive and hence able to translocate to the nucleus via an additional import pathway. For this experiment we used NIH 3T3 cells because p120*ctn* displays a robust LMB sensitivity in these cells, possibly because of the absence of E-cadherin, which generally sequesters p120*ctn* at cell junctions. Indeed, while the p120*ctn*-NLSmut proteins localized to the cytosol in 92% of untreated NIH 3T3 cells (Fig. 4Di), the subcellular distribution of this p120*ctn* mutant was altered with LMB treatment. The majority of transfected cells (63.7%) displayed nuclear and cytosolic localization (Fig. 4Dii) whereas less than 40% transfected cells displayed a cytosolic localization of p120*ctn*-NLSmut. In conclusion, although our identified NLS clearly impacts p120*ctn* subcellular localization

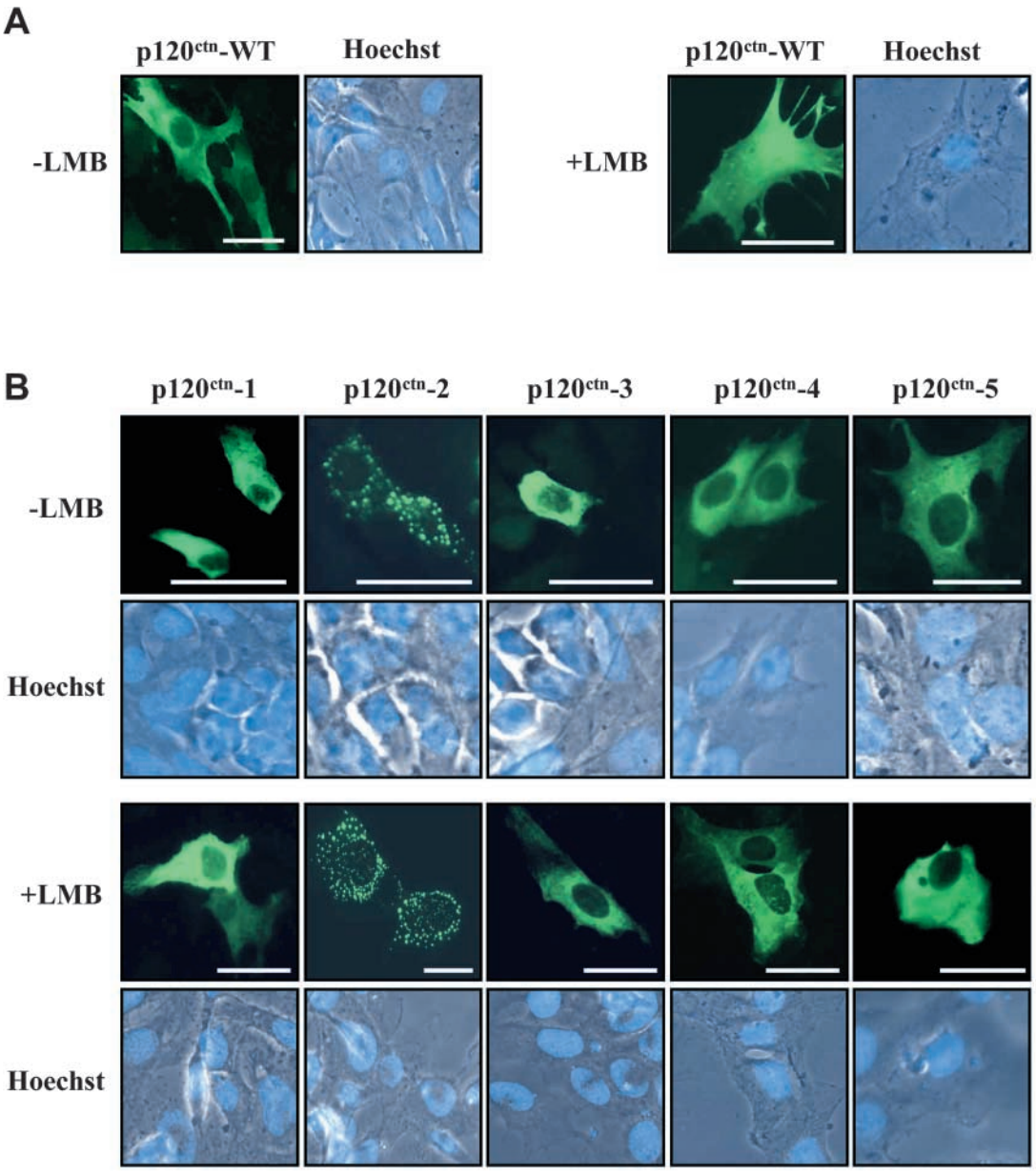

Fig. 3. Leptomycin B insensitivity of p120*ctn* deletion mutants. NIH 3T3 cells were transfected with our panel of p120*ctn*-deletion mutants and treated with LMB for 18 hours before fixation and imaging. Wild-type GFP-fused p120*ctn* displayed marked LMB sensitivity (A), but none of the p120*ctn* deletion mutants used for NLS analyses were LMB sensitive (B). Bars, 20 μm.

at steady state, our findings point to the existence of an alternate import pathway that may additionally regulate p120*ctn* nuclear import in cells. Such a pathway may reflect the ability of p120*ctn* to associate with an array of import receptors through differential use of its Armadillo repeats. Alternatively, our findings may be hinting at an intrinsic p120*ctn* import activity akin to importin-α. Recently it was demonstrated that deletion of the putative p120*ctn* NLS in isoform 3A failed to inhibit the nuclear localization of that splice variant (Roczniak-Ferguson and Reynolds, 2003). This is not surprising considering that the p120*ctn* isoforms are hypothesized to participate in distinct cellular processes and suggests that different p120*ctn* isoforms likely utilize multiple pathways for nucleocytoplasmic trafficking.

p120*ctn* inhibition of Kaiso-mediated transcriptional repression is NLS-dependent

