Phosphorylation and Intracellular Localization of p120 Catenin

by

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Abstract

p120 catenin (p120ctn) is an ARM-repeat protein and a component of cadherin adhesion complexes. In contrast to its role in mediating cell-cell adhesion at the plasma membrane, overexpression of p120ctn in the cytoplasm stimulates cell migration and causes actin cytoskeleton rearrangement via the regulation of Rho family GTPases. In addition, p120ctn localizes to the nucleus where it may regulate transcription by binding to the transcription factor Kaiso. p120ctn thus has different functions depending on the cellular compartment. The identification of mechanisms regulating the intracellular localization of p120ctn will be crucial for gaining a better understanding of how the diverse functions of p120ctn are coordinated.

The state of p120ctn serine/threonine phosphorylation varies between different cellular compartments, but the relevance of these phosphorylation events for p120ctn function and localization are unclear. A number of novel serine/threonine phosphorylation sites were identified and their involvement in regulating p120ctn localization was investigated using phospho/non-phospho-mimicking mutants. In addition, a panel of kinase and phosphatase inhibitors was tested for their effects on p120ctn phosphorylation and localization.

The ARM-repeat domain of p120ctn mediates the interaction with cadherins and is involved in the regulation of Rho GTPases. Several point mutations in this region were identified that target p120ctn to microtubules, leading to microtubule reorganization and stabilization. Binding of p120ctn to microtubules was inversely related to its ability to regulate Rho GTPases. Mutant p120ctn also localized to the mitotic spindle and centrosomes, implicating p120ctn in the regulation of mitosis.

Hepatocyte growth factor (HGF) treatment of MDCK cells induces adherens junction disassembly and stimulates cell migration. HGF stimulation caused relocalization of p120ctn from adherens junctions into the cytoplasm and led to a transient accumulation of p120ctn in the nucleus. The identification of HGF as a physiological stimulus of p120ctn nuclear translocation suggests a role for p120ctn in HGF-induced transcriptional changes. Finally, a novel interaction of p120ctn with the tight junction protein ZO-1 was identified, pointing towards a more complex role of p120ctn in modulating intercellular adhesion.
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1 Introduction

1.1 Preface

p120 catenin (p120ctn) is the founding member of a subfamily of ARM repeat domain proteins found at cell-cell junctions and in the nucleus (Anastasiadis and Reynolds, 2000). Originally identified as an excellent substrate of the tyrosine kinase Src (Reynolds et al., 1989), p120ctn was soon found to be a member of cadherin adhesion complexes (Daniel and Reynolds, 1995; Reynolds et al., 1994; Staddon et al., 1995) and termed p120 CAS (for cadherin-associated Src substrate), though later renamed p120ctn (p120 catenin) (Daniel and Reynolds, 1997) to avoid confusion with another Src substrate, p130 CAS (Crk-associated substrate) (Sakai et al., 1994). Most studies on p120ctn have focused on its role in regulating cadherin-mediated cell-cell adhesion, but the recent discovery that p120ctn is a regulator of Rho family GTPases (Anastasiadis and Reynolds, 2000; Anastasiadis and Reynolds, 2001; Grosheva et al., 2001; Noren et al., 2001), thereby affecting cytoskeletal organization and cell migration, have ascribed p120ctn an additional signalling function. p120ctn is frequently found to localize to the nucleus (van Hengel et al., 1999), where it may regulate gene transcription via an interaction with the transcription factor Kaiso (Daniel and Reynolds, 1999). The different functions of p120ctn appear to correlate with changes in its intracellular localization. A better understanding of how p120ctn is targeted to different cellular compartments may be crucial for elucidating how the various functions of p120ctn are coordinated.

1.2 p120ctn structure

p120ctn contains a central Armadillo domain consisting of 10 imperfect copies of a structural motif called ARM repeat (Anastasiadis and Reynolds, 2000)(Figure 1.1). ARM repeats are tandemly repeated sequences containing approximately 45 amino acids (Peifer et al., 1994). They were first identified in the Drosophila melanogaster β-catenin homologue armadillo (Riggleman et al., 1989) (for the structure of the β-catenin ARM repeat domain, see Figure 3.21) and are found in other proteins, such as γ-catenin/plakoglobin (Franke et al., 1989), the tumour suppressor adenomatous polyposis coli (APC) (Peifer et al., 1994), and the nuclear transport proteins of the importin family (Gorlich et al., 1994). ARM repeat domains are protein-protein interaction domains (Andrade et al., 2001) and in p120ctn this domain mediates the interaction with cadherins.
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Introduction

(Daniel and Reynolds, 1995), the transcription factor Kaiso (Daniel and Reynolds, 1999) and there is evidence that the ARM repeat domain is involved in binding to the GTPase RhoA (Anastasiadis et al., 2000). In addition to the central ARM repeat domain, p120ctn contains a putative coiled-coil domain (Anastasiadis and Reynolds, 2000) and a tyrosine phosphorylation domain (Mariner et al., 2001) within its N-terminus.

1.3 p120ctn is expressed in multiple isoforms

p120ctn is expressed in a wide range of tissues, including intestine, brain, uterus, blood vessels, skin, lung, kidney and heart (Golenhofen and Drenckhahn, 2000; Keirsebilck et al., 1998; Montonen et al., 2001). The expression profile of p120ctn however is complex. N-terminal splicing events lead to the use of four different ATG start codons (Keirsebilck et al., 1998), resulting in the expression of isoforms 1, 2, 3 or 4 (Figure 1.1). In addition, alternative splicing can lead to the inclusion of three further exons: Exon A, encoding 21 amino acids, and Exon B, encoding 29 amino acids, are located near the C-terminus of p120ctn. Exon C encodes 6 amino acids and its expression leads to the disruption of a basic motif situated on a looped-out structure inserted within ARM repeat 6. The use of the alternative start codons in combination with the differential expression of exons A, B and C generates 32 potential p120ctn isoforms but it is not known whether all possible isoforms are expressed in vivo. Little is known about the relevance of differential isoform expression for p120ctn function. However, exon B has been shown to contain a nuclear export signal (see Section 1.7) and there is evidence for tissue-specific expression of p120ctn isoforms: motile cells, such as macrophages and fibroblasts, preferentially express the longer isoform p120ctn-1A, whereas epithelial cells express the shorter p120ctn-3A isoform. Transcripts of isoforms 2 and 4 are found in a variety of cell types, but they are not abundant. Human transcripts rarely lack exon A and often contain exon B, whereas exon C is rarely used except in brain tissue, where it is highly expressed (Keirsebilck et al., 1998). Src transformation of MDCK cells leads to strong upregulation of isoform 1 expression, suggesting that the pattern of isoform expression can be altered by cell transformation (Mo and Reynolds, 1996). However, the pattern of isoform expression is very heterogeneous in human cancer cell lines (Keirsebilck et al., 1998; Skoudy et al., 1996; Wu et al., 1998) and there is no clear correlation between the expression of particular p120ctn isoforms and cell transformation.
Figure 1.1  Domain structure of p120ctn

The N-terminal domain of p120ctn (amino acids 1 to 351) contains a short putative coiled-coil (CC) domain near the N-terminus followed by a tyrosine phosphorylation (PY) domain. The central ARM repeat domain contains 10 imperfect copies of the ARM repeat motif. Repeats 4, 6 and 9 are interrupted by short looped-out sequences. Four alternative start codons give rise to p120ctn isoforms 1, 2, 3 and 4. The use of the alternative start codons in combination with the differential expression of exons A, B and C has the potential to generate up to 32 different isoforms. p120ctn isoform 1-4 containing exons A, B and C (ABC) are shown.
Chapter 1

Introduction

1.4 p120ctn is part of the E-cadherin adhesion complex

1.4.1 The cadherin superfamily

Cadherins are a large superfamily of glycoproteins mediating Ca\(^{2+}\) dependent cell-cell adhesion in all solid tissues of the organism (Ivanov et al., 2001). Cadherin-mediated adhesion plays a key role in tissue and organ development during embryogenesis and in maintenance of normal tissue structure in the adult organism. The expression of cadherins is tightly controlled during development, and each tissue shows a characteristic pattern of cadherin molecules (Nollet et al., 2000). In their extracellular portion, all cadherins contain multiple cadherin repeats which mediate Ca\(^{2+}\)-dependent, homophilic interactions, but the number of cadherin repeats varies between 4 and 34 for different cadherins. Ca\(^{2+}\) ion binding to a linker region between the cadherin repeats allows cadherin molecules to arrange into a rigid and organized structure (Steinberg and McNutt, 1999). Under these conditions, cadherins are able to form both cis and trans dimers, leading to cadherin clustering. Removal of extracellular Ca\(^{2+}\) by EGTA chelation leads to loss of the ordered cadherin structure and loss of cadherin-mediated adhesion.

Based on their domain structure, cadherins can be classified into four groups: classical cadherins, desmosomal cadherins, protocadherins and other cadherin-related proteins. Members of the classical cadherin subfamily contain five cadherin repeats in their extracellular domain and this includes E-cadherin, the main mediator of adhesion in epithelial cells (see next section), N- and R-cadherin, predominantly expressed in neuronal tissues, and VE-cadherin which mediates adhesion between endothelial cells. Further members of the classical cadherin subfamily are P-cadherin, a cadherin expressed in mouse placenta, C-cadherin, the Xenopus homologue of E-cadherin, and the somewhat less related M-cadherin, found in skeletal muscle.

1.4.2 The E-cadherin complex is the main mediator of adhesion in epithelial cells

The main adhesive contacts between epithelial cells are adherens junctions with the E-cadherin complex as their principle component. Several proteins are associated with E-cadherin on the intracellular side and together they form the E-cadherin complex (Figure 1.2). β-catenin and plakoglobin/γ-catenin bind in a mutually exclusive way to the cytoplasmic domain of E-cadherin. α-catenin in turn binds β-catenin or plakoglobin/γ-catenin and links the E-cadherin complex to the actin cytoskeleton either directly
(Herrenknecht et al., 1991; Nagafuchi et al., 1991; Rimm et al., 1995) or indirectly via α-actinin (Knudsen et al., 1995; Nieset et al., 1997), vinculin (Watabe-Uchida et al., 1998; Weiss et al., 1998) and possibly ZO-1 (Takeda et al., 1995; Yonemura et al., 1995).

1.4.3 p120ctn associates with the cytoplasmic domain of E-cadherin via its ARM repeat domain

β-catenin associates with the cytoplasmic tail of E-cadherin via its ARM repeat domain (Hulsken et al., 1994). The discovery that p120ctn contains an ARM repeat domain (Reynolds et al., 1992) and the fact that in epithelial cells p120ctn colocalizes with E-cadherin at sites of cell-cell contact (Reynolds et al., 1994) suggested that p120ctn may also interact with E-cadherin. The association of p120ctn and E-cadherin was demonstrated by immunoprecipitation experiments (Reynolds et al., 1994; Shibamoto et al., 1995; Staddon et al., 1995) and the direct interaction of the p120ctn ARM repeat domain with the E-cadherin cytoplasmic tail was confirmed using the yeast two-hybrid system (Daniel and Reynolds, 1995). Although both β-catenin and p120ctn use their ARM repeat domains to bind to E-cadherin, their binding sites on the cytoplasmic tail of E-cadherin are distinct (Figure 1.2). Whereas β-catenin binds to the so-called catenin-binding domain (CBD) at the C-terminus of E-cadherin (Huber and Weis, 2001; Nagafuchi and Takeichi, 1989; Ozawa et al., 1990; Stappert and Kemler, 1994), p120ctn binds to the juxtamembrane domain (JMD) of E-cadherin (Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998b; Thoreson et al., 2000; Yap et al., 1998). The core p120ctn-binding site on E-cadherin comprises approximately 10 residues (Ferber et al., 2002; Ohkubo and Ozawa, 1999; Thoreson et al., 2000). p120ctn and β-catenin do not interact with each other (Daniel and Reynolds, 1995), nor does binding of p120ctn to the cytoplasmic tail of E-cadherin require simultaneous β-catenin binding or vice versa (Daniel and Reynolds, 1997; Roura et al., 1999). Binding of p120ctn and β-catenin to E-cadherin therefore appear to be independent events.
Figure 1.2 The E-cadherin complex

Schematic representation of the E-cadherin complex. The positions of the juxtamembrane domain (JMD) and the catenin-binding domain (CBD) on the E-cadherin are indicated. Adapted from Nollet et al., 2000.
1.4.4 Positive effects of p120ctn on cadherin-mediated cell-cell adhesion

Unlike β-catenin, γ-catenin/plakoglobin or α-catenin, p120ctn does not act to link the cadherin complex to the actin cytoskeleton and its requirement for cadherin-mediated adhesion is therefore not intuitively obvious. Nevertheless, by regulating cadherin clustering, p120ctn is thought to be crucial for proper cadherin-mediated adhesion (Anastasiadis and Reynolds, 2000). Several studies propose a positive role for the JMD, and therefore for p120ctn, in regulating cadherin clustering. Introduction of wild-type E-cadherin into E-cadherin-negative A431D cells causes tight aggregation of the cells and restores the epithelial phenotype, whereas expression of E-cadherin proteins carrying minimal mutations in the JMD that uncouple it from p120ctn failed to organize the cells into compact colonies (Thoreson et al., 2000). In a different assay, CHO cells stably expressing C-cadherin lacking the CBD or the JMD were plated on immobilized extracellular domains of C-cadherin (Yap et al., 1998). Deletion of the CBD (ΔCBD) did not prevent cadherin clustering and the establishment of strong adhesion, although the ΔCBD protein is unable to interact with the actin cytoskeleton. In contrast, cells expressing C-cadherin with a deleted JMD showed no cadherin clustering despite the fact that the mutated C-cadherin could associate with the actin cytoskeleton.

1.4.5 Evidence for p120ctn as a negative regulator of cadherin-mediated cell-cell adhesion

Other reports suggest an inhibitory effect of p120ctn on cadherin clustering (Aono et al., 1999; Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998b). Whereas expression of E-cadherin lacking the CBD (ΔCBD-E-cadherin) fails to induce cell-cell adhesion in E-cadherin-negative K562 cells, the additional deletion of the JMD in ΔCBD-E-cadherin rescues its adhesive properties (Ozawa and Kemler, 1998b). Two studies suggest p120ctn serine/threonine phosphorylation as a mechanism for regulating cadherin clustering (Aono et al., 1999; Ohkubo and Ozawa, 1999): Colo205 cells express all the known components of the E-cadherin complex but do not form cadherin-mediated cell-cell contacts. Interestingly, p120ctn is hyperphosphorylated on serine/threonine residues in these cells. Treatment with the serine/threonine kinase inhibitor staurosporine reduces p120ctn serine/threonine phosphorylation and this correlates with the induction of cadherin-mediated cell aggregation (Aono et al., 1999). Furthermore, expression of N-terminally-truncated p120ctn (i.e. of a protein corresponding to p120ctn isoform 4),
which lacks the majority of the p120ctn serine/threonine phosphorylation sites, also restores cell-cell adhesion. Similarly, Ohkubo and Ozawa show that staurosporine treatment or coexpression of N-terminally truncated p120ctn restores cadherin-mediated adhesion in L cells expressing adhesion-incompetent ΔCBD-E-cadherin (Ohkubo and Ozawa, 1999).

1.4.6 p120ctn is not essential for E-cadherin-mediated adhesion in Drosophila

Whereas the above studies suggest an essential role for p120ctn in cadherin-mediated adhesion in mammalian cell lines, the requirement of the Drosophila p120ctn homologue p120/δ-catenin for Drosophila epithelial cadherin (DE-cadherin)-mediated adhesion has recently been questioned. p120/δ-catenin-null mutants are viable and fertile and show no substantial change in junction structure or function (Myster et al., 2003). During dorsal closure p120/δ-catenin-null mutants occasionally showed minor irregularities in cell arrangement at the leading edge. However, dorsal closure was neither delayed nor impaired in these mutants. Consistent with these results, Drosophila DE-cadherin mutants carrying minimal mutations that uncouple it from p120/δ-catenin efficiently substituted for wild-type DE-cadherin activity in a number of DE-cadherin-dependent processes, such as maintenance of epithelial integrity and border cell migration (Pacquelet et al., 2003). Because p120/δ-catenin is believed to be the only p120ctn family member homologue in Drosophila (Myster et al., 2003), compensation by other p120ctn family members for p120/δ-catenin function cannot explain these surprising findings. p120/δ-catenin is therefore not an essential adherens junction component in Drosophila.

p120/δ-catenin and mammalian p120ctn show considerable identity within their ARM repeat domain (43%, see Figure 1.3) but show little homology between their C- and N-termini. In addition, the N-terminus of p120/δ-catenin is much shorter than that of p120ctn and may lack potential protein-protein interaction domains, such as the coiled-coil domain. Furthermore, the tyrosine phosphorylation sites and the majority of serine/threonine phosphorylation sites map to the p120ctn N-terminus and those sites may be missing in p120/δ-catenin. Thus, in mammals p120ctn may play a more complex role in regulating cadherin-mediated adhesion than in flies, perhaps reflecting the higher mechanical stress adherens junctions are subjected to in organisms of greater size and complexity.
Figure 1.3 The ARM repeat domains of mammalian p120ctn and Drosophila p120/δ-catenin share a high degree of identity

Comparison of the domain structure of mammalian (murine) p120ctn isoform 1 and Drosophila p120ctn/δ-catenin. The overall structure of the ARM repeat domain, including the short insertions in repeats 4, 6 and 9, is well preserved. The sequence similarity between the two ARM repeat domains is high with 43% identity (Myster et al., 2003). However, there is little homology between the C- and N-terminal regions flanking the ARM repeat domain. The N-terminal region of p120/δ-catenin lacks a coiled-coil domain (CC) and may not contain a tyrosine phosphorylation domain (PY).
1.4.7 p120ctn may protect E-cadherin from degradation and endocytosis

Interestingly, in flies lacking p120/δ-catenin, the phenotypes of different mutations in the DE-cadherin gene that lead to reduced DE-cadherin expression are strongly enhanced, suggesting that p120/δ-catenin becomes an important positive regulator of DE-cadherin-mediated adhesion when DE-cadherin levels are reduced (Myster et al., 2003). One way in which p120ctn could exert its positive effect on E-cadherin-mediated adhesion is by stabilizing the E-cadherin protein and there is now some evidence that p120ctn functions in this way. SW48 colon carcinoma cells, which have lost the ability to establish a normal epithelial morphology, express mutant forms of p120ctn and these only at low levels (Ireton et al., 2002). Cadherin-mediated adhesion can be re-established in these cells by restoring normal wild-type p120ctn levels. Interestingly, reintroduction of p120ctn coincides with an increase in E-cadherin levels. Messenger RNA (mRNA) levels of E-cadherin are unaffected by p120ctn expression, but E-cadherin half-life is more than doubled, suggesting that the presence of p120ctn stabilizes the E-cadherin protein.

A hypothesis for how p120ctn could stabilize E-cadherin has recently been presented based on the findings (1) that Presenilin1 (PS1), a member of the γ-secretase complex cleaving transmembrane proteins within or near their transmembrane domain (De Strooper, 2003), competes with p120ctn for binding to the JMD of E-cadherin (Baki et al., 2001) and (2) that PS1 cleaves E-cadherin at the membrane/cytosol interface, thereby releasing the intracellular domain of E-cadherin and inducing adherens junction disassembly (Marambaud et al., 2002). p120ctn may therefore protect E-cadherin from proteolytic cleavage by preventing PS1/γ-secretase binding to the JMD of E-cadherin.

Endocytosis of the E-cadherin complex has also been suggested as a mechanism to downregulate E-cadherin-mediated adhesion (Le et al., 1999). In addition to stabilizing E-cadherin, p120ctn could promote E-cadherin-mediated adhesion by preventing endocytosis of the E-cadherin complex. Hakai, an E3 ubiquitin-ligase, interacts with the JMD of E-cadherin in a tyrosine phosphorylation-dependent manner and promotes ubiquitination and subsequent endocytotic removal of E-cadherin from the plasma membrane (Fujita et al., 2002). By binding to the JMD, p120ctn could prevent tyrosine phosphorylation of E-cadherin and consequently Hakai-mediated endocytosis.

During apoptosis, the cytoplasmic domain of newly synthesized E-cadherin becomes O-glycosylated and this modification prevents the transport of E-cadherin to the plasma
membrane, resulting in reduced intercellular adhesion (Zhu et al., 2001). Although there are no potential O-glycosylation sites within the minimal p120ctn-binding site, O-glycosylation prevents the interaction of E-cadherin with p120ctn, but not β- or γ-catenin. It is unknown if O-glycosylation of E-cadherin occurs in other instances of adherens junction disassembly and if p120ctn binding to the JMD can block such O-glycosylation, thereby permitting transport of E-cadherin to the plasma membrane and strengthening E-cadherin-mediated adhesion.

1.4.8 Interaction of p120ctn with other classical cadherins
Interestingly, the JMD is the most highly conserved region among classical cadherins (Nollet et al., 2000), allowing p120ctn to interact with N-cadherin, P-cadherin (Reynolds et al., 1996) and VE-cadherin (Lampugnani et al., 1997) in addition to E-cadherin. Little is known about the function of p120ctn when it is in a complex with cadherins other than E-cadherin. However, p120ctn has been implicated in the regulation of N-cadherin-promoted neurite outgrowth (Riehl et al., 1996). Plating of neuronal cell lines on purified N-cadherin substrates or transfection of N-cadherin promotes the extension of neurites (Bixby and Zhang, 1990; Matsunaga et al., 1988; Payne et al., 1992). The mechanism by which N-cadherin promotes such neurite outgrowth is poorly understood but probably involve an unusual heterophilic interaction of N-cadherin with the fibroblast growth factor receptor (FGFR), resulting in FGFR activation (Saffell et al., 1997; Williams et al., 2001). Interestingly, expression of a dominant-negative N-cadherin mutant, containing just the transmembrane region and the JMD, but not the CBD, inhibits neurite outgrowth (Riehl et al., 1996), assigning an essential function to the JMD, and possibly p120ctn, in this process. However, as is the case for the E-cadherin JMD, additional proteins may interact with the JMD of N-cadherin, which could be responsible for the inhibitory effect on neurite outgrowth.
1.5 p120ctn regulates Rho GTPases

1.5.1 Rho Family GTPases

1.5.1.1 Rho GTPases are key regulators of the actin cytoskeleton

Rho family GTPases are key regulators of the actin cytoskeleton and they are involved in coordinating a variety of cellular processes, including cell polarity, cell migration and cell adhesion (Hall, 1998; Ridley, 2001a). Generally, Rho proteins exist in an active, GTP-bound, or in an inactive, GDP-bound, state (Figure 1.4). GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho proteins, thereby converting them from the GTP-bound form to the GDP-bound form and inactivating them. Guanine nucleotide exchange factors (GEFs), on the other hand, promote the exchange of GDP for GTP and consequently activate Rho proteins. Furthermore, in the absence of an activating stimulus, GDP-bound Rho proteins are predominantly locked in the inactive, GDP-bound form by binding to guanine nucleotide dissociation inhibitors (GDIs) localized in the cytoplasm.

More than 20 members of the Rho GTPase family have been identified in mammals (Ridley, 2001a), but Rho, Rac and Cdc42 have been most widely studied for their effect on the actin cytoskeleton (Hall, 1998; Ridley, 2001b). In fibroblasts, activation of the highly homologous isoforms RhoA, RhoB or RhoC stimulates the assembly of actomyosin contractile filaments known as stress fibres and the formation of focal adhesions (Ridley and Hall, 1992), essential structures for creating cell contractility. In contrast, Rac and Cdc42 activation promotes lamellipodial and filopodial extensions (Nobes and Hall, 1995; Ridley et al., 1992), allowing cells to polarize, chemotax and migrate (Figure 1.5).

Lamellipodia are sheet-like membrane extensions containing an extensive mesh of actin filaments. They form at the leading edge of cells and are important for cell migration. The ability of Rac to induce such structures is believed to involve the activation of the Arp2/3 complex via Scar/Wave proteins (Cory and Ridley, 2002; Eden et al., 2002; Miki et al., 2000). The Arp2/3 complex then facilitates the formation of new actin filaments and branching of existing filaments (Pollard et al., 2000). In addition, Rac is thought to stimulate actin polymerisation by promoting the uncapping of actin filaments. By activating phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase), an enzyme which converts PtdIns(4)P (PIP) into PtdIns(4,5)P₂ (PIP₂), active Rac is able to increase the amount of PIP₂ at the plasma membrane. By binding to PIP₂, actin filament capping
proteins, such as CapZ, are then recruiting away from the ends of actin filaments, allowing filament extension (Carpenter et al., 1999; Tolias et al., 2000). Furthermore, Rac and Cdc42 may also be able to decrease the rate of actin depolymerisation. Via their target p21-activated kinase (PAK), Rac and Cdc42 stimulate LIM-kinase (Edwards et al., 1999; Stanyon and Bernard, 1999), which in turn inactivates cofilin, a protein that can promote actin depolymerisation (Stanyon and Bernard, 1999).

Filopodia are fine protrusions containing bundles of actin filaments and extend from the leading edge of cells. In fibroblasts, Cdc42 is able to induce the formation of filopodia by stimulating the Arp2/3-complex-mediated actin polymerization activity of N-WASP (Miki et al., 1998). Filopodia also extended from the growth cones of neuronal cells, where they may be involved in exploring the extracellular surrounding and guiding the growth cone.

Stress fibres are the force-generating element in actomyosin-mediated cell contractility (Mitchison and Cramer, 1996) and are formed from the interaction of actin cables with myosin II filaments. Non-muscle myosin II is a motor protein which forms filaments upon phosphorylation. The control of actomyosin-mediated contractility by Rho involves the activation of Rho-kinases (ROCKs), which in turn regulate myosin light chain (MLC), the regulatory subunit of myosin II, both by inhibiting MLC phosphatase and by phosphorylating MLC (Amano et al., 2000; Kaibuchi et al., 1999). Rho thus controls stress fibre assembly by regulating the state of myosin II phosphorylation.

To translate stress fibre contraction into cell tension and movement, the ends of stress fibres have to be attached firmly to the ECM. This is achieved by integrin-containing adhesion complexes termed focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996) and Rho GTPases have been shown to be involved in the formation and the turnover of these structures (Petit and Thiery, 2000). By inducing the bundling and contraction of actomyosin filaments, Rho is thought to cause the clustering of integrin complexes into focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996), whereas Rac activation promotes the disassembly of these structures in a PAK-mediated manner (Zhao et al., 2000).
1.5.1.2 **Rho GTPases are emerging as regulators of the microtubule network**

Although Rho GTPases are best studied for their effect on the actin cytoskeleton, they are now also emerging as important regulators of microtubule dynamics (Wittmann and Waterman-Storer, 2001). Activation of RhoA in fibroblasts causes microtubule stabilization (Cook et al., 1998; Palazzo et al., 2001), whereas Rac1 promotes the extension of microtubules into the leading edge of migrating cells (Wittmann et al., 2003). Rac and Cdc42 may regulate microtubule stability through stathmin, which can be phosphorylated by PAK (Daub et al., 2001). Stathmin is a microtubule-destabilizing protein which can be inactivated by phosphorylation (Andersen, 2000; Larsson et al., 1997). By inducing PAK-mediated phosphorylation of stathmin, Rac and Cdc42 may thus increase microtubule stability. Recently, Rac and Cdc42 have been shown to tether microtubule ends to the plasma membrane by binding to IQGAP1, which in turn interacts with the microtubule (+) end-binding protein CLIP-170 (Fukata et al., 2002). Cdc42 is involved in polarizing the microtubule network in migrating cells (Nobes and Hall, 1999). For example, in migrating astrocytes, polarization involves Cdc42-dependent regulation of glycogen synthase kinase-3β and localization of APC to microtubule (+) ends (Etienne-Manneville and Hall, 2003).

Microtubule dynamics, in turn, can regulate the activity of Rho GTPases. Nocodazole-induced microtubule depolymerisation induces RhoA activation (Liu et al., 1998). Interestingly, the RhoA-specific exchange factor p190GEF associates with microtubules (van Horck et al., 2001), and it has been hypothesized that microtubule depolymerisation may cause the release of exchange factors, for instance p190GEF, from the microtubule lattice, enabling them to promote guanine nucleotide exchange on RhoA. The microtubule network could therefore function to maintain low RhoA activity by sequestering Rho-specific GEFs. In contrast, nocodazole washout activates Rac (Waterman-Storer et al., 1999). Several Rac GEFs interact with microtubules (Glaven et al., 1999; Ren et al., 1998), but it is not known how this affects their activity towards Rac.

1.5.1.3 **Rho GTPases and E-cadherin-mediated adhesion**

In addition to their role in remodelling the actin cytoskeleton and the microtubule network, Rho proteins are involved in the establishment and maintenance of cadherin-independent cell-cell adhesion (Braga, 2000; Fukata and Kaibuchi, 2001). Inhibition of Rac1 or RhoA prevents the formation of cadherin-containing intercellular junctions.
between keratinocytes upon restoration of extracellular Ca$^{2+}$ levels (Braga et al., 1997). Furthermore, inhibition of Rho leads to rapid removal of E-cadherin from mature junctions (Braga et al., 1997). Expression of activated Rac, on the other hand, increases the amount of E-cadherin accumulating at sites of cell-cell contact in MDCK cells (Hordijk et al., 1997; Jou and Nelson, 1998; Takaishi et al., 1997) and both Rac1 and Cdc42 are necessary to maintain E-cadherin-mediated cell-cell adhesion in these cells (Kodama et al., 1999; Kuroda et al., 1997). Interestingly, Rac1 activation is also required during HGF-induced adherens junction disassembly (Potempa and Ridley, 1998), demonstrating that Rac1 is involved in positive as well as negative regulation of cadherin-mediated cell-cell adhesion.

