Specific sequences in p120ctn determine subcellular distribution of its multiple isoforms involved in cellular adhesion of normal and malignant epithelial cells

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Summary

P120 catenin (p120ctn) belongs to the Armadillo family of proteins, which is implicated in cell-cell adhesion and signal transduction. Owing to alternative splicing and multiple translation initiation codons, several p120ctn isoforms can be expressed from a single gene. All p120ctn isoforms share the central Armadillo repeat domain but have divergent N- and C-termini. Little is known about the biological functions of the different isoforms. In this study, we examined the distribution of various p120ctn isoforms and the consequences of their expression in cultured cells of epidermal origin. Immunohistochemical analysis and western blotting revealed that melanocytes and melanoma cells primarily express the long isoform 1A, whereas keratinocytes express shorter isoforms, especially 3A, which localize to cell-cell adhesion junctions in a calcium-dependent manner. The shortest isoform 4A, which was detected in normal keratinocytes and melanocytes, was generally lost from cells derived from squamous cell carcinomas or melanomas. The C-terminal alternatively spliced exon B was present in the p120ctn transcripts in the colon, intestine and prostate, but was lost in several tumor tissues derived from these organs. To test whether p120ctn isoforms serve in distinct biological functions, we transiently transfected the expression constructs into melanoma cells (1205-Lu) and immortalized keratinocytes (HaCaT). Indeed, distinct domains of p120ctn are responsible for its different biological functions. The prominent branching phenotype was induced equally by isoforms 1A, 2A and 3A, whereas the shortest isoform 4A, which was devoid of the N-terminal domain, completely lacked this ability. Also, the exon-B-encoded sequences, as in the isoform 1AB, were sufficient to abolish the branching phenotype as induced by the isoform 1A. The induction of the branching phenotype cosegregated with the nuclear localization of the p120ctn isoforms 1A, 2A and 3A, whereas the isoforms 4A and 1AB, which were excluded from the nucleus, did not induce the branching phenotype. The N-terminal sequences that contain seven out of eight tyrosine residues, recently characterized as potential candidates for phosphorylation by Src kinase, are required for the nuclear localization and for the formation of the branching phenotype. Finally, expression of the p120ctn isoforms, which caused the branching phenotype, was associated with cellular relocalization of E-cadherin in HaCaT cells. Collectively, we have identified sequences within the p120ctn N-terminus that are prerequisites for both nuclear localization and the p120ctn-induced branching phenotype. Loss of the cytoplasmic pool of p120ctn from tumor cells suggests an important function for such isoforms in normal cells and tissues.

Key words: Catenin, Cadherin, Cell-cell adhesion, Cell morphology, Alternative splicing, Protein domains

Introduction

Catenins form a family of proteins implicated in regulation of cadherin-based cell-cell adhesion and transcription. Early interest in the catenins was spurred by the observations that the prototypic member, β-catenin, has oncogenic properties and that β-catenin-dependent transcription is negatively regulated by the APC tumor suppressor gene (Nollet et al., 1999; Provost and Rimm, 1999; Semb and Christofori, 1998). Similar to β-catenin, p120 catenin (p120ctn) contains an Armadillo repeat (Arm) domain and directly interacts with cadherins (Daniel and Reynolds, 1995; Reynolds et al., 1994; Shibamoto et al., 1995; Staddon et al., 1995). However, several differences between β-catenin and p120ctn have become obvious in recent years, supporting the notion that these molecules serve non-redundant and possibly quite different roles in cell biology. Although both molecules can bind to the cytoplasmic tail of E-cadherin, their binding sites are different. Specifically, whereas β-catenin interacts with the catenin-binding domain (CBD) of cadherins, p120ctn binds to the immediate juxtamembrane domain (JMD) of E-cadherin (Ohkubo and Ozawa, 1999; Thoreson et al., 2000; Yap et al., 1998). Furthermore, both molecules physically associate with a number of transcription factors. However, β-catenin activates LEF1/TCF-dependent transcription by physical association with LEF (Behrens et al., 1996), whereas p120ctn interacts with the transcription factor KAISO (Daniel and Reynolds, 1999). The functional consequences of the latter interaction are poorly understood at present.

Unlike β-catenin, multiple p120ctn isoforms are generated through extensive splicing and alternative usage of translation

initiation codons (Keirsebilck et al., 1998; Mo and Reynolds, 1996). The Armadillo domain of p120ctn facilitates interaction with the intracellular JMD of E-, N- and C-cadherins within the adhesion complexes (Reynolds et al., 1996; Thoreson et al., 2000). Originally p120ctn was identified as a major substrate for tyrosine phosphorylation (Reynolds et al., 1989). Recently, eight tyrosine residues within the N-terminal domain were identified as major sites of Src-induced p120ctn phosphorylation (Mariner et al., 2001). The N-terminus of p120ctn is responsible for the interaction with the cytoplasmic tyrosine kinase FER (Kim and Wong, 1995). Also, the RPTPμ-interaction domain of p120ctn maps to its unique N-terminus, a region distinct from the cadherin-interacting domain (Zondag et al., 2000). The p120ctn isoform 3A was identified as a main interaction partner of the protein-tyrosine phosphatase SPH-1 (Keilhack et al., 2000). The N-terminus of the isoform 3 also interacts with the N-terminal globular domain of BPAG2/BP180/type XVII collagen, a transmembrane protein primarily located to hemidesmosomal attachment complexes in epidermal basal keratinocytes (Aho et al., 1999).

Small GTPases of the Rho subfamily are signaling molecules primarily involved in remodeling of the cytoskeleton, but they also contribute to cadherin-mediated cell adhesion (Braga, 2000). Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia (Nobes and Hall, 1995). The interaction of p120ctn with Rho family GTPases has been recently described (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). Transfection experiments have shown that p120ctn can induce a dramatic effect on the morphology of fibroblasts (Reynolds et al., 1996). Specifically, ectopic overexpression of p120ctn was associated with the formation of cellular extensions reminiscent of filopodia and dendrites. The intact Armadillo domain and the C-terminal domain were required for the induction of the branching phenotype (Reynolds et al., 1996). Thus p120ctn can regulate cell adhesion and motility through the actin cytoskeleton via Rho family GTPases.

An infrequent splicing event inserts an exon C between the Armadillo repeats 6 and 7 of p120ctn, thus disrupting a potential nuclear localization signal (Keirsebilck et al., 1998). Furthermore, splice variants of the extreme C-terminus of p120ctn have been identified (Keirsebilck et al., 1998; Mo and Reynolds, 1996). Murine transcripts appear either with or without a C-terminal exon A (Mo and Reynolds, 1996), although the exon A is regularly included in human transcripts (Keirsebilck et al., 1998). A putative nuclear export signal with a characteristic leucine-rich motif is encoded by another alternatively spliced C-terminal exon, exon B (van Hengel et al., 1999).

In this study, we took advantage of the branching cell phenotype to investigate whether different p120ctn isoforms serve distinct roles in cell adhesion and motility. We show that both the N-terminal and C-terminal sequences are essential for this phenotype, as is the Arm domain. Furthermore, we provide evidence that nuclear localization of p120ctn cosegregates with the branching phenotype and that the presence of a C-terminal exon B abolishes both nuclear localization and the branching phenotype. The opposite effects of p120ctn isoforms on cellular morphology may have physiological relevance, as we demonstrate lineage-specific expression patterns in two major epithelial cell types, that is, keratinocytes and melanocytes, and disclose alterations of p120ctn expressed in tumor cells.

Materials and Methods

Generation of expression plasmids

A cDNA encoding p120ctn isoform 1A was generated using PCR. The control template from a Multiple Tissue cDNA Panel (Clontech) was used as a template for PCR. The sequence encoding the FLAG-tag (underlined) was incorporated into the isoform-1-specific primer 5′-ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG ATG GAC GAC TCA GAG GTG GA-3′. The reverse primer 5′-CCC AGA TGG AAC GGA GAT-3′ was complementary to the sequence 8 to 25 nucleotides 3′ from the p120ctn translation stop codon. The PCR-product was ligated into *Eco*RV digested dideoxy-T tailed pcDNA3. The FLAG-tagged p120ctn isoforms 2, 3 and 4 were generated using the 5′-primers with the FLAG-tag sequence as above, joined to the isoform-specific 5′ sequence at either Met 55, Met 102 or Met 324 (according to the GenBank #AF062341). A shortened pcDNA3, with a 1.6-kb *Nae*I fragment deleted, was used as a cloning vector to generate expression constructs 2A and 3A.

The expression constructs for the N-terminal domains (N1, N3) were generated with the FLAG-tagged isoform-specific primers as above and with the reverse primer 5′-ATC CGA TGG CAC CTC CTC A-3′, which was complementary to the N-terminal sequences of p120ctn isoform 4. For the expression studies, the PCR products were ligated into pcDNA3 vector as above. The N-terminal construct of isoform 1 (pcDNA3-N1) was further truncated by *Apa*I digestion and religation, which generated a cDNA encoding a polypeptide of 258 amino acids.