To date the strongest clues regarding nuclear p120*ctn* function have stemmed from p120*ctn*'s association with the bimodal POZ-ZF transcriptional repressor, Kaiso (Daniel and Reynolds, 1999). Kaiso is a nuclear phosphoprotein belonging to the family of POZ-zinc finger transcription factors implicated in tumorigenesis and embryonic development (Albagli et al., 1995; Bardwell and Treisman, 1994). The relevance of the p120*ctn*-Kaiso interaction was recently revealed when we discovered that p120*ctn* inhibits Kaiso DNA-binding (Daniel et al., 2002) and Kaiso-mediated transcriptional repression of target genes (our unpublished data). Although the inhibition of Kaiso-mediated transcriptional repression by p120*ctn* was most likely due to steric hindrance of Kaiso DNA-binding, it remained possible that p120*ctn* exerted its inhibitory effect by sequestering Kaiso in the cytosol. To elucidate whether the nuclear localization of p120*ctn* was required for its inhibitory effect on Kaiso transcriptional activity, we performed minimal promoter studies in HeLa cells (Fig. 5A) using our p120*ctn* NLS-mutant with a double point mutation of the identified monopartite NLS (KK622AA). Wild-type and NLS-mutated p120*ctn* were co-transfected with Kaiso and the pGL3-4xKBS plasmid, which carries four tandem copies of the consensus Kaiso binding site upstream of the *luciferase* reporter gene. Wild-type p120*ctn* inhibited Kaiso-mediated repression of luciferase expression by approximately 90%, but the p120*ctn* NLS-mutant did not affect luciferase expression levels (Fig. 5A, compare 4× KBS + WT and 4× KBS + NLS Mut) despite similar expression levels relative to wild-type p120*ctn* (Fig. 5B). These experiments were also performed in Cos-1 cells and similar results were obtained (data not shown). To further show that the p120*ctn* de-repression effect required p120*ctn* nuclear localization, we repeated these experiments using our p120*ctn* NLS-mutant tagged with the SV40-LTAg NLS to restore the nuclear translocation of this mutant protein. As seen in Fig. 5, inhibition of Kaiso-mediated transcriptional repression by p120*ctn* was fully rescued by fusing the SV40-LTAg NLS to the carboxy-terminus of NLS-defective p120*ctn* (Fig. 5A, compare 4× KBS + NLS Mut with 4× KBS + NLS Mut + SV40 NLS). To stimulate the nuclear import of wild-type p120*ctn* in our assays, we likewise fused one copy of the SV40-LTag NLS to the C-terminus of p120*ctn* and monitored its effect on Kaiso-mediated transcriptional repression. Over repeated trials, the inhibition of Kaiso-mediated