It is unknown which effectors mediate the effect of RhoA on cadherin-containing junctions. However, more is known about how Rac and Cdc42 may modulate E-cadherin activity. IQGAP1, a scaffolding protein, negatively regulates E-cadherin-mediated adhesion by binding to β-catenin, thereby displacing it from α-catenin and severing the link between the E-cadherin complex with the actin cytoskeleton (Kuroda et al., 1998). IQGAP1, however, also interacts with Rac1 and Cdc42 in their activated state (Kuroda et al., 1996). By recruiting IQGAP1 away from β-catenin, Rac1 and Cdc42 are able to positively regulate E-cadherin-mediated adhesion (Fukata et al., 1999).

Rho proteins may regulate E-cadherin-mediated adhesion in ways that are more indirect. Endocytosis has been suggested as a mechanism regulating the amount of E-cadherin at the plasma membrane (Le et al., 1999). Rho GTPases are known to regulate endocytic transport (Ellis and Mellor, 2000) and may in this way control the amount of E-cadherin able to engage in cell-cell adhesion at the plasma membrane. Consistent with this idea, expression of dominant active forms of Rac1 and RhoA blocks HGF-induced endocytosis of E-cadherin in MDCK cells (Kamei et al., 1999).

The studies described above clearly show a role for GTPases in regulating E-cadherin function. Interestingly, E-cadherin engagement in turn is able to regulate Rho GTPases (Fukata and Kaibuchi, 2001). For instance, initiation of E-cadherin-mediated cell-cell contacts in MCF-7 cells results in the rapid activation of Cdc42 (Kim et al., 2000). Likewise, Rac1 is activated upon formation of E-cadherin-mediated cell-cell adhesion in MDCK cells (Nakagawa et al., 2001). In both cases, Rac or Cdc42 activation coincides with their recruitment to the forming junctions. RhoA appears to be inversely regulated to Rac1 and Cdc42 during the formation of E-cadherin-mediated cell-cell contacts: RhoA
activity is elevated in low density cultures but decreases as E-cadherin-containing junctions mature (Noren et al., 2001). In contrast, increased levels of Rac1 and Cdc42 activity are maintained even after epithelial cells have developed mature junctions and reached confluency. It is not known how E-cadherin signals to Rac and Cdc42. However, because of its localization to intercellular junctions (Hordijk et al., 1997), the Rac exchange factor Tiam1 has been suggested as a candidate protein (Fukata and Kaibuchi, 2001).

1.5.2 pl20ctn regulates cell morphology and stimulates cell migration via Rho family GTPases

Initial experiments showed that overexpression of pl20ctn in fibroblasts cell lines induces a so-called “branching phenotype”, characterized by the extension of branching dendrite-like protrusions, and that the pl20ctn ARM repeat domain, and possibly the C-terminus, are necessary for this change in morphology (Reynolds et al., 1996). Subsequently, it was shown that pl20ctn induces the branching phenotype by regulating small GTPases of the Rho family (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001; Grosheva et al., 2001; Noren et al., 2000). pl20ctn appears to regulate Rho GTPases in at least two different ways: it inhibits RhoA (Anastasiadis et al., 2000) and it activates Rac and Cdc42 (Grosheva et al., 2001; Noren et al., 2000). pl20ctn downregulates RhoA activity by inhibiting its GDP/GTP exchange and therefore has been proposed to function in a manner comparable with a RhoGDI (Anastasiadis et al., 2000), although pl20ctn contains no sequence homology to other GDIs. A basic motif in a looped-out-structure inserted within pl20ctn ARM repeat 6 has been shown to be essential for the inhibition of RhoA by pl20ctn, implicating the ARM repeat domain in the regulation of RhoA. Although immunoprecipitation or yeast two-hybrid assays have so far failed to demonstrate a direct interaction of mammalian pl20ctn and RhoA, the direct interaction of Drosophila p120/β-catenin and Rho1, the Drosophila homologue of RhoA, has recently been shown using GST-fusion protein pulldown experiments (Magie et al., 2002). Pulldown experiments using the Rac/Cdc42-binding (CRIB) domain of PAK1 showed that pl20ctn overexpression in fibroblast cell lines increases the level of active Rac1 and Cdc42 (Grosheva et al., 2001; Noren et al., 2000). Furthermore, pl20ctn is in a complex with the GEF Vav2 and Rac1 and Cdc42 activation has been suggested to occur via binding of pl20ctn to Vav2 (Chauvet et al., 2003; Noren et al., 2000). However, Vav2 has exchange
activity towards Rac/Cdc42 as well as RhoA both in in vitro assays (Abe et al., 2000) and in vivo (Liu and Burridge, 2000). The mechanisms by which p120ctn binding stimulates Vav2 exchange activity specifically towards Rac1 and Cdc42 but not RhoA remain to be clarified.

Both the inhibition of RhoA and the activation of Rac and Cdc42 are necessary for the induction of the branching phenotype (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000), whereas overexpression of constitutively active Rac1 or Cdc42 does not induce comparable cellular branching, possibly because they need to cycle between active and inactive forms to stimulate extension (Albertinazzi et al., 1999). However, the p120ctn-induced branching phenotype is indistinguishable from a RhoA inhibition phenotype (Anastasiadis et al., 2000). The activity levels of Rac1 and RhoA are frequently inversely related, with increased Rac activity leading to decreased RhoA activity (Sander and Collard, 1999; Sander et al., 1999), and inhibition of RhoA signalling inducing an increase in Rac activity (Tsuji et al., 2002). Therefore, p120ctn could induce the branching phenotype primarily by inhibiting RhoA, whereas the increase in Rac1 and Cdc42 activity could be a secondary event resulting from RhoA inhibition. Alternatively, inhibition of RhoA in p120ctn-transfected cells could be the result of Cdc42 and Rac1 activation.

In addition to the induction of the branching phenotype, overexpression of p120ctn causes focal adhesion disassembly and stimulates cell migration (Grosheva et al., 2001; Noren et al., 2000), additional activities which involve the regulation of Rho proteins (Grosheva et al., 2001; Noren et al., 2000; Ridley, 2001a). Cell migration requires the activation of Rac1 specifically at the front of the cell, whereas p120ctn is homogeneously distributed throughout the cytoplasm. In order for p120ctn to stimulate cell migration, additional signals must restrict its activating effect on Rac1 to the front of the cell. The effect of p120ctn on Rho GTPases can be suppressed by the overexpression of E-cadherin (Grosheva et al., 2001), but not by the overexpression of mutant E-cadherin unable to bind p120ctn (Anastasiadis et al., 2000). p120ctn is therefore thought to affect Rho protein activity only when it is in a cadherin-uncoupled, cytoplasmic state (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000; Noren et al., 2001). Interestingly, in sparse MDCK cultures, a substantial fraction of p120ctn localizes to the cytoplasm, whereas it is quantitatively recruited to the plasma membrane upon junctional maturation and cell compaction (Grosheva et al., 2001).
Chapter 1

Introduction

Based on the above observations, a model has been proposed in which p120ctn functions to control the balance between sessile and motile behaviour of cells depending on their density (Anastasiadis and Reynolds, 2001; Grosheva et al., 2001) (Figure 1.6). In sparse cultures lacking cadherin-mediated cell-cell adhesion, p120ctn is localized in the cytoplasm, but activates Racl and Cdc42 specifically at the front of the cell, allowing polarized protrusive activity and cell motility. With increasing cell density, p120ctn is recruited to newly forming cadherin-mediated cell-cell junctions. As the cytoplasmic concentration of p120ctn gradually decreases, the activities of Rac1 and Cdc42 are in turn decreased and cells consequently become less motile. In addition, upon binding of p120ctn to E-cadherin, RhoA may be released from the inhibitory effect of p120ctn, resulting in increased RhoA activity, which may further repress protrusive activity by increasing cell contractility. However, this model does not take into account the requirement for Rac1 and Cdc42 activity in the maintenance of E-cadherin-mediated cell-cell adhesion (see Section 1.5.1.3), nor does it explain the finding that RhoA activity is high in subconfluent epithelial cells, but gradually decreases as cellular junctions mature (Noren et al., 2001). In addition, the model does not address the fact that cell migration requires cell contractility and therefore at least basal levels of RhoA activity. The model could be reconciled with the above findings if one assumes that in subconfluent cells, p120ctn only decreases RhoA activity to a level that still allows cell contractility, whereas it activates a pool of Rac1/Cdc42 (presumably at the front of the cells) which is specifically involved in promoting cell migration. The activity of this pool may indeed decrease as p120ctn is recruited to nascent cadherin-mediated cell-cell contacts, whereas a separate, junctional Rac1/Cdc42 population may now be activated by p120ctn-independent mechanism, for instance by junctional GEFs like Tiam1. It is important to point out that the regulation of Rho GTPases by p120ctn has only been studied in fibroblasts. The results of these studies were then applied to explaining the behaviour of epithelial cells. p120ctn, however, may regulate Rho GTPases differently depending on the cell type.

As mentioned in Section 1.5.1.3, cadherin engagement leads to Rac1/Cdc42 activation, but downregulation of RhoA. Although this activation profile matches the effect of p120ctn on Rho GTPases in fibroblasts (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000), it is unlikely that p120ctn is involved in the pathway from cadherins to Rho proteins because of the mutual exclusivity of p120ctn cadherin-binding and Rho
GTPase regulation. Nevertheless, Goodwin et al. implicate p120ctn directly in the signalling from E-cadherins to Rac1 by showing that engagement of wild-type E-cadherin increases Rac activity, whereas engagement of E-cadherin carrying minimal mutations in the JMD that uncouple it from p120ctn fails to do so (Goodwin et al., 2003). However, the possibility cannot be excluded that the p120ctn-uncoupling mutations also prevent the binding of other proteins to the JMD, which could be potential regulators of Rho GTPases.
Figure 1.4 GTPase cycle

Rho GTPases cycle between an inactive, GDP-bound and an active, GTP-bound state. Guanine nucleotide exchange factors (GEF) facilitate the exchange of GDP for GTP on Rho GTPases, thereby activating them, whereas GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho GTPases, transferring them into the inactive, GDP-bound state. In addition, Rho GTPases are stabilized in their inactive form by forming a complex with guanine nucleotide dissociation inhibitors (GDIs). Effector proteins become activated upon specifically binding to the GTP-bound form of Rho GTPases.
Figure 1.5  Rho GTPase control the organization of the actin cytoskeleton

In fibroblasts, activated RhoA induces the formation of stress fibres and focal adhesion and increases cell contractility. In contrast, activated Rac1 induces sheet-like membrane extensions known as lamellipodia important for cell migration. Active Cdc42 induces finger-like extensions termed filopodia and controls cellular polarity and chemotaxis. Images of Swiss 3T3 fibroblasts (stained for F-actin with phalloidin) adapted from Hall, 1998.
In isolated epithelial cells, p120ctn (in green) is localized in the cytoplasm, where it stimulates cell motility and protrusive activity by activating Rac1 and Cdc42 (possibly via the exchange factor Vav2) and by decreasing RhoA (left panel). As the cell density increases, p120ctn is recruited to newly formed E-cadherin adhesion complexes (right panel). RhoA is displaced from p120ctn and the inhibitory effect is terminated, whereas Rac1 and Cdc42 are no longer activated by p120ctn. Consequently, cell motility and protrusive activity are suppressed. However, the model does not offer an explanation for the fact that cadherin engagement in epithelial cells promotes activation of Rac1 and Cdc42 and deactivation of RhoA (see Section 1.5.1.3).
1.6 Phosphorylation of p120ctn

1.6.1 Tyrosine phosphorylation

p120ctn was originally identified as a substrate of the Src tyrosine kinase (Reynolds et al., 1989). The Src-targeted sites have recently been mapped and are all localized within amino acids 96 to 302 of the N-terminus of p120ctn (Mariner et al., 2001; Ozawa and Ohkubo, 2001). Whereas p120ctn is not or only weakly tyrosine phosphorylated in resting cells (Reynolds et al., 1994), tyrosine phosphorylation of p120ctn is strongly induced upon expression of activated Src mutants (Reynolds et al., 1994; Reynolds et al., 1989). Because elevated p120ctn tyrosine phosphorylation in Src-transformed MDCK cells coincides with loss of cell-cell junctions (Behrens et al., 1993), tyrosine phosphorylation of p120ctn has been suggested as a mechanism to regulate adherens junction disassembly (Daniel and Reynolds, 1997; Reynolds et al., 1994). However, expression of activated Src mutants also induces tyrosine phosphorylation of additional junctional proteins, such as β-catenin, plakoglobin/γ-catenin, ZO-1 and E-cadherin (Behrens et al., 1993; Matsuyoshi et al., 1992; Reynolds et al., 1994; Takeda et al., 1995; Tsukita et al., 1991), making it difficult to elucidate the specific contribution of p120ctn tyrosine phosphorylation to junctional remodelling.

Neither hyperphosphorylation (Reynolds et al., 1994) nor dephosphorylation (Mariner et al., 2001) of the Src sites in p120ctn disrupts its interaction with E-cadherin, demonstrating that tyrosine phosphorylation is neither required for nor does it block the p120ctn/E-cadherin interaction. However, in in vitro assays using recombinant proteins, Src-mediated tyrosine phosphorylation of p120ctn increases its affinity for E-cadherin (Roura et al., 1999). Although tyrosine phosphorylation of p120ctn does not appear to be essential for the binding to E-cadherin, it may fine-tune the affinity between the two proteins. Masayuki and Ohkubo propose that Src-dependent phosphorylation of p120ctn on tyrosine 217 reduces the adhesive activity of the E-cadherin complex. However, tyrosine 217 was not identified as a phosphorylation site in a phosphopeptide mapping experiment identifying eight major Src phosphorylation sites (Mariner et al., 2001).

Other members of the Src kinase family have been reported to phosphorylate p120ctn. Fyn is constitutively bound to p120ctn, whereas Yes associates with p120ctn upon activation and both efficiently phosphorylate p120ctn (Calautti et al., 1998; Piedra et al., 2003). In Fyn-negative differentiating keratinocytes, p120ctn and β-catenin have strongly decreased tyrosine phosphorylation levels. Interestingly, these cells are unable to form
normal adherens junctions, suggesting that under physiological conditions Src family kinase-dependent tyrosine phosphorylation of p120ctn may play a positive role in cell adhesion (Calautti et al., 1998).

In addition to Src family kinases, several other tyrosine kinases affect the state of p120ctn phosphorylation. The non-receptor tyrosine kinase FER phosphorylates p120ctn and associates constitutively with the longest p120ctn isoform 1 (Kim and Wong, 1995). Fyn- and FER-dependent phosphorylation of p120ctn has been suggested to increase its affinity for E-cadherin, whereas Fyn and FER-dependent β-catenin phosphorylation disrupts its interaction with α-catenin (Piedra et al., 2003). p120ctn may therefore act as a docking protein recruiting tyrosine kinases to the E-cadherin complex where the kinases act on other catenin proteins, thereby modulating E-cadherin complex-mediated adhesion.

p120ctn tyrosine phosphorylation increases in response to stimulation with a number of growth factors, such as EGF, PDGF, HGF, VEGF, CSF-1 or NGF (Cozzolino et al., 2000; Downing and Reynolds, 1991; Esser et al., 1998; Shibamoto et al., 1994), but it is not known whether the receptor tyrosine kinases stimulated by these growth factors directly phosphorylate p120ctn or via the recruitment of associated tyrosine kinases, such as Src. Stimulation with these growth factors also promotes junctional disassembly and cell migration in a variety of cell types (Seedorf, 1995; Wells et al., 2002), further implicating p120ctn tyrosine phosphorylation in these processes.

The tyrosine phosphorylation state of p120ctn is also controlled by a number of tyrosine phosphatases. The receptor protein tyrosine phosphatases (RPTP) RPTPμ (Zondag et al., 2000) and DEP-1 (Holsinger et al., 2002) directly bind to and dephosphorylate p120ctn. Both RPTPs colocalize with p120ctn at sites of cell-cell contact, suggesting that p120ctn may recruit these RPTP to the E-cadherin complex, where they may be involved in regulating the adhesive properties of the complex by dephosphorylating p120ctn and possibly other members of the complex. Whereas the interaction with RPTPμ did not require p120ctn tyrosine phosphorylation (Zondag et al., 2000), the interaction with DEP-1 is tyrosine phosphorylation-dependent. DEP-1 does not contain SH2 domains and the mechanisms by which p120ctn tyrosine phosphorylation promotes association with DEP-1 are unknown.

Upon EGF stimulation, p120ctn associates with the non-receptor tyrosine phosphatase SHP-1 (Keilhack et al., 2000). SHP-1 contains tandem SH2 (Src homology 2) domains in
its N-terminal region. Using these SH2 domains, SHP-1 associates with p120ctn in a tyrosine phosphorylation-dependent manner and differentially associates with p120ctn isoforms correlating with the extent of their EGF-induced tyrosine phosphorylation. For instance, p120ctn isoforms containing the short exon C are less efficiently phosphorylated and consequently bind only low levels of SHP-1. Interestingly, the association of SHP-1 and p120ctn is only abrogated if all eight Src phosphorylation sites identified by Marriner et al. are mutated to non-phosphorylatable phenylalanines, suggesting that these sites function redundantly in mediating the SHP-1/p120ctn interaction (Mariner et al., 2001). Consistent with this idea, the consensus binding sequence for the terminal SH2 domain of SHP-1 (Tamir et al., 2000) partially overlaps with several of the p120ctn phosphomotifs (Mariner et al., 2001).
Eight Src-induced tyrosine phosphorylation sites (tyrosines 96, 112, 228, 257, 280, 291, 296 and 302) identified in a phosphopeptide mapping study are situated within the N-terminal region of p120ctn (Mariner et al., 2001). Ozawa and Ohkubo propose another tyrosine phosphorylation site at tyrosine 217 (Ozawa and Ohkubo, 2001). The use of the third and fourth ATG start codon generates p120ctn molecules which lack some or all Src-induced tyrosine phosphorylation sites.
Chapter 1

1.6.2 Serine/threonine phosphorylation

In addition to tyrosine phosphorylation, p120ctn is phosphorylated on serine and (to a lesser extent) on threonine residues (Aono et al., 1999; Downing and Reynolds, 1991; Ratcliffe et al., 1997; Ratcliffe et al., 1999; Wong et al., 2000). Treatment with the kinase inhibitor staurosporine reduces p120ctn serine/threonine phosphorylation (Ratcliffe, 1997, 1999), suggesting that p120ctn is targeted by class AGC (cAMP-dependent protein kinase/protein kinase C extended family) kinases or Ca^{2+}/calmodulin-dependent kinases (CaMK), all of which are potently inhibited by staurosporine (Herbert et al., 1991; Omura et al., 1977; Tamaoki et al., 1986). In addition, both inhibition (Ratcliffe, 1997) and activation (Ratcliffe et al., 1999; Wong et al., 2000) of protein kinase C (PKC) causes a decrease in the level of p120ctn serine/threonine phosphorylation, implicating PKC-modulated kinases and/or phosphatases in the control of the p120ctn phosphorylation state.

Little is known about the function of p120ctn serine/threonine phosphorylation, but the finding that the p120ctn serine/threonine phosphorylation state depends on its intracellular localization could be an important step towards a better understanding of the relevance of these phosphorylation events. Upon recruitment to the E-cadherin complex, p120ctn becomes serine/threonine phosphorylated (Ohkubo and Ozawa, 1999; Reynolds et al., 1994; Thoreson et al., 2000), whereas p120ctn is only weakly phosphorylated when it is localized in the cytoplasm (Ohkubo and Ozawa, 1999; Thoreson et al., 2000). p120ctn therefore appears to be targeted by kinases associated with the E-cadherin complex at the plasma membrane and it is likely that these phosphorylation events are important in regulating the E-cadherin complex. Consistent with this idea, serine/threonine phosphorylation events within the N-terminal region of p120ctn have been implicated in negatively regulating E-cadherin-mediated adhesion (see 1.4.5) (Aono et al., 1999; Ohkubo and Ozawa, 1999). However, these studies failed to identify specific phosphorylation events and other effects mediated by the N-terminal region of p120ctn may account for the negative effect on cadherin clustering.

In endothelial cells, p120ctn is dephosphorylated on serine/threonine residues in response to treatment with vascular endothelial growth factor (VEGF), histamine or PKC-activating phorbol esters (Wong et al., 2000), i.e. stimuli that increase vascular permeability (Lum and Malik, 1994; Neufeld et al., 1999). Although vascular permeability is normally regulated by tight junctions, the establishment of adherens
junction is a prerequisite for tight junction formation (Denker and Nigam, 1998; Mitic et al., 2000). p120ctn may therefore be involved in regulating vascular permeability in the endothelium by modulating VE-cadherin adhesiveness.

1.7 Nuclear localization of p120ctn

In fibroblasts or in cancer cells that have lost E-cadherin expression, p120ctn is frequently found in the nucleus (van Hengel et al., 1999), p120ctn contains a nuclear export sequence (NES) in exon B and isoforms containing this exon are excluded from the nucleus (van Hengel et al., 1999). Less is known about the mechanisms by which p120ctn enters the nucleus. p120ctn family members share a highly conserved basic motif inserted into ARM repeat 6 that resembles a nuclear localization sequence (NLS) and has been proposed to play a role in nuclear localization of these proteins (Lu et al., 1999). Other studies implicate the N-terminal domain of p120ctn family members in mediating nuclear import: Aho et al. propose the presence of a putative bipartite nuclear localization sequence just upstream of the fourth start codon of p120ctn (Aho et al., 2002), whereas Mariner et al. demonstrate that the sequences for nuclear localization of the p120ctn family member ARVCF (see Section 1.9) reside within its N-terminal half (Mariner et al., 2000).

The discovery that the ARM repeat domain of p120ctn binds the transcription factor Kaiso suggests a role for p120ctn in regulating transcription. Kaiso is a putative transcriptional repressor of the POZ/ZF (Pox virus and zinc finger) family and is a dual specificity DNA-binding protein that recognizes the specific consensus sequence TCCTGCNA as well as methyl-CpG dinucleotides (Daniel et al., 2002; Prokhortchouk et al., 2001). Two copies of the Kaiso consensus sequence are present in the promoter of the Matrilysin/MMP7 gene (Daniel et al., 2002), implicating Matrilysin/MMP7 as a target gene for Kaiso. In many cancers E-cadherin expression is downregulated by methylation of CpG dinucleotides in the promoter region (Graff et al., 1995), suggesting an intriguing model in which p120ctn would control E-cadherin expression via Kaiso. However, as long as the Kaiso target genes have not been identified, the significance of the Kaiso/p120ctn interaction remains unknown.
1.8 p120ctn interacts with BP180

Using the yeast two-hybrid system, Aho et al. have demonstrated that p120ctn interacts with the 180 kDa bullous pemphigoid antigen (BP180) (Aho et al., 1999). BP180, also known as type XVII collagen, is an unusual transmembrane protein with collagenous extracellular domain (Hirako and Owaribe, 1998). BP180 is a key component of hemidesmosomes, adhesion structures responsible for linking keratin intermediate filaments of stratified and complex epithelia to components of the extracellular matrix, such as collagen fibrils. The significance of the p120ctn/BP180 interaction is unknown. However, this finding raises the interesting possibility that p120ctn is involved in the regulation of cell-matrix as well as cell-cell adhesion.

1.9 p120ctn family members

The p120ctn family consists of seven members: p120ctn, ARVCF (Armadillo repeat gene deleted in Velo-Cardio-Facial Syndrome) (Sirotkin et al., 1997), δ-catenin/NPRAP (neural plakophilin-related Armadillo protein) (Paffenholtz and Franke, 1997; Zhou et al., 1997), p0071 (Hatzfeld and Nachtsheim, 1996) and plakophilin 1, 2 and 3 (Bonne et al., 1999; Hatzfeld et al., 1994; Heid et al., 1994; Kowalczyk et al., 1999; Mertens et al., 1996; Schmidt et al., 1999).

All members of the p120ctn family contain the characteristic central armadillo domain consisting of 10 ARM repeats, but differ in the degree of similarity they share with p120ctn in their ARM repeat domain. ARVCF, δ-catenin/NPRAP and p0071 share approximately 45% identity in their ARM repeat domain with p120ctn, whereas plakophilins are more distantly related with about 30% identity in this region (Anastasiadis and Reynolds, 2000). The lower degree of conservation of the plakophilin ARM repeat domains coincides with functional differences: whereas p120ctn, ARVCF, and δ-catenin/NPRAP all interact with classical cadherins at adherens junctions (Lu et al., 1999; Mariner et al., 2000; Paffenholtz et al., 1999; Reynolds et al., 1994), plakophilins localize to desmosomes through interactions with desmosomal cadherins mediated by their N-terminal domains (Hatzfeld et al., 2000). Plakophilins also interact with keratins (Bonne et al., 2003; Klymkowsky, 1999; McGrath et al., 1997; Smith and Fuchs, 1998) and may help to link desmosomes to keratin filaments (Bonne et al., 2003; Nachtsheim, 1994). Interestingly, p0071 localizes both to desmosomes (Hatzfeld and Nachtsheim, 1996; Jaulin-Bastard et al., 2002) and cadherin complexes (Calkins et al., 2003; Hatzfeld et al., 2000).
et al., 2003) and may thus combine functions of plakophilins and the more p120ctn-like family members.

Of the p120ctn family members, ARVCF is most closely related to p120ctn. The exon boundaries between p120ctn and ARVCF are preserved, demonstrating an ancient evolutionary relationship (Keirsebilck et al., 1998). There are functional overlaps but also important differences between p120ctn and ARVCF. Like p120ctn, ARVCF associates with cadherins and when overexpressed, competes with p120ctn for the JMD of classical cadherins (Mariner et al., 2000). However, ARVCF is at least ten times less abundant than p120ctn in a variety of cell types, making it unlikely that it functionally competes with p120ctn in these cells. Rather, ARVCF may modulate the adhesive properties of cadherin complexes in a way that does not require high expression levels. Nevertheless, it is possible that ARVCF expression is higher in some cell types, allowing it to compete more efficiently with p120ctn for binding to the JMD of classical cadherins. Interestingly, despite the high degree of similarity between p120ctn and ARVCF, the latter fails to induce the branching phenotype when overexpressed in fibroblasts (Mariner et al., 2000), suggesting that ARVCF is either unable to regulate Rho GTPases or that this regulation requires posttranslational modifications or splicing events. ARVCF frequently displays nuclear localization under conditions were p120ctn is excluded from the nucleus (Mariner et al., 2000), suggesting that nuclear translocation of p120ctn and ARVCF is differently regulated.

Whereas p120ctn is a ubiquitously expressed protein, δ-catenin/NPRAP appears to be exclusively expressed in neural tissues (Kawamura et al., 1999; Paffenholz and Franke, 1997), where it binds to the JMD of N-cadherin (Lu et al., 1999). Overexpression of δ-catenin/NPRAP in fibroblasts induces a p120ctn-like branching phenotype, whereas overexpression in primary neurons enhances dendritic morphogenesis (Kim et al., 2002). This points towards a role for δ-catenin/NPRAP in regulating processes that determine neuronal morphology, such as neurite extension. The p120ctn-induced branching phenotype observed in fibroblasts morphologically resembles the extension of neurites during neuronal differentiation, suggesting that p120ctn and δ-catenin/NPRAP may function concertedly in regulating neuronal morphology. This hypothesis is supported by the fact that p120ctn is highly expressed in the developing CNS of Drosophila and rat (Myster et al., 2003); (Chauvet et al., 2003) and that p120ctn localizes to dendritic spines in cultured hippocampal neurons (Chauvet et al., 2003).
Interestingly, ARVCF, δ-catenin/NPRAP and p0071, but not pl20ctn, contain a C-terminal PDZ-binding motif. Recently a number of PDZ-containing binding partners for these motifs have been identified. For instance, ARVCF, δ-catenin/NPRAP (Laura et al., 2002) and p0071 (Izawa et al., 2002b; Jaulin-Bastard et al., 2002) interact in a PDZ domain-dependent manner with the ErbB2 receptor-interacting protein ERBIN (Borg et al., 2000). Likewise, δ-catenin/NPRAP associates with Densin-180 (Izawa et al., 2002a), a protein found in the postsynaptic density (Apperson et al., 1996), a specialization of the submembranous cytoskeleton seen at postsynaptic sites in the central nervous system (CNS). In addition, δ-catenin/NPRAP interacts with the novel scaffolding protein Papin (Deguchi et al., 2000) and with S-SCAM, another postsynaptic scaffolding protein (Hirao et al., 1998; Ide et al., 1999). The lack of the C-terminal PDZ-binding motif constitutes an important difference between p120ctn its three closest relatives in the p120ctn family.

However, all p120ctn family members can localize to the nucleus (Bonne et al., 1999; Mariner et al., 2000; Mertens et al., 1996; Schmidt et al., 1997; van Hengel et al., 1999), suggesting that communication of signals from cellular junctions to the nucleus is a common function of these proteins.