The C-terminally deleted isoform 1ΔX (1A deletion *Xho*I) was generated from pcDNA3-1A by *Xho*I digestion and religation, which truncated the C-terminus from the seventh Armadillo repeat at amino acid 653. The isoform 1AB was obtained by replacing an *Xho*I fragment from pcDNA3-1A with an *Xho*I fragment containing the alternatively spliced exon B generated by PCR with primers 5′-GTG TTT GCC TTC TTC GGA ATC-3′ and 5′-CCC AGA TGG AAC GGA GAT-3′, and using the colon adenocarcinoma CX-1 cDNA as a template (Tumor Tissue MTC Panel, Clontech). The PCR-product was first ligated into Bluescript, from which the *Xho*I fragment was separated and subcloned into *Xho*I-digested pcDNA3-1A. The pcDNA3-1AB was used as a template for in situ mutagenesis using the QuickChange Mutagenesis kit (Stratagene). The double mutant L951A, L953A was generated as described previously (van Hengel et al., 1999).

An N-terminal deletion construct 2AΔNS (2A deletion *Nae*I-*Sma*I) was prepared from pcDNA3-2A through deleting a 510 bp *Nae*I-*Sma*I fragment, which resulted in the deletion of amino acids 63-232. Another N-terminal deletion construct 3AΔAK (3A deletion *Avr*II-*Kas*I) was prepared from pcDNA3-3A through deleting a 341 bp *Avr*II-*Kas*I fragment. The ligation, after filling the ends with the Klenow-fragment of DNA polymerase I, resulted in the in-frame expression construct with amino acids 205-316 omitted. The putative bipartite nuclear localization signal, encoded by amino acids 306-319, was deleted from expression plasmids pcDNA3-2A and pcDNA3-3A. The plasmids were linearized with *Kas*I, treated with S1 nuclease and recircularized with T4 DNA ligase. The in-frame ligations resulted in the expression constructs pcDNA3-2AΔNLS (2A deletion nuclear localization signal), encoding isoform 2A with amino acids 310-326 deleted, and pcDNA3-3AΔNLS (3A deletion nuclear localization signal), encoding isoform 3A with amino acids 305-325 deleted.

The N-terminal 1-kb *Eco*RI-fragment from Ubinuclein (Aho et al., 2000) was joined into the *Eco*RI site at the 5′-end of cDNA encoding p120ctn isoform 4 in the expression plasmid pcDNA3-4A. The in-frame fusion produced an expression plasmid pcDNA3-4A+Ubi NLS. As the second approach, the complementary oligonucleotides

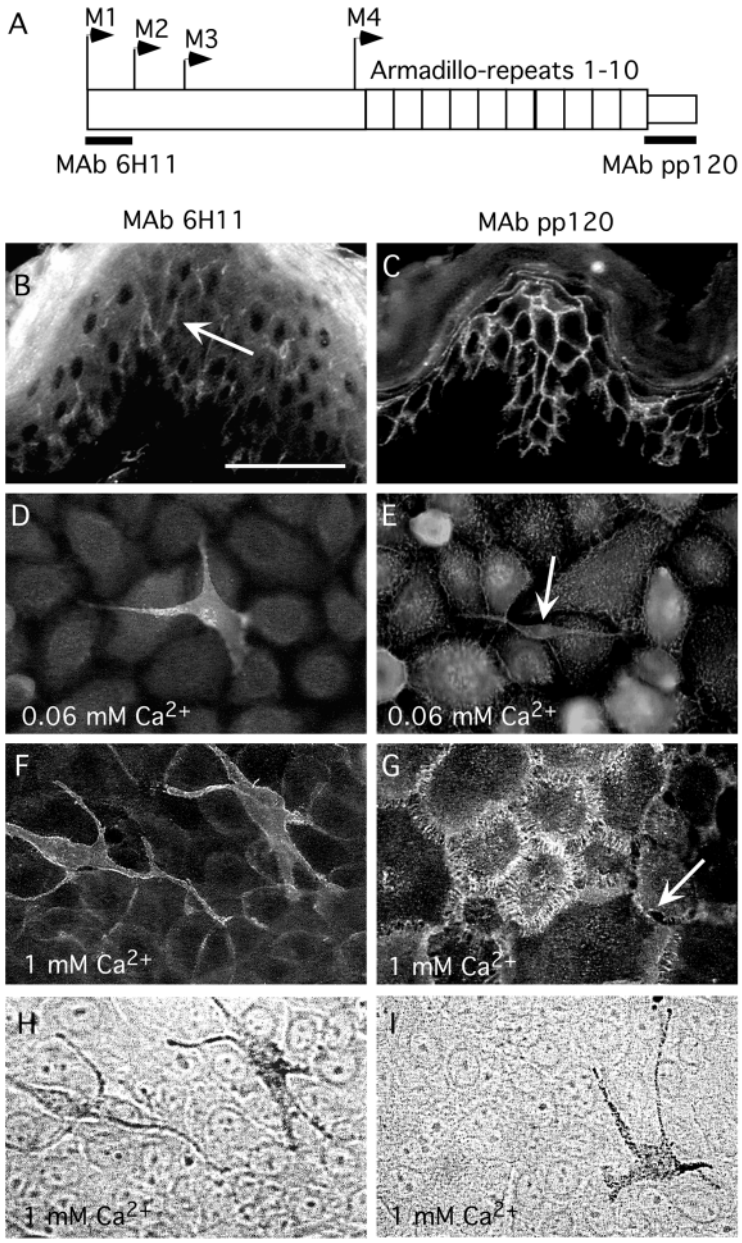


Fig. 1. Differential expression of p120ctn isoforms in epidermal keratinocytes and melanocytes. Horizontal bars along the schematic structure of p120ctn (A) show the domains recognized by MAb 6H11 and MAb pp120. Cryosections of adult human epidermis (B,C) showed prominent immunofluorescence with MAb pp120 at the cell-cell adhesion junctions (C). Melanocytes residing within the basal cell layer, and their dendrites extending up to the upper spinous layer, were positive for MAb 6H11 (B, arrow). The prominent staining of the cornified layer with MAb 6H11 was considered non-specific and was not characterized further. Keratinocytes in primary cultures, when grown in KGM containing 60 μM Ca^{2+} were negative for MAb 6H11 signal (D), whereas MAb pp120 stained intracellular structures and gave a weak signal at the cell-cell adhesion junctions (E). Keratinocyte cultures grown in the presence of 1 mM Ca^{2+} (F-I) were also negative for MAb 6H11 (F), whereas pp120 brightly stained keratinocyte cell-cell adhesion junctions (G). Melanocytes (arrows in E and G) were difficult to distinguish with MAb pp120 owing to the intense staining of keratinocytes. Bright staining of melanocytes with MAb 6H11 (D,F) revealed that 1A is the major p120ctn isoform in melanocytes. Phase contrast images (H,I) of panels (F) and (G) demonstrate the pigmented melanocytes against the keratinocyte background.

encoding three copies of SV40 bipartite nuclear localization signal were annealed and ligated at the 5′-end of the cDNA encoding isoform 4A, producing the expression construct pcDNA3-4A+SV40 NLS.

The cDNAs produced by PCR were sequenced and compared with the cDNA sequence in the GenBank (Accession number AF062341). DNA sequences were obtained using the PRISM Ready Reactions DyeDeoxy Terminator Cycle sequencing Kit and the Applied Biosystems Models 373A and 377 DNA sequencing systems (The Nucleic Acids Facilities, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). The sequences were edited and analyzed using MacVector 6.0 (Oxford Molecular).

Cell cultures and transfections

Normal human keratinocytes (NHK) were derived from neonatal foreskins and propagated in KGM medium, adjusted to 60 μM calcium, and supplemented with growth factors (Bullet kit) as supplied by the manufacturer (Clonetics). HaCaT keratinocyte cultures (Boukamp et al., 1988) were maintained in the W489 medium supplemented with 2% FCS (Rodeck et al., 1987). FM516Sv3/3 human immortalized melanocytes were described previously (Melber et al., 1989). Human melanoma cell line 1205-Lu is a variant of WM164 cells, selected for metastatic capacity through serial passages in nude mice (Juhasz et al., 1993). The squamous carcinoma cell lines SCC12, A253, FADU and DET562 were a generous gift from J. Rheinwald. A431 squamous carcinoma cells were obtained from ATCC (Rockville, MD).

For the transient transfections, cells were plated on the chamber slides or on the 35 mm dishes, 1205-Lu cells for 4-6 hours and HaCaT cells for 6-8 hours. Transfections were performed using FUGENE-6 according to the manufacturer's instructions (Roche). 1205-Lu cells were further incubated in W489 medium supplemented with 2% FCS for 16 hours before preparing for immunofluorescence detection. HaCaT cells were also incubated in W489 medium supplemented with 2% FCS for 16 hours, then the serum-free KGM-medium with 1 mM Ca^{2+} was changed and the incubation was continued for additional 8 hours.