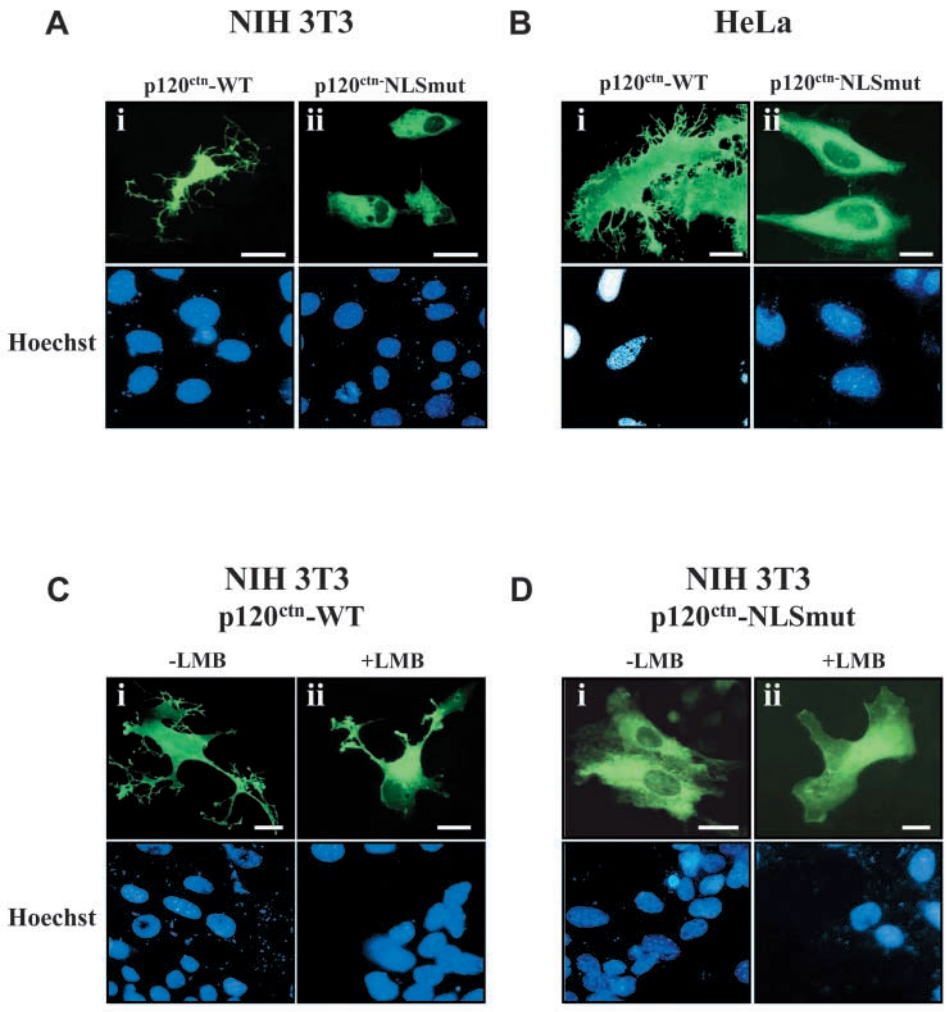

Fig. 4. An intact NLS in p120*ctn* is integral for p120*ctn* nuclear localization and the p120*ctn*-induced branching phenotype. (A) Expression of wild-type eGFP-fused p120*ctn* (p120ctn-WT) causes the characteristic branching phenotype in NIH 3T3 cells (Ai) and HeLa cells (Bi). Expression of NLS-defective p120*ctn* (p120*ctn*-NLSmut) is largely restricted to the cytosol and unable to induce the branching phenotype (Aii and Bii). (C) An 18 hour LMB treatment caused the nuclear retention of both p120*ctn*-WT (Cii) and p120*ctn*-NLSmut (Dii). Results are indicative of at least 100 cells observed for each construct. Bars, 10 μm.

transcriptional repression by NLS-fused wild-type p120^{ctn} was only slightly augmented. This is possibly because the effect by p120^{ctn} is dose-dependent (our unpublished data) and had reached its saturation point. Collectively our data show that nuclear localization of p120^{ctn} is necessary for the p120^{ctn} inhibitory effect on Kaiso-mediated transcriptional repression. Furthermore, our findings support the idea that the inhibition of Kaiso-mediated transcriptional repression by p120^{ctn} is due to steric hindrance of Kaiso DNA-binding rather than to p120^{ctn} sequestering Kaiso in the cytosol. However, it remains possible that nuclear p120^{ctn} also hinders an interaction between Kaiso and the HDAC corepressor complex that is involved in facilitating Kaiso-mediated transcriptional repression (our unpublished data) (Yoon et al., 2003).