1.10 A possible involvement of p120ctn in cancer

An estimated 85% of cancers are believed to originate from epithelia with aberrant adhesive properties. The E-cadherin complex is the main mediator of adhesion between epithelial cells and there is a strong correlation between aberrant expression of members of the E-cadherin complex, in particular E-cadherin and β-catenin, and malignancy (Nollet et al., 1999; Van Aken et al., 2001). Because p120ctn is an important regulator of E-cadherin-mediated adhesion, it is likely to contribute to tumour growth and/or invasion.

In a number of tumours, p120ctn localizes to the cytoplasm and the nucleus (Gold et al., 1998; Jawhari et al., 1999; Lo Muzio et al., 2002), which is probably caused by loss of E-cadherin expression. Complete loss of p120ctn expression has been reported in a number of human tumours and is in some cases statistically linked to an aggressive tumour phenotype (Dillon et al., 1998; Gold et al., 1998; Shimazui et al., 1996; Skoudy et al., 1996; Syrigos et al., 1998). The expression profile of p120ctn isoforms is very heterogeneous in human cancer cell lines (Keirsebilck et al., 1998; Skoudy et al., 1996; Wu et al., 1998), but the significance of altered isoform expression pattern for cellular transformation is unknown. The oncogenic potential of increased nuclear β-catenin levels
is due to the aberrant activation of TCF/LEF transcription factors, resulting in the increased transcription of TCF/LEF-controlled genes regulating cell proliferation, such as cyclin D1 and c-myc (Nollet et al., 1999; Novak and Dedhar, 1999). Since the Kaiso target-responsive genes have not been identified, it is unknown whether nuclear accumulation of p120ctn contributes to malignancy in a similar manner. Because increasing the cytoplasmic levels of p120ctn stimulates cell migration (Grosheva et al., 2001; Noren et al., 2000), p120ctn may contribute to the invasive behaviour of tumours of cancer cells that have lost E-cadherin expression and consequently are unable to sequester p120ctn at the plasma membrane.

1.11 Hepatocyte growth factor

Hepatocyte growth factor (HGF), also known as scatter factor, is a secreted morphogen, mitogen and motogen that plays multiple roles in embryogenesis, wound healing and is implicated in the development of cancer (Birchmeier and Gherardi, 1998; Zarnegar and Michalopoulos, 1995). HGF is normally secreted by cells of mesenchymal origin and acts through its receptor, the Met tyrosine kinase, which is expressed by many cell types including epithelial and endothelial cells. HGF-treatment of MDCK cells has been used as a model system to study adherens junction disassembly. HGF initially induces centrifugal spreading of MDCK cells in colonies and subsequently stimulates cell-cell dissociation, allowing each cell to scatter or detach from colonies and migrate independently of other cells (Ridley et al., 1995; Stoker and Perryman, 1985). HGF-induced adherens junction disassembly requires both the activation of p42/p44 mitogen-activated protein kinase (MAPK) and PI 3-kinase by Ras (Potempa and Ridley, 1998), but the mechanisms at the level of the junctions are unclear. HGF induces a shift of the adherens junction proteins E-cadherin and β-catenin form the NP-40-insoluble to the NP-40-soluble fraction of MDCK cell lysates (Potempa and Ridley, 1998). Detergent insolubility is indicative of association with the actin cytoskeleton and adherens junctions (Nathke et al., 1994) and therefore, it can be concluded that HGF promotes the loss of these proteins from adherens junctions. HGF-induced tyrosine phosphorylation of β-catenin, plakoglobin/γ-catenin and p120ctn has been suggested as a mechanism to modulate cadherin-mediated adhesion (Hiscox and Jiang, 1999; Shibamoto et al., 1994). Lastly, HGF stimulation induces co-endocytosis of E-cadherin with the HGF receptor c-
Met (Kamei et al., 1999). HGF may therefore regulate E-cadherin-mediated cell-cell adhesion by promoting the removal of E-cadherin from the plasma membrane.
1.12 Aims of the project

p120ctn is involved in a variety of cellular functions. At the plasma membrane it is involved in modulating cadherin-mediated cell-cell adhesion, whereas in the cytoplasm it promotes cell migration and cytoskeletal rearrangement by regulating Rho GTPases. Furthermore, in the nucleus it interacts with the transcription factor Kaiso and may be involved in regulating gene transcription. The coordination of the different functions of p120ctn requires the precise regulation of its intracellular localization. There is evidence that changes in the intracellular localization of p120ctn coincide with changes in its state of serine/threonine phosphorylation, but the relevant phosphorylation sites are unknown.

The aim of this study was to identify mechanisms that regulate the intracellular localization of p120ctn. To this end, serine/threonine phosphorylation sites were to be identified and tested for their involvement in controlling the localization of p120ctn within the cell. Furthermore, using GST-pulldown experiments and protein identification by mass spectrometry, new p120ctn interaction partners were to be identified and tested for their ability to recruit p120ctn to different cellular compartments. In addition, the behaviour of p120ctn during HGF-induced adherens junction disassembly was to be investigated.
## Materials and Methods

### 2.1 Materials

#### 2.1.1 Reagents and Kits

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<td>PE Applied Biosystems (<a href="http://home.appliedbiosystems.com">http://home.appliedbiosystems.com</a>)</td>
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<td>DNA markers II and VI</td>
<td>Roche (<a href="http://www.roche.com">http://www.roche.com</a>)</td>
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<td>ECL – enhanced chemiluminescence substrate</td>
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<td>PDA, 2D gel crosslinker</td>
<td>Bio-Rad Laboratories (<a href="http://Bio-Rad.com">http://Bio-Rad.com</a>)</td>
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</table>
Chapter 2 Materials and Methods

Multiphor II  
Amersham Pharmacia  
(http://www4.amershambiosciences.com)

GIBCO OptiMEM transfection medium  
Invitrogen Life Technologies  
http://www.invitrogen.com/

DyeEx™ 2.0 spin columns for dye-terminator removal  
Qiagen Inc  
(http://www.qiagen.com)

INFαF supercompetent E. coli  
Invitrogen Life Technologies  
(http://www.invitrogen.com)

Trypsin/EDTA  
Gibco  
(http://www.invitrogen.com)

Coolview 12 cooled CCD camera  
Photonic Science  
(http://www.photonic-science.ltd.uk)

Moviol  
Calbiochem  
(http://www.calbiochem.com)

HGF (recombinant)  
R & D Systems  
http://www.rndsystems.com/

2.1.2 Buffers and Solutions

1% NP-40 lysis buffer  
1% NP-40, 150 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 1 mM Na₃VO₄, 25 mM NaF, 1% phosphatase inhibitor cocktail II (Sigma) and 0.1 tablet/ml Mini EDTA-free protease inhibitor cocktail

2D equilibration buffer  
6 M urea, 2% SDS, 1% DTT, 30% glycerol, 50 mM Tris pH 6.8

2D gel acrylamide stock  
30% acrylamide (29.2 g), 2.67% PDA (0.8 g), (add 100 ml)

2D lower buffer solution  
10 mM H₃PO₄

2D lysis buffer  
8 M Urea, 2 M Thiourea, 4% CHAPS, 65 mM, 1% phosphatase inhibitor cocktail II (Sigma) and 0.1 tablet/ml Mini EDTA-free protease inhibitor cocktail

2D overlay buffer  
8 M urea, 1% ampholine (ampholine pH 5 to 8), 5% NP-40, 100 mM DTT

2D tube gel monomer solution  
3.34 M urea (9.6 M), 0.834 ml 2D acrylamide stock, 0.335 ml 2D tube gel stock B, 200 µl Ampholines pH 3 to 10, 135 µl Ampholines pH 5 to 8, 2.333 ml H₂O, 10 µl TEMED, 17 µl APS (10%)
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<tr>
<th><strong>2D tube gel, stock B</strong></th>
<th>0.3 g CHAPS, 0.9 ml NP-40, 0.9 ml H₂O</th>
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<td><strong>2D upper buffer solution</strong></td>
<td>20 mM NaOH</td>
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<td><strong>5 x SDS-PAGE loading buffer</strong></td>
<td>250 mM Tris-HCl pH6.8, 50% glycerol, 5% SDS, 0.2% bromophenol blue, 5% β-mercaptoethanol</td>
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<td><strong>50 x TAE</strong></td>
<td>2 M Tris-HCL, pH 8.0, 50 mM EDTA, pH 8.0, 5.7% (v/v) acetic acid</td>
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<td><strong>6 x DNA loading buffer</strong></td>
<td>0.25% bromophenol blue, 0.25% xylene cyanol in a 25% (w/v) Ficoll 400 solution</td>
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<td><strong>Ampicillin 100 x stock</strong></td>
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<td><strong>Coomassie destain solution</strong></td>
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<td><strong>Coomassie stain solution</strong></td>
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<td><strong>High salt wash buffer</strong></td>
<td>0.5 M LiCl, 10 mM Tris pH 7.5</td>
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<td><strong>Kanamycin A (monosulphate) 100 x stock</strong></td>
<td>50 mg/ml in ddH₂O</td>
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<tr>
<td><strong>LB</strong></td>
<td>1.5% Bacto-Tryptone, 1% Bacto-Yeast, 1% NaCl (w/v)</td>
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<td><strong>LB-Agar</strong></td>
<td>1.5% (w/v) bacterial agar in L-Broth</td>
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<tr>
<td><strong>LMB buffer (Rac pull down lysis buffer)</strong></td>
<td>25 mM Heps pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM NaF, 1 mM Na₃VO₄</td>
</tr>
<tr>
<td><strong>Nuclear extraction buffer A</strong></td>
<td>10 mM Heps pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 0.1 tablet/ml Mini EDTA-free protease inhibitor cocktail</td>
</tr>
<tr>
<td><strong>Nuclear extraction buffer C</strong></td>
<td>20 mM Heps pH 8.0, 1.5 mM MgCl₂, 25% glycerol, 420 mM NaCl, 0.2 EDTA, pH 8.0, 0.1 tablet/ml Mini EDTA-free protease inhibitor cocktail</td>
</tr>
<tr>
<td><strong>Nuclear extraction buffer D</strong></td>
<td>20 mM Heps pH 8.0, 20% glycerol, 100 mM KCl, 0.2 mM EDTA pH 8.0, 0.1</td>
</tr>
</tbody>
</table>
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PAW: First dimension running buffer for TLC
mM KCl, 0.2 mM EDTA pH 8.0, 0.1 tablet/ml Mini EDTA-free protease inhibitor cocktail

PAWB: Second dimension running buffer for TLC
pyrridine:acetic acid:ddH₂O (10:100:1890)

PBS
pyrridine:acetic acid:ddH₂O:butanol (15:3:12:10)

Running buffer
137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 2 mM K₂HPO₄

Silverstain developing buffer
0.04% (w/v) formaldehyde, 2% Na₂CO₃

TBS
20 mM Tris-HCl pH 7.6, 120 mM NaCl

Time lapse medium
5ml Hanks Balanced Salts (10x), 0.235g Hepes (added first), 0.260 g Hepes/Sodium salt, 5 ml FCS, 40 ml H₂O

Transfer buffer
20% (v/v) methanol, 1.44% (w/v) glycine, 0.3025% (w/v) Tris

10x TTBS
29.22 g NaCl, 20 ml 1 M Tris pH 8.0, 1 ml Tween-20, 0.5 ml azide (20%), add 100 ml ddH₂O

PKA kinase buffer
10 mM Tris/HCl pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT

2.1.3 Antibodies

mouse anti-VSV glycoprotein clone P5D4 Sigma Aldrich Company Ltd (http://www.sigmaaldrich.com)
mouse monoclonal anti-β-tubulin TUB 2.1 Sigma Aldrich Company Ltd (http://www.sigmaaldrich.com)
mouse monoclonal anti-acetylated tubulin clone 6-11B-1 Sigma Aldrich Company Ltd (http://www.sigmaaldrich.com)
rabbit polyclonal anti-γ-tubulin Sigma Aldrich Company Ltd (http://www.sigmaaldrich.com)
mouse monoclonal anti-p120ctn Transduction Laboratories (http://wwwbdbiosciencescom/pharmingen)
fluorescein (FITC)-conjugated donkey anti-mouse IgG
Cy5-conjugated goat anti-mouse
TRITC-conjugated goat anti-rabbit
sheep HRP-conjugated anti-mouse IgG
mouse monoclonal anti-β-catenin
mouse monoclonal anti-α-catenin
mouse monoclonal anti-γ-catenin
mouse monoclonal anti-E-cadherin
mouse monoclonal anti-N-cadherin
mouse monoclonal anti-phosphotyrosine PY99
mouse monoclonal anti-Rac1
mouse monoclonal anti-HA tag

2.1.4 Primers
2.1.4.1 Mutagenesis Primers

S225AF: 5' GGTTATCCAGGTGGCGCTGACAACTACGGCAG 3'
S225AR: 5' CTGCCGTAGTTGTCAGCGCCACCTGGATAACC 3'
S230AF: 5' GACAACTACGGCGCTCTGTCCCGGGTGACCCG 3'
S230AR: 5' CGGGTCACCCCGGGACAGACCGCCGTAGTTGTC 3'

kindly provided by Dr Maria Balda, UCL
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S232AF  5' GACAACCTACGGCAGTCTGGCCCGGGTGACCCG 3'
S232AR  5' CGGGTCACCCGGCCAGACTGCCGTAGTTGTC 3'
SS230232AAF: 5' GACAACCTACGGCAGTCTGGCCCGGGTGACCCG 3'
SS230232AAR: 5' CGGGTCACCCGGCCAGACTGCCGTAGTTGTC 3'
S230DF  5' GACAACCTACGGCAGTCTGGCCCGGGTGACCCG 3'
S230DR  5' CGGGTCACCCGGCCAGACTGCCGTAGTTGTC 3'
S232DF  5' GACAACCTACGGCAGTCTGGCCCGGGTGACCCG 3'
S232DR  5' CGGGTCACCCGGCCAGACTGCCGTAGTTGTC 3'
S230232DDF 5' GACAACCTACGGCAGTCTGGCCCGGGTGACCCG 3'
S230232DDR 5' CGGGTCACCCGGCCAGACTGCCGTAGTTGTC 3'
S244AF: 5' CGGTATAGGGCCCCCATGGAAGGCTACCGG 3'
S244AR: 5' CGGTATAGGGCCCCCATGGAAGGCTACCGG 3'
S252AF: 5' GGCTACCAGGCCCCGTAGACAAGATGTC 3'
S252AR: 5' GACATCTTGTCTAGCGGGTGCCCGGTAGCC 3'
S252DF  5' GGCTACCAGGCCCCGTAGACAAGATGTC 3'
S252DR  5' GACATCTTGTCTAGCGGGTGCCCGGTAGCC 3'
SS268269AAF: 5' CAGGTTCCAGTGATAGGTGAGCCGCTGTGGACCTGCATCG 3'
SS268269AAR: 5' CGATGCAGGTCCACAGCCGCTCCACCTACTCGAACCTG 3'
SS268269DDF 5' CAGGTTCCAGTGATAGGTGAGCCGCTGTGGACCTGCATCG 3'
SS268269DDR 5' CGATGCAGGTCCACAGCCGCTCCACCTACTCGAACCTG 3'
S288AF: 5' GAGGATGACCAGCGCCCATGGAAGGCTATGATGAC 3'
S288AR: 5' GTCATCATAGCCCATGGCGCGCTGGTCATCCTC 3'
S300AF: 5' CTGATTACGGCAGTGGCTCGTATTATGGTGACTGCC 3'
S300AR: 5' GGCAGTACATCAATGCTCAGGCCATGCGCATGCTGAGA 3'
S480/481AF 5' GGAACCTCTGTGGAATCTCGCAGCCCATGATTCAATC 3'
S480/481AR 5' GATTGAATCATGGGCTGCGAGATTCCACAGGTTCC 3'
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S506AF 5’ GTTATCATTTCCACATGCTGGGTGGGAGAGAGA 3’
S506AR 5’ GTTCTCTCTCCCCACCAGCATGTGGAAGAATGATAAC 3’
S538AF 5’ CTGCCCTCGGAAGCTAGGCTCCAGAAGAGTGAAG 3’
S538AR 5’ CTTCACTCTCTCTCTGAGGCTACGTCTCCGAAGGGCAG 3’
S539AF 5’ CTGCCCTCGGAACAGGAAGAGGAGGTGAAG 3’
S539AR 5’ CTTCACTCTCTCTCTGAGGCTACGTCTCCGAAGGGCAG 3’
S538/539AF 5’ CTGCCCTCGGAACAGGAGGAGGAGGTGAAG 3’
S538/539AR 5’ CTTCACTCTCTCTCTGAGGCTACGTCTCCGAAGGGCAG 3’
SS538/39DDF 5’ CTGCCCTCGGAACAGGAGGAGGAGGTGAAG 3’
SS538/39DDR 5’ CTTCACTCTCTCTCTGAGGCTACGTCTCCGAAGGGCAG 3’
S587AF 5’ CCTCCCTGGGAACCTAGCATAACGCGATTCATCG 3’
S587AR 5’ CGATGAACCTGTGATGCTAAGGCTCCGGAAGAGG 3’
S587DF 5’ CCTCCCTGGGAACCTAGCATAACGCGATTCATCG 3’
S587DR 5’ CGATGAACCTGTGATGCTAAGGCTCCGGAAGAGG 3’
S587PF 5’ CCTCCCTGGGAACCTAGCATAACGCGATTCATCG 3’
S587PR 5’ CGATGAACCTGTGATGCTAAGGCTCCGGAAGAGG 3’
S889AF 5’ GACCACAACAGAGACCTGGACCGATCTGGG 3’
S889AR 5’ CCCAGATCGGTCCAGTGCTCTGTTGCTGTC 3’
S889DF 5’ GAGACCACAACAGAGACCTGGACCGATCTGGG 3’
S889DR 5’ CCCAGATCGGTCCAGTGCTCTGTTGCTGTC 3’
LLGKGKKKF 5’ CTGCCAGTTGTGGAGGCCCCTGCTGGGCAAAGGG 3’
LLGKGKKKFR 5’ CCCCCTGCACGGGCTCACCAGCAACTGAGCAG 3’
KKKGKLLF 5’ GGGCAAGGGGTACGCTACAGGAGGTCCAGAAATG 3’
KKKGKLLFR 5’ CATTGCTGGATCTCTGTGACAGTACCCTTTGCC 3’
LLGLGGLLF 5’ GAGCCCTGCTGAGGCTAGGTTACTGCTAC 3’
LLGLGGLLFR 5’ GTAGGCACTAACCCTAAGGGCCAGCGGCTC 3’
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DeltaKF
5’ GTTGCTTTGGAGCCCCTACAGAGGATCCAG 3’

DeltaKR
5’ CTGGATCCTCTGTAGGGGCTCCAAAGCAAC 3’

SSS346/349/352AAA F
5’ GCATGAGCGGCGCTTTAGCAGCCTTGGATGCTT- TGGCAAAGGGAATGC 3’

SSS346/349/352AAAR
5’ GCATTTCCCTTTGCACAAAGCACAAGGCTGCTAAGC- GCGCCCAGCTCATGC 3’

SSS346/349/352DDDF
5’ GCATGAGCGGCGATTAGCAGACTTGGATGATTTG- GGCAAAGGGAATGC 3’

SSS346/349/352DDDR
5’ GCCATTTCCCTTTGCACAAAGCACAAGGCTGCTAAGC- GCGCCCAGCTCATGC

LLL347/350/353AAAF
5’ GAGCGGGCGAGCTGAGATGAGTGGCTCATCGGCCG- CGGAAAGGGAATG 3’

LLL347/350/353AAAR
5’ CATCCCCTTTTTGCCCTCCTCTGGAGAACCAGCAT- CATCTTTGGCCCTTCTTGG 3’

ExonCF
5’ CCAAGAAGCCGCAAGTGAGATGCTTCTCCAGAGG- GAAAGGCAAACGCATACAG 3’

ExonCR
5’ CTGTAGCGCTTTTTCCCTCTGGAGATCGCTCATC- CATCTTTGGCCCTTCTTGG 3’

2.1.4.2 Sequencing Primers

ARMF
5’ CTGCTCTGGGTCCGATTGCTC 3’

ARMR
5’ GCATCCACAGCCAGATTTCTCAG 3’

ARMFF
5’ CAGGAGCTATGAAGACATGATTG 3’

Primer 1
5’ GTGGACGCCTAGCAGATGATTG 3’

Primer 2
5’ GCCGTGGCTTACAGTCTTCATTAG 3’

2.1.4.3 Cloning primers

p120VSVF
5’ CGCTGAGAAGCTTACAAGGCTG 3’

p120VSVR
5’ GCTAGCTAGCAATCTTCTGCATCAAGGGTGCTCC 3’

ntermseq
5’ GTTCCTAGGAAGTGCGTAGCTGC 3’

EGFP EcoRI F
5’ CCGGAATTCATCCCATGATTCAATCAATCAATG 3’

EGFP KpnI R
5’ CGCGGTACCCTAACTAGTTTCTTATGGGAAATCCAC 3’

BamHIF
5’ CTAGGATCCTCCCTAGTATCATAAACATGGAGATTG 3’

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SmaIR 5' GAGCCCGGGCTAACTAGTTCTTTTAGGAAAATCCAC 3'
HindIIIIF 5' CTACGAAGCTTTGATTTCCCATGATTCATCAATCGGAAATGGAGG 3'
EGFPKpn1R new 5' GTCGGTACCCGACTAGTTCTTTTAGGAAATCCAC 3'
GSTBamHIIF 5' CAAGGATCCATGGACGACTCAGAGGTG 3'
GSTBstEII 5' GATTGGTCACCCGGGACAGACTG 3'
GSTHindIIIIR 5' CTATCCAAGCTTGCTAAACTGCCC 3'
GSTBglIIIF 5' CTAAGATCTTCCCATGATTCATCAATCGGAGATTG 3'

2.1.5 Software

Quantity One Bio-Rad Laboratories Ltd
(http://Bio-Rad.com)


NetPhos 2.0 http://www.cbs.dtu.dk/services/NetPhos/

Adobe Photoshop 6.0 Adobe Systems Inc.
(http://www.adobe.com/main.html)

LSM 5 image browser Carl Zeiss
(http://www.zeiss.com/)

Image Pro Plus v 3 Media Cybernetics
(http://www.mediacy.com)
2.2 Methods

2.2.1 Cell culture methods

2.2.1.1 Growing and passaging cells

MDCK, MDA-MB-231, HB4A and Cos-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine fetal calf serum (FCS), pen/strep (penicillin (100 IU/ml) and streptomycin (100 μg/ml)). For NIH 3T3 fibroblasts, FCS was substituted with donor calf serum (DCS). PC12 cells were grown in DMEM containing 10% heat-inactivated (1 h, 56°C) horse serum and 5% heat-inactivated FCS and pen/strep. Cells were grown in 75 cm² tissue culture flasks in a humidified atmosphere containing 10% CO₂ (MDCK, COS-7) or 5% CO₂ (MDA-MB-231, PC12) and passaged every 3 to 4 days or before reaching confluence. For passaging, cells were rinsed once with PBS and incubated with 2 ml trypsin/EDTA at 37°C until cells detached. 8 ml of growth medium was added to inactivate the trypsin and the cells were diluted according to their growth rate between 1:5 and 1:10.

2.2.1.2 Freezing and thawing of cells

Cells were pelleted at 200 g for 5 min, resuspended at 10⁶ –10⁷ cells/ml in freezing solution (cell culture medium containing 10% dimethyl sulphoxide (DMSO) and 20% FCS) and aliquoted into cryovials. Cryovials were inserted into a cell freezer and stored at -70°C to allow slow freezing overnight. After 24 h, the cells were transferred to liquid nitrogen for long-term storage.

To thaw cells, an aliquote was removed from liquid nitrogen and thawed rapidly at 37°C. After addition of fresh growth medium, cells were pelleted at 200 g for 5 min, resuspended in 10 ml of fresh growth medium, and transferred to a 75 cm² tissue culture flask. Cells were usually passaged at least two times before being used for experiments.

2.2.1.3 HGF stimulation of MDCK cells

MDCK cells were grown to no more than 30% confluence or so that most colonies comprised about 20 to 30 cells. Before stimulation with HGF, no medium was added to the cells. Cells were stimulated with 10 ng/ml HGF for 1 to 16 h.
2.2.1.4 Calcium switch experiment
MDCK cells were seeded in DMEM/10% FCS at $5 \times 10^4$ per coverslip or $5 \times 10^5$ cells per 10 cm tissue culture dish and grown for 48 h. Subsequently cells were transferred to medium containing 4 mM EGTA. After 3 h, cells were transferred back to DMEM/10% FCS containing a physiological calcium level (1.8 mM). Cells were fixed or lysed at different time points after the switch to physiological calcium levels.

2.2.1.5 Differentiation of PC12 cells
For differentiation, PC12 cells were plated on collagen-coated glass coverslips. Collagen coating of coverslips was performed by first rinsing them extensively in ddH$_2$O, followed by a rinse with 100% ethanol. After the coverslips had dried completely, they were precoated in 1mg/ml poly L-lysine (PPL) under constant rocking at RT for 30 min. Subsequently, the PPL-coated coverslips were rinsed 5 times in ddH$_2$O, washed once in 100% ethanol and dried in a sterile incubator. Finally, the coverslips were coated with Collagen type IV (100μg/ml) for 30 min at 37 °C, washed 5 times with calcium and magnesium free PBS and once with tissue culture medium. To initiate differentiation, the medium was switched from growth medium to DMEM containing 1% horse serum and 100 ng/ml nerve growth factor (NGF). Differentiation was carried out for up to 72 h.

2.2.1.6 Treatment with kinase and phosphatase inhibitors
Protein kinase and phosphatase inhibitors were used as follows: staurosporine (100 nM), H-89 (100 μM), calyculin A (100nM), cantharidic acid (100 μM), KT5720 (1 μM), LiCl (20 mM), dibutyryl-(db)-cAMP (1 or 1.5 mM) and PP2 (1 μM). For treatment of cells with pervanadate, 333 μl of a 3% H$_2$O$_2$ solution were added to 1 ml of a 20 mM Na$_3$VO$_4$ solution. After incubation at RT for 5 min, this solution was used at a final concentration of ~50 μM pervanadate. The nuclear export inhibitor leptomycin B was used at 5 ng/ml. For control reactions, cells were incubated with vehicle alone.

2.2.2 Transfection of cells
2.2.2.1 Electroporation
MDCK or Cos-7 cells were grown in 15cm tissue culture dishes to 95% confluence and split 1:2 the day before transfection. Plasmid DNA (10 μg) was electroporated into $2 \times 10^7$
cells in electroporation buffer (120 mM KCl, 10 mM K$_2$PO$_4$/ KH$_2$PO$_4$ pH 7.6, 2 mM MgCl$_2$, 25 mM Hepes pH 7.6, 0.5% Ficoll 400) using a Biorad Genepulser at 250 V, 960 mF. Subsequently, cells were diluted into 10 ml growth medium and plated into 10 cm tissue culture plates. For immunofluorescence studies, cells were further diluted 1:500 in growth medium and 0.5 ml of this dilution was added per coverslip in 4-well plates. Cells were lysed or fixed after expression for 16 to 24 h.

2.2.2.2 Lipofectamine 2000 and Polyfect transfection

MDCK, MDA-MB-231 and Cos-7 cells were seeded at 5 x 10$^4$ cells per coverslip in 4-well plates the day before transfection. For Lipofectamine 2000 transfection, 0.8 µg plasmid DNA and 2 µl Lipofectamine 2000 reagent were each diluted in 50 µl OptiMEM per coverslip, incubated at RT for 5 min and combined and incubated for a further 20 min at RT to allow complex formation. In the mean time, cells were washed 2x with PBS and 0.5 ml growth medium containing FCS, but no antibiotics, was added per well. 100 µl Lipofectamine 2000/DNA complexes were added per well and the cells left to express for 16 to 24 h.

PC12 cells were transfected as above, with the exception that they were plated on collagen-coated coverslips (see section 2.2.1.4) at 2 x 10$^4$ cells per coverslip 48 h before transfection. After transfection, PC12 cells were transferred immediately into differentiation medium and grown for 48 h.

Polyfect transfection reagent was used to transfect NIH 3T3 cells. Cells were seeded at 2 to 4 x 10$^4$ cells per coverslip in 4-well plates the day before transfection. Per cover slip (per 10 cm dish), 0.25 µg (4 µg) plasmid DNA were diluted in 17 µl (300 µl) OptiMEM, followed by addition of 4 µl (25 µl) Polyfect reagent and complex formation for 10 min at RT. Cells were washed 2x with PBS, followed by addition of 250 µl (7 ml) growth medium per well (dish). 100 µl (1 ml) growth medium was added to the Polyfect/DNA complexes and 110 µl (1.3 ml) of this mixture added drop-wise to the cells. The cells were left to express for 16 to 24 h.
2.2.3 DNA methods

2.2.3.1 Extraction of plasmid DNA from bacteria

For small-scale production of up to 10 µg plasmid DNA, the QIAprep Miniprep Kit was used. LB (5 ml) containing 100 µg/ml ampicillin or 50 µg/ml kanamycin was inoculated with a single colony from an agar plate and incubated overnight with shaking at 37°C. Bacterial cells from 1.5 ml of this culture were pelleted at 10000 g for 1 min and resuspended in 250 µl cell resuspension buffer (50 mM Tris/HCl, pH 8.0; 10 mM EDTA; 100 µg/ml RNAse A). After addition of 250 µl lysis buffer (200 mM NaOH, 1% SDS (w/v)) and 350 µl of neutralization buffer (3 M potassium acetate, pH 5.5), cell debris and proteins were removed by centrifugation at 10000 g for 10 min. The supernatant was applied to QIAprep spin columns and the columns were centrifuged at 10000 g for 1 min. After a wash with 750 µl wash buffer (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v), the DNA wash eluted with 50 µl elution buffer (1.25 M NaCl; 50 mM Tris/HCl, pH 8.5; 15% isopropanol (v/v)) by centrifugation of the column at 10000 g for 2 min. The minipreps typically yielded between 5 and 10 µg plasmid DNA.