Before immunological detection, cells grown on the chamber slides or tissue culture dishes were washed three times with PBS (phosphate buffered saline).

Immunological detection

A FLAG-tag-specific monoclonal antibody M2 (Stratagene) was used at 1:1000 dilution for both immunoblotting and indirect immunofluorescence (IIF). A rabbit polyclonal antibody against FLAG-tag was obtained from Abcam Ltd. (www.abcam.com) and used in 1:500 dilution for IIF. The pp120 monoclonal antibody, detecting all p120ctn isoforms, was purchased from Transduction Laboratories and used in 1:2000 dilution. Monoclonal antibody 6H11 was a generous gift from A. Reynolds (Vanderbilt University, Nashville, TN). It was used at 1 pg/ml to detect the long p120ctn isoform 1 (Wu et al., 1998). The E-cadherin monoclonal antibody was obtained from Transduction Laboratories and used at a 1:1000 dilution. The species-specific anti-mouse and anti-rabbit secondary antibodies, conjugated to FITC, Texas Red or horseradish peroxidase were from Jackson Laboratories (West Grove, PA).

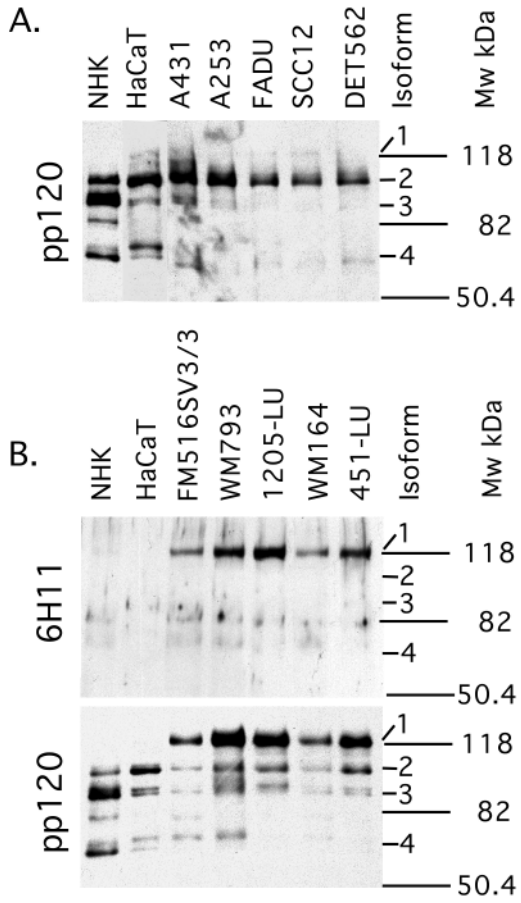


Fig. 2. Western blot analysis of p120ctn isoforms in normal and malignant epithelial cells. Cell lysates of neonatal human keratinocytes (NHK), HaCaT cells, squamous cell carcinomas (A431, A253, FADU, SCC12, DET562) (A) and normal and malignant melanoma cells (FM516SV3/3, WM793, 1205-LU, WM164, 451-LU) (B) were resolved on 6% PAGE and proteins detected with the MAb pp120 (A, and lower panel in B) and with MAb 6H11 (B, upper panel). The position of the bands corresponding to p120ctn isoforms 1 to 4 are shown on the right side of the blot together with the positions of molecular weight markers. Note that the isoform 1 is specific for melanocytic cells, whereas normal keratinocytes express predominantly isoform 3. A slower migrating band either corresponding to isoform 2 or to the phosphorylated form of isoform 3 is prominent, especially in squamous carcinoma cells. Isoform 4 is present in normal keratinocytes but is largely lost from the tumor cells.

For western blotting, cells were lysed directly on the tissue culture dishes, and proteins were extracted using SDS-loading buffer (Bio-Rad). Proteins were separated by SDS-PAGE and transferred onto the PVDF membrane (New England Nuclear Life Science, Boston, MA). P120ctn polypeptides were detected with primary antibodies as indicated in the figure legends, followed by the anti-mouse HRP-conjugated secondary antibody (Jackson Laboratories, West Grove, PA), and visualized using the Renaissance western blot chemiluminescence reagent (New England Nuclear Life Science Products).

A section of human adult skin, embedded and frozen in the OCT compound, was cut into 7 μm cryosections, which were air dried and stored at –20°C. Slides with cryosections, or the chamber slides with cells, were washed three times with PBS and fixed in methanol at –20°C for 5 minutes. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature, washed three times with PBS and blocked with 1% BSA (bovine serum albumin) in PBS for 1 hour at room temperature. The primary antibodies were applied on the samples overnight at 4°C. After four washes with PBS, samples were incubated for 1 hour at room temperature with the species-specific secondary antibody conjugated to FITC or Texas Red and 0.00002% DAPI, washed four times with PBS and mounted for viewing. Sections and slides were evaluated by fluorescence microscopy (Axioskop; Carl Zeiss, Inc.), images were stored with ImagePro Plus 4.0 imaging software (Media Cybernetics) and processed with Photoshop 5.0 (Adobe Systems Inc.) and Canvas 5 (Deneba Software).

Expression studies by PCR

Human multiple tissue cDNA panels (Human MTC Panels I and II and Human Tumor MTC Panel) were obtained from Clontech and used as templates for PCR analysis. The p120ctn-specific primers, 5′-GAAGCCAGAGCAGTCATTCA-3′ and 5′-CCCAGATGGAACGGAGATA-3′, produced a PCR fragment of 273 bp, when exon B was not included in the template, and a fragment of 360 bp, when exon B was present in the cDNA. PCR conditions were 2 minutes at 94°C, followed by 38 cycles of 94°C for 20 seconds, 58°C for 30 seconds and 72°C for 1 minute. PCR was conducted using Taq DNA Polymerase and the Q-solution provided with the kit (Qiagen). The PCR-products were separated on 1.5% agarose-TBE gels.

Results

Divergent p120ctn isoform expression patterns in keratinocytes and melanocytes

Previous work has shown that different p120 catenin isoforms are expressed in a tissue- and cell-type-specific manner (Aho et al., 1999; Mo and Reynolds, 1996; Montonen et al., 2001). To investigate p120ctn expression in human epidermis, we used two antibodies that recognize either a C-terminal epitope shared between all known p120ctn isoforms (MAb pp120) or an epitope exclusive to the longest p120ctn isoform (MAb 6H11; see for schematic view of epitope specificity Fig. 1A). Immunohistochemical analysis of adult human epidermis with these two antibodies revealed distinct staining patterns (Fig. 1B,C). Whereas the MAb pp120 strongly and uniformly stained cell-cell adhesion junctions throughout the epidermis, MAb 6H11 immunoreactivity was generally weaker and irregularly distributed. To further investigate the cause for these divergent staining patterns, we performed immunocytochemistry on primary keratinocyte cultures. These experiments were done using different Ca^{2+} concentrations (60 μM versus 1 mM) as calcium at concentrations >100 μM is required to enable E-cadherin function and recruitment of p120ctn to the cell membrane (Owens et al., 2000). As expected, strong staining of keratinocyte cell membranes and cell-cell adhesion junctions was observed with the pan p120ctn antibody MAb pp120 at 1 mM Ca^{2+}, whereas at low Ca^{2+} concentrations (0.06 mM) p120ctn staining was primarily cytoplasmic and punctuated (Fig. 1G,E, respectively). Consistent with the in situ staining pattern in the epidermis, MAb 6H11 showed only weak reactivity at either low or high ambient Ca^{2+} levels. The primary cultures used in these experiments also contained melanocytes with characteristic dendritic morphology. The identity of these cells as melanocytes was confirmed by the presence of brownish pigment when examined by phase contrast microscopy (Fig.