NLS-defective p120^{ctn} does not induce the branching phenotype

Ectopic expression of p120^{ctn} in various cell lines results in a dramatic arborization phenotype that is thought to represent an exaggerated manifestation of the normal p120^{ctn} cellular function (Aho et al., 2002; Reynolds et al., 1996). Other independent studies also showed that moderate p120^{ctn} overexpression causes significantly increased cellular migration and enhanced formation of filopodia and/or lamellipodia (Grosheva et al., 2001). Since its initial characterization, the branching phenotype has been attributed at least in part to the inhibition of the RhoA GTPase (Anastasiadis et al., 2000). However, since the region of p120^{ctn} responsible for RhoA inhibition is the same region that exhibits NLS activity in our study, it remained possible that the nuclear translocation of p120^{ctn} was a contributory factor in the branching phenotype. This hypothesis was first proposed by Aho et al. (Aho et al., 2002), who noted that the branching phenotype correlated with the incidence of nuclear p120^{ctn}.

Over the course of our studies to identify and characterize the p120^{ctn} NLS, we noted that, in contrast to wild-type p120^{ctn}, the expression of p120^{ctn}-NLSmut displayed a dramatic reduction in the characteristic p120^{ctn}-overexpression branching phenotype. As shown previously (Reynolds et al., 1996), both NIH 3T3 and HeLa cells displayed the p120^{ctn}-induced branching phenotype upon forced expression of wild-type p120^{ctn} (Fig. 4Ai and 4Bi). By contrast, cells expressing p120^{ctn}-NLSmut displayed a marked reduction in branching in NIH 3T3 and HeLa cells (Fig. 4Aii and 4Bii). Consistent with the findings of Aho et al. (Aho et al., 2002), these data suggested that the p120^{ctn}-induced branching and cell migration occur in conjunction with the nuclear localization of p120^{ctn}. We therefore attempted to rescue the branching phenotype via the expression of a p120^{ctn}-NLS mutant fused C-terminally to the heterologous NLS of the SV40-large-T antigen. Interestingly, this mutant was unable to induce branching, although it was strongly targeted to the nucleus (Fig. 6Ei and 6Eii). This suggests that an intact NLS in p120^{ctn} is essential for branching to occur and that nuclear localization of p120^{ctn} alone is insufficient for induction of the characteristic p120^{ctn}-branching phenotype. To definitively ascertain whether the branching phenotype is a downstream consequence of p120^{ctn} nuclear localization, wild-type p120^{ctn} was targeted to the nucleus of NIH 3T3 cells via a direct fusion with the SV40-LTAg NLS (Fig. 6Ci and 6Cii). This p120^{ctn} mutant localized strongly to nuclei but was insufficient to induce branching, despite having an intact NLS and putative Rho-inhibition domain. Our findings are consistent with an earlier study by Anastasiadis et al. (Anastasiadis et al., 2000), who reported that a p120^{ctn} mutant lacking amino acids 622-628 (i.e. the NLS we identified) failed to induce branching and was unable to inhibit RhoA GTPase activity. Our findings now suggest that p120^{ctn}