For larger scale plasmid DNA purification of up to 700 µg, the Qiagen Maxi Kit was used. 1 ml of a 5 ml starter culture was diluted 1:100 and grown overnight. After pelleting of the bacterial cells at 3000 rpm in a Sorvall RC5C centrifuge (Rotor SS-34) for 30 min, the pellet was resuspended, lysed and neutralized using in each case 10 ml of buffers identical to the miniprep procedure. After centrifugation at 3000 rpm for 20 min, the supernatant was applied to a pre-equilibrated column (equilibration buffer: 750 ml NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton X-100) and washed 2x with a medium salt buffer (1 M NaCl; 50 mM MOPS, pH 7; 15% isopropanol (v/v)). The DNA was eluted with 15 ml of a high salt buffer (1.25 M NaCl; 50 mM Tris/HCl, pH 8.5; 15% isopropanol (v/v)). To precipitate the DNA, 0.7 volumes (10.5 ml) isopropanol was added and the preps centrifuged at 3000 rpm for 30 min. After decanting the supernatant, the pellet was washed with 5 ml 70% ethanol and air-dried. When resuspended in 200 µl ddH₂O, the preps typically yielded concentration of around 2.5 µg/µl, corresponding to a total of around 500 µg plasmid DNA.
2.2.3.2 Determination of DNA concentration

The DNA concentration of aqueous solutions was determined spectrophotometrically. An OD (optical density) of 1 at 260 nm was taken to be equivalent to 50 μg/ml DNA. In practice, 2 μl of DNA solution were diluted in 1 ml ddH2O and the OD$_{260}$ was determined. The DNA concentration was calculated as follows:

\[
\text{Conc}_{\text{DNA}} = \text{OD}_{260} \times 25 \mu\text{g/μl}
\]

2.2.3.3 Restriction enzyme digestion of DNA

Generally 1 to 4 μg plasmid DNA was digested using 10 units/μg enzyme for 1 to 3 h. Restriction enzymes BamHI, KpnI, NheI, BstEII, EcoRI, BsmBI, PflMII, BplI, Bsu36I and BglII were from New England Biolabs (NEB). They were used in the buffers recommended by the manufacturer. Double digests were performed in the buffer indicated in the NEB catalogue, or when necessary, sequentially after phenol/chloroform extraction of the first restriction product. After each digest, a fraction of the restriction product as well as a similar amount of undigested DNA were separated on a 1% agarose gel to determine complete digestion.

2.2.3.4 Ligation of DNA/CIP-treatment

DNA fragments were ligated using 200 units T4 DNA ligase (NEB) in the supplied ligation buffer at 16°C for 16 h. For “sticky end” ligations, a 3-fold molar excess of insert over vector DNA was used. For “blunt end” ligations, the vector digest was followed by treatment with 10 units of calf intestinal alkaline phosphatase (CIP) for 30 min to avoid vector religation and 10-fold insert excess was used. For three-way-ligations, the molar amounts of both inserts were determined as if they were used in a two-way ligation.

2.2.3.5 Phenol/Chloroform extraction of DNA

To remove protein contaminations that might interfere with subsequent DNA manipulation reactions, the DNA-containing solutions were mixed with an equal volume of phenol/H$_2$O-saturated chloroform (1:1). After centrifugation, the aqueous (upper) phase was removed to a new reaction tube and mixed with an equal volume of H$_2$O-
saturated chloroform. After centrifugation, the DNA was usually ethanol-precipitated from the aqueous phase.

2.2.3.6 Ethanol precipitation of DNA
For DNA purification and concentration, 1/10 volume sodium acetate pH 5.2 was added to the sample solution, followed by 2.5 volumes of cold 100% ethanol. After incubation for 20 min at -20°C, the precipitated DNA was recovered by centrifugation at 13000 g for 15 min. The pellet was washed with 0.75 ml 70% (v/v) ethanol, air-dried and finally resuspended in ddH₂O.

2.2.3.7 Polymerase Chain Reaction (PCR)
For DNA amplification by PCR, 10 to 50 ng of template DNA, 50 μM of both forward and reverse primers and 200 μM of each dNTP in a total of 99.5 μl 1x ThermoPol reaction buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% Triton X-100) were heated to 94°C for 1 min, then to 80°C for 5 min, during which period 0.5 μl Vent polymerase was added to the reaction to allow complete DNA denaturation before polymerase addition (Hot start). Reactions were carried out for 30 cycles, each cycle consisting of denaturing (94°C for 30 sec), annealing (55°C for 30 sec) and extension (72°C for 1 min). The PCR product was analysed by agarose gel electrophoresis and when necessary purified and concentrated using chloroform/phenol extraction and ethanol precipitation.

2.2.3.8 Agarose gel electrophoresis
DNA was analysed by size separation in 1% agarose gels. To prepare gels, 1 g of electrophoresis grade agarose was resuspended in 100 ml 1x TAE and carefully dissolved by boiling in a microwave oven. After cooling to 50°C, 0.5 μg/ml ethidium bromide was added to allow post-separation visualization of the DNA under UV light (254 nm) and the solution poured into the gel tank containing the well comb. After solidifying the DNA samples supplemented with 6x DNA loading buffer were applied to the wells and the gel electrophoresed at 5 to 10 Volts/cm until sufficient separation was achieved. To estimate the size of DNA fragments, either a 1 kb DNA ladder or Markers II and IV were run along side the samples.
2.2.3.9 Extraction of DNA from agarose gels
Following agarose gel electrophoresis, fragments of interest were identified under UV light (254 nm) and excised from the gel with a sterile blade. For DNA recovery the GeneClean kit was used. The gel piece was incubated with three volumes of NaI (sodium iodide) at 50°C until the agarose had completely dissolved. 10 μl of glass milk (a slurry of glass micro beads) per 1 μg DNA were added and the mixture incubated on a rotating wheel for 10 min. The glass milk beads with the bound DNA were pelleted by centrifugation and washed three times with the supplied wash buffer. Beads were left to air-dry and subsequently the DNA was eluted into ddH₂O.

2.2.3.10 Site-directed mutagenesis
Single or double base changes or deletions of multiple bases were performed following the guidelines of the QuikChange Site-Directed Mutagenesis Kit. For each mutagenesis reaction a pair of forward and complimentary reverse primers were designed with a melting temperature ($T_m$) of not below 78°C, using the following formula

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%\text{mismatch},$$

whereby GC is the percentage of guanidine and cytosine bases and N the total number of bases. PCRs were performed using 10 nM of template DNA, 200 μM of each dNTP, 125 pmol of each primer and 2.5 units of Pfu Turbo polymerase in a total reaction volume of 50 μl of 1 x Pfu buffer. For single and double base changes, 16 cycles of denaturation (30 sec at 95°C), annealing (1 min at 50°C) and extension (2 min/kb plasmid) were used, whereas for the deletion of multiple bases 18 cycles were used. Subsequently, methylated parental DNA was digested by adding 10 units of the restriction enzyme DpnI directly into the PCR reaction mix and incubation at 37°C for 1 h. DNA containing the mutation was transformed into INFαF E.coli (section 2.2.3.12) and the correct sequence alterations changed verified by sequencing.
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2.2.3.11 Sequencing
DNA constructs were sequenced using the ABI Rhodamine DyeTerminator kit. In a reaction volume of 20 µl, 1 µg DNA, 3.2 pmol sequencing primer and 8 µl of the sequencing mix were subjected to 24 cycles of denaturation (94°C, 30 sec), annealing (50°C, 30 sec) and extension (60°C, 4 min). Unincorporated dNTPs were removed from the reactions using DyeEX™ 2.0 spin columns and the vacuum-dried samples were submitted to the in-house sequencing facility.

2.2.3.12 Transformation of E. coli
Generally between 10 and 100 ng of plasmid DNA, 10 µl of ligation reactions or 5 µl of mutagenesis PCR products were added to 100 µl competent INFαF E. coli in Falcon 352059 tubes (14 ml) and incubated on ice for 30 min. The bacterial cells were heat-shocked at 42°C for 45 sec, and then put on ice for 2 min. After addition of 900 µl LB (containing no antibiotics) the cells were grown at 37°C for 1 h, pelleted and resuspended in 100 µl LB and plated on LB agar plates containing the appropriate antibiotic and incubated at 37°C over night. Plasmids from individual colonies were analysed by restriction digest or DNA sequencing.

2.2.3.13 Generation of GST-p120 expression vector
A mammalian GST-p120ctn expression vector, pEF-Bos GST-p120ctn, was made using the pEF-Bos vector. The GST sequence from pGEX-2T was amplified by PCR with primers including XbaI sites and ligated with XbaI-digested pEF-Bos. Using the pEGFP-p120ctn plasmid (Noren et al., 2000) as a template and primers GST-BamHI and GST-HindIII, the p120ctn N-terminus (amino acids 1 to 349) was amplified by PCR, inserting a BamHI restriction site upstream of the first p120ctn start codon. pEGFP-p120ctn was kindly provided by K.Burridge (University of North Carolina). The PCR product was subjected to a BstEII/BamHI double digest. In addition, the pEGFP-p120 plasmid (Noren et al., 2000) was sequentially digested with BstEII and SmaI. The resulting 2.1 kb C-terminal fragment was ligated in tandem with the N-terminal BstEII/BamHI fragment into the pEF-Bos-GST vector using the polylinker BamHI and SmaI sites downstream of the GST sequence. The N-terminal p120ctn sequence up to the BstEII restriction site was verified by sequencing.
2.2.3.14 Generation of a p120ctn-VSV expression vector

A VSV-tag (an eleven amino acid epitope (YTDIEMNRLGK) derived from the vesicular stomatitis virus G protein) was introduced at the C-terminus of p120ctn. To this end, 
p120ctn-GFP (Noren et al.,) was digested first with BsmBI at 55°C for 1 h and then with EcoRI at 37°C for 1 h, yielding a 2.4 kb N-terminal fragment of p120ctn (Figure 2.1). Using primers p120VSVF and p120VSVR, the C-terminal end of p120ctn was amplified and the KpnI restriction site was replaced with an NheI site by PCR. A subsequent sequential digest with BsmBI (55°C, 1 h) and NheI (37°, 1 h) produced a fragment containing the last 424 bases of p120ctn1A, followed by the NheI site. In addition, vector PW5 (pcDNA3, into which a VSV-tag, preceded by an NheI site, had been incorporated, kindly provided by Dr G. Cory, UCL) was digested with EcoRI and NheI (37°, 1h), resulting in a 5.5 kb fragment into which the EcoRI/BsmBI and BsmBI/NheI fragments of p120ctn were ligated in tandem. The correct sequence of the construct (p120ctn-VSV) was verified by sequencing.
Figure 2.1  Schematic representation of the construction of plasmid p120ctn-VSV
2.2.3.15 Generation of p120ctn-4A-VSV (ΔN-p120ctn-VSV)
The first 350 base pairs in p120ctn, which contain the first three start codons, were deleted by doubly digesting the p120ctn-VSV construct with Bsu36I and EcoRI. The resulting recessed ends were filled in by adding of 1 unit Klenow polymerase per μg DNA and dNTPs (33 μM each) and incubation at 25°C for 15 min, followed by heat inactivation of the polymerase at 70°C for 20 min. The vector containing the N-terminally truncated p120ctn was purified by agarose gel electrophoresis, extracted from the gel and blunt-end religated. The correct sequence of the construct was checked by sequencing over the joining point between the pcDNA3 multiple cloning site and the p120ctn sequence just upstream of the remaining fourth start codon. The obtained construct is equivalent to a p120ctn4A-VSV expression construct.

2.2.3.16 Generation of GFP-ARM-456-p120ctn and GST-ARM-456-p120ctn
To fuse GST to the ARM repeats 4, 5 and 6 (amino acids 481 to 645), the pEF-Bos-GST vector was digested with BamHI and SmaI, separated by agarose electrophoresis and gene cleaned. The ARM repeats were amplified from the p120ctn-GFP (WT or SSS538/539/587AAA) vector using primers GSTBglIIIF and EGFPKpnlR. The PCR product was digested with Kpnl and incubated with Mung bean nuclease (10 units) for 30 min at 30°C to blunt the restriction site. After phenol/chloroform extraction and ethanol precipitation, the fragment was digested with BglII and purified again. Since BglII and BamHI digests produce compatible ends, the BglII/blunt end fragment could be ligated into the BamHI/SmaI digested vector. Similarly, to make GFP-ARM-456-p120ctn constructs, repeats 4, 5 and 6 were amplified by PCR using primers EcoRIF and KpnIR. After an EcoRI/Kpnl digest, the fragment was ligated into EcoRI/KpnI-digested pEGFP-C1 vector, fusing EGFP N-terminally to the ARM repeats. Clones were checked by sequencing for in-frame joining and absence of PCR-introduced mutations.

2.2.3.17 Generation of GST-N-term
A construct containing GST fused to the first 349 amino acids of p120ctn was made by PCR-amplifying the N-terminus of p120ctn, using primers GST-BamHI and GST-HindIII. After a HindIII digest, the recessed end was filled in using Klenow polymerase and the purified product was then restricted with BamHI and ligated into BamHI/SmaI-
digested pEF-Bos. The part of the vector containing the N-terminus of p120ctn was sequenced in its entirety.

2.2.4 Protein preparation and analysis
2.2.4.1 Preparation of cell lysates
Cells growing in tissue culture dishes were washed once with PBS and then lysed on ice in an appropriate volume of lysis buffer (e.g. in 1 ml per 10 cm dish). After incubation on ice for 10 min, the cells were scraped into a micro-centrifuge tube and centrifuged at 13000 g in a micro-centrifuge at 4°C for 10 min. The supernatant (soluble fraction) was removed and transferred to a new tube and either used immediately or stored at –20°C.

2.2.4.2 Immunoprecipitation
For anti-p120ctn or anti-VSV immunoprecipitations (IP) MDCK, Cos-7, MDA-MB-231 or NIH 3T3 cells were grown in 10 cm dishes up to 90% confluence, washed twice with ice-cold PBS and scrapped into 1 ml ice-cold 1% NP-40 lysis buffer. After centrifugation in a minicentrifuge at 13000 g for 10 min to remove insoluble components, the supernatant was transferred to a reaction vessel containing 30 μl of Protein A beads equilibrated in NP-40 lysis buffer. For each IP, 2 μl of pp120 antibody were added and the precipitation carried out on a rotating wheel at 4°C for 2 h. Subsequently, the beads were pelleted by pulse centrifugation and washed 5 times in 1 ml NP-40 lysis buffer. Any excess fluid was removed and the beads were resuspended in 15 μl SDS sample buffer when used for SDS-PAGE or in 2D-lysis buffer when intended for 2D-gel electrophoresis.

2.2.4.3 SDS-PAGE
Proteins were fractionated according to their size by SDS-PAGE, using a minigel apparatus (Mini-Protean II Cell, Biorad) and gels of 0.75 or 1.5 mm thickness. Separating gels with a final polyacrylamide content of 6%, 7.5% or 10% were prepared by using different volumes of the 40% acrylamide stock (37.5:1 acrylamide: bisacrylamide). In addition, the gel solutions contained 375 mM Tris-HCl pH 8.8 and 0.1% SDS and water in a total volume of 10 ml. For polymerisation, 5 μl of TEMED and 50 μl of freshly prepared 10% APS solution were added. A stacking gel was layered on
top of the set separating gel, containing 4% acrylamide, 190 mM Tris-HCl pH 6.8, 0.1% SDS in a volume of 5 ml. To initiate polymerisation, 5 µl TEMED and 25 µl 10% APS were used. A comb was inserted to form wells into which protein samples could be loaded.

After addition of 1/5 sample volume of 5 x SDS sample buffer, proteins were heated to 95°C for 5 min to guarantee complete protein denaturing. Samples were loaded into the wells along with rainbow markers (RPN 756, Amersham) to determine the molecular weight of proteins. Gels were run at 160 V in an electrophoresis tank containing running buffer.

2.2.4.4 Two-dimensional gel electrophoresis

For 2-dimensional separation of proteins, cells were lysed in 2D-lysis buffer at RT to avoid precipitation of the urea. p120ctn immunoprecipitation beads were resuspended in 20 µl 2D-lysis buffer following the final wash with NP-40 lysis buffer. A Mini-Protean II Cell containing a 2-D Tube Cell was used to separate the proteins first according to their pI (first dimension), then according to their molecular weight (second dimension). Tube gels were prepared from the gel monomer solution in bulks of 20 according to the manufacturers instructions. Between 20 and 50 µl of sample were applied onto the gels using the sample reservoirs and the samples were carefully overlaid with 30 µl 2D-gel overlay buffer. After insertion of the 2D Tube Cell into the Mini-Protean II Cell containing 800 ml lower 2D gel buffer, the upper buffer chamber was filled with upper 2D gel buffer. Electrophoresis was performed in two steps: 10 min at 500 V, followed by 4 h at 750 V, giving a total of roughly 3000 Volt h. Tube gels were removed and equilibrated in SDS-containing 2D equilibration buffer for 10 min and either stored at -20°C or applied to 6% acrylamide separating gels for size fractionation analogous to standard SDS-PAGE.

2.2.4.5 Western Blotting

Following SDS-PAGE, proteins were transferred onto PVDF membrane (Immobilon-P). Gels and the membranes (pre-wetted in methanol for 30 sec) were equilibrated in transfer buffer and then sandwiched between two sheets of Whatman 3MM paper and two sponges. The case was then inserted into a Mini-Protean II Cell and filled with transfer
buffer. Proteins were transferred at 100 V for 1 h. After blotting membranes were blocked in 20 ml of non-fat dried milk in TBS containing 0.1% Tween 20 on a rocking platform for 1 h. The blocking solution was replaced by 5 ml of 5% non-fat dried milk/0.1% Tween-20/TBS solution containing 0.1 - 0.5 μg/ml primary antibody and left for 1 h on a roller. Blots were washed 3 x for 10 min in TBS/0.1% Tween-20. The membranes were then incubated for 1 h in 5 ml of 5% non-fat dried milk/0.1% Tween-20/TBS solution containing the secondary HRP-conjugated antibody at half the concentration of the primary antibody. Finally, the membranes were washed 2 times with TBS/0.1% Tween-20, once with PBS for 10 min each and developed in ECL reagent for 1 min and exposed to X-ray film.

2.2.4.6 Stripping of Western blots
To allow reprobing, PVDF membranes were washed in western blot stripping buffer for 2 times 20 min at 65°C. After two washes in TBS/0.5% Tween-20, blots were reblocked in 5% non-fat dried milk powder for 1 h and then developed according to the standard western blot protocol.

2.2.4.7 Coomassie and Silver staining
When protein levels allowed visualization by Coomassie Brillant Blue (R250) staining (>100 ng per band), gels were incubated in Coomassie stain solution for 1 h, followed by several washes in Coomassie destaining solution. Visualization of proteins separated on SDS-PAGE by silver staining was performed when the protein amount was below the sensitivity of Coomassie staining (>100ng). Gels were fixed for 1 h in fixing buffer and washed 2 x in ddH2O for 15 min. After sensitisation in 0.02% sodium thiosulfate buffer for 1 min and two 1 min rinses with ddH2O, the gels were stained with cold 0.1% AgNO3 solution for 10 min, followed by two 1 min rinses with ddH2O. Gels were developed in developing buffer until bands became visible. The reaction was stopped with two rinses in 5% acetic acid.

2.2.4.8 GST-pull-downs
Cos-7 cells were transfected by electroporation with various pEF-Bos-GST constructs and lysed in 1 ml NP-40 lysis buffer after 24 h. The soluble fractions of the lysates were
incubated with 30 μl glutathione-sepharose 4B beads on a rotating wheel at 4°C for 1 h. Beads were washed 4 times with 1 ml of lysis buffer for 10 min and finally resuspended in 20 μl SDS sample buffer. After SDS-PAGE on 7.5% gels and blotting, the membranes were probed with antibodies against α-tubulin, stripped and reprobed with antibodies against GST. For mass spectrometric analysis of coprecipitating proteins, cells were lysed in CHAPS lysis buffer and protein complexes were resolved on 10% polyacrylamide gels. Proteins were visualized by silver staining and bands of interested were excised from the gel and stored at -40°C.

2.2.4.9 Purification of GST-p120ctn for Mass Spectrometry
The pEF-Bos GST-p120ctn plasmid (10 μg) was electroporated into 2x10^7 Cos-7 cells. After expression for 24 h, the cell were incubated with the serine/threonine phosphatase inhibitor Calyculin A (100 nM) for 30 min and subsequently lysed in CHAPS lysis buffer. The lysate was centrifuged for 10 min at 13000 g and the supernatant was incubated with 20 μl glutathione-sepharose 4B beads for 1 h. The beads were removed and the lysate incubated with an additional 20 μl beads for 1.5 h. Beads were combined and washed three times with CHAPS lysis buffer, two times with a high-salt buffer (0.5 M LiCl/10 mM Tris pH 7.5), and two times with 1 mM Tris/HCl pH 8.0. After removal of excess buffer, the beads were stored at -40°C.

2.2.4.10 Phosphatase treatment of p120ctn immunoprecipitation complexes
p120ctn was immunoprecipitated as described in section 2.2.4.2. Before SDS-sample buffer was added, however, immunoprecipitation complexes were washed twice with buffer NEB 3 for calf intestinal alkaline phosphatase (CIP) treatment, or LAR buffer for LAR tyrosine phosphatase treatment. Immunoprecipitation complexes were then incubated with 10 units LAR at 30°C or 20 units CIP at 37°C for 1 h under constant agitation. Immunoprecipitation complexes were subsequently washed twice with lysis buffer and finally resuspended in 20 μl SDS-sample buffer.
2.2.4.11 Rac pull-downs

NIH 3T3 cells were seeded at 5 x 10^5 cells in 10 cm tissue culture plates. After 24 h cells were transfected reagent with pcDNA3 vector (control), p120ctn-VSV or 538/539/587AAA-p120-VSV using Polyfect transfection. After expression for 24 h cells were quickly washed with ice-cold PBS, lysed in 0.5 ml 1 x LMB an immediately centrifuged for 3 min at 13000 g. Soluble supernatant (50 μl) was kept to determine total Rac levels in the cell lysate; the rest of the supernatant was transferred to a new tube containing 10 μl of PAK-1-PBD agarose beads and incubated on a rotating wheel for 1 h. For in vitro GTPyS/GDP loading of lysates as positive and negative controls, 20 μl 0.5 M EDTA, pH 8.8 and either GTPyS (final concentration 100 μM) or GDP (final concentration 1 mM) was added to 0.5 ml lysate. After loading for 15 min at 30°C, nucleotide exchange was stopped by adding MgCl2 to a final concentration of 60 mM and Rac-GTP was precipitated by incubation of the loaded lysates with 10 μl of PAK-1-PBD agarose beads at 4°C for 30 min. All beads were washed 3 times in LMB buffer and resuspended in 15 μl SDS sample buffer. Protein concentrations of the lysates were determined by Bradford protein assay and volumes containing equal amounts of proteins were loaded onto 10% polyacrylamide gels. Following SDS-PAGE and western blotting, Pull down samples and lysate samples were probed with antibodies against Rac1. Membranes carrying the lysate samples were stripped and reprobed with antibodies to the VSV epitope to determine expression levels of the transfected p120ctn.

2.2.4.12 Nuclear fractionation

MDCK cells were seeded at 5 x 10^5 cells per 15cm dish. On day 4, cells were treated with 10 ng/ml HGF for 4 h, 16 h or left untreated (control). Cells were harvested by incubating them in 5 ml trypsin/EDTA for 2 min. After three washes with PBS, cells were resuspended in one PCV (Packed Cell Volume) of Nuclear Extraction Buffer A (NEB A) and left to swell for 15 min on ice. To disrupt the cell membrane, the cell suspension was put through a 21-gauge needle approximately 50 to 100 times until most nuclei had been separated from the plasma membrane (monitored using a light microscope). Whole cells were spun out at 100 g for 5 min and lysed in 50 μl boiling SDS sample buffer. The supernatant was centrifuged at 12000 g for 20 sec to produce a crude nuclear pellet and a postnuclear supernatant. The nuclear pellet was resuspended in 2/3 PCV (determined at time of cell lysis) of NEB C and incubated on ice for 30 min.
Nuclear debris was pelleted at 12000 g for 5 min. The supernatant (nuclear extract) was dialyzed against buffer D for 2 h. The protein concentrations of the nuclear extract and the postnuclear supernatant were determined by Bio-Rad protein assay and equal amounts of protein were resolved on a 10% SDS-polyacrylamide gel and subsequently blotted and probed with antibodies against p120ctn and E-cadherin.

2.2.4.13 PKA kinase assay
MDCK cells were grown to confluency in 10 cm dishes and lysed in NP-40 lysis buffer. p120ctn was immunoprecipitated as described in section 2.2.4.2. For control precipitations, lysate was incubated with protein A beads without antibodies. Immunocomplexes and control beads were subsequently washed twice with high salt wash buffer to remove coprecipitating proteins, and then three times with PKA kinase buffer. The supernatant was removed and the beads were incubated for 15 min at 37°C with 40 µl kinase buffer containing 50 units PKA and 25 µM ATP/2.5 µCi [\(^{32}\)P]–ATP. As a positive control for the kinase activity, 2 µg histone H1 was added to 40 µl kinase buffer containing 20 units PKA and 25 µM ATP/2.5 µCi [\(^{32}\)P]–ATP.

2.2.4.14 Metabolic labeling of cells and 2D Peptide mapping
Cos-7 cells transfected with p120-VSV constructs were grown in phosphate-free DMEM for 8 h. The medium was replaced with 1.5 ml phosphate-free DMEM supplemented with 0.5 mCi [\(^{33}\)P] orthophosphate. After labelling for 8 h, cells were lysed in NP-40 lysis buffer and an anti-VSV immunoprecipitation was performed. IPs were resolved by SDS-PAGE and proteins transferred onto Immobilon-P membranes. Membranes were blocked and subsequently dried and exposed to X-ray film. Protein levels were assessed by probing the membranes with antibodies to the VSV epitope. Band intensities were quantitated using Bio-Rad’s Quantity One software.

2.2.4.15 2D Phosphopeptide mapping
Phosphopeptides were analysed by 2D phosphopeptide mapping. Radioactive bands were cut out of polyacrylamide gels, washed four times in acetone, once in ether and left to air-dry. Gel pieces were reswollen in 0.5 ml 50 mM ammonium bicarbonate (ABC) and washed twice in ABC. Proteins were in-gel digested in 0.5 ml ABC containing 10 µg
trypsin on a rotating wheel at room temperature overnight. The supernatant with the tryptic peptides was dried down in a speed-vac, resuspended in ddH$_2$O, dried down again and stored at 4°C. For separation of the peptides by TLC, samples were resuspended in 10 μl PAW first dimension running buffer, spotted onto TLC plates (in the middle at a distance of 1.5 cm from the lower end). Plates were inserted into a Multiphore II equilibrated with PAW buffer and electrophoresed at 50 mA for 20 min. Plates were then air-dried and inserted into a TLC chamber equilibrated with PAWB second dimension running buffer. After chromatographic separation of the peptides, the plates were air-dried and exposed onto phospho-imager screens for 4 h to 14 days before being scanned in a phosphoimager.

2.2.4.16 Cell supernatant protein analysis
To analyse proteins in the supernatant of HGF-stimulated MDCK and HB4A cells, $10^5$ cells were seeded into T75 flasks in 10% FCS/DMEM. After 2 days cells were washed twice with PBS and the medium was replaced with 5 ml of serum-free DMEM. Cells were grown for 96 h and stimulated with 10 ng/ml HGF every 24 h. Bovine serum albumin (10 μg/ml), corresponding to the amount of albumin present in the HGF preparation, was added to control cells. The medium was collected and concentrated to about 1 ml using Ultrafree Biomax-5K filter concentrator devices. Samples were twice diluted to 20 ml with ddH$_2$O, concentrated again to decrease the salt concentration, and ultimately dried completely in a speed-vac. After resuspension in 50 μl ddH$_2$O, protein concentrations were determined and equal protein amounts were loaded onto 10% SDS-polyacrylamide gels. After western blotting, membranes were probed with a panel of anti-MMP antibodies.

2.2.4.17 Bio-Rad protein assay
When appropriate the protein concentration of lysates was determined using the Bio-Rad protein assay. The assay is derived from the Bradford protein assay and is based on the fact that the absorption of light of wavelength 695 nm of Coomassie Blue G dye changes upon binding to protein under acidic conditions. Usually, 5 μl of sample were diluted in 795 μl of ddH$_2$O, followed by addition of 200 μl Bio-Rad protein assay reagent. After
vortexing and incubation at room temperature for 10 min, the OD\textsubscript{695} was measured and the protein concentration determined using a bovine serum albumin standard curve.