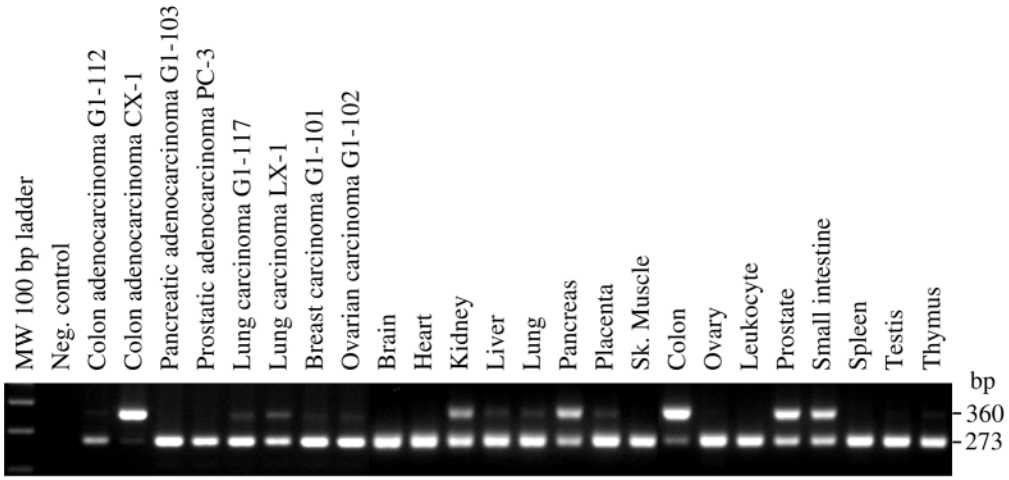
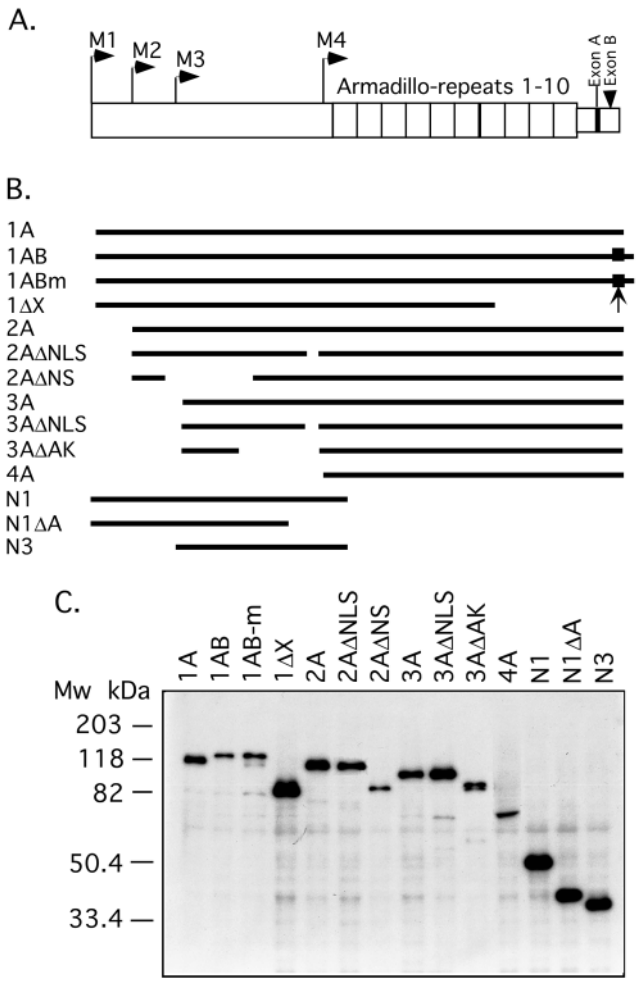


Fig. 3. RT-PCR analysis demonstrating the tissue-specific expression of p120ctn transcripts containing sequences encoding the C-terminal exon B. The tumor tissue cDNA panel and Human Multiple Tissue cDNA panels I and II (Clontech) were used as templates for PCR. The primers produced a 360 bp product, when exon B was included, and a 273 bp fragment when exon B was not present in the transcripts encoding p120ctn. While present in normal pancreas, prostate and colon, exon B is lost from the adenocarcinomas originating from the corresponding tissues.

1H,I). In contrast to the keratinocytes, these cells reacted strongly with the MAb 6H11 in both low and high Ca^{2+} (Fig. 1D,F), suggesting that melanocytes primarily express the longest p120ctn isoform. In high Ca^{2+} the 6H11 staining localized to the melanocyte cell membrane, suggesting a role for both p120ctn isoforms in E-cadherin-mediated cell-cell adhesion in the epidermis.



Expression of multiple p120ctn isoforms in normal and malignant epithelial cells

To further evaluate the expression of different p120ctn isoforms in keratinocytes and melanocytic cells, we performed immunoblot analysis using extracts of these cells and transformed cells derived from these lineages. As show in Fig. 2, the MAb pp120 recognized four major p120 bands in the immunoblot analysis. Consistent with the immunohistochemical results, three major protein species, excluding isoform 1, were identified in neonatal keratinocyte lysate (NHK). Bands 2, 3 and 4 appeared to be tightly spaced doublets, either owing to the alternative usage of exons A, B or C or as a result of post-translational modifications. HaCaT cells, which are immortalized but non-tumorigenic keratinocytes, contained a similar set of isoforms. A panel of five squamous carcinoma cell lines expressed mainly band 2 but not bands 3 and 4. Consistent with the immunohistochemical results in Fig. 1 and in contrast to keratinocytes, immortalized melanocytes (FM516SV3/3) and a panel of four melanoma cell lines expressed primarily the long p120ctn isoform 1 (Fig. 2B, upper panel), although they coexpressed shorter isoforms to a lesser degree (Fig. 2B, lower panel). The monoclonal antibody 6H11 detected a single band corresponding to the isoform 1 only in melanocyte and melanoma cell extracts but not in extracts from either normal keratinocytes or HaCaT cells. This finding confirms the notion that the expression of p120ctn isoform 1 is characteristic of melanocytes.

Fig. 4. Expression constructs encoding p120ctn isoforms, mutants and domains. Organization of p120ctn (A). Four alternative translation initiation sites within the N-terminal globular domain are shown as M1 to M4. The central part of p120ctn contains 10 Armadillo repeats. Two alternatively spliced exons, A and B, reside within the C-terminal domain. (B) Expression constructs encoding the p120ctn isoforms, deletions and domains, attached to the N-terminal FLAG-tag, were cloned into pcDNA3-vector. Proteins expressed by 1205-Lu cells, transiently transfected with each expression construct, were separated on a 9% PAGE, and the MAb M2 was used to demonstrate the FLAG-tagged proteins on the western blot (C). MW, molecular mass markers.

C-terminal sequences encoded by exon B contribute to additional p120ctn isoforms

Additional p120ctn isoforms are generated through the alternative splicing of an exon B, which encodes a 29 amino acid peptide including a nuclear export signal (van Hengel et al., 1999). Our RT-PCR results indicated that exon B was not included in p120ctn transcripts either from the epidermal derived keratinocytes or the squamous carcinoma cells (data not shown), although it has been generally detected in most epithelial cells (van Hengel et al., 1999). RT-PCR analysis of Multiple Tissue cDNA panels demonstrated exon B sequences prominently present in p120ctn transcripts from pancreas, prostate, colon and small intestine, whereas the tumor tissues derived from colon (G1-112), pancreas (G1-103) and prostate (PC-3) had lost exon-B-containing transcripts (Fig. 3). A weak signal also indicated the presence of exon B in normal kidney, liver, lung and placenta, as well as in lung, breast and ovarian carcinomas (Fig. 3).

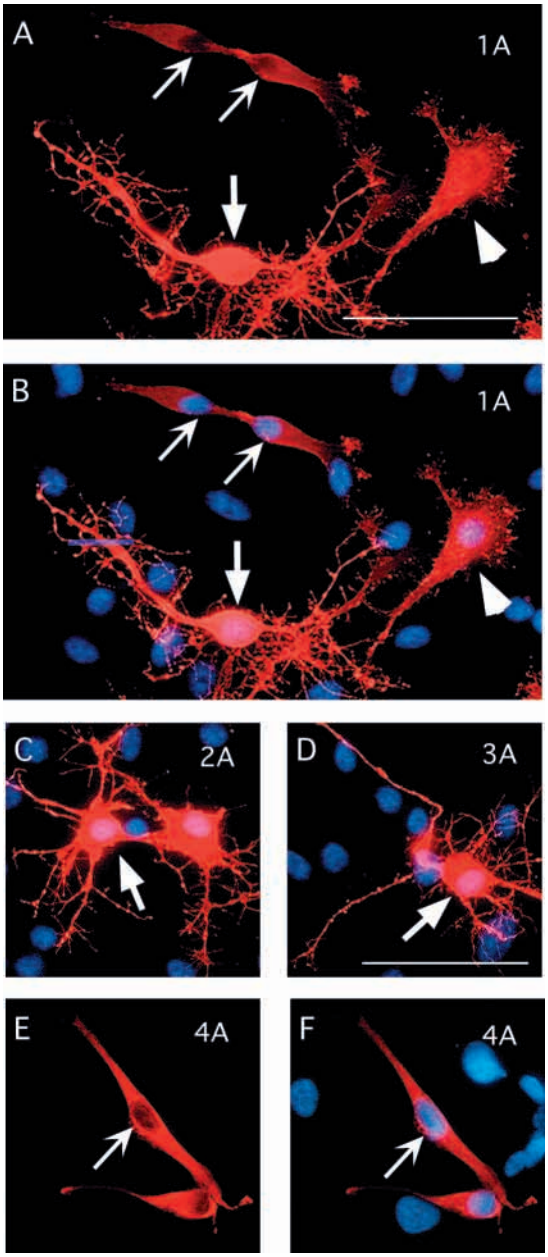


Fig. 5. The phenotype induced by p120ctn isoforms transiently transfected into 1205-Lu melanoma cells. The expression of the FLAG-tagged polypeptides was demonstrated with MAb M2 16 hours after transfection (red signal), and nuclei were visualized with DAPI staining (blue signal). Isoforms 1A (A, B), 2A (C) and 3A (D) gave a strong signal throughout the cell, including the nucleus, and induced prominent branching phenotype (thick arrows). In some cells the signal was excluded from the nucleus (A,B,E,F; thin arrows), and these cells did not show the branching phenotype. The intermediate phenotype showed nuclear localization and lamellipodia (arrowheads in panels A,B). The isoform 4A was cytoplasmic and clearly excluded from the nucleus and did not induce the branching phenotype (E, F). Bar, 50 μm.