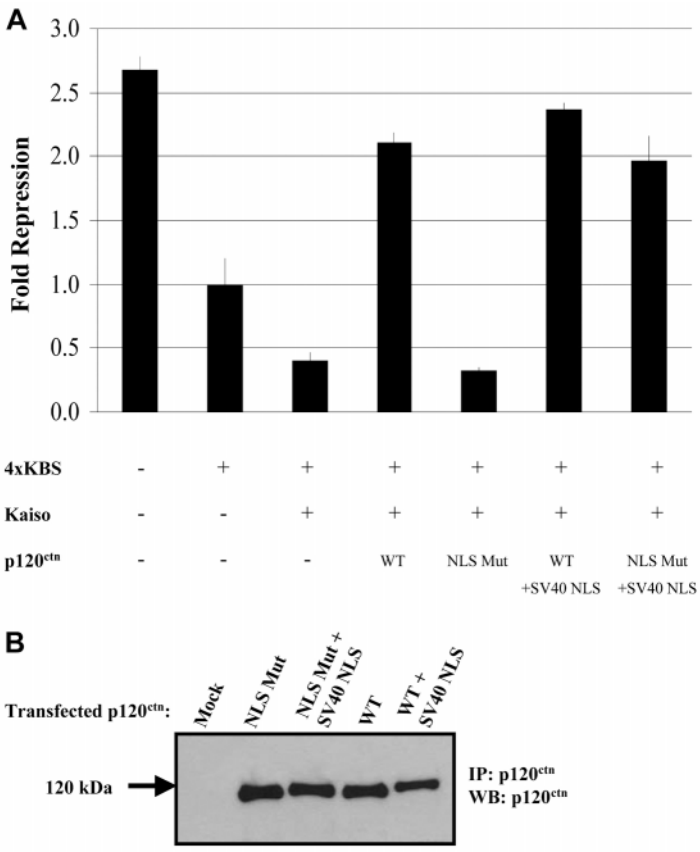

Fig. 5. NLS-defective p120^{ctn} does not inhibit Kaiso-mediated transcriptional repression. (A) Artificial promoter assays in HeLa cells revealed that endogenous Kaiso repressed luciferase expression from the 4× KBS-luciferase construct approximately twofold (compare pGL3 Control to 4× KBS). Overexpression of wild-type Kaiso (4× KBS + Kaiso) repressed luciferase expression an additional twofold. Overexpression of wild-type p120^{ctn} inhibited Kaiso-mediated repression of the luciferase reporter, whereas the NLS-defective p120^{ctn} mutant (NLS Mut) failed to inhibit Kaiso-mediated transcriptional repression despite similar expression levels to wild-type p120^{ctn}, as shown in (B). Expression levels of transfected p120^{ctn} were detected by immunoprecipitation with p120^{ctn}-specific antibody 8D11, which recognizes exogenous mouse, but not endogenous human, p120^{ctn}. Inhibition of Kaiso-mediated transcriptional repression by p120^{ctn} was fully rescued by fusing the SV40-LTAg NLS to the carboxy-terminus of NLS-defective p120^{ctn} (compare 4× KBS + NLS Mut with 4× KBS + NLS Mut + SV40 NLS). Migration of p120^{ctn} fused C-terminally to the SV40-LTAg NLS was impeded slightly on SDS-PAGE due to the addition of the heterologous NLS. Similar results were also obtained in Cos-1 cells (data not shown).