### 2.2.4.18 Generation and purification of phosphopeptide-specific antibodies

Antibodies were raised against phosphopeptides corresponding to sequences adjacent to the potential phosphorylation sites at serines 538/539 and 587 using Eurogentec's Double X program, during which each of two rabbits is immunized with two phosphopeptides simultaneously (details at http://www.eurogentec.be/hp/hp.htm). The following peptides were selected according to Eurogentec's instructions for maximizing antigenicity.

- **peptide 1 (pSpS):** CLRNVpSpSERSE
- **peptide 2:(pS):** NLpSYQVHRE

Final bleeds were collected three weeks after the final booster immunization. For affinity-purification of phospespecific antibodies, the respective phosphopeptides were covalently coupled to CNBr-sepharose. First, 1 g of beads were swollen and washed 5 times in 50 ml ddH\textsubscript{2}O, followed by washing and activation in 50 ml 0.1 M NaHCO\textsubscript{3} (3x). 1 ml of the bead suspension was incubated with 5 mg phosphopeptide (resuspended in 0.5 ml of 0.1 M NaHCO\textsubscript{3}) at 37\textdegree C for 16 h. Beads were washed 2 times with 5 ml of 0.1 M \(\beta\)-mercaptoethanol to inactivate reactive amines. Beads were subjected to three rounds of alternating washes with 10 ml of 0.2 M glycine, pH 2.8 and 10 ml of 0.1 M NaCO\textsubscript{3} pH 8.5, 0.5 M NaCl. After a final wash with 25ml TTBS, beads were stored in TTBS at 4\textdegree C.

Using 10x TTBS, final bleeds were equilibrated in 1x TTBS and filtered by passing them through a 0.2 \(\mu\)m pore size membrane. 5 ml of this solution was incubated with the peptide beads on a rotator at 4\textdegree C for 16 h. The supernatant was removed and the beads were washed 2 times with TTBS. The beads were subsequently packed into an Econocolumn (BioRad) and washed with TTBS until the OD\textsubscript{280} was less than 0.01. Antibodies were eluted with 0.2 M glycine, pH 2.8 and collected in 400 \(\mu\)l fractions in reaction vessels containing 25 \(\mu\)l 1.5 M Tris to allow rapid neutralization of the eluent. The OD\textsubscript{280} of each fraction was measured and the three fractions with the highest OD were pooled and dialysed o/n against PBS/0.2% azide. The antibodies were stored at 4\textdegree C or aliquotted for long-term storage at -20\textdegree C.
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For dot-blot analysis, 500 ng of peptide were applied to PVDF membrane using a dot-blot applicator. Membranes were probed with 1 to 200 dilutions of the purified antibodies using standard western blotting procedures.

2.2.5 Staining procedures and image generation

2.2.5.1 Phase contrast
Phase contrast micrographs of fixed cells were taken with a Photonic Science Coolview 12, 1024x1024 pixel, 12 bit cooled CCD camera using a x40 NA 0.75 objective on an axiophot microscope (Zeiss). The data sets were collected using Image Pro Plus v 3 from Media Cybernetics and processed in Adobe Photoshop v 6.0.

2.2.5.2 Immunofluorescence and confocal microscopy
Cells were fixed with 4% paraformaldehyde dissolved in PBS for 20 min at RT and permeabilized for 5 min with 0.2% Triton X-100. Primary antibodies were diluted in 0.5% BSA /PBS (anti-β-tub, anti-ac-tub, γ-tub, β-catenin, pp120, HECD, anti-VSV), as were secondary antibodies (TRITC-conjugated, FITC-conjugated, Cy5-conjugated). Staining was performed for 1 h per layer, followed by six washes with 0.5% BSA/PBS. Actin filaments were visualized by incubation with 0.8 nM TRITC-phalloidin for 1 h. Coverslips were mounted onto slides using 5 μl Mowiol.

Confocal laser scanning microscopy was carried out with an LSM 510 (Zeiss) mounted over an affinity corrected Axioplan microscope (Zeiss) fitted with a x10 eyepiece, using a x 40 1.3 NA oil immersion objective. Image files were collected as a matrix of 1024 x 1024 pixels describing the average of 8 frames scanned at 0.062 Hz where FITC, TRITC and Cy5 were excited at 488 nm, 543 nm, and 633 nm and visualized with a 540 +/- 25, 608 +/- 32 and 690 +/- 30 nm bandpass filter, respectively, where the levels of interchannel crosstalk were insignificant.

2.2.5.3 Time lapse microscopy
For time-lapse experiments, Cos-7 cells were transfected with plasmids pEGFP-N1 or p120-GFP by electroporation as described (section 2.2.2.1) and seeded onto glass coverslips in 35mm tissue culture dishes. After 24 h cells were switched to HEPES/Hanks’ balanced salts based medium to allow air buffering during the collection
of the time series. Frames were collected every 30 sec over a period 60 min using a LSM 510 confocal microscope with FITC configuration (as above) and a 25 x water immersion objective (Zeiss).
3 p120ctn and Microtubules

3.1 Introduction

The central region of the p120ctn molecule consists of ten copies of a structural unit known as an ARM repeat (see Fig in introduction). Although ARM repeat domains are found in proteins with such diverse functions as nucleocytoplasmic transport (importin α family), cell adhesion (β-catenin, plakoglobin and p120ctn), guanine nucleotide exchange (exchange factor SmGDS) or tumor suppression (APC), in all of these cases they are believed to mediate protein-protein interactions (Andrade et al., 2001; Hatzfeld, 1999). In the case of p120ctn, the ARM repeat domain has been shown to mediate binding to cadherins (Daniel and Reynolds, 1995; Shibamoto et al., 1995). Furthermore, it is required for the induction of the branching phenotype in fibroblasts (Reynolds et al., 1996) and therefore likely to be involved in the regulation of the small GTPases RhoA, Rac1 and Cdc42 (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). Binding of p120ctn to cadherins, thereby promoting cell-cell adhesion, and regulation of the GTPases, leading to increased cell migration and branching, are believed to be mutually exclusive events (Noren et al., 2000) and it is of great interest to understand how p120ctn can switch between the two functions. In order to identify regions of the ARM repeat critical for either of the two functions, a number of mutations were introduced into the ARM repeat domain of p120ctn and tested for their effect on cadherin binding and GTPase signalling.

3.2 The intracellular localization of p120ctn is affected by point mutations in its ARM domain region

3.2.1 Introducing serine→alanine mutations in the ARM repeat domain of p120ctn

Phosphorylation on tyrosine, serine or threonine residues regulates a vast number of protein-protein interactions. Since the ARM repeat region of p120ctn mediates interaction with cadherins and Rho GTPases, phosphorylation events in this region could be a mechanism through which p120ctn’s affinity for its different interaction partners is regulated. The major tyrosine phosphorylation sites have recently been mapped (Mariner et al., 2001) and all reside outside of the ARM repeat domain. In an effort to find potential serine phosphorylation sites in the ARM repeat domain, a number of candidate serines were identified. There are more than 50 serines or threonine residues in the ARM
repeat domain of p120ctn and the number of candidate serine/threonine residues was narrowed down in two steps:

- serines conserved between p120ctn and ARVCF, its closest relative, were identified (Figure 3.1 A)

- conserved serines were analysed for their probability to be phosphorylated using the NetPhos 2.0 software and residues with a probability score of 0.5 or higher were chosen for mutational analysis (Figure 3.1 B)

Five serine residues (three single and one double serine) matched the chosen criteria (S488, S505, S538/539, S587) and were changed to non-phosphoserine-mimicking alanine residues in p120ctn-GFP and p120ctn-VSV constructs by site-directed mutagenesis as described in Material and Methods.
Figure 3.1 Identification of potential serine phosphorylation sites in the ARM repeat domain of pl20ctn

**Fig A:** The ARM repeat domains of pl20ctn and its closest relative, ARVCF (based on Anastasiadis and Reynolds, 2000), were aligned using the BioEdit 5.0.9 software. Identical (dark grey background) and similar (light grey background) residues are indicated. Conserved serines and threonines are highlighted in red. Asterisks indicate the border between ARM repeats. **Fig B:** Phosphorylation probability scores of conserved serine residues were calculated using the NetPhos 2.0 software. Phosphorylation probability scores range from 0 to 1, with residues scoring below 0.5 considered unlikely to be phosphorylated. Serines within the pl20ctn ARM repeat domain with a score of 0.5 or higher were chosen for serine→alanine mutations.
3.2.2  *p120ctn mutants are still able to interact with E-cadherin*

To assess whether the introduced serine→alanine mutations affected the ability of p120ctn to bind to E-cadherin, p120ctn-GFP constructs carrying the various mutations were transfected into MDCK cells. In most transfected cells, mutant p120ctn proteins localized to the sites of cell-cell contact (as indicated by the staining for cortical actin) similarly to wild-type p120ctn (Figure 3.2). Since E-cadherin is necessary and sufficient to recruit p120ctn to the plasma membrane (Daniel and Reynolds, 1995; Thoreson et al., 2000), these results suggest that binding to E-cadherin was not affected by the mutations. Because p120ctn uses large parts of the ARM repeat domain (ARM repeats 1, 2, 3, 4, 5 and 7 (Ireton et al., 2002) to interact with E-cadherin, localization of the mutants to cell-cell contacts, which requires E-cadherin-binding, also suggests that the overall three-dimensional structure of each mutant protein’s ARM domain was not substantially altered.
Figure 3.2  Point mutations in the ARM repeat domain of p120ctn do not abrogate E-cadherin binding

MDCK cells were transfected with wild-type-p120ctn-GFP (GFP), S480A-p120ctn-GFP (480A), S506A-p120ctn-GFP (506A), SS538/539AA-p120ctn-GFP (538/539AA) or S587A-p120ctn-GFP (587A) using Lipofectin 2000. After 24 h cells were fixed and stained for the actin cytoskeleton with TRITC-phalloidin.
3.2.3 Two serine→alanine point mutations in the ARM domain cause association of p120ctn with interphase microtubules

To investigate whether the p120ctn mutants were able to induce the branching phenotype (Reynolds et al., 1996), the panel of constructs was overexpressed in Cos-7 cells. Whereas wild-type p120ctn caused extensive arborisation, the mutants SS538/539AA and S587A failed to induce any branching (Figure 3.3). Rather, the cells were flattened out and spread. Surprisingly, both mutants localized along microtubules in about 10% of transfected cells (near-perfect overlap of p120-GFP and anti-β-tubulin staining), a phenomenon never observed with wild-type p120ctn in these cells. Combination of the two mutations (SS538/539AA and S587A) in one construct (SSS538/539/587AAA or AAA-p120ctn) increased the percentage of transfected cells showing p120ctn/microtubule colocalization to approximately 25%. The proteins containing the single mutations S538A or S539A did not localize along microtubules (data not shown).

In Cos-7 cells, the association of p120ctn with microtubules did not appear to depend primarily on the expression levels of the mutants. Cells expressing similar amounts of AAA-p120ctn (as judged by comparing fluorescence intensities of neighbouring cells) often displayed contrasting localization of the overexpressed protein, though individual cells generally showed either near-complete p120ctn/microtubule colocalization or an entirely diffuse cytoplasmic and sometimes nuclear distribution of p120ctn. In MDCK cells, however, AAA-p120ctn, and to a lesser degree also wild-type p120ctn, was found to colocalize with microtubules exclusively at high expression levels achieved only by electroporation (see Chapter 3.3.2). The wild-type and mutant proteins showed nuclear localization to a varying degree, which did not correspond to either localization along microtubules or diffuse cytoplasmic distribution.

In some cells showing a low level of AAA-p120ctn expression, the staining for p120ctn along microtubules was not evenly distributed but was concentrated in distinctive dots (Figure 3.4). This particular pattern could only be observed in some extremely well spread cells. Such dot-like decoration of microtubules often occurs when proteins or vesicles interact with microtubules via motor proteins such as kinesins or dyneins. It is therefore quite possible that mutant p120ctn is transported actively along microtubules.
Figure 3.3  Specific alanine mutations in the ARM repeat domain cause association of p120ctn with interphase microtubules

Cos-7 cells were electroporated with p120ctn-GFP, SS538/539AA-p120ctn-GFP (538/539AA), S587A-p120ctn-GFP (587A), or SSS538/539AAA-p120ctn-GFP (538/539/587AAA) plasmids and fixed after expression for 24 h. Cells were then stained with antibodies to β-tubulin and confocal images were collected. Bar 5 μm.
Figure 3.4 Mutant p120ctn localizes in distinct puncta along microtubules

Confocal image of Cos-7 cells expressing SSS538/539AAA-p120ctn-GFP (AAA-p120ctn-GFP). Cells were fixed 24 h after transfection and stained with the β-tubulin antibody. Bar 2 μm.
3.2.4 During cell division mutant p120ctn and a fraction of endogenous p120ctn localizes to centrosomes and the mitotic spindle

Association of AAA-p120ctn with tubulin-containing structures was not restricted to interphase microtubules. In Cos-7 cells, mutant p120ctn localized to both the mitotic spindle and to centrosomes during metaphase (Figure 3.5 A). Most of the exogenous p120ctn appeared to be associated with the mitotic spindle but there was also a fraction of mutant p120ctn arrayed in dots around the circumference of the cell. These structures may be points where astral microtubules meet the plasma membrane. However, this could not be examined since costaining for microtubules (anti-β-tubulin staining) was not possible because of the species of antibodies available. The use of the GFP-fusion constructs was not informative because GFP itself showed non-specific localization to centrosomes (not shown). In contrast to the clear localization to the two mitotic centrosomes, mutant p120ctn could never be detected at the single interphase centrosome, suggesting that the association of p120ctn with centrosomes occurs exclusively during cell division.

Consistent with this observation, a fraction of endogenous p120ctn could be detected at centrosomes in MDA-MB-231 cells during mitosis (Figure 3.5 B). The recruitment of endogenous p120ctn to the centrosomes in these cells suggests a physiological role for the interaction of p120ctn with tubulin-containing structures during cell division. During interphase, no clearly defined centrosomes could be identified in these cells by γ-tubulin staining. It was therefore impossible to establish whether the p120ctn/centrosome association also occurs in interphase. Localization of endogenous p120ctn to centrosomes could not be observed in Cos-7 cells. Possibly the expression level of p120ctn in Cos-7 cells is too low to allow detection of a centrosome-associated pool. In addition, MDA-MD-231 cells contract and round up considerably during cell division which may facilitate the detection of p120ctn at centrosomes.
Figure 3.5 Mutant p120ctn localizes to the centrosomes and the mitotic spindle in Cos-7 cells and a fraction of endogenous p120ctn localizes to the centrosomes during mitosis in MDA-MB-231 cells.

**Fig A:** Cos-7 cells were transfected with AAA-p120-VSV and fixed after 24 h. Metaphase cells were identified on account of their condensed chromosomes by Hoechst staining (not visible in the confocal image). Centrosomes were visualized by staining for γ-tubulin and mutant p120ctn by anti-VSV staining. Bar 5 μm. **Fig B:** MDA-MB-231 cells were fixed and stained for endogenous p120ctn and γ-tubulin. Mitotic cells were identified because of their spherical, contracted morphology and by Hoechst staining. Images were collected at the plane of the centrosomes. Note the different magnification for interphase and mitosis images (Bar 5 μm).
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3.3 The phosphorylation state of mutant p120ctn

3.3.1 Mutant p120ctn is less strongly phosphorylated than wild-type p120ctn

The introduction of serine→alanine point mutations at residues 538/539 and 587 drastically changed the intracellular localization of p120ctn. However, the initial motivation for the point mutations had been to identify phosphorylation sites in the ARM domain of p120ctn. To assess whether the change in the intracellular localization of AAA-p120ctn indeed coincided with decreased phosphorylation, Cos-7 cells were transfected with WT-p120ctn-VSV or AAA-p120ctn-VSV constructs and metabolically labelled with $[^{33}P]PP_i$. Overexpressed proteins were immunoprecipitated with the anti-VSV antibody, subjected to SDS-PAGE and transferred onto PVDF membrane. The membranes were exposed to X-ray film to measure the incorporation of the radioactive label. Relative amounts of precipitated protein were subsequently determined by blotting for the VSV-epitope. Quantitation of the bands showed that AAA-p120ctn was approximately 20% less phosphorylated than wild-type p120ctn (Figure 3.6). p120ctn is only weakly tyrosine-phosphorylated in these cells (not shown) and any decrease in overall phosphorylation can be attributed to loss of serine/threonine phosphorylation.
Figure 3.6 Mutant p120ctn is less strongly phosphorylated compared to wild-type p120ctn

**Fig A:** Cos-7 cells transfected with AAA-p120ctn-VSV or wild-type p120ctn-VSV were metabolically labelled with $[^{32}\text{P}]-\text{PP}_{1}$. After immunoprecipitation with anti-VSV antibodies, proteins were separated by SDS-PAGE and transferred onto PVDF membrane. The membranes were exposed to X-ray film directly to measure the relative incorporation of radioactive label. Blots were then probed with anti-VSV antibodies to determine protein levels. A representative blot is shown.

**Fig B:** For quantitation, label incorporation was normalized for the amount of precipitated protein. Results of three independent experiments are shown. The decrease in phosphorylation of AAA-p120ctn-VSV compared to wild-type p120ctn-VSV was statistically highly significant ($P \leq 0.007$).
3.3.2 A phosphoserine-mimicking aspartate mutant efficiently suppresses association of p120ctn with microtubules

The fact that the mutant protein AAA-p120ctn-VSV, carrying non-phosphoserine-mimicking serine→alanine exchanges at positions 538/539 and 587, localized to microtubules suggests that association of p120ctn with microtubules requires dephosphorylation at residues SS538/539 and/or S587 or, in other words, that phosphorylation at these residues prevents association of p120ctn with microtubules. Serine→aspartate mutations are commonly used to mimick the negative charge added to serines upon their phosphorylation. Serine→aspartate exchanges at residues SS538/539 or S587 should therefore prevent association of p120ctn with microtubules. When MDCK cells were transfected by electroporation to achieve high expression levels, AAA-p120ctn localized to microtubules in nearly all the cells (Figure 3.7 A and B). Wild-type p120ctn showed a similar localization only in the most highly overexpressing cells and was normally recruited to adherens junctions. Mutant S587D, on the other hand, only extremely rarely associated with microtubules, showing that the introduction of a negative charge at position 587, mimicking a phosphoserine residue, efficiently prevented binding of p120ctn to microtubules. Interestingly, in most cells S587D-p120ctn showed even more pronounced junctional localization than wild-type p120ctn, suggesting that the ARM repeat region functions as a switch between junctional, E-cadherin-associated and microtubule-associated localization. Figure 3.7 B shows a quantitation of the percentage of cells transfected with the different constructs displaying microtubule/p120ctn colocalization.
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p120cfn and Microtubules

Figure 3.7  The phosphoserine-mimicking mutant serine 587 to aspartate efficiently prevents association of p120cfn with microtubules

Fig A: MDCK cells were electroporated with wild-type p120cfn-VSV (WT), SSS538/539/567AAA-p120cfn-VSV (AAA) or S587D-p120cfn-VSV (587D) plasmids, fixed after 24 h and stained for the VSV-epitope. Wild-type p120cfn associates with microtubules in highly overexpressing cells, whereas AAA-p120cfn localizes to microtubules in most cells. 587D-p120cfn-VSV is prevented from associating with microtubules and shows mostly junctional localization with low cytoplasmic staining. Asterisks (*) indicate cells with p120cfn localization to microtubules. Fig B: Quantitation of microtubule association of the different constructs in percent of transfected cells. 100 cells per construct were counted in one experiment representative of a total of three experiments.
3.3.3 Phosphopeptide-specific antibodies fail to recognize purified p120ctn

The facts that the introduction of serine→alanine mutations at positions 538/539 and 587 leads to a reduction in the total phosphorylation of p120ctn (Figure 3.6) and that the phosphoserine-mimicking mutation 587D efficiently prevents p120ctn from interacting with microtubules (Figure 3.7) point to serines 538/539 and 587 as potential phosphorylation sites. To further investigate this hypothesis, phosphospecific antibodies were raised against two peptides corresponding to the sequences flanking the potential phosphorylation sites and containing phosphoserines at the positions corresponding to serines 538/539 and 587:

peptide 1 (pSpS):  
CLRNVpSpSERSE

peptide 2:(pS):  
NLpSYQVHRE

The extend of the flanking sequences was determined according to Eurogentec's instructions for guaranteeing optimal antigenicity, though peptide 2 was predicted to be considerably less antigenic then peptide 1. Antibodies were affinity-purified from the rabbit serum against each of the phosphopeptides. The specificities of the purified antibody fractions were tested on dot blots. Antibodies purified against peptide 1 strongly reacted with the phosphopeptide (pSpS), (see Figure 3.8, upper panel), but not with the non-phosphorylated version of the peptide (SS). No reacting antibodies could be purified against peptide 2. Possibly no immune response had been elicited because of the low antigenicity of the peptide.

To test the ability of the affinity-purified "pSpS" antibody to recognize full-length protein, wild-type-, SS538/539AA- and SS538/539DD-p120ctn-GST fusion proteins were purified from overexpressing Cos-7 cells, separated on SDS-PAGE and transferred onto PVDF membrane. Although large amounts of protein were present on the membrane, the antibodies did not recognize wild-type or mutant p120ctn. This could be because in the full-length protein the sequence covered by the phosphopeptide is not accessible to the antibodies because of structural reasons. On the other hand, residues 538/539 may not be phosphorylation sites and therefore not be recognized by the anti-phospho antibody.
The specificity of the antibodies purified against the phosphopeptide pSpS was tested on dot blots carrying 500 ng of the phosphopeptide (pSpS) or the nonphosphopeptide (SS) (top panel). Antibodies reacted strongly with the phosphopeptide (pSpS), but not with the non-phosphopeptide (SS). However, the antibodies failed to recognize wild-type GST-p120ctn (p120 WT), GST-SS538/539AA-p120ctn (p120 AA) or GST SS538/539DD-p120ctn (p120 DD) purified from Cos-7 cells (middle panel), although considerable amounts of protein were present (Coomassie stain of the membrane shown in the bottom panel).
3.4 p120ctn and Rac activation

3.4.1 Mutant p120ctn does not induce the branching phenotype in fibroblasts

As described in Chapter 3.3.1, in Cos-7 cells AAA-p120ctn did not induce the branching phenotype characteristic of wild-type p120ctn overexpression. Instead, cell expressing mutant p120ctn appeared to be more spread than untransfected cells. The p120ctn-induced branching phenotype is especially strong in fibroblasts (Reynolds et al., 1996). NIH 3T3 fibroblasts were therefore chosen for quantitation of the ability of wild-type and mutant p120ctn to cause branching. In preliminary experiments VSV-tagged p120ctn was found to be far more potent in eliciting the branching phenotype than GFP-p120ctn protein. Consequently, wild-type p120ctn-VSV and AAA-p120ctn-VSV constructs were used for transfection.

Overexpression of wild-type p120ctn-VSV induced branching in more than 70% of transfected cells (Figure 3.9 A and B). The cell body was highly constricted around the nucleus and each cell showed a number of extremely long protrusions, some of them extending for more than twenty times the cell diameter. The ends of the extensions often showed extensive arborisation reminiscent of neurites. In contrast, AAA-p120ctn-VSV caused branching in less than ten percent of transfected cells and the cells appeared spread out (Figure 3.9 A and B). The mutant p120ctn showed localization consistent with microtubule association in more than 90% of the cells.
Figure 3.9  Mutant p120ctn does not cause branching in NIH 3T3 cells

**Fig A:** NIH 3T3 cells were transfected with wild-type p120-VSV (WT) or AAA-p120-VSV (AAA), fixed after expression for 16 h and stained with anti-VSV antibodies. Bar 20 μm. **Fig B:** Quantitation of branching. Cells were counted as showing branching if they showed two or more cellular extensions each at least three times the diameter of the nucleus. Results from three independent experiments are shown, with at least 100 cells counted per construct and experiment.
3.4.2 Mutant p120ctn is unable to activate Rac1

Overexpression of p120ctn in fibroblasts activates Rac and Cdc42 (Noren et al., 2000; Grosheva et al., 2001) and inhibits RhoA (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). Rac1 and Cdc42 are required for branching: coexpressing dominant-negative versions of Rac1 or Cdc42 with p120ctn efficiently blocks branching (Grosheva et al., 2001; Noren et al., 2000). To find out whether the reason for the failure of AAA-p120ctn to induce branching is the loss of its ability to activate Rac1, a series of Rac pulldown experiments were performed. Rac pulldown experiments allow the level of active (GTP-bound) Rac in the cells to be measured by incubating cell lysates with agarose beads linked to the Rac-GTP binding domain of PAK (PAK-RBD). NIH 3T3 cells were transiently transfected with either wild-type p120ctn-VSV, AAA-p120ctn-VSV or with empty vector as a control. In a parallel transfection of p120ctn-GFP, the transfection efficiency was estimated to be between 60 and 70 percent. The pulldown experiments showed that wild-type p120ctn increased the levels of active Rac1 in the cells (Figure 3.10). Mutant p120ctn, however, did not raise active Rac1 levels above the levels obtained when vector alone was transfected. Because of the low transfection efficiency, the true level of Rac1 activation in response to wild-type p120ctn expression is underrepresented in the pulldown experiments, which shows active Rac1 levels averaged over both transfected and untransfected cells. A quantitative comparison of the active Rac1 levels of wild-type and mutant p120ctn-overexpressing cells is therefore not feasible. These results suggest that mutant, microtubule-associated p120ctn fails to induce branching because it is unable to activate Rac1.
Figure 3.10  Wild-type p120ctn, but not mutant p120ctn activates Rac

NIH-3T3 cells were transfected with empty vector (vec), wild-type p120ctn-VSV (wt) or AAA-p120ctn-VSV (AAA). As positive and negative controls, lysates were loaded with GTP$_\gamma$S or GDP. Rac-GTP was precipitated using PAK1-RBD beads. Total Rac levels in the lysates were checked by western blotting. Lanes marked “pulldown” show the amount of active, GTP-bound Rac, whereas the “lysate” lanes show the amount of total Rac in the lysates. Lysate blots were reprobed against the VSV-epitope to show equal expression levels of the VSV-constructs. The blot is representative of three independent experiments.
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3.5 Association of p120ctn with microtubules leads to their stabilization

3.5.1 Wild-type and mutant p120ctn stabilize microtubules in different patterns

The recruitment of AAA-p120ctn to microtubules depended on an intact microtubule network, but was entirely independent of the actin cytoskeleton. AAA-p120ctn-expressing Cos-7 cells were treated with nocodazole to completely depolymerise microtubules or with the actin filament-disrupting drug Cytochalasin D (Figure 3.11 A). Whereas AAA-p120ctn was diffusively distributed following microtubule depolymerisation, it still showed extensive colocalization with microtubules when only actin filaments were disrupted. Although prolonged incubation with nocodazole eventually led to an almost complete loss of microtubule filaments, association of AAA-p120ctn-GFP with microtubules appeared to render microtubules more resistant to nocodazole-induced depolymerisation. Time-lapse analysis of AAA-p120ctn-GFP-expressing cells showed that individual microtubules, visualized by their association with AAA-p120ctn-GFP, resisted nocodazole treatment for up to one hour (Figure 3.11 B and Movie 2, supplementary material). Interestingly, nocodazole treatment led to a very rapid (within 2 min after nocodazole addition) accumulation of a fraction of AAA-p120ctn-GFP in the perinuclear region.

p120ctn-associated microtubules were not only more resistant to nocodazole treatment, they also displayed a changed morphology: they formed thick and curly bundles, were in some cases arranged in circles around the nucleus and often lacked a clearly defined end and beginning (Figure 3.3, cells expressing 538/539/587AAA-p120ctn). This is in contrast with typical interphase microtubules, which originate from the MTOC and extend their (+) ends towards the plasma membrane.

Microtubule bundling into large multi-filament structures has been linked to microtubule stabilization (Lee and Rook, 1992). During their maturation from highly dynamic, unstable to stabilized microtubules, the α-tubulin subunit of the tubulin dimer undergoes a series of modifications (Webster and Borisy, 1989; Webster et al., 1987). First, the lysine at position +6 from the C-terminus (consensus sequence x-x-lys-x-x-glu-glu-tyr) becomes acetylated, followed by the loss of the C-terminal tyrosine ("detyrosination"). Antibodies raised against either of the two modified C-termini allow identification of mature, stabilized microtubules. An anti-acetylated α-tubulin antibody was used to confirm that p120ctn binding to microtubules had a strong stabilizing effect on
microtubules (Figure 3.12). Only little acetylated tubulin could be detected in untransfected cells, whereas the thick microtubule cables in the AAA-p120ctn expressing cells stained very strongly for acetylated tubulin (top four images). Expression of wild-type p120ctn did not increase the anti-acetylated-α-tubulin signal in the body of the cell (bottom four images). The p120ctn-driven cellular extensions, however, showed strong staining for acetylated tubulin. These extensions, by analogy to neurite extensions, which depend on microtubule polymerization for their formation (Nobes and Hall, 1999; Tanaka et al., 1995), may be at least partly driven by tubulin polymerisation and one could therefore speculate that p120ctn promotes these extensions by binding to and stabilizing microtubules.
Figure 3.11  The microtubule disrupting drug nocodazole, but not the F-actin depolymerising drug Cytochalasin D alters the localization of AAA-p120ctn.