N-terminal sequences of p120ctn are required for the branching cell phenotype

The distinct expression patterns of p120ctn in epidermal cell types raised the question of whether the observed differences in the composition of the p120ctn N-terminus have functional consequences. To address this question we turned to a bioassay based on the capacity of the p120ctn to induce extensive cellular extensions when overexpressed in fibroblasts (Reynolds et al., 1996). To this end, FLAG-tagged expression constructs were developed: (a) p120ctn isoforms 1AB, 1A, 2A, 3A and 4A; (b) N-terminal sequences N1, N3 and N1ΔA; (c) the isoform 4A equipped with additional nuclear localization sequences; and (d) the isoforms containing both N-terminal and C-terminal modifications (Fig. 4) and transiently transfected into 1205-Lu melanoma cells and immortalized keratinocytes (HaCaT). A FLAG-tag-specific antibody was used to demonstrate by immunoblotting that each construct was correctly expressed after transient transfection into melanoma cells 1205-Lu (Fig. 4C) and HaCaT cells (not shown).

Similar to previously published observations in fibroblasts (Anastasiadis et al., 2000; Reynolds et al., 1996), p120ctn isoforms 1A, 2A and 3A induced prominent cellular extensions resembling dendrites, filopodia and lamellipodia in melanocytic cells (Fig. 5A-D). As demonstrated with isoform 1A, the intensity of the signal showed a positive correlation with the cellular phenotype. Lower expression of the transgene was detected only in the cytoplasm, although in a cell with more intense staining, the FLAG-tagged protein expressed from the transgene entirely filled the nucleus, lamellipodia and filopodial extensions. Finally, a cell demonstrating an extremely strong signal appeared as a rounded cell body from which dendritic-like extensions and numerous filopodia protruded across the field of neighboring cells (Fig. 5A,B). The isoform 4A did not induce dendrites, filopodia or lamellipodia, and the staining was detected exclusively in cytoplasm (Fig. 5E,F).

Multiple targeting signals determine the subcellular localization of p120ctn

The presence of a nuclear localization sequence encoded by the N-terminal sequences of p120ctn was confirmed by using FLAG-tagged expression constructs encoding the N-terminus of p120ctn isoform 1 (N1), isoform 3 (N3) and N1 truncated after amino-acid residue 258, deleting 88 amino acids upstream

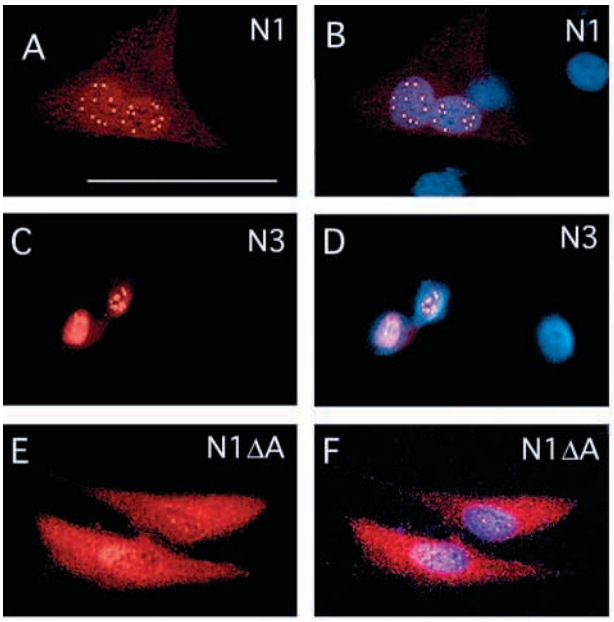


Fig. 6. Demonstration of functional nuclear localization signal within the N-terminus of p120ctn. Both expression constructs, N1 and N3, localized in the nucleus (A-D), whereas the C-terminally deleted N1ΔA (see Fig. 4) demonstrated diffuse staining throughout the cell (E,F). Transiently transfected 1205-Lu cells were detected with MAb M2 (A,C,E) and the nuclei demonstrated with DAPI (B,D,F). Bar, 50 μm.

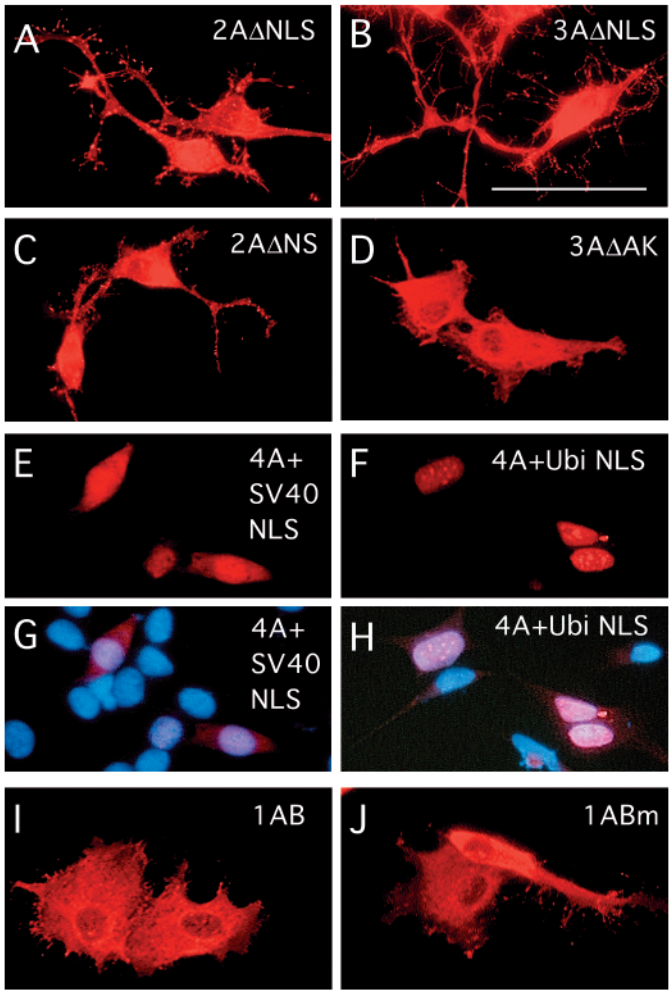


Fig. 7. Effects of deletion mutants and modifications of p120ctn on their subcellular distribution and on the morphology of 1205-Lu cells. The branching phenotype induced by isoforms 2A and 3A was not affected by the deletion of the N-terminal nuclear localization signal (A,B). The deletion of amino acids 63-232 from isoform 2A (2AΔNS) did not completely abolish the branching phenotype (C), but the deletion construct of isoform 3A (3AΔAK), with amino acids 205-316 deleted, showed predominantly cytoplasmic localization and only a few lamellipodial protrusions of transiently transfected 1205-Lu cells (D). The nuclear localization of isoform 4A, induced either through the SV40 nuclear localization signal (E,G) or ubinuclein nuclear localization domain (F,H) joined to the amino terminus of 4A, did not induce the branching phenotype in transiently transfected 1205-Lu cells. When the alternatively spliced exon B was included in the transcript (1AB), it abolished both the nuclear localization and the prominent branching phenotype induced by isoform 1A (I). However, the site-directed mutagenesis of the nuclear export signal within exon B (1ABm) was not sufficient to restore the phenotype induced by isoform 1A (J). Red signal, MAb M2. Blue signal, DAPI. Bar, 50 μm.

from the Armadillo domain (N1ΔA). Upon transfection into 1205-Lu cells, the N1 and N3 polypeptides were observed in the nucleus (Fig. 6A-D). The C-terminally deleted N1 showed primarily cytoplasmic localization, although diffuse nuclear staining was observed as well (Fig. 6E,F). Detailed examination of the amino-acid sequence within the 88 amino-acid stretch revealed a putative bipartite nuclear localization signal at amino acids 306-319 to be responsible for the nuclear localization of N1 and N3. The diffuse nuclear staining detected with N1ΔA is probably due to the fact that small proteins (less than 40 kDa) do not possess a specific targeting signal and can enter through the nuclear pores without a specific nuclear localization signal.