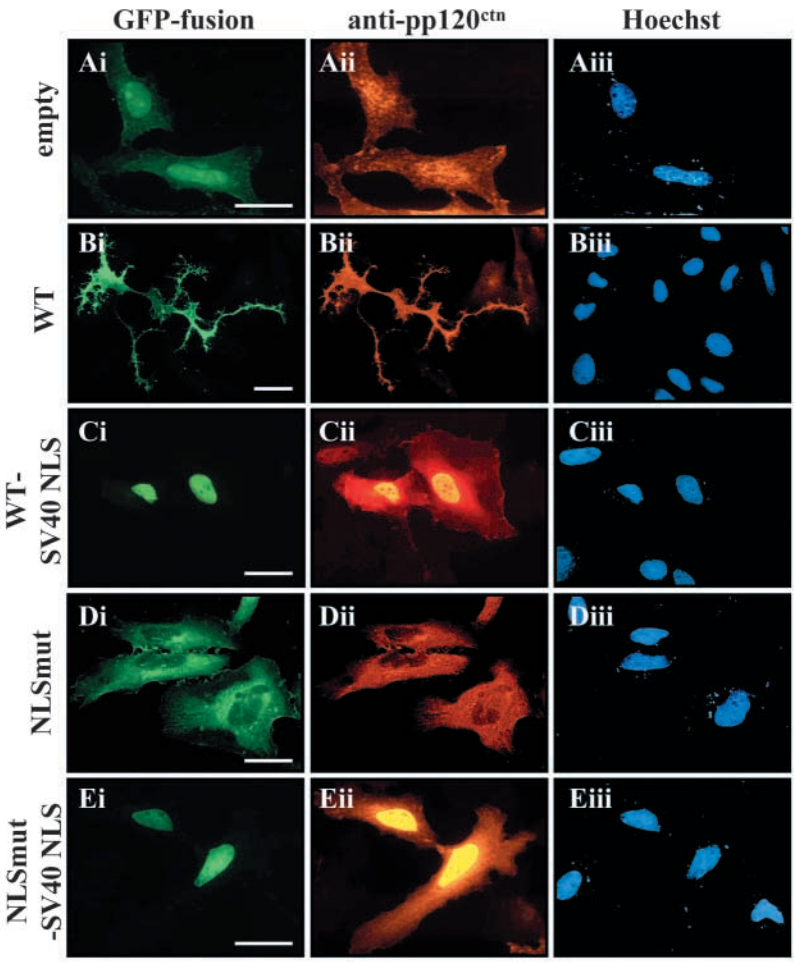

Fig. 6. p120^{ctn} nuclear translocation does not cause the branching phenotype. To address whether the nuclear translocation of p120^{ctn} stimulates the branching phenotype, we directed ectopic p120^{ctn} into the nucleus of HeLa cells via a direct C-terminal fusion to the SV40-LTag NLS. In contrast to wild-type p120^{ctn} (Bi-iii), which strongly induces branching, nuclear-targeted p120^{ctn} is unable to induce branching (Ci-iii). NLS-defective p120^{ctn} localizes to the cytosol and does not induce branching (Di-iii). Direct fusion of NLS-defective p120^{ctn} to the SV40-LTAg NLS is insufficient to rescue branching (Ei-iii), indicating that an intact NLS in p120^{ctn} is required for this phenomenon. Endogenous and exogenous p120^{ctn} are detected by pan-specific p120^{ctn} antibody pp120^{ctn} (Aii, Bii, Cii, Dii, Eii) to highlight the cell boundaries. Empty vector expressing GFP alone (empty) has no effect on branching and freely diffuses into the nucleus (Ai-iii). Similar results were obtained in NIH 3T3 cells. Bars, 20 μm.

nuclear translocation is insufficient to cause the branching phenotype and perhaps that the nuclear localization of p120^{ctn} is a consequence rather than the cause of the branching phenotype. More comprehensive future studies will be required to test this latter hypothesis.

In conclusion, we report on the identification of a functional, highly conserved monopartite NLS within the Armadillo repeat domain of the catenin p120^{ctn}. Our data clearly show NLS activity associated with amino acids 622-629 of p120^{ctn} in the context of both full-length p120^{ctn} (Fig. 4) and in the nuclear import of heterologous proteins (β-gal, Fig. 1). Furthermore, we show that the mutation of key positively charged residues within the p120^{ctn} NLS abolishes its functionality, thereby establishing it as a bona fide NLS. To date, a potential nuclear role for p120^{ctn} is to inhibit Kaiso-mediated transcriptional repression (our unpublished data). Using minimal promoter assays we now show that this effect is dependent on the efficient nuclear localization of p120^{ctn}. These findings support a novel, NLS-dependent nuclear role for p120^{ctn} in regulating Kaiso-mediated transcriptional repression. Consistent with previous independent studies, we found that expression of NLS-defective p120^{ctn} in mammalian cells yields a marked shift of the GFP-fused protein to the cytosol, and causes a drastic reduction in the previously observed p120^{ctn}-induced branching phenotype. Our results indicate, however, that the nuclear translocation of p120^{ctn} does not cause or stimulate the branching phenotype. It is noteworthy that recent studies have observed increased nuclear localization of p120^{ctn} in cancers of the pancreas (Mayerle et al., 2003) and mouth (Muzio et al., 2002), and in colorectal polyps (Valizadeh et al., 1997). This is consistent with the fact that decreased cadherin expression, a frequent observation in human cancers, leaves p120^{ctn} stranded in the cytosol (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999) and perhaps competent for nuclear translocation. The significance of nuclear-localized p120^{ctn} will become increasingly evident as we identify and characterize gene targets of its nuclear binding partner, the transcriptional repressor Kaiso.

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