**Fig A:** Cos-7 cells transfected with AAA-p120ctn-GFP were treated with 1 μM nocodazole for 30 min or with 50 ng/ml Cytochalasin D for 1 h. Cells were fixed and stained for β-tubulin and F-actin. **Fig B:** Time series (1 frame/min) of Cos-7 cells expressing AAA-p120ctn-GFP. After 30 min, 1 μM nocodazole was added (N). The arrowheads indicate p120ctn accumulation. Bars 10 μm. (See Movie 2, Suppl. Material)
Figure 3.12 Wild-type and mutant p120ctn stabilize microtubules in different patterns

MDA-MB-231 cells were transfected with 538/539/587AAA-p120ctn-VSV or wild-type p120ctn-VSV and fixed after 24 h. Stable microtubules were visualized by staining for acetylated tubulin and p120ctn was stained using the anti-VSV antibody. F-actin staining was used to show the outline of cells. Mutant (AAA) p120ctn association causes stabilization (acetylation) of microtubules (top 4 images) throughout the cell body. Wild-type p120ctn (WT) fails to induce a similar effect on microtubules in the cell body. However, there are stabilized microtubules in the p120ctn-induced cellular extensions (bottom four images). Bar 10 μm.
3.5.2 AAA-p120ctn does not affect NGF-induced neurite extension

The branching phenotype caused by overexpression of p120ctn in fibroblasts (Reynolds et al., 1996) strikingly resembles the morphology of neurons (Figure 3.13). The long cellular protrusions induced by p120ctn expression are reminiscent of neurite extensions and the highly motile, finger-like structures along these protrusions (see movie 1) are similar to so-called spines found along axons. Finally, p120ctn-induced extensions often terminate in growth cone-like structures. As seen in the previous section, p120ctn-driven extensions contain stabilized microtubules. Neurite extensions have been shown to require microtubule polymerization (Nobes and Hall, 1999; Tanaka et al., 1995). A possible role for p120ctn, and/or its closely related neuronal family member NPRAP/δ-catenin, could therefore be to facilitate neurite outgrowth by stabilizing microtubules in these extensions.

Overexpression of wild-type p120ctn-GFP in neuronal PC12 cells induced neurite outgrowth-like branching (Figure 3.14). Interestingly, the extensions often contacted neighbouring cells. As in fibroblasts, overexpression of AAA-p120ctn-GFP did not induce any cellular extensions in PC12 cells. Having established that overexpressed wild-type p120ctn is able to induce branching in neuronal cells, the influence of the expression of different p120ctn constructs on NGF-induced branching was investigated. NGF (Nerve growth factor) causes neuronal differentiation and stimulates neurite outgrowth in PC12 cells (Figure 3.15). If expression of AAA-p120ctn in PC12 cells stabilizes microtubules (not in cellular extensions but) throughout the cell body similar to what was observed in MDA-MB-231 cells (Figure 3.12), and if microtubule stabilization selectively within extensions is important for promoting neurite outgrowth, AAA-p120ctn-expressing cells should be unable to form neurite extensions in response to NGF. However, wild-type and AAA-p120ctn-expressing cells were equally able to form neurite extensions following NGF treatment (Figure 3.15). Therefore, the failure of AAA-p120ctn to induce branching may be due to its inability to activate Rac1 rather than to its indiscriminate effect on microtubule stabilization. However, it must be stressed that because of the small size of PC12 cells, individual microtubules were difficult to distinguish (see anti-β-tubulin staining in Figure 3.15) and it was therefore not possible to assess whether AAA-p120ctn-GFP bound to (and stabilized) microtubules in these cells.
Figure 3.13  

**p120ctn-overexpressing Cos-7 cells morphologically resemble neurons**

Overexpression of p120ctn in Cos-7 cells changes their morphology, making them resemble neurons. Cos-7 cells were transfected with wild-type p120ctn. p120ctn expression induces long cellular protrusions reminiscent of neurite extensions, finger-like structures along those protrusions resembling neuritic spines (arrows) and growth cone-like termini (arrowhead). The Cos-7 cell image is part of time-lapse movie 1. The picture of the neuron was taken from Alberts et al. 1996, Molecular Biology of the Cell.
Figure 3.14 Overexpression of wild-type p120ctn, but not of AAA-p120ctn, induces a branching phenotype in PC12 cells

Undifferentiated PC12 cells were transfected with wild-type p120ctn-GFP (WT) or AAA-p120ctn-GFP (AAA) and fixed after 24 h. Wild-type overexpression induces cellular extensions, which frequently contact neighbouring cells. AAA-p120ctn-GFP expression had no visible effect on cellular architecture. Bar 10 μm.
Figure 3.15 Expression of AAA-p120ctn does not block NGF-induced neurite extension

PC12 cells were left untreated or differentiated with NGF for 48 h (top two panels). Cells transfected with wild-type p120ctn-GFP (WT), AAA-p120ctn-GFP (AAA) or GFP as control before differentiation with NGF (lower three panels) formed neurite extensions in the same way as untransfected cells. Bar 20 μm.
3.6 The colocalization of p120ctn with microtubules depends on sequences both in the ARM repeat region and in the N-terminus

3.6.1 Constructs containing only the central three ARM repeats or lacking the N-terminus of p120ctn are not targeted to microtubules

The punctate decoration of microtubules observed with mutant p120ctn suggests that p120ctn interacts with microtubules via an ancillary protein, for example a motor protein such as kinesin. However, it cannot be excluded that p120ctn is also able to bind directly to microtubules. In order to determine which domains of p120ctn are required for the interaction with microtubules, a number of deletion constructs were generated (Figure 3.16). Since point mutations at residues 538/539 and 587 regulate the association with microtubules when introduced into the full-length protein, it was assumed that the ARM domains containing the mutated residues are involved in microtubule binding. GFP-constructs containing only ARM repeats 4, 5 and 6 were generated. The N-terminus of p120ctn contains a coiled-coiled and a phosphotyrosine domain. Both are domains known to be involved in protein-protein interaction. The N-terminus was deleted to investigate whether the N-terminus is required for microtubule binding.

Neither the N-terminally truncated construct ΔN-p120ctn-VSV, nor the construct AAA-ΔN-p120ctn-VSV, missing the N-terminus of p120ctn but containing the alanine→serine mutations that target the full length protein to microtubules, localized to microtubules (Figure 3.17 A). Likewise, constructs containing just ARM repeats 4, 5 and 6 with or without the alanine→serine mutations showed no association with microtubules (Figure 3.17 B). It was therefore concluded that sequences within the ARM repeat domain as well the N-terminus are required for p120ctn to interact with microtubules.
To map regions required for p120ctn to bind to microtubules, a series of deletion and truncation constructs was generated. (ARM repeat, tyrosine phosphorylation domain, coiled coil domain). Vector information: FL (full length p120ctn), ΔN-p120ctn-VSV (amino acids upstream of start codon 4 deleted), ΔK-p120ctn-VSV (amino acids 622 to 626 deleted), GST-N-term (GST fused to the first 351 amino acids of p120ctn), GST-ARM-456 (GST fused to amino acids 481 to 645), GFP-ARM-456 (EGFP fused N-terminally to amino acids 481 to 645). The pEF-Bos-GST (made by Dr Giles Cory) vector was used to generate the GST constructs and vector C1-pEGFP for GFP constructs. In addition, serine→alanine mutations at positions 538/539 and 587 were introduced into constructs ΔN-p120ctn-VSV, GST-ARM-456 and GFP-ARM-456 to yield constructs AAA-ΔN-p120ctn-VSV, GST-ARM-456-AAA and GFP-ARM-456-AAA, respectively.
Figure 3.17  Both the N-terminus and the ARM repeat region are required for p120ctn to interact with microtubules

**Fig A:** MDCK cells were electroporated with N-terminally truncated constructs ΔN-p120ctn-VSV or AAA-ΔN-p120ctn-VSV and fixed after 24 h. Neither protein localized to microtubules, suggesting that the N-terminus of p120ctn is required for interaction with microtubules. **Fig B:** GFP-ARM-456 and GFP-ARM-456-AAA, containing just ARM repeats 4, 5 and 6 with or without alanine mutations at positions 538/539 and 587 likewise were not recruited to microtubules. Bar 10 μm.
3.6.2 GST-fusion proteins containing either the central three ARM repeats or the N-terminus precipitate α-tubulin

p120ctn appears to require sequences both in the N-terminus and in the ARM repeat domain to bind to microtubules (see above). To test whether the N-terminus and ARM repeats 4, 5 and 6 are able to bind α-tubulin, expression vectors for GST fusion proteins of the N-terminus (GST-N-term) and of ARM repeats 4, 5 and 6 (GST-ARM-456) were created, using the pEF-Bos-GST vector for expression of GST-fusion proteins in mammalian cells (Figure 3.16). Expression of the fusion proteins in mammalian cells was chosen over bacterial expression to allow eukaryotic posttranslational modifications, in particular serine/threonine phosphorylation. To test for their ability to bind α-tubulin, the GST-fusion proteins were expressed in Cos-7 cells and purified on glutathione-sepharose beads from the NP-40 soluble lysate fraction. Both GST-N-term and GST-ARM-456-WT precipitated α-tubulin. Surprisingly, GST-ARM-456-AAA, containing alanine mutations at positions corresponding to serines 538/539 and 587, precipitated less α-tubulin, although the corresponding mutations in the full length protein increased its colocalization with microtubules in cells (Figure 3.3). This suggests that the abilities of p120ctn to bind monomeric α-tubulin or to associate with microtubules are inversely related. However, the interpretation of the results is complicated for several reasons: cell lysis in these experiments was not performed under microtubule-stabilizing conditions and it is therefore impossible to determine whether the precipitated α-tubulin had originally been part of the soluble, monomeric α-tubulin pool of the cell or had been incorporated into microtubules. In addition, microtubules resisting depolymerisation in the lysis buffer are likely to sediment in the insoluble fraction, potentially coprecipitating associated GST-fusion proteins. Precipitation experiments involving abundant cytoskeletal proteins (tubulins are among the most abundant cellular proteins) often pose a specificity problem. However, the interaction of the GST-fusion proteins with monomeric tubulin was specific since GST itself did not precipitate any α-tubulin.
Figure 3.18 The N-terminus of p120ctn and a construct comprising ARM repeats 4, 5 and 6 of p120ctn are able to bind α-tubulin

Cos-7 cells were electroporated with pEF-Bos-GST, GST-N-term or GST-ARM-456-wt or -AAA plasmids. After expression for 24 h, cells were lysed and GST-fusion proteins purified on glutathione-sepharose beads. Both ARM repeat-containing constructs and the N-terminus of p120ctn, but not GST on its own, precipitated α-tubulin.
3.6.3 Deletion of a basic motive in the ARM repeat domain of p120ctn prevents association with microtubules

The N-terminus and ARM repeats 4, 5 and 6 were analysed for the presence of putative tubulin-binding motifs. The known tubulin-binding motifs vary greatly between the different tubulin-interacting proteins (an overview of tubulin-binding sites is available on the microtubule-interaction site [http://server.nbio.uni-heidelberg.de/Groups/WWW_Brandt/MT.html]). However, a number of MAPs (microtubule-associated proteins) possess basic motifs with a characteristic repetition of lysine-rich stretches. No putative tubulin-binding motifs were found in the N-terminus, but analysis of ARM repeats 4, 5 and 6 showed that repeat 6 best matches the criteria for such a motif (Table 3.1). Repeat 6 is distinctly more basic than ARM repeats 4 and 5 and it has the highest percentage of lysine and glycine residues among the three repeats. A cluster of lysines between amino acids 622 to 626 of p120ctn, situated on a looped-out structure within ARM repeat 6 (Anastasiadis and Reynolds, 2000), resembles the tubulin-binding motifs of MAP-1B and the yeast protein EBF5, although in these proteins the lysines are mainly interspersed with negatively charged aspartate and glutamate residues instead of apolar glycines:

\[
\begin{align*}
\text{p120ctn amino acids 622 to 626:} & \quad 622\text{KKGKGGK}626 \\
\text{tubulin-binding motive of EBF5:} & \quad \ldots\text{KKDKKEKKEKKDKEKKEKKEKKEKKRKAD}\ldots \\
\text{tubulin-binding motive of MAP-1B:} & \quad \ldots\text{KKEDKTPIKKEEKPKKEKEVKEVKEIKEKKEKKEKKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEK
Isoleucines are commonly used to eliminate the positive charge of the lysine side-chain amino group, while retaining similar sterical properties.

Deletion of the entire basic motif prevented localization of p120ctn to microtubules in MDCK cells (Figure 3.19). Although several thousand ΔK-p120ctn-overexpressing cells were analysed, no cell was found to show p120ctn/microtubule colocalization. Similarly, substitution of lysines 622/623 with isoleucines also blocked microtubule association. Construct KKKLL-p120ctn, on the other hand, associated with microtubules to the same degree as the wild-type p120ctn, showing that lysines 627/628 are not required for microtubule binding.

All p120ctn mutants were still able to bind to cell-cell junctions (for instance mutant ΔK-p120ctn in Figure 3.19), implying that the overall structure of the ARM repeat domain was not compromised. Interestingly, the ΔK-p120ctn and LLKKK-p120ctn showed little or no nuclear localization (Figure 3.19), whereas the wild-type p120ctn and KKKLL-p120ctn showed nuclear staining in about half of the cells. It remains to be investigated whether there is a functional connection between microtubule association and nuclear import or export.

3.6.4 Inclusion of exon C disrupts the basic motif but does not abrogate the ability of p120ctn to induce the branching phenotype or to associate with microtubules

Exon C encodes for 6 amino acids inserted within ARM repeat 6 (Keirsebilck et al., 1998). Interestingly, inclusion of exon C disrupts the basic motif between amino acids 622 and 628:

\[
\text{basic motif} \\
\text{(+) exon C: } 619\text{FGAKKGKEWFSRGKKPTE}_{631} \\
\text{(-) exon C: } 619\text{FGAKKGK}_{631}
\]

The basic motif is required for the interaction with microtubules and for the inhibition of RhoA (Anastasiadis et al., 2000). In p120ctn isoforms containing exon C the basic motif
is disrupted and these isoforms may therefore not be able to associate with microtubules 
or inhibit RhoA. To test whether inclusion of exon C abrogates the ability of p120ctn to 
regulate Rho proteins, and therefore to induce the branching phenotype, an 18 base pair 
sequence corresponding to exon C was incorporated into the wild-type p120ctn-VSV 
construct after the base pair triplet coding for amino acid 625 by PCR. The resulting 
construct, encoding for a protein corresponding to p120ctn isoform 1AC, was transfected 
into NIH 3T3 cells and after expression for 16 h the morphology of expressing cells was 
analysed. The exon C-containing mutant efficiently induced the branching phenotype, 
indicating that inclusion of exon C does not affect the ability of p120ctn to inhibit RhoA 
(Figure 3.20, upper panel). Likewise, incorporation of exon C into the AAA-p120ctn 
mutant did not prevent its association with microtubules (Figure 3.20, lower panel), 
demonstrating that the ability of the basic motif to mediate the association with 
microtubules is not affected by the presence of exon C.
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## Table 3.1 Comparison of amino acid composition and pI between ARM repeats 4, 5 and 6 and classical tubulin-binding domains

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Classical tubulin binding domain</th>
<th>ARM 456</th>
<th>ARM 4</th>
<th>ARM 5</th>
<th>ARM 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>11 - 44%</td>
<td>6%</td>
<td>3%</td>
<td>6%</td>
<td>10%</td>
</tr>
<tr>
<td>P</td>
<td>3 - 17%</td>
<td>6%</td>
<td>5%</td>
<td>0%</td>
<td>10%</td>
</tr>
<tr>
<td>G</td>
<td>0 - 22%</td>
<td>5%</td>
<td>3%</td>
<td>4%</td>
<td>7%</td>
</tr>
<tr>
<td>pI</td>
<td>9 - 11</td>
<td>5.8</td>
<td>5.1</td>
<td>5.2</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Classical tubulin binding domains contain a high percentage of lysines and glycines and are often very basic. Of ARM repeat 4, 5 and 6, repeat best matches these criteria: It has the highest proportion of lysines and glycines it is far more basic than the other repeats. Expasy’s “Compute pI/Mw” tool [http://ca.expasy.org/tools/pi_tool.htm] was used to calculate the pI of the different domains. The values for the classical tubulin-binding domain were taken from the web page of Martin Brandt’s laboratory, University of Osnabrück [http://server.nbio.uni-heidelberg.de/Groups/WWW_Brandt/MT.html].
Figure 3.19  Deletion of a basic motive in ARM repeat 6 prevents colocalization of p120ctn with microtubules

MDCK cells were transfected by electroporation with wild-type p120ctn-VSV (WT), ΔK-p120ctn-VSV (ΔK), LLKKK-p120ctn-VSV (LLKKK) or KKKLL-p120ctn-VSV (KKKLL). Cells were fixed after 24 h and stained with anti-VSV antibodies and with TRITC-phalloidin to visualize the actin cytoskeleton. Inserts in the bottom two panels show junctional localization of the respective mutants at lower expression levels. Bars 10 μm.
Figure 3.20  Inclusion of exon C does not prevent p120ctn from associating with microtubules or from inducing the branching phenotype

NHI 3T3 cells were transfected with p120ctn-1AC-VSV (1AC-VSV) or AAA-p120ctn-1AC-VSV (1AC-AAA-VSV). After expression for 16 h, cells were fixed and stained with anti-VSV antibodies and phalloidin to visualize F-actin. Bar 25 μm.
3.7 Discussion

3.7.1 Mutations in the ARM repeat domain of p120ctn reveal a novel colocalization with microtubules and the centrosome and add it to the list of ARM repeat proteins associating with tubulin-containing structures

Introduction of serine->alanine mutations at positions 538/539 and 587 induced p120ctn to colocalize with interphase microtubules or with the mitotic spindle during cell division. This effect could be observed in fibroblast and epithelial cells, though required higher expression levels in the latter cells. Association with microtubules is a novel function for p120ctn and adds another facet to its known roles in adhesion, cell migration and transcription. However, there are other ARM repeat proteins that interact with microtubules and this interaction could emerge as a general feature of ARM repeat-containing proteins. Endogenous APC localizes to the tips of microtubules in plasma membrane extensions (Nathke et al., 1996) and has been suggested to anchor them to the plasma membrane via interaction with the microtubule (+) end-binding protein EB-1 (Barth et al., 2002; Mimori-Kiyosue et al., 2000a; Mimori-Kiyosue et al., 2000b; Mogensen et al., 2002; Nakamura et al., 2001). APC also localizes to kinetochores, the microtubule attachment sites of chromosomes (Fodde et al., 2001; Kaplan et al., 2001). The related APC2 protein and armadillo (the drosophila homologue of β-catenin) have been shown to control the positioning of the mitotic spindle by tethering it to cortical actin filaments (McCartney et al., 2001). β-catenin interacts with the motor protein dynein and may tether microtubules at adherens junctions (Ligon et al., 2001), whereas APC travels along microtubules by binding to kinesins (Jimbo et al., 2002).

In addition, a number of HEAT repeat containing proteins are known to associate with tubulin structures (Neuwald and Hirano, 2000). Although HEAT repeats consist of only two α-helices, compared to the three α-helices found in ARM repeats, both motifs are structurally similar and believed to be evolutionary related (Andrade et al., 2001; Andrade et al., 2000). Members of the Dis1/TOG family of HEAT proteins are able to bind directly to (+) ends of microtubules, thereby increasing their elongation rate (Charrasse et al., 1998; Gard and Kirschner, 1987), and associate with centrosomes and kinetochores (reviewed in Ohkura et al., 2001).

At this point, one can only speculate about the physiological significance of the p120ctn/microtubule interaction. Based on the knowledge about the microtubule-related functions of APC/APC2 and β-catenin and the particular cellular effects of p120ctn, there
are a number of possibilities: p120ctn could link microtubule tips to the E-cadherin complex, anchoring it more firmly to the cytoskeleton. Alternatively, it could support the formation of microtubule-driven cellular extensions, (e.g. neurites) by binding to and stabilizing microtubules. Finally, it could be involved in the proper positioning of the mitotic spindle during cell division. These points will be discussed in detail in the following section.

3.7.2 Possible physiological relevance of the p120ctn/microtubule interaction

3.7.2.1 Tethering of interphase microtubules to the plasma membrane

β-catenin has been proposed to link microtubule tips to the plasma membrane via the motor protein dynein (Ligon et al., 2001). However, p120ctn is unlikely to exert a similar function. Whereas most microtubules originate from the MTOC and extend towards the plasma membrane, overexpression of mutant p120ctn shapes the microtubules into very long filaments circling the nucleus. In both fibroblast and epithelial cells, only few p120ctn-associated microtubules extend towards the plasma membrane. Therefore it appears as if p120ctn binding reduces rather than supports membrane tethering of microtubules. In addition, in MDCK cells adherens junctions and p120ctn-associated microtubules do not colocalize (not shown). p120ctn requires the ARM repeat domain to interact with both microtubules (this chapter) and E-cadherin (Daniel and Reynolds, 1995; Ireton et al., 2002; Shibamoto et al., 1995). Unless each interaction use a different part of this domain, the cadherin and microtubule binding would compete with each other. However, as described below, the looped-out structures within ARM repeats 4 and 6 could facilitate parallel binding of cadherins and microtubules. In preliminary studies, AAA-p120ctn also failed to coprecipitate the dynein intermediate chain (not shown).

3.7.2.2 Microtubule stabilization and neurite outgrowth

The association of mutant p120ctn with microtubules leads to their remodelling into thick, curly bundles and to their stabilization as indicated by the increase in α-tubulin acetylation. Expression of wild-type p120ctn does not cause stabilization of microtubules in the cell body. Microtubules within the p120ctn-driven extensions, on the other hand, show a high degree of acetylation. It is therefore possible that wild-type p120ctn interacts with and stabilizes microtubules exclusively in cellular extensions. The formation of
these extensions is the result of overexpressing p120ctn to a considerable degree and may not represent a physiological role of p120ctn. However, they strikingly resemble neurite extensions in neuronal cells. Neurite outgrowth requires microtubule polymerisation (Nobes and Hall, 1999; Tanaka et al., 1995), and Rac1 activation (Altun-Gultekin and Wagner, 1996; Yuan et al., 2003). In addition, microtubules become stabilized during neurite growth, correlating with an increase in their acetylation (Falconer et al., 1989; Ferreira and Caceres, 1989; Shea, 1999). p120ctn and its closely related neuronal family member NPRAP/β-catenin could therefore have a role in driving neurite outgrowth by stabilizing microtubules and activating Rac1 (Grosheva et al., 2001; Noren et al., 2000). NPRAP/β-catenin has recently been shown to induce neurite outgrowth in neuronal cells (Kim et al., 2002; Lu et al., 2002), although it is not known whether it is able to interact with microtubules. Wild-type p120ctn, but not AAA-p120ctn, induces cellular protrusions in neuronal PC12 cells that are morphological indistinguishable from neurite extensions. If the stabilization of microtubules by p120ctn specifically in the neurite extensions were essential for their formation, one would expect AAA-p120ctn, which in fibroblasts interacts with microtubules aberrantly and does not induce branching, to block NGF-induced neurite extensions. This, however, is not the case: both wild-type and AAA-p120ctn-expressing PC12 cells form neurite extensions when treated with NGF. This suggests that mutant p120ctn has lost its ability to activate Rac but exerts no dominant-negative effect on independent NGF-induced Rac activation. Since in these cells no clear localization of AAA-p120ctn along microtubules could be observed, mutant p120ctn may not affect microtubule stabilization in neuronal cells.

As mention above, the branching induced by p120ctn in PC12 cells is morphologically similar to the neurite extensions formed during the differentiation process into mature neurons. It would be interesting to see whether the two kinds of cellular protrusions only coincidently resemble each other, or whether p120ctn overexpression itself induces differentiation into neuronal cells. Neuronal differentiation following p120ctn expression could not be detected using antibodies against neuronal markers such as MAP1B (not shown). The identification of p120ctn/Kaiso-regulated genes (Daniel and Reynolds, 1999), for instance through microarray analysis, may clarify this issue.
3.7.2.3 Localization of p120ctn to centrosomes and the mitotic spindle

AAA-p120ctn shows clear localization to the mitotic spindle and to the centrosomes in Cos-7 cells. In addition, endogenous p120ctn localizes to centrosomes in MDA-MB-231 cells. The localization to centrosomes may therefore be a true physiological role for p120ctn, whereas the association with microtubules could be an artefact of overexpression.

Although epithelial adherens junctions between dividing cells and surrounding interphase cells are largely retained (Baker and Garrod, 1993), the cytoplasmic concentration of the intracellular components of the E-cadherin complex, the catenin proteins, is transiently increased during mitosis (Bauer et al., 1998). The accumulation of catenin proteins in the cytoplasm could be either the result of endocytosis of the entire, intact E-cadherin complex or of a transient disassembly of the complex. During mitosis p120ctn is distributed diffusely in the cytoplasm (Bauer et al., 1998), rather than in the punctate pattern typical for the endocytosed, endosome-associated E-cadherin complex (Le et al., 1999), suggesting that p120ctn is released from the E-cadherin complex into the cytoplasm. On the other hand, endosomes tend to accumulate around the centrosomes during mitosis (Bergeland et al., 2001) and the centrosomal p120ctn staining could be caused by the recruitment of p120ctn-containing endosomal vesicles to the centrosomes. However, this is unlikely because during mitosis the p120ctn staining overlapped perfectly with centrosome staining (Figure 3.5), contrasting with the staining pattern of p120ctn around, but not at, the centrosomes following H-89 treatment (Figure 4.6), which is most likely to be caused by the accumulation of vesicular p120ctn (Section 4.2.4).

As the cytoplasmic level of p120ctn rises, it could increasingly localize to the centrosomes, and signal to the cell division machinery that sufficient junctional disassembly has occurred to progress through mitosis. It must be pointed out, however, that localization of p120ctn to centrosomes could only be observed in the E-cadherin-deficient cell lines Cos-7 and MDA-MB-231. These cells have high levels of cytoplasmic p120ctn, with only a fraction of the total p120ctn being recruited to the plasma membrane (Figure 3.5, interphase cells), possibly by other cadherins such as N-cadherin or cadherin-11 (Pishvaian et al., 1999). In E-cadherin-positive MDCK cells, p120ctn is mainly sequestered at the plasma membrane and the cytoplasmic pool of p120ctn available for centrosome association may be too low to allow detection by immunofluorescence.
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p120ctn and Microtubules

There are no reports of ARM proteins other than p120ctn localizing to centrosomes. The APC-interacting protein EB-1 associates with centrosomes during mitosis (Berrueta et al., 1998; Morrison et al., 1998; Rehberg and Graf, 2002), but APC itself has not been observed at centrosomes. However, all HEAT proteins of the Dis1/TOG family of proteins localize to centrosomes, depending on the organism either permanently (budding yeast, *Dictyostelium discoideum*) or specifically during mitosis (human cells, *Xenopus laevis*) (Charrasse et al., 1998; Graf et al., 2000; Popov et al., 2001; Tournebize et al., 2000; Wang and Huffaker, 1997). Depletion of the human member of the Dis1/TOG family (ch-TOG) from Hela cells by RNA interference (RNAi) led to the formation of highly disorganized mitotic spindles (Gergely et al., 2003). Using RNAi experiments, it would be interesting to investigate whether p120ctn also has a similar effect on spindle organization.

Several ARM proteins associate with the mitotic spindle: APC localizes to kinetochores, the points where microtubules attach to microtubules (Fodde et al., 2001; Kaplan et al., 2001). In *Drosophila melanogaster* both APC2 and armadillo have been suggested to anchor the mitotic spindle to cortical actin (McCartney et al., 2001) (Allan and Nathke, 2001). In Cos-7 cells, AAA-p120ctn showed some punctate staining around the cell cortex during anaphase and could similarly be involved in anchoring astral spindle microtubules to the cortex.

Members of the importin family of ARM proteins are best known for their role in mediating Ran GTPase dependent nuclear import (Chook and Blobel, 2001). However, they also associate with the mitotic spindle (Gruss et al., 2001; Wiese et al., 2001) and both Ran and the importins are emerging as important regulators of spindle assembly (reviewed in Kahana and Cleveland, 2001; Marshall and Kahana, 2001). Importins bind and inhibit several spindle assembly proteins (Dasso, 2002; Gruss et al., 2001; Nachury et al., 2001). Binding of Ran-GTP to importins in the vicinity of chromosomes releases the spindle assembly factors and promotes spindle assembly and microtubule growth. It will be interesting to investigate whether p120ctn is able to regulate small GTPases that are not members of the Rho family, such as Ran. The Ran/importin interaction is an example of regulation of ARM proteins by a small GTPase, rather than vice-versa. Whereas the focus has so far been on the regulation of small GTPases by p120ctn, small GTPases may in turn regulate p120ctn itself.
Interestingly, pl20ctn is the only known component of the E-cadherin complex to undergo a change in its phosphorylation state during mitosis, demonstrating a cell cycle-dependent regulation of pl20ctn (Bauer et al., 1998). Using the MPM-2 antibody, which recognizes serines phosphorylated specifically during mitosis (Ding et al., 1997; Vandre et al., 1986), pl20ctn was found to be phosphorylated during mitosis. This phosphorylation event did not coincide with a mobility shift of pl20ctn on SDS-PAGE, suggesting that serine(s) targeted by the cell cycle-regulated kinase or phosphatase are different from the subset of serines phosphorylated upon recruitment of pl20ctn to the E-cadherin complex (see Chapter 4).