Because the N-terminal sequences of p120ctn isoform 3A seemed to be important for both nuclear localization and branching phenotype, we postulated that the N-terminal nuclear localization signal is responsible for these functions. However, the cellular phenotype obtained by the isoforms 2A and 3A from which the N-terminal nuclear localization signal was deleted, 2AΔNLS and 3AΔNLS (Fig. 7A,B), were indistinguishable from the wild-type isoforms 2A and 3A (compare with Fig. 5C,D). The deletion of amino acid 63-232 (2AΔNS) (Fig. 7C) resulted in milder phenotype compared with that of isoform 2A (Fig. 5C), whereas the deletion of amino acids 205-316 (3AΔAK) abolished the nuclear localization and the branching phenotype, although some lamellipodia were still detectable at the lateral cell membranes (Fig. 7D).

Although the isoform 4A contained intact Armadillo- and C-terminal domains, its location was found to be strictly cytoplasmic and did not confer a distinct branching phenotype (see Fig. 5E,F). To test the hypothesis that bringing the isoform 4A into the nucleus would induce the branching phenotype, three copies of the SV40 nuclear localization sequences were attached N-terminally to the p120ctn isoform 4A. The corresponding protein (4A+SV40 NLS; Fig. 7E,G) localized both in the nucleus and in the cytoplasm, but it did not induce the branching phenotype. Also, when the N-terminus of ubinuclein was attached to the p120ctn isoform 4A, the fusion

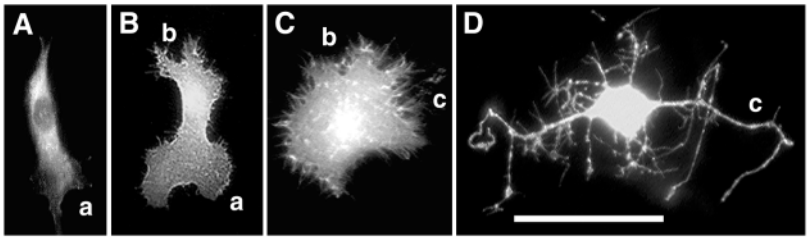


Fig. 8. The spectrum of cellular phenotypes induced by p120ctn isoforms. P120ctn isoform 1A, expressed in 1205-Lu cells, fills up the entire cell volume, but less dense staining is evident in the cell nucleus (A) and in the lamellipodial extensions (a in panels A,B). Some cells demonstrate flattening and extensive lamellipodial protrusions (B,C). Thin filopodia (b in panels B,C) emerge from the cell body. The extending filopodia form branches (c in panel C). In the fully developed branching phenotype (D), dendritic extensions send out secondary branches (c in panel D). 1205-Lu cells grown in the chamber slides were transiently transfected with p120ctn isoforms and deletion constructs in parallel. Slides were processed for indirect immunofluorescence 16 hours later. Cells positive for the FLAG-tag-specific antibody M2 in each 0.7 cm^2 well were counted and divided into four categories (A-D) on the basis of their appearance. The percentage of cells in each category is shown (E). The total number of cells assessed in each well varied from 150 to 350. Bar, 50 μm.

E Percentage of cells displaying cellular phenotypes A-D

Construct	A	B	C	D
1A	20	21	15	44
2A	27	25	16	32
3A	24	19	21	36
4A	100	0	0	0
1AB	92	5	2	1
1ABm	89	8	3	0
2AΔNLS	26	19	30	25
3AΔNLS	48	16	11	25
2AΔNS	64	20	5	11
3AΔAK	78	17	2	3

protein (4A+Ubi NLS; Fig. 7F,H) was detected in the nuclei but no branching phenotype was induced. These results showed that neither the previously characterized nuclear localization sequence within the Armadillo-repeat region, between the repeats 6 and 7, nor the putative nuclear localization sequence within the N-terminus are sufficient or necessary for the nuclear localization. Instead, the critical region for nuclear localization is located between amino acids 233 and 303, the region that contains five out of eight tyrosine residues phosphorylated by Src in p120ctn (Mariner et al., 2001).

Previously, a nuclear export signal has been identified in human exon B of p120ctn (van Hengel et al., 1999). Consistent with this earlier report, exon B, when included in an expression construct encoding the p120ctn isoform 1A, led to nuclear exclusion of the transgene (isoform 1AB; Fig. 7I). Importantly, the p120ctn isoform 1AB did not induce cellular branching, whereas some flattened cells with lamellipodia were detected. This is consistent with the observation of nuclear localization cosegregating with the branching phenotype. When the nuclear export signal was altered through site-directed mutagenesis as described previously (van Hengel et al., 1999), a slight change in the cellular phenotype was detected (1ABm, Fig. 7J), but the striking branching phenotype of isoform 1A was not observed.

Cells transfected with p120ctn isoform 1A displayed a spectrum of phenotypes, suggesting that the branching phenotype develops gradually (Fig. 8A-D). Although some cells displayed the normal 1205-Lu phenotype, flattened cells with lamellipodia and filopodial extensions were also detected. The percentage of cells displaying long dendrites with multiple branches was time dependent (data not shown). When 1205-Lu cells were harvested for immunofluorescence 24 hours after transfection, a considerable number of cells expressing isoform 1A was detached, and only traces of branches could be detected on the slide. The population analysis of the 1205-Lu cells 16 hours after transfection with isoforms 1A, 2A and 3A revealed that 20-25% of transfected cells showed normal phenotype, and the rest of the transfected cells displayed the altered cell phenotype (Fig. 8E). As shown in Fig. 5 (E,F), the isoform 4A completely lacked the ability to induce a phenotypic change. Also, when the exon-B-encoded sequences were included in the p120ctn, the isoform 1AB lacked the ability to induce a branching phenotype, and only about 10% of cells developed lamellipodial and filopodial protrusions. As shown in Fig. 7 (A,B), the deletion of the putative nuclear localization signal immediately upstream from the Armadillo domain did not abolish completely the branching phenotype but resulted in a switch towards a milder phenotype, especially with isoform 3AΔNLS became evident (Fig. 8E). Further deletions within the N-termini of isoforms 2A and 3A resulted in 84% and 95% of cells, respectively, displaying milder phenotypes, as exemplified in categories A and B (Fig. 8).

Overexpression of p120ctn in HaCaT keratinocytes interferes with E-cadherin expression disrupting the cell-cell adhesion

In the epithelial cells, p120ctn binds to the juxtamembrane region of E-cadherin and thus localizes to the cell membrane in a Ca^{2+}-dependent manner. Keratinocytes primarily expressed shorter p120ctn isoforms, especially isoform 3A, which is known as an epithelial p120ctn isoform. Surprisingly, all three isoforms, 1A, 2A and 3A, and also the deletion construct 2AΔNS, colocalized with E-cadherin at the cell-cell junctions (Fig. 9B,J,P,K), but the overexpression of these transgenes downregulated the E-cadherin signal both in the cytoplasmic pool and at the cell-cell junctions (Fig. 9F,M,T,N). HaCaT cells, expressing high levels of these isoforms, lost the cell-cell contact and demonstrated the prominent branching phenotype (Fig. 9A,I,O), loosing the E-cadherin signal as well (Fig. 9E,L,S). This event is clearly demonstrated in Fig. 9, panels K and N, where a cell in the process of loosing the cell-cell contact on one side is negative for E-cadherin but on the opposite side still demonstrates E-cadherin staining along the cell-cell contact regions.

Isoforms 4A (Fig. 9R) and 1AB (Fig. 9C), as well as the deletion construct 3AΔAK (Fig. 9Q), were detected only in the

cytoplasmic pool and did not interfere with cell-cell contact and did not demonstrate visible effects on E-cadherin expression level (Fig. 9V,G,U, respectively). The C-terminally truncated p120ctn isoform 1 showed both nuclear and cytoplasmic staining (Fig. 9D) and did not interfere with E-cadherin subcellular localization (Fig. 9H).

Collectively, these results revealed that the N-terminal amino acids 233-303, the Armadillo-repeat region and the C-terminus devoid of exon B are necessary for p120ctn to interact with E-cadherin are necessary for p120ctn to interact with E-cadherin. Isoforms 1A and 2A, as well as the epithelial isoform 3A, when expressed at high levels, can either downregulate E-cadherin expression or relocate it from the cell-cell junctions, thus destabilizing cell-cell adhesion and resulting in the prominent branching phenotype.

Nuclear localization cosegregates with the capacity of p120ctn constructs to induce a branching phenotype

While monitoring the expression of the p120ctn constructs in HaCaT cells, we noticed distinct subcellular distribution patterns for the FLAG-tagged proteins. In each case they accumulated in the cytoplasm. However, as shown in Fig. 10, only those transgenes (1A, 2A, 2AΔNS and 3A) that were able to induce the branching cellular phenotype were present in the nuclei of the transiently transfected cells (Fig. 10A,D,E,F). By contrast, p120ctn isoforms 4A and 1AB, as well as 3AΔAK, were excluded from the nucleus upon transfection into either HaCaT cells (Fig. 10H,B,G) or melanoma cells (Fig. 5E; Fig. 7I,D) and did not possess a capacity to induce the branching phenotype in either cell type. Construct 1ΔX (Fig. 10C), with a C-terminal deletion, but with intact nuclear localization signals both in the N-terminus and within the Arm-domain, extensively accumulated into the nucleus but was not able to induce a branching cellular phenotype.

Discussion

Among the catenins, p120ctn stands out because multiple protein isoforms can be expressed from the same gene. These isoforms are composed of divergent sets of domains encoded by alternatively spliced exons, in conjunction with the use of alternative translation initiation codons. This report demonstrates that different p120ctn isoforms have distinct, and even opposite, effects on the cellular phenotype. A direct correlation was observed between the nuclear localization of each isoform and its capacity to induce the prominent branching phenotype. The extent of the cellular phenotype was cell-type dependent, thus attesting to how p120ctn isoforms, depending on the cellular background, may function in the fine tuning of cell motility and adhesion.

Upon cloning the human *p120ctn* gene, Keirsebilck et al. (Keirsebilck et al., 1998) recognized that different cell types contain multiple p120ctn transcripts because of alternative splicing and translation start codon usage. These authors concluded that theoretically 32 different p120ctn isoforms may exist and provided RT-PCR evidence for the expression of 11 distinct p120ctn mRNA species in various cell lines derived from epithelial and mesenchymal tissues. They also noted that much of the heterogeneity of p120ctn isoforms is due to N-termini of different length, which results from the usage of alternative start codons. In our earlier work, we discovered that the splicing pattern of p120ctn transcripts is unique for each human tissue and was consistent between each fetal and adult tissue but was random in the tumor tissues regardless of their origin (Aho et al., 1999).

Utilizing the monoclonal antibody specific for the isoform 1, we recently reported that the long isoform 1 is specifically expressed at the vascular-endothelial junctions in blood vessels, at the cell-cell junctions in the serosal epithelium lining the internal organs, in the choroid plexus of brain, in the pigment epithelium of retina and in the structures such as the outer limiting membrane of retina and intercalated discs of cardiomyocytes (Montonen et al., 2001). In those tissues either VE-cadherin or N-cadherin are the major adhesion molecules. The analysis of p120ctn isoforms in a variety of murine cell lines has revealed that motile cells, such as fibroblasts and macrophages, preferentially express the long isoform, and epithelial cells preferentially express the short isoform, whereas non-adherent cells do not express detectable levels of p120ctn (Mo and Reynolds, 1996). The cell-lineage-specific patterns of p120ctn isoform expression emerged also during this investigation. Specifically, isoform 1 is prominently expressed in melanocytic cells but not in normal or malignant keratinocytes. In normal human skin, E-cadherin is expressed on the surfaces of all epidermal cells, including keratinocytes and melanocytes, but is lost from melanoma cells, which during tumorigenesis switch to express N-cadherin (see references within Herlyn et al. (Herlyn et al., 2000)). These results suggest that the tissue- and cell-type-specific expression of p120ctn isoform 1A does not directly correlate with certain cadherin type, but the functional difference between the isoforms 1A and 3A, perhaps in signal transduction, remains to be studied.

We observed shifts in the expression pattern of p120ctn between normal keratinocytes and their counterparts with malignant potential. Specifically, the latter cells generally retained expression of band 2 but not of bands 3 and 4, which were expressed robustly by normal keratinocytes. Upon western blot analysis, the isoform 1A could be detected with MAb 6H11, which specifically recognize the N-terminal region of 1A. Other isoforms were identified on the basis of their molecular size and comigration with the transiently expressed tagged proteins. Thus band 4 represents isoform 4 and band 3 the epithelial isoform 3, but band 2 represents either isoform 2, translated from Met-55, or the phosphorylated form of isoform 3. The cell-type-specific phosphorylation, although not addressed in this study, may thus be responsible for the band shift detected through the western blotting. The activation of protein kinase C in epithelial cells results in dephosphorylation of serine and threonine residues of p120ctn (Ratcliffe et al., 1997). The disruption of tight junctions and the increase in permeability of cell monolayers was accompanied by the dephosphorylation event. However, in v-Src-transfected L cells tyrosine phosphorylation of p120ctn was dependent on its association with E-cadherin and resulted in reduced cell adhesion (Ozawa and Ohkubo, 2001). On the other hand, in differentiating keratinocytes, tyrosine phosphorylation plays a positive role in the control of cell adhesion (Calautti et al., 1998). In regard to our observation of the slower migrating band in the carcinoma cells, which usually are more motile than their normal counterparts, the higher phosphorylation

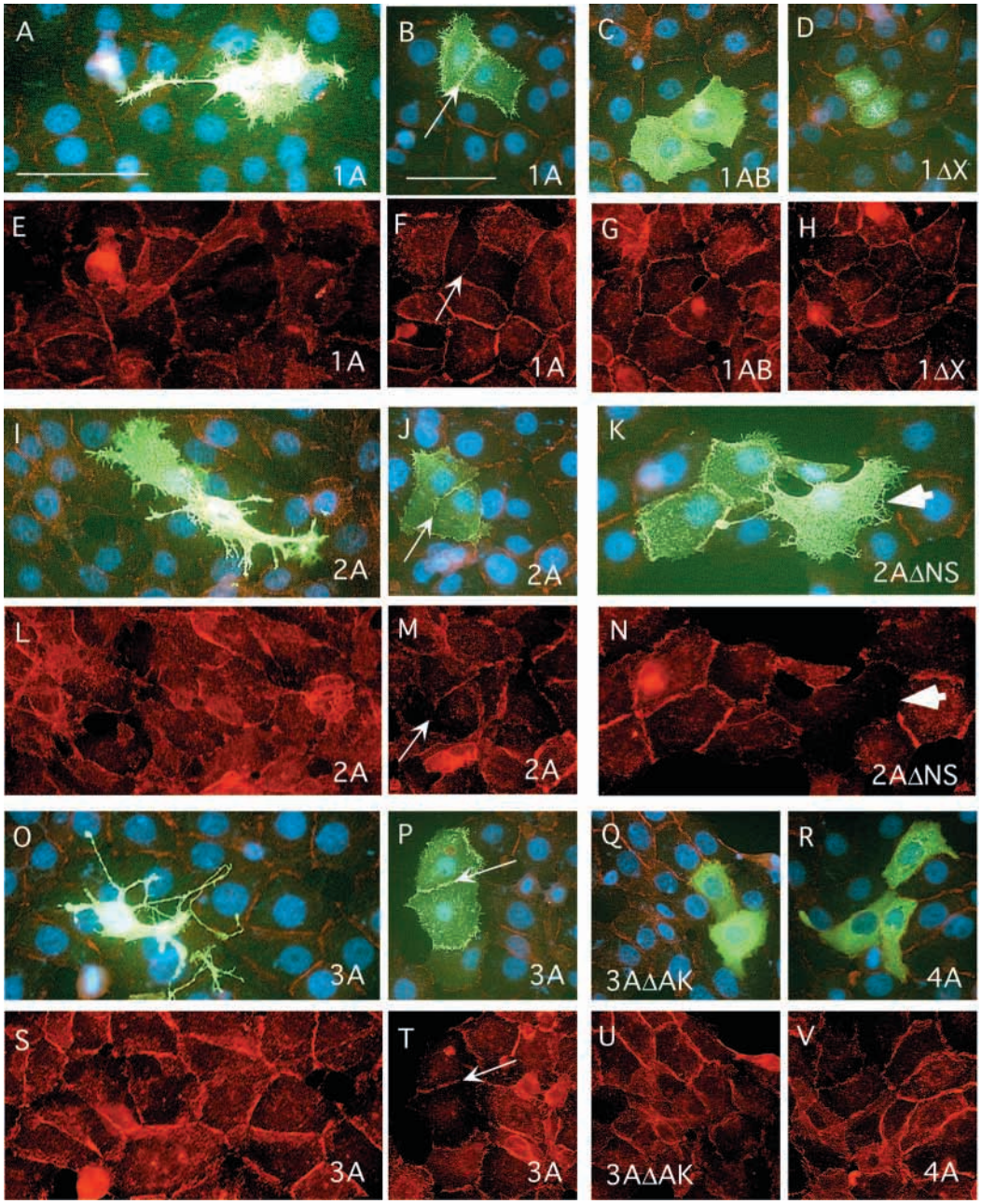


Fig. 9. The phenotype induced by p120ctn isoforms and deletion constructs transiently transfected into HaCaT cells. 16 hours after the transfection, a serum-free medium containing 1 mM Ca^{2+} was added and cells were incubated for 8 hours before preparing for the immunofluorescence staining. The expression of the FLAG-tagged polypeptides was demonstrated with FLAG-tag-specific rabbit antibody (FITC, green signal), endogenous E-cadherin is shown as red signal (Texas Red), and nuclei were visualized with DAPI staining (blue signal). Isoforms 1A (A,B,E,F), 2A (I,J,L,M) and 3A (O,P,S,T), as well as the deletion construct 2AΔNS (K,N), colocalized with E-cadherin at the cell-cell junctions, but the overexpression of each transgene resulted in the branching phenotype and the simultaneous disappearance of the E-cadherin staining both from the cell-cell adhesion borders as well as from the cytoplasmic pool (arrowheads in K and N). The isoforms 4A (R,V) and 1AB (C,G), as well as the deletion construct 3AΔAK (Q,U), displayed cytoplasmic localization and did not interfere with the endogenous E-cadherin signal. The C-terminally truncated isoform 1, 1ΔX, (D,H) localized to the nucleus but did not affect the E-cadherin signal. Bar, 50 μm. The bar in (A) applies to panels E,I,K,L,N,O,S, and the bar in (B) applies to panels C,D,F,G,H,J,M,P,Q,R,T,U,V.

level of p120ctn would correlate with decreased cell adhesion. We also observed that the isoform 4A, although present in normal cells, was lost in tumor cells.