3.7.3 Regulation of Rho GTPases and microtubule association

AAA-pl20ctn did not induce the branching phenotype characteristic of wild-type pl20ctn expression in fibroblasts. Pl20ctn-induced branching depends on the activation of Rac1 and Cdc42 (Grosheva et al., 2001; Noren et al., 2000) and possibly on the inhibition of RhoA (Anastasiadis et al., 2000). It was therefore obvious to investigate whether AAA-pl20ctn failed to induce branching because it had lost the ability to activate Rac. Expression of AAA-pl20ctn indeed did not increase active Rac1 levels.

The loss of the ability of AAA-pl20ctn to activate Rac1 coincided with its association with microtubules. This could occur because microtubules and a guanine-nucleotide exchange factor, such as Vav2 (Noren et al., 2000), bind mutually exclusively to pl20ctn. In PC12 cells, however, AAA-pl20ctn did not induce branching despite the fact that mutant pl20ctn did not clearly localize to microtubules. This would suggest that binding of an exchange factor is also affected by the point mutations. Alternatively, it is theoretically possible that binding of pl20ctn to microtubules releases a Rac activator normally sequestered by microtubules. However, because of the contracted morphology of undifferentiated PC12 cells, individual microtubules were difficult to visualize in these cells. Thus, localization of AAA-pl20ctn along microtubules may occur in PC12 cells but may be difficult to detect.

Interestingly, the basic motive KKGKGKK, which appears to be required for pl20ctn/microtubule interaction, has previously been shown to be essential for the inhibition of RhoA by pl20ctn (Anastasiadis et al., 2000). This suggests that microtubule binding and RhoA inhibition are linked. They would be mutually exclusive events if
RhoA, or a RhoGDI, and microtubules compete for binding to the motif. They could also be concurrent events if p120ctn only inhibits RhoA when it is microtubule-associated, or if p120ctn is only targeted to microtubules when RhoA is inhibited. However, the latter two scenarios are unlikely because \textit{in vitro} experiments showed a direct inhibitory effect of p120ctn on RhoA activation (Anastasiadis et al., 2000), and inhibition of RhoA, by expression of a dominant-negative version, has not been reported to cause association of p120ctn with microtubules. Hence, binding of p120ctn to microtubules appears to be inversely related to its ability to regulate Rho GTPases.

3.7.4 Are serines 538/539 and 587 phosphorylation sites?

Although the point mutations introduced in the p120ctn ARM repeat domain dramatically shifted the intracellular localization of p120ctn towards an association with microtubules, the mechanisms underlying this change in localization remain unclear. The original motivation for introducing the serine–alanine mutations had been to identify phosphorylation sites in the ARM repeat domain. Several observations favour the interpretation that serines 538/539 and/or 587 are indeed phosphorylation sites. Firstly, total phosphorylation of AAA-p120ctn in Cos-7 cells was reduced compared to wild-type p120ctn by approximately 20%. Tryptic phosphopeptide mapping analysis yielded up to 20 different phosphopeptides (see Chapter 4). Assuming stoichiometric phosphorylation of all the sites and the occurrence of just one phosphorylation site per tryptic peptide, loss of three out of 20 phosphorylation sites (15%) would give a similar decrease in overall phosphorylation of the protein. Secondly, replacing serine 587 with an aspartate residue efficiently prevented association of p120ctn with microtubules, reversing the microtubule-targeting effect of the alanine mutation. These opposite effects of the phosphoserine-mimicking aspartate and the non-phosphoserine-mimicking alanine mutations are consistent with serine 587 being a phosphorylation site which regulates the association of p120ctn with microtubules.

On the other hand, the phospho-specific antibodies generated against a phosphopeptide corresponding to the potential phosphorylation site at serines 538/539 failed to recognize p120ctn on western blots. It is a common phenomenon that antibodies raised against a peptide do not react with the corresponding peptide sequence when it is part of the polypeptide chain of a protein, possible because the peptides exist in different conformations in the two cases. However, this is a problem more frequently encountered
when such antibodies are used in cell staining applications or immunoprecipitations, which require recognition of the protein in its native state. Under the denaturing conditions of SDS-PAGE, the epitope should be accessible to the antibody. In addition, a polyclonal antibody should give a certain redundancy by containing antibody populations binding slightly different parts of the peptide. Therefore, the phosphospecific antibody may not recognize p120ctn because serines 538/539 are not phosphorylated. No conclusion could be drawn from these experiments about the phosphorylation state of serine 587 because no antibodies were obtained against the corresponding phosphopeptide.

Mass spectrometric analysis of p120ctn did not identify any serine phosphorylation sites located in the ARM repeat domain (see Chapter 4). Although the technique is by no means exhaustive, the absence of any mass peaks corresponding to peptides containing phosphoserines 538/539 or 587 is an indication that phosphorylation may not occur at these residues. The results of the mass spectrometrical analysis and the failure of the generated phosphospecific antibodies to recognize purified p120ctn do not rule out the possibility that serines 538/539 or 587 are phosphorylation sites, but, at the same, they also deny proof that phosphorylation at these sites indeed occurs. The question whether serines 538/539 and 587 are phosphorylation sites could therefore not be conclusively answered.

### 3.7.5 Potential mechanisms of regulation of the p120ctn/microtubule interaction

#### 3.7.5.1 Regulation by phosphorylation

Phosphorylation of bipartite serine motifs is a known mechanism by which some protein-protein interactions are regulated. For instance, 14-3-3 protein dimer can bind neighbouring phosphoserine-containing consensus sequences within the same protein (Yaffe and Elia, 2001; Yaffe and Smerdon, 2001). However, neither the sequences around serine 538/539 and 587 nor their spacing matches the consensus for 14-3-3 protein binding, making it unlikely that 14-3-3 proteins are involved in regulating p120ctn localization/function.

Tau proteins represent a family of microtubule-associated phosphoproteins. Hyperphosphorylation (or the introduction of phosphorylation-mimicking glutamate clusters) prevents the association of tau with microtubules (Billingsley and Kincaid, 1997;
Buee et al., 2000; Eidenmuller et al., 2001). The relevant serine and threonine residues are situated in a proline-rich region adjacent to microtubule-binding repeats (Hanger et al., 1998; Morishima and Ihara, 1994). Although p120ctn lacks such a proline-rich domain and comparable microtubule-binding repeats, there may be an interesting analogy between tau and p120ctn regarding the effect of phosphorylation on their association with microtubules.

The amino acid sequences directly preceding serines 538/539 and 587 are remarkably similar (the positions of the amino acids in the ARM repeat consensus sequences are indicated):

538/539SS: \[\ldots L_{32}R_{33}N_{34}V_{35}S_{36}S_{37}\ldots\]

587S: \[\ldots L_{32}R_{33}N_{34}L_{35}S_{36}Y_{37}\ldots\]

This similarity suggests that these sequences may be consensus sites for a protein kinase phosphorylating both sites. However, motifs similar to LRN(L/V) are found at the end of many ARM repeats in different ARM proteins and are important for proper ARM repeat folding (Andrade et al., 2001). Especially the two leucines/valines (at positions 32 and 35 within the 42 amino acid ARM repeat consensus sequence) are structurally crucial because they are part of the hydrophobic core that stabilizes the ARM repeat domain. In addition, residues at positions 34 are highly conserved hydrophilic residues, most commonly asparagines, which are involved in substrate recognition by importin-α (Conti et al., 1998) and which are also required for the binding of β-catenin to the E-cadherin tail (Graham et al., 2000). The similarity between the sequences preceding serines 538/539 and 587 may therefore be the result of the requirements for ARM repeat folding rather than reflect a kinase consensus sequence.

3.7.5.2 Conformational changes

If phosphorylation does not regulate the p120ctn/microtubule interaction, then structural effects caused by the introduction of alanine mutations must account for the increased affinity of p120ctn for microtubules. This could be either because interaction with a protein preventing p120ctn from binding to microtubules is lost, or because a microtubule-binding site normally concealed is revealed in the mutant. Conformational changes could also be responsible for the decreased level of phosphorylation of AAA-
p120ctn compared to wild-type p120ctn if the mutations affect the efficiency of phosphorylation at additional phosphorylation sites.

Serines 538/539 and 587 are situated towards the end of the ARM repeats 4 and 5, respectively. Their exact structural context is unknown because the structure of the p120ctn ARM repeat domain has not been determined. However, the structure of the ARM repeat domain of the related protein β-catenin has been solved (Huber et al., 1997). In β-catenin there are corresponding serine residues at the end of ARM repeats 4 and 5. Although in p120ctn these serines are separated by 48 amino acids, based on the β-catenin structure they are expected to be in close proximity in the folded protein (Figure 3.21). Because of this juxtaposition, they are likely to be involved in the same protein-protein interactions, which would explain why mutation of either set of the serine residues has an identical effect on p120ctn localization.

It is nevertheless unlikely that the serine→alanine mutations cause major conformational changes in the ARM repeat domain. The AAA-p120ctn mutant is still able to bind to cell-cell junctions, an interaction that requires a largely intact p120ctn ARM repeat domain (Daniel and Reynolds, 1995; Ireton et al., 2002). In addition, the consensus amino acid at position 36 in ARM repeats of the p120ctn protein family is serine or alanine (Anastasiadis and Reynolds, 2000), demonstrating that alanine residues at these positions are compatible with proper ARM repeat structure.

Since p120ctn does not always interact with microtubules, one could hypothesize that the tubulin binding sites are either hidden or exposed, depending on the state of the cell. The ARM repeats are probably fairly rigid structural units (Andrade et al., 2001) that are less likely to become substantially unfolded to reveal a tubulin-binding domain. However, there are linker regions within ARM repeats 4 and 6 of 17 or 15 amino acids, respectively. These linkers are absent in β-catenin, for instance, and could enable p120ctn to interact with proteins in addition to classical binding partners for the ARM repeat domain such as E-cadherin. Also, the linkers are expected to be more flexible than the core ARM repeats and could more easily change their conformation to reveal a protein-binding site. Interestingly, the basic motif KKGK, which resembles some known microtubule-binding domains and appears to be required for p120ctn/microtubule association, is part of such a loop. This basic motif is disrupted when exon C is expressed (Keirsebilck et al., 1998), suggesting that inclusion of exon C could be an additional way
of regulating tubulin binding by disrupting a tubulin-binding motif. However, the ability of the AAA-p120ctn mutant to associate with microtubules was not abrogated by the inclusion of exon C. Exon C is inserted just before the two C-terminal lysines (residues 627 and 628) of the basic motif, leaving the N-terminal part of the motif (residues 622 to 625) unchanged. Since mutation of lysines 627/628 to isoleucines did not prevent microtubule association either, the functionality of the basic motif in regard to mediating the association with microtubules appears to reside within its N-terminal part.

In addition to sequences in ARM repeats 4, 5 and 6, the N-terminus of p120ctn is also able to bind α-tubulin and is required for microtubule association in cells, though no putative tubulin-binding domains could be identified within the N-terminus of p120ctn. Occasionally AAA-p120ctn was observed to colocalize with microtubules in a dot-like fashion, suggesting that it may bind to microtubules via additional proteins such as motor proteins. The APC protein contains several different domains involved in interacting with microtubules, including two independent tubulin-binding domains and binding sites for the microtubule-associated protein EB-1 (reviewed in Allan and Nathke, 2001) and the kinesin superfamily-associated protein 3 (KAP3) (Jimbo et al., 2002). It is feasible that p120ctn too is able to interact with microtubule in a number of different ways.

In conclusion, highly overexpressed wild-type p120ctn occasionally localized to microtubules in MDCK cells, whereas p120ctn carrying specific serine to alanine mutations in the ARM repeat domain (AAA-p120ctn) frequently associated with interphase microtubules or the mitotic spindle and the centrosomes in a variety of epithelial and fibroblast cell lines. In addition, endogenous p120ctn was found to localize to the centrosomes in MDA-MB-231 cells during mitosis. The localization of AAA-p120ctn to the microtubule network coincided with the loss of the ability of AAA-p120ctn to activate Rac1 and a marked reduction of the branching phenotype in fibroblasts. In addition to the ARM repeat domain, the N-terminus of p120ctn was required for the interaction of p120ctn with microtubules. The association with AAA-p120ctn stabilized microtubules throughout the cell body, whereas wild-type p120ctn-expressing cells contained stabilized microtubules exclusively in p120ctn-driven cellular extensions. The association with the microtubule network and the centrosomes suggests an unexpected role for p120ctn during mitosis and in regulating cell morphology beyond its effects on the actin cytoskeleton.
Figure 3.21  The ARM repeat domain structure of β-catenin

**Fig A:** Space-fill model of the ARM repeat domain of β-catenin (Huber et al., 1997). The image on the right displays a magnified section (contained within the white frame) of the image on the right. White arrows point out serines (green) in corresponding positions to serines 539 and 587 in p120ctn.  **Fig B:** Secondary structure of the same model, magnification and arrows as in A. The proximity of the two serines in the tertiary structure becomes obvious. The model uses data set 3BCT (mmdbld:6960) and was viewed in NCBI’s CnD3 structure viewer.
Chapter 4

p120ctn and Phosphorylation

4.1 Introduction

p120ctn was originally identified as a Src kinase substrate (Reynolds et al., 1989) and the major Src-induced tyrosine phosphorylation sites have recently been mapped (Mariner et al., 2001; Ozawa and Ohkubo, 2001). In addition to tyrosine phosphorylation, p120ctn is extensively phosphorylated on serine and threonine residues (Aono et al., 1999; Downing and Reynolds, 1991; Ratcliffe et al., 1997; Ratcliffe et al., 1999; Wong et al., 2000). p120ctn becomes serine/threonine phosphorylated upon recruitment to the E-cadherin complex (Thoreson et al., 2000) and serine/threonine phosphorylation has been proposed to negatively regulate E-cadherin-mediated adhesion (Aono et al., 1999; Ohkubo and Ozawa, 1999). The serine/threonine kinases involved are still unknown. However, incubation of epithelial cells with Gö6967, an inhibitor of protein kinase C (PKC) isoforms α and β1 (Martiny-Baron et al., 1993; Qatsha et al., 1993), causes dephosphorylation of p120ctn (Ratcliffe et al., 1997), suggesting that these PKC isoforms may phosphorylate p120ctn. Interestingly, incubation with phorbol esters, which activate a subset of PKC isoforms, including the α and β1 types, also leads to dephosphorylation of p120ctn in epithelial (Ratcliffe et al., 1997) and endothelial cells (Ratcliffe et al., 1999; Wong et al., 2000). PKCs may therefore also function upstream of phosphatases and/or kinases acting on p120ctn. The identification of serine/threonine kinases phosphorylating p120ctn and the corresponding phosphorylation sites may offer more insight into phosphorylation-dependent p120ctn functions, such as modulation of cadherin adhesion and microtubule association. Therefore, the serine/threonine phosphorylation state of p120ctn was investigated in detail using a panel of kinase and phosphatase inhibitors, by 2D phosphopeptide mapping and by IMAC/MS analysis.

4.2 Characterization of the serine/threonine phosphorylation state of p120ctn in cell lines of epithelial origin

4.2.1 The p120ctn phosphorylation state differs between cadherin-positive and -negative cells

An initial screen of a panel of 27 breast cancer cell lines (cell lysates kindly provided by Dr. Rob Harris and Prof Mike O'Hare, UCL, London) showed very heterogeneous expression levels for members of the E-cadherin complex (Figure 4.1). The expression of E-cadherin and α- and γ-catenin was sometimes decreased or lost, whereas β-catenin...
expression varied substantially. Some cell lines showed a switch from E-cadherin to N-cadherin expression. In contrast to the other catenins, the level of p120ctn expression was more constant throughout the tested cell lines, with much reduced expression observed in only one cell line (Du-4475). All cell lines showed expression of two isoforms of p120ctn, most likely isoforms 1 and 3 (see Figure 1.1), the isoforms most commonly found in human cells (Keirsebilck et al., 1998). However, there was some variation of the apparent sizes of the two isoforms within the panel of cell lines, which could be the result of differential inclusion of Exons A, B and C, or because of the use of alternative start codons which yield isoforms 2 or 4. In addition, the shape of the p120ctn bands on SDS-PAGE varied significantly between cell lines. In cell lines expressing E-cadherin, p120ctn usually migrated as two diffuse bands, whereas in cell lines with loss of E-cadherin expression it migrated as two condensed bands.

The diffuse band pattern of p120ctn has been shown to result from phosphorylation on serine/threonine residues (Ratcliffe et al., 1997; Ratcliffe et al., 1999; Thoreson et al., 2000; Wong et al., 2000). Consistent with this, treatment of p120ctn immunoprecipitations from E-cadherin-positive MDCK cells with calf intestinal phosphatase (CIP), a phosphatase that removes phosphates from tyrosine as well as serine/threonine residues, reduced p120ctn to two major (lower) and two minor (upper) condensed bands in conventional SDS gel electrophoresis (Figure 4.2, 1D), or to one spot per isoform on 2D gels (Figure 4.2, 2D). Incubation with the phosphotyrosine-specific phosphatase LAR, on the other hand, had little or no influence on the migration pattern of p120ctn on either 1D or 2D gels, re-emphasising that serine/threonine, but not tyrosine, phosphorylation is responsible for the diffuse migration of p120ctn in SDS-PAGE. The condensed bands following CIP treatment resemble the band pattern of p120ctn purified from E-cadherin-negative MDA-MB-231 cells, although the bands differed in size, which could reflect the expression of different sets of isoforms in the two cell lines (Figure 4.2, 1D). The finding that p120ctn is serine/threonine phosphorylated in most E-cadherin-positive cell lines is in agreement with a report showing that reintroduction of E-cadherin into a cadherin-negative cell line induces p120ctn serine/threonine phosphorylation (Thoreson et al., 2000). It therefore emerges as a general pattern that the presence of E-cadherin is a prerequisite for serine/threonine phosphorylation of p120ctn.
Figure 4.1  Expression of cadherin and catenin proteins in a panel of breast cancer cell lines

Lysates (50 µg of total protein) of each of 27 breast cancer cell lines were separated on 10% SDS-polyacrylamide gels and transferred onto PVDF membrane. The membranes were probed with antibodies to E-cadherin (C37020), stripped, and subsequently reprobed with antibodies to N-cadherin and the four catenin proteins. Cell lines were arranged based on their expression characteristics of E- and N-cadherin, with E-cadherin-positive cells on the left, N-cadherin-positive cells in the middle and cadherin-negative cells on the right.
Serine/threonine phosphorylation induces mobility shifts in p120ctn

p120ctn was immunoprecipitated from E-cadherin-expressing MDCK cells or from the E-cadherin negative cell line MDA-MB-231 (231). After three washes with lysis buffer, beads were washed once with CIP buffer (NEB 3 buffer) or with LAR buffer and then incubated in fresh CIP or LAR buffer containing the respective phosphatase. No phosphatase was added to CIP buffer-washed beads carrying p120ctn precipitated from MDA-MD-231 cells or for the control. Beads were subsequently washed with lysis buffer, immunoprecipitated proteins eluted off the beads by boiling in 2x SDS sample buffer or addition of 2D sample buffer and subjected to SDS-PAGE (1D) or two-dimensional gel electrophoresis (2D), followed by western blotting using the pp120 antibody.
Cadherins are sufficient and necessary to recruit p120ctn to the plasma membrane (Daniel and Reynolds, 1995; Thoreson et al., 2000) and it can therefore be assumed that p120ctn is recruited to the plasma membrane in cadherin-expressing cells and localized primarily in the cytoplasm in cadherin-negative cells. This would suggest that the kinases responsible for p120ctn serine/threonine phosphorylation are present only at the plasma membrane. In addition, there may be cytoplasmic serine/threonine phosphatases that keep cadherin-uncoupled p120ctn in a dephosphorylated state (Figure 4.3). Interestingly, in N-cadherin-positive/E-cadherin-negative cells p120ctn was hypophosphorylated, suggesting that recruitment to the plasma membrane alone is not sufficient to induce serine/threonine phosphorylation of p120ctn. The kinase(s) acting on p120ctn appear to be associated with the E-cadherin complex, but not with N-cadherin adhesion complexes. Alternatively, the switch of E-cadherin to N-cadherin expression is believed to indicate epithelial to mesenchymal transition (EMT) (Cavallaro et al., 2002). N-cadherin-expressing cells may not express the p120ctn-targeting kinases if these kinases are specific for epithelial cells.
Figure 4.3  Model for the action of serine/threonine kinases and phosphatases on p120ctn at the plasma membrane or in the cytoplasm

Upon recruitment to the plasma membrane by E-cadherin, p120ctn becomes phosphorylated on multiple serine/threonine residues by staurosporine-sensitive and other kinases (see Figure 4.4). In the absence of E-cadherin, p120ctn is localized in the cytoplasm, where it may be maintained in a hypophosphorylated state by serine/threonine phosphatases of the PP-1 class (see Figure 4.4).
4.2.2 The effects of broad spectrum serine/threonine kinase and phosphatase inhibitors on p120ctn phosphorylation

In order to investigate which kinases or phosphatases affect p120ctn phosphorylation, different broad-spectrum serine/threonine kinase and phosphatase inhibitors were tested for their effect on p120ctn mobility on 1D and 2D gels. In addition to the previously reported effect of the general ACG kinase inhibitor staurosporine on p120ctn phosphorylation (Ratcliffe et al., 1997), the protein kinase A (PKA) inhibitor H-89 (Chijiwa et al., 1990) was found to cause dephosphorylation of p120ctn in MDCK cells, as judged by the mobility increase of p120ctn in conventional SDS-polyacrylamide gels (Figure 4.4). The p120ctn band pattern after H-89 treatment differed from that following staurosporine treatment, suggesting that both inhibitors affect different subsets of phosphorylation sites. This was even more apparent on 2D gels: H-89 and staurosporine treatment led to distinctive arrays of spots. 2D-gel analysis also showed that there are staurosporine- and H-89-insensitive phosphorylation sites in p120ctn because in both cases more than one spot per isoform was discernable, whereas complete dephosphorylation by CIP treatment reduced the pattern to one spot per isoform (compare Figure 4.2). This demonstrates that not all serine/threonine phosphorylation or dephosphorylation events can be analysed by looking for vertical mobility shifts of p120ctn bands on conventional 1D gels and underlines the usefulness of 2D gel analysis.

Of the two serine/threonine phosphatase inhibitors tested, cantharidic acid and calyculin A, only calyculin A was able to increase serine/threonine phosphorylation of p120ctn in MDA-MB-231 cells as seen by the dramatic hypershifting of the p120ctn bands (Figure 4.4). Calyculin inhibits mainly PP-1 phosphatases, whereas cantharidic acid has a greater potency towards PP-2A phosphatases (Wera and Hemmings, 1995), suggesting that p120ctn is dephosphorylated by PP-1 phosphatases. Prolonged incubation (>30 min) with both phosphatase inhibitors caused cell detachment from the tissue culture dish, leading to a decrease in total protein concentration in the lysates generated from these cells.
Figure 4.4  Effect of broad-specificity serine/threonine kinase and phosphatase inhibitors on p120ctn phosphorylation

p120ctn was immunoprecipitated from MDCK (A and C) or MDA-MB-231 (B) cell lysates. MDCK cells were treated with 100 nM staurosporine (stauro), 100 μM H-89 (H-89) or vehicle as control (con) for 30 min or 3 h (A) or 3 h (C) prior to lysis. MDA-MB-231 cells were incubated with 100 μM cantharidic acid (canth a), 100 nM calyculin A (caly A) or vehicle as control (con) for 30 min or 3 h. Immunoprecipitated p120ctn proteins were separated on 6% SDS-polyacrylamide gels (A and B). For two-dimensional gel electrophoresis (C), proteins were first separated according to their pI as described in Section 2.2.4.4, followed by conventional SDS-PAGE (6% polyacrylamide gels).
4.2.3 Serine/threonine phosphorylation of p120ctn is the result of E-cadherin binding but is not essential for this interaction

Binding to E-cadherin induces serine/threonine phosphorylation of p120ctn as judged by the mobility shift on SDS-PAGE (Thoreson et al., 2000). To investigate whether this serine/threonine phosphorylation of p120ctn merely coincides with its binding to E-cadherin or is actually required for the p120ctn/E-cadherin interaction, MDCK cells with well-formed, mature junctions (3 days after seeding), in which p120ctn localized almost exclusively to cell-cell junctions and can be assumed to be in a complex with E-cadherin, were treated with the kinase inhibitor staurosporine (Figure 4.5). Although staurosporine treatment caused dephosphorylation of p120ctn in MDCK cells (Figure 4.4), p120ctn still localized to intercellular junctions and can therefore be assumed to have retained the ability to bind E-cadherin. Phosphorylation of the staurosporine-sensitive serine/threonine sites of p120ctn is therefore a result of binding to E-cadherin, but this phosphorylation is not required for the interaction. Staurosporine treatment affected the architecture of the junctions, making them appear more irregular and ragged, although cell-cell adhesion and overall monolayer integrity were maintained. Since staurosporine is a broad specificity kinase inhibitor, affecting the phosphorylation state of many proteins, it is impossible to determine whether the altered morphology of the junctions is caused by dephosphorylation specifically of junctional p120ctn.
Figure 4.5  Staurosporine treatment affects junctional morphology but does not prevent p120ctn from binding to intercellular junctions

MDCK were seeded at 5 x 10^4 cells per coverslip, grown for 3 days and then treated with 100 nM staurosporine (stauro) or vehicle (control) for 3 h. After fixing the cells were stained with antibodies to p120ctn. F-actin was visualized by staining with TRITC-phalloidin. Bar, 50 μm.
4.2.4 Treatment with the PKA inhibitor H-89 affects p120ctn phosphorylation and localization

Incubation with the serine/threonine kinase inhibitor H-89 increased the mobility of p120ctn on SDS-PAGE and changed the distribution of p120ctn isoforms on 2D gels (Figure 4.4), most likely resulting from a decrease in serine/threonine phosphorylation. To investigate whether the H-89-induced decrease in phosphorylation coincided with a change in the intracellular localization of p120ctn, H-89-treated MDCK cells were fixed and the localization of p120ctn determined by immunofluorescence staining. Whereas in untreated cells p120ctn localized predominantly to sites of cell-cell contact along the basolateral membrane, in H-89-treated cells a fraction of p120ctn also localized around centrosomes in both dividing and interphase cells (Figure 4.6 A). However, p120ctn did not associate with centrosomes directly, but accumulated in the pericentrosomal region, leaving an area of negative staining at the position of the centrosome itself (Figure 4.6 B). This pattern of localization differed from the direct centrosomal localization of AAA-p120ctn in Cos-7 cells or of endogenous p120ctn in MDA-MB-231 cells (see Figure 3.5). Other members of the E-cadherin complex, such as β- and γ-catenin, also localized to the pericentrosomal region in H-89-treated MDCK cells (not shown). This pericentrosomal localization of catenin proteins in H-89-treated cells is probably the result of impaired protein trafficking through the Golgi apparatus, leading to their accumulation in the Golgi apparatus in the vicinity of the centrosomes and is unlikely to represent a specific regulation of their intracellular localization by PKA (see discussion section of this chapter).

4.2.5 p120ctn is not a PKA substrate

To establish whether p120ctn is a PKA substrate, MDCK cells were treated with an alternative PKA inhibitor, KT5720 (Kase et al., 1987; Simpson and Morris, 1995) (Figure 4.7 B). In contrast to H-89 treatment, KT5720 treatment caused no change in the electrophoretic mobility of p120ctn and therefore had presumably no effect on p120ctn phosphorylation, indicating that p120ctn is not a PKA substrate. Consistent with this observation, treatment of E-cadherin-negative MDA-MB-231 cells, which contain unphosphorylated p120ctn, with dibutyryl-cAMP (db-cAMP), a cell permeable analogue of the PKA activator cAMP (Posternak and Weimann, 1974), did not increase p120ctn phosphorylation as indicated by the unchanged electrophoretic mobility of p120ctn.
Figure 4.6  A fraction of p120ctn accumulates around centrosomes in H-89-treated MDCK cells

(A) Confluent MDCK cells were treated with vehicle (control) or with 100 μM H-89 for 3 h. Cells were fixed and stained for p120ctn and γ-tubulin. Confocal images were collected at the plane of the centrosomes. In H-89-treated cells, p120ctn accumulated around centrosomes of mitotic (arrows) and interphase (arrowhead) cells. Bar, 10 μm.