Previous work has demonstrated that the overexpression of p120ctn isoform 1A in both fibroblasts and in epithelial cells caused the formation of extensive cellular extensions (Anastasiadis et al., 2000; Noren et al., 2000; Reynolds et al., 1996). We observed that this effect of p120ctn 1A extends to epidermal keratinocytes and cells of melanocytic origin. In the earlier work, Reynolds et al. (Reynolds et al., 1996) showed that deletion of amino acids 28-233 of p120ctn did not affect the branching phenotype, but the expression construct with the deletion of amino acids 233-387 was not able to induce the branching phenotype. Because the Armadillo domain starts from amino acid 347, and additional deletions within the Armadillo-repeat domain abolished the phenotypic effect, it was concluded that the intact Armadillo domain is indispensable for the branching phenotype. While investigating the isoform-specific phenotype, we found that the isoform 4A clearly did not induce the branching phenotype. Thus, N-terminal sequences of p120ctn impart distinct functional properties to p120ctn, at least as measured by the changes in cell shape after forced expression. The N-terminal domain, immediately preceding the N-terminus of isoform 4 (amino acids 233-323) contains five out of eight Src phosphorylation sites in the p120ctn (Mariner et al., 2001). As the catalytic activity of the Src family kinases is required to disrupt cadherin-dependent cell-cell contacts (Owens et al., 2000), the phosphorylation of tyrosine residues 257, 280, 291, 296 and 302, and also to a lesser extent tyrosine of residues 112 and 228, may play a role in the induction of the branching phenotype by p120ctn. Colon carcinoma cells Colo 205 show

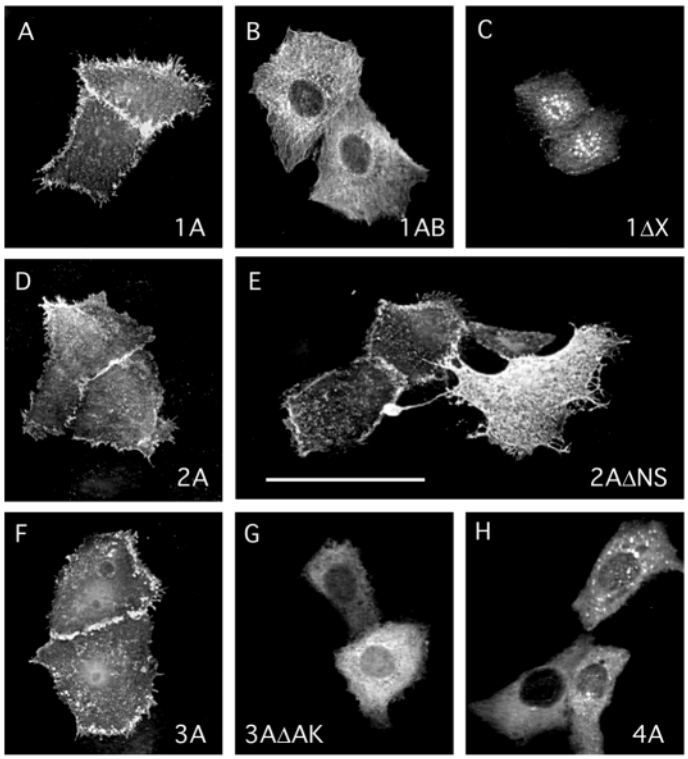


Fig. 10. The nuclear localization of p120ctn isoforms correlates with their capacity to induce the branching phenotype. The isoforms 1A (A), 2A (D) and 3A (F) as well as the deletion construct 2AΔNS (E) localized equally in the cytoplasm and in the cell nucleus. In addition to the prominent signal at the cell-cell adhesion junctions, filopodial structures are starting to emerge. Isoforms 1AB (B), 4A (H) and the deletion construct 3AΔAK (G) were excluded from the nucleus but localized to the cytoplasm. The C-terminally truncated 1A, 1ΔX, showed nuclear localization but was not sufficient for the induction of branching cellular phenotype (C). Bar, 50 μm.

defective cell adhesion, possibly owing to the aberrant p120ctn phosphorylation. The overexpression of p120ctn with deletion of amino acids 245-324 appeared to compete with the endogenous p120ctn, abolishing the defect and inducing cell aggregation (Aono et al., 1999).

The requirement for N-terminal sequences outside of the Armadillo repeat domains for the branching phenotype is novel, as previous studies have focused on sequences within the Arm repeat domain as a prerequisite for branching. Mutational analysis unequivocally demonstrated that the Arm repeat domain is essential for branching to occur in fibroblasts, as the double point mutation (KK/AA) substitution of residues 622-623 contained in Arm repeat 6 abolished the induction of the branching phenotype (Anastasiadis et al., 2000).

The N-terminal end of the isoform 1A contains a coiled-coil region, which is highly homologous with other Armadillo family members (Anastasiadis and Reynolds, 2000). This coiled-coil sequence appears not to be critical for branching because the p120ctn 2A and 3A were also capable of inducing this phenotype. Nevertheless, the function of the coiled-coil domain remains open to discussion and cannot be linked to the branching phenotype. The keratinocytes form stable cell-cell adhesion complexes throughout the layers of stratified epithelia. However, they mainly express the p120ctn isoform 3A, which is as potent inducer of the branching phenotype as the isoform 1A. This leads us to the conclusion that either the expression levels of p120ctn or other p120ctn isoforms play an important role in balancing and regulating stable cell adhesions.

Recent studies have elucidated a molecular mechanism required for the branching phenotype to occur. Specifically, three independent reports described that p120ctn inhibits RhoA activity, an effect that appears to be due to p120ctn binding to the Rho family exchange factor Vav2 (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). At least two distinct nuclear localization signals and one nuclear export signal have been reported to contribute to the subcellular localization of p120ctn. Our results indicate that the putative nuclear localization signal in Arm repeat 6 is not sufficient for nuclear localization, as the isoform 4A showed cytoplasmic but not nuclear localization. A second putative nuclear localization signal, located immediately upstream from the Armadillo domain, was functional in directing the N-terminal domain of p120ctn into the nucleus, but deletion of this sequence from isoforms 2A and 3A resulted in a less prominent branching phenotype. However, deletion of the N-terminal sequences containing the tyrosine residues phosphorylated by Src (Mariner et al., 2001) abolished both the nuclear localization and the branching phenotype. Conversely, if the transgenic proteins were excluded from the nuclei no branching was observed. The presence of exon B containing a nuclear export signal, as in p120ctn 1AB, resulted in nuclear exclusion and lack of branching phenotype despite the presence of all sequences necessary for branching to occur (long N-terminus, Arm repeat domain, C-terminus). Although these results do not provide understanding of the precise molecular mechanisms leading to the branching phenotype, they do provide a strong argument in support of the notion that nuclear accumulation not only cosegregates with the branching phenotype but is also essential for it.

We demonstrated the loss of the isoform 4A from tumor cells although it is present in normal keratinocytes. Although exon B was not present in epidermal cells or tumor cells of epidermal origin (data not shown), its presence in several internal organs with a prominent epithelial component was confirmed by RT-PCR. Because the expression pattern of p120ctn isoforms is characteristic to each tissue or cell type, the loss of isoform 1AB from tumors of the colon, pancreas and prostate may be significant, although it remains to be confirmed with additional material. The opposing effects observed for p120ctn in cell adhesion and mobility can be explained through the isoform-specific effects. Our results clearly support a tumor-suppressor role for the p120ctn isoforms 4A and 1AB, and the isoforms capable of nuclear localization may be considered as oncogenes, at least under certain circumstances.

In conclusion, p120ctn isoforms display different functions. The combination of p120ctn isoforms expressed by a single cell, their expression levels, and the cell-type-specific proteins interacting with p120ctn isoforms all potentially contribute to the cellular phenotype.

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