(B) Pericentrosomal localization of p120ctn at higher magnification. p120ctn does not localize directly to the centrosome (arrow). Bar, 2 μm.
Figure 4.7  p120ctn is not a PKA substrate

(A) MDA-MD-231 cells were grown to confluency in 6-well dishes. The cells were subsequently incubated with 1 or 1.5 mM dibutyryl-cAMP (db-cAMP) or vehicle (control) for the indicated times. Cells were lysed in 1% NP-40 lysis buffer and equal amounts of proteins were separated on 6% SDS-polyacrylamide gels. After western blotting, p120ctn was visualized with the pp120 antibody. (B) MDCK cells were grown to confluency in 6-well plates and incubated with 1 μM KT5720 or vehicle (control) for the indicated times. Cell lysis and protein analysis as in A. C: Recombinant PKA (50 units) was incubated with p120ctn immunoprecipitated from confluent 10 cm dishes of MDCK cells (IP p120ctn), 2 μg histone (positive control) or protein G beads only (negative control) in the presence of 25 μM ATP/2.5 μCi [γ-32P]-ATP at 37°C for 15 min. Reaction mixes were separated on SDS-PAGE. The gel was dried and exposed to X-ray film.
(Figure 4.7 A). Finally, PKA did not phosphorylate immunoprecipitated p120ctn in an *in vitro* kinase assay (Figure 4.7 C). These experiments taken together suggest that p120ctn is a substrate not of PKA but of other H-89-sensitive kinase(s). Although H-89 is best known for his inhibition of PKA (IC50=135 nM), it inhibits at least three kinases with equal or higher efficiency than PKA: S6 kinase 1 (IC50=80 nM), mitogen- and stress-activated kinase 1 (IC50=120 nM) and Rho-kinase II (IC50=270 nM) (Davies et al., 2000). However, neither indirect inactivation of S6 kinases via inhibition of their upstream activator mTOR with rapamycin (Lorenz and Heitman, 1995) nor Rho-kinase inhibition with the inhibitor Y-27632 (Uehata et al., 1997) changed the electrophoretic mobility of p120ctn (data not shown), suggesting that H-89 does not block p120ctn phosphorylation via inhibition of S6 or Rho-kinases.
4.3 p120ctn phosphorylation and E-cadherin binding are not affected during junctional disassembly in low calcium conditions

The calcium-dependent homophilic interaction between E-cadherin molecules of neighbouring cells is essential for epithelial cell-cell adhesion. Depletion of free extracellular calcium by EGTA leads to rapid internalisation of E-cadherin complexes and loss of epithelial cell-cell adhesion, whereas reconstitution of extracellular calcium causes restoration of junctions (Gumbiner and Simons, 1986; Volberg et al., 1986). These so-called “calcium switch” experiments are widely used as a model for junctional dis- and reassembly.

Recruitment of p120ctn to the plasma membrane by E-cadherin leads to an increase in serine/threonine phosphorylation of p120ctn (Thoreson et al., 2000). To investigate whether the serine/threonine phosphorylation state of p120ctn changes during junctional remodelling in calcium switch experiments, MDCK cells were allowed to form mature junctions by growing them for 48 h after seeding and were then transferred to medium containing a sufficient concentration of EGTA to chelate extracellular calcium. After 3 h, calcium levels were restored and cells allowed to recover for 2 h. The effectiveness of the calcium switch was monitored by fixing cells at various time points and staining for F-actin and p120ctn (Figure 4.8). During junctional disassembly, cell-cell contacts were completely lost and p120ctn changed from a junctional to a predominantly cytoplasmic localization, although a fraction of p120ctn remained membrane-associated. Reconstitution of extracellular calcium caused rapid reformation of junctions and recruitment of p120ctn to the basolateral membrane. To investigate the serine/threonine phosphorylation state of p120ctn during the different stages of the calcium switch, cells were lysed at different time points and the migration patterns of total p120ctn and E-cadherin-complexed p120ctn on SDS-PAGE were analysed (Figure 4.9). The diffuse pattern of migration, indicative of serine/threonine phosphorylation of total p120ctn (lysate), remained constant throughout the entire calcium switch experiment. Junctional disassembly under low calcium conditions therefore does not itself lead to p120ctn dephosphorylation. At all stages of the calcium switch experiment, similar amounts of p120ctn were bound to E-cadherin (IP E-cadherin), implying that p120ctn is removed from sites of cell-cell adhesion in a complex with E-cadherin. The serine/threonine phosphorylation of the E-cadherin-bound p120ctn pool remained constant, suggesting that
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Figure 4.8  p120ctn is released into the cytoplasm during junctional disassembly under low extracellular calcium.

MDCK cells were grown in DMEM/10% FCS for 48 h and subsequently transferred to medium containing 4 mM EGTA to disrupt (calcium-dependent) cadherin-mediated cell-cell adhesion. After 3 h, cells were transferred back to medium containing a physiological calcium level (1.8 mM). At the indicated time points, cells were fixed and stained with antibodies to p120ctn. F-actin was visualized with TRITC-phalloidin.

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Figure 4.9  The electrophoretic mobility of p120ctn does not change during the calcium switch

MDCK cells were grown in DMEM/10% FCS in 6-well plates to 90% confluency and then transferred to medium containing 4 mM EGTA to deplete extracellular calcium. After 3 h the EGTA-containing medium was removed and replaced with standard medium for 2 h. At the indicated time points, cells were lysed and samples containing equal amounts of total protein were prepared for SDS-PAGE. For E-cadherin immunoprecipitations, the lysates were adjusted to contain equal concentrations of total protein and incubated with 2 μg E-cadherin antibody (clone 34) coupled to Protein A-sepharose beads for 2 h. Total lysates (lysate) and immunoprecipitated proteins (E-cadherin IP) were resolved on 6%-polyacrylamide gels, transferred to PVDF membranes and probed with antibodies to p120ctn.
E-cadherin-binding rather than recruitment to the plasma membrane is crucial for p120ctn phosphorylation (see discussion section of this chapter).

4.4 **p120ctn stability and localization is not regulated by GSK-3**

Phosphorylation of β-catenin by glycogen synthase kinase 3β (GSK3β) (Rubinfeld et al., 1996; Yost et al., 1996) marks it for degradation by the ubiquitin/proteasome pathway (Aberle et al., 1997). Inhibition of GSK3β with Li⁺ ions leads to accumulation of β-catenin in the cytoplasm and eventually to its translocation into the nucleus (Hedgepeth et al., 1997). To investigate whether stability and intracellular localization of p120ctn are similarly regulated by GSK-3β, MDCK cells were incubated with concentrations of Li⁺ ions sufficient for GSK-3β inhibition (Hedgepeth et al., 1997). Intracellular localization and total protein levels of p120ctn and β-catenin were compared. Whereas inhibition of GSK-3β caused accumulation of β-catenin in the cytoplasm and nucleus (Figure 4.10 A) and increased β-catenin levels (Figure 4.10 B) and, it had no effect on p120ctn levels or intracellular localization. This demonstrates a clear difference in the regulation between the two catenin proteins.
Figure 4.10 Protein stability of p120ctn is not regulated by GSK3β

MDCK cells were grown to 60-90% confluency on coverslips for immunofluorescence studies or 6-well dishes for protein analysis by western blotting. (A) After treatment with 20 mM LiCl for 90 minutes, cells on coverslips were fixed and stained for p120ctn or β-catenin and F-actin. Bar 50 μm. (B) Cells grown in 6-well dishes were incubated with 20 mM LiCl for 30 or 60 min, lysed and samples containing equal amounts of protein were separated on 6% SDS-polyacrylamide gels. After western blotting, membranes were first probed for p120ctn, followed by stripping of the blot and probing for β-catenin.
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4.5 Characterization of p120ctn serine/threonine phosphorylation by IMAC/MS

4.5.1 Principles of IMAC/MS with β-elimination and Michael addition

The identification and subsequent mutation of p120ctn serine/threonine phosphorylation sites may reveal how potentially phosphorylation-dependent functions of p120ctn, such as clustering of E-cadherin molecules or the interaction with microtubules, are regulated. An attempt was undertaken to identify p120ctn phosphorylation sites using immobilized metal ion affinity chromatography/mass spectrometry (IMAC/MS). IMAC/MS is a powerful method allowing enrichment of phosphopeptides from a mixture of tryptic peptides and subsequent identification of the phosphopeptides. The IMAC/MS analysis was performed in collaboration with Dr Andrew Thompson, LICR/UCL. The methods have been described (Thompson et. al., 2003) and here there will be only a brief description of the underlying principles.

The purified protein of interest is reduced with dithiothreitol (DTT) to break potential disulphide bonds, followed by alkylation of free cysteines using iodoacetamide to prevent reformation of disulphide bonds. The fusion protein is then digested with trypsin, a protease cleaving specifically after arginine and lysine residues. The resulting tryptic peptides are subsequently incubated with iron(III)-nitrilotriacetic acid (NTA) sepharose beads (IMAC beads). The IMAC beads generally bind phosphopeptides with higher affinity than unphosphorylated peptides through ionic interactions but often also retain other negatively charged peptides, such as peptides rich in acidic residues.

In order to identify IMAC-binding peptides containing phosphoserine or phosphothreonine residues, the IMAC-bound peptides are first subjected to a β-elimination step, which specifically derivatizes serine and threonine phosphorylated peptides at the phosphorylated residue, but does not modify unphosphorylated peptides. The β-elimination of phosphate from phosphoserine and phosphothreonine generates dehydroalanine and dehydro-2-aminobutyric acid, respectively (Figure 4.11 A). The elimination of the phosphate group leads to a characteristic reduction of the peptide mass by 98 Da. In a second step, the site of the original phosphorylation can be further derivatized in a Michael addition of 2-aminoethanethiol, leading to a mass addition of 77 Da (Figure 4.11 B). The Michael addition step prevents non-specific addition reactions and yields products with improved fragmentation characteristics during MS analysis. The combination of β-elimination and Michael addition leads to the net addition of a mass of...
Peptides retained by the IMAC beads were subjected to β-elimination in the presence of Ba(OH)$_2$, leading to the elimination of phosphate groups exclusively from phosphoserine and -threonine residues. The phosphate elimination leads to a mass loss of 98 Da. In a second step, the resulting dehydroalanine and dehydro-2-aminobutyric acid residues were reacted with 2-aminooethanethiol in a Michael addition, leading to a mass addition of 77 Da. The combination of β-elimination and Michael addition leads to a net mass loss of 98 Da + 77 Da = -21 Da.
approximately 21 Da (+ 98 Da − 77 Da = + 21 Da) per serine/threonine phosphorylation site.

4.5.2 Purification of hyperphosphorylated GST-p120ctn from mammalian cells for IMAC/MS analysis

Although MS is a sensitive peptide detection method, the preceding IMAC step requires considerable amounts of purified protein as starting materials. The protein to be analysed should also be phosphorylated with high stoichiometry. Therefore a strategy had to be devised for the production of sufficient amounts of hyperphosphorylated p120ctn. In vitro phosphorylation of bacterial produced p120ctn was not possible since the kinase(s) phosphorylating p120ctn are unknown. In order to generate sufficient amounts of hyperphosphorylated p120ctn, p120ctn was produced as a GST-p120ctn fusion protein in calyculin A-treated mammalian cells. Expression in mammalian cells allowed serine/threonine phosphorylation of the fusion protein to occur, calyculin A-treatment guaranteed high phosphorylation stoichiometry and the GST-tag enabled efficient purification of the fusion protein. To generate a high expression construct, p120ctn cDNA was cloned into the pEF-Bos-GST expression vector (kindly provided by Dr Giles Cory). The pEF-Bos vector contains part of the elongation factor 1 (EF1) promoter and allows very high protein expression levels in mammalian cells (Mizushima and Nagata, 1990). A large number (2x10⁷) of Cos-7 cells were transfected with the pEF-Bos-GST-p120ctn plasmid by electroporation. After expression for 24 h the cells were incubated with 100 nM calyculin A for 30 min and subsequently lysed in an IMAC/MS-compatible buffer (containing 1% CHAPS). To demonstrate the effectiveness of calyculin A, lysates of untreated control cells and calyculin A-treated cells were subjected to SDS-PAGE followed by western blotting for p120ctn. The decrease in the electrophoretic mobility of p120ctn following calyculin A treatment indicates hyperphosphorylation (Figure 4.12).

GST-p120ctn was purified on glutathione-sepharose beads. Separation of the isolated proteins by SDS-PAGE followed by silver staining showed the purity of the preparation (Figure 4.13). In addition to GST-p120ctn, only two bands were prominent on the gel. These bands probably represent different isoforms of endogenous GST precipitated by the glutathione-sepharose beads and do not present a contamination problem since the fusion protein itself contains a GST moiety. Comparing the intensity of the GST-p120ctn band to that of defined amounts of a control protein (albumin), the amount of purified GST-
Figure 4.12  Hyperphosphorylation of GST-p120ctn in the presence of calyculin A

Cos-7 cells were electroporated with plasmid pEF-Bos-GST-p120ctn. After 24 h cells were treated with calyculin A (100 nM) or vehicle for 30 min. Cells were subsequently lysed and samples containing equal amounts of protein were subjected to SDS-PAGE (6% SDS-polyacrylamide gel). p120ctn was detected by western blotting.
Cos-7 cells ($2 \times 10^7$ cells) were transfected with pEF-Bos or pEF-Bos-GST-p120ctn plasmids by electroporation. After 24 h, cells were incubated with calyculin A (100 nM) for 30 min and then lysed in 1% CHAPS lysis buffer. GST and GST-p120ctn were purified from the cell lysates with glutathione-sepharose beads. Beads were washed 3 times with lysis buffer and twice with a high salt buffer (0.5 M LiCl) to remove contaminating proteins. Beads were boiled in sample buffer for protein analysis by SDS-PAGE or equilibrated in 1 mM Tris/HCl pH 8.0 and stored at $-40$ °C for IMAC/MS analysis.

Figure 4.13  Purification of GST-p120ctn from Cos-7 cells
p120ctn fusion protein was estimated to be at least 0.5 μg, corresponding to about 4 picomoles for the ~130-kDa protein. This amount is well above the minimum sample amount (low femtomole range) required for the IMAC/MS method applied (Thompson et al., 2003).

Because of the purity of the protein preparation, the tryptic digest of GST-p120ctn was performed directly on the sepharose beads (“on-resin”) rather than on fusion protein extracted from the SDS-polyacrylamide gel. Digestion on beads increases the yield of tryptic peptides compared to in-gel digestion and minimizes the loss of post-translational modifications (phosphorylation) but is only suitable when there are low levels of contaminating proteins.
4.5.3 IMAC/MS analysis of tryptic p120ctn peptides

In a preliminary attempt to estimate the total number of phosphopeptides generated by a tryptic digest of p120ctn, a 2D thin layer chromatography (TLC) phosphopeptide-mapping (PPM) experiment of tryptic p120ctn peptides was performed. Approximately 20 phosphopeptides could be separated by PPM (Figure 4.14). In comparison, the IMAC beads retained 19 potential phosphopeptides. The number of peptides retained by the IMAC resin and the number of phosphopeptides separated in the PPM experiment therefore corresponded well. This suggests that the IMAC resin retained only few, if any, non-phosphorylated peptides. β-elimination in combination with Michael addition confirmed the presence of single, double or triple phosphoserine residues within 10 of the 19 tryptic peptides. For example, MS analysis of the tryptic peptides yielded a peak at 1777.84 Da, a mass corresponding to a peptide carrying one phosphate group within amino acids 216-233 (HYEDGYGGSDNYGSLR) (Figure 4.15 A). After β-elimination/Michael addition, the peak was reduced to 1756.92 Da (Figure 4.15 B). The characteristic drop by approximately 21 Da confirmed the presence of a phosphorylated residue within the original peptide. When there were only single serine or threonine residues present within the peptide, or when the total number of phosphorylated residues equalled the sum of serine and threonine residues, the sites of phosphorylation were unequivocally identified with the method described above. For phosphopeptides containing a higher number of serine and threonine residues than phosphorylation sites, the site of phosphorylation was determined by Dr Andrew Thompson by peptide sequencing using matrix-assisted laser desorption/ionization mass spectrometry (MALDI)(Thompson et al., 2003). In total, eight novel phosphorylation sites, seven phosphoserines and one phosphothreonine, were identified (Figure 4.16). The exact site of phosphorylation could not be determined for 5 tryptic peptides that were retained by the IMAC resin and were confirmed to contain phosphoserines or -threonines by β-elimination/Michael addition. This was because the peptides contained a higher number of serine/threonine residues than phosphorylation sites and the peptide amounts were too low to allow peptide sequencing by MALDI.
Cos-7 cells were electroporated with plasmid p120ctn-VSV and grown in 10 cm dishes for 24 h. Cells were subsequently grown in phosphate-free DMEM for 8 h. For metabolic labelling, the medium was replaced with 1.5 ml phosphate-free DMEM supplemented with 0.5 mCi $[^{32}P]$ orthophosphate. After labelling for 8 h, cells were lysed in NP-40 lysis buffer and an anti-VSV immunoprecipitation was performed. The immunocomplexes were subjected to several high salt washes to remove coprecipitating proteins and finally separated on 10%-SDS-polyacrylamide gels. Labelled p120ctn-VSV was extracted from the gel and digested with trypsin. Tryptic peptides were separated on a TLC plate in the first dimension according to their charge and then based on their solvent solubility in the second dimension. (A) The TLC plate was exposed to X-ray film for 10 days. (B) The same image as in (A) but with circles indicating which spots of the 2D peptide map were judged to represent discreet phosphopeptides. In total, approximately 20 phosphopeptides could be distinguished.
Figure 4.15 Confirmation of the phosphorylation state of IMAC-isolated peptides using β-elimination/Michael addition by mass spectrometry

MALDI analysis of an IMAC-isolated peptide before (A) and after β-elimination/Michael addition (B). Peptides that contained phosphoserines or -threonines show a characteristic net mass loss of 21 Da (-98 Da + 77 Da = -21 Da) after the β-elimination/Michael addition step. This mass addition provides unequivocal proof for the presence of a phosphoserine or -threonine residue in the original peptide. The peptide in the example corresponds to amino acids 216 to 233 of murine p120ctn isoform 1A. In the mass spectra the abscissas show the peptide mass m over the peptide charge z (m/z). The depicted peaks correspond to peptides carrying a single positive charge (z = 1) as the result of the ionisation process during MALDI analysis. Therefore the numerical value of the position of the peptide peak on the abscissa equals the peptide mass in Da. This figure was generated by Dr Andrew Thompson (LICR-UCL).
Figure 4.16 IMAC/MS analysis identified eight novel phosphorylation sites in p120ctn

(A) MALDI analysis of IMAC purified peptides after β-elimination/Michael addition confirmed the presence of phosphoserine or -threonine residues in 10 tryptic peptides. For two peptides, peptide sequencing identified the position of the phosphorylated residue (S230 and S252). When the number of serine and threonine residues in a peptide equaled the number of phosphorylated residues, the phosphorylation sites could be directly assigned (S268/S269, S346/S349/S352). Peptides containing phosphoserines and/or threonines in green, potential serine/threonine phosphorylation sites in blue and identified phosphorylation sites in red. (B) The positions of the identified phosphorylation sites in murine p120ctn isoform 1A. The green bars indicate the positions of the phosphopeptides containing phosphorylation sites that could not be identified. CC, coiled-coiled domain, PY, tyrosine phosphorylation domain.
4.6 Preliminary characterization of the novel phosphorylation sites

4.6.1 Electrophoretic mobility of p120ctn proteins carrying serine→alanine mutations at the phosphorylation sites

p120ctn becomes serine/threonine phosphorylated upon binding to E-cadherin or in the presence of serine/threonine phosphatase inhibitors, and these phosphorylation events coincide with a decrease in its electrophoretic mobility (Ratcliffe et al., 1997; Thoreson et al., 2000). To investigate whether any of the newly identified serine/threonine phosphorylation sites contribute to this change in electrophoretic mobility, constructs carrying serine/threonine to alanine mutations at the respective residues were expressed in Cos-7 cells. After expression for 24 h, serine/threonine phosphorylation was increased by calyculin A treatment. Under these conditions, mutation of a serine/threonine residue should prevent the change in electrophoretic mobility, if phosphorylation at this site normally causes the upward shift of p120ctn on SDS-PAGE. However, all serine/threonine→alanine mutants were shifted up to similar degree as wild-type p120ctn on SDS-polyacrylamide gels (Figure 4.17), suggesting that shift-inducing phosphorylation sites were not identified in the IMAC/MS analysis. Alternatively, several phosphorylation sites could concertedly contribute to the electrophoretic shift of p120ctn and mutation of only single sites may not noticeably affect p120ctn migration in gels.

4.6.2 Activating or inactivating mutations of the newly identified phosphorylation sites do not affect known p120ctn functions

To investigate whether the identified phosphorylation sites are involved in regulating known p120ctn functions, such as E-cadherin-binding, induction of the branching phenotype or nuclear translocation, nonphosphoserine/threonine-mimicking alanine and phosphoserine/threonine-mimicking aspartate mutants of the respective phosphorylation sites were expressed in epithelial cells or fibroblasts and the intracellular localization of p120ctn was analysed. However, none of the mutant proteins showed any differences in their localization from wild-type p120ctn: all mutant proteins localized to the plasma membrane at low expression levels in MDCK cells, suggesting that the ability to bind to E-cadherin was not affected by the mutations. Likewise, at higher expression levels in MDCK cells or when expressed in fibroblasts, mutant proteins showed no clear changes in the amount of p120ctn translocating to the nucleus. Finally, all mutants were able to
Figure 4.17 Serine/threonine→alanine mutations of the novel phosphorylation sites do not block the calyculin A-induced decrease in electrophoretic mobility

Cos-7 cells were electroporated with constructs S230A-p120ctn-VSV (S230A), S252A-p120ctn-VSV (S252A), SS268/269AA-p120ctn-VSV (SS268/269AA), SSS346/349/352AAA-p120ctn-VSV (SSS346/349/352AAA), T889A-p120ctn-VSV (T889A) or wild-type p120ctn-VSV (wt). After expression for 24 h, cells were treated with calyculin A (100 nM) for 30 min. Cells were lysed in NP-40 lysis buffer and samples containing equal amounts of protein separated on 6% SDS-polyacrylamide gels. After western blotting, membranes were probed for p120ctn.
induce the branching phenotype to a similar degree as wild-type p120ctn. For example, p120ctn containing an inactivating alanine or an activating aspartate residue at the position of the phosphorylation site serine 230 showed localization patterns in the above scenarios that were indistinguishable from wild-type p120ctn (Figure 4.18). The novel phosphorylation sites therefore appear to be involved in hitherto unknown functions of p120ctn. Alternatively, if phosphorylation sites act additively or redundantly to regulate p120ctn function, mutation of several sites would be necessary to detect changes p120ctn function.
Mutation of phosphorylation site serine 230 to alanine or aspartate does not change known functions of p120ctn.

Cos-7 cells or MDCK cells were transfected with S230A-p120ctn-VSV (230A) or S230D-p120ctn-VSV (230D). After expression for 24 h, p120ctn was visualized by staining for the VSV epitope. Both mutants induced the branching phenotype in Cos-7 cells similar to wild-type p120ctn. In MDCK cells, both mutants localized to adherens junctions at low expression levels, whereas they associated with microtubules at high expression levels. p120ctn molecules carrying analogous mutations of the other phosphorylation sites showed an identical pattern of localization. Bar, 10 μm.
4.7 Discussion

p120ctn is phosphorylated on multiple tyrosine (Mariner et al., 2001) and serine/threonine residues (Aono et al., 1999; Downing and Reynolds, 1991; Ratcliffe et al., 1997; Ratcliffe et al., 1999; Wong et al., 2000). Tyrosine phosphorylation of p120ctn has been shown to mediate the binding of the tyrosine phosphatase SHP-1 to p120ctn (Keilhack et al., 2001; Mariner et al., 2001) and to increase the affinity of p120ctn for E-cadherin in vitro (Roura et al., 1999). p120ctn becomes phosphorylated on serine/threonine residues upon recruitment to the plasma membrane by E-cadherin (Thoreson et al., 2000) and serine/threonine phosphorylation within the N-terminus of p120ctn has been suggested to negatively regulate E-cadherin-mediated adhesion (Aono et al., 1999; Ohkubo and Ozawa, 1999) but the exact function of the E-cadherin-dependent serine/threonine phosphorylation of p120ctn is unknown.

The serine/threonine kinase(s) phosphorylating p120ctn are unknown. Since p120ctn is highly phosphorylated when it is recruited to the plasma membrane, but hypophosphorylated when it is localized in the cytoplasm, the p120ctn kinase(s) appear to be membrane-associated. Alternatively, the kinase(s) could be distributed uniformly throughout the cell and p120ctn is kept in a dephosphorylated state by phosphatases excluded from membrane-proximal areas.

Interestingly, during junctional disassembly under low extracellular calcium conditions ("calcium switch"), a large fraction of p120ctn was released from the plasma membrane, but stayed in a complex with E-cadherin and remained serine/threonine phosphorylated. This suggests that E-cadherin-binding rather than association with the plasma membrane is required for p120ctn phosphorylation. Alternatively, the E-cadherin/p120ctn complex could be endocytosed together with a membrane-associated kinase that could maintain the phosphorylation state of p120ctn in endocytotic vesicles. In contrast, during HGF-induced junctional disassembly, p120ctn is released into the cytoplasm and later translocates to the nucleus (see Section 5.2.1), suggesting the disassembly of the E-cadherin complex.

To investigate p120ctn serine/threonine phosphorylation, two protein kinase inhibitors, staurosporine and H-89, were used. Treatment of epithelial cells with the broad-spectrum kinase inhibitor staurosporine decreases p120ctn serine/threonine phosphorylation (Aono et al., 1999; Ratcliffe et al., 1997; Ratcliffe et al., 1999) but does not completely abolish it. The fact that p120ctn still localized to sites of cell-cell contact in staurosporine-treated
epithelial cells suggests that phosphorylation of the staurosporine-sensitive sites is not required for p120ctn to bind to E-cadherin. However, intercellular junctions appeared ragged and discontinuous in staurosporine-treated cells. p120ctn has been suggested to control the clustering of E-cadherin molecules into larger complexes (Anastasiadis and Reynolds, 2000) and serine/threonine phosphorylation of p120ctn may regulate this process (Aono et al., 1999; Ohkubo and Ozawa, 1999). p120ctn is therefore a good candidate for mediating the effect of staurosporine on the morphology of cadherin-containing junctions. However, since staurosporine inhibits a range of kinases (Ruegg and Burgess, 1989), staurosporine treatment is likely to affect the phosphorylation state of other proteins regulating junctional morphology. Once it has been established which phosphorylation sites in p120ctn are staurosporine-sensitive, it will be interesting to test whether the expression of mutant p120ctn proteins carrying inactivating alanine or activating aspartate residues at these phosphorylation sites affects the morphology of E-cadherin-containing junctions.

Treatment of MDCK cells with the PKA inhibitor H-89 led to dephosphorylation of p120ctn and to an increase in its electrophoretic mobility in SDS-polyacrylamide gels, but the pattern of changes induced by H-89 was clearly different from staurosporine. Similarly, staurosporine- and H-89-treatment changed the array of p120ctn isoforms on 2D-gels in different patterns. Each inhibitor therefore appears to target different subsets of phosphorylation sites. This also suggests that there are at least two different serine/threonine kinases phosphorylating p120ctn in MDCK cells.

In MDCK cells, H-89 treatment caused a fraction of p120ctn (and other catenin proteins) to accumulate in the pericentrosomal region, but not directly at the centrosomes. PKA is an important regulator of protein trafficking through the Golgi apparatus (Pimplikar and Simons, 1994), and PKA inhibition by H-89 blocks protein transport through the Golgi apparatus (Muniz et al., 1996). The Golgi apparatus localizes to the pericentrosomal region (Rios and Bornens, 2003) and the pericentrosomal localization of p120ctn could therefore indicate generally impaired protein transport through the Golgi apparatus as the result of PKA inhibition rather than a specific role of p120ctn dephosphorylated on H-89-sensitive phosphorylation sites.

p120ctn is phosphorylated on multiple serine and threonine residues, as revealed by tryptic phosphopeptide mapping and IMAC/MS analysis. Two-dimensional mapping of tryptic p120ctn peptides separated at least 20 different phosphopeptides. On two-
dimensional maps of peptides containing several phosphorylation sites, the number of discreet peptide spots may exceed the number of phosphorylation sites, depending on the stoichiometry of phosphorylation of the individual sites. The total number of serine/threonine phosphorylation sites may therefore be lower than 20. Nevertheless, the peptide map demonstrates the presence of a large number of serine/threonine phosphorylation sites.

IMAC/MS analysis (performed by Dr Andrew Thompson, LICR-UCL), demonstrated the presence of at least 13 phosphorylated serine/threonine residues in p120ctn. The exact position of eight of these phosphorylated residues could be determined. Although artificially hyperphosphorylated GST-p120ctn, purified from calyculin A-treated cells, was used for the identification of the phosphorylation sites, the phosphorylation events can be considered physiologically relevant. In experiments using overexpression of kinases or \textit{in vitro} phosphorylation to obtain hyperphosphorylated proteins, residues may become phosphorylated which are not efficiently targeted by the kinase under physiological conditions. In contrast, incubation with the phosphatase inhibitor calyculin A only prevented the removal of phosphates from p120ctn that were added under physiological conditions. On the other hand, sites not affected by calyculin A or sites phosphorylated only in a specific cellular context, for instance during cell division, may be missed with this approach.

The majority of the serine/threonine phosphorylation sites map to the N-terminus of p120ctn. At least 6 serine/threonine phosphorylation sites are localized in or near the tyrosine phosphorylation domain between residues 228 and 302 (Mariner et al., 2001). This domain therefore appears to be a serine/threonine as well as tyrosine phosphorylation domain. Simultaneous phosphorylation of these serine/threonine residues and of the six Src-targeted tyrosine residues in this domain (Mariner et al., 2001) would create a highly negatively charged region in p120ctn which could have profound effects on the conformation of p120ctn. The proximity of the serine/threonine and tyrosine phosphorylation sites also suggests that the phosphorylation domain may integrate signalling pathways involving both tyrosine and serine/threonine phosphorylation of p120ctn.

p120ctn isoforms 1, 2, 3 and 4 are generated through the use of four alternative ATG start codons and differ in the length of their N-termini (see Figure 1.1). The N-terminus of p120ctn contains two potential protein-protein interaction domains: a coiled-coiled
domain and the phosphorylation domain. The longest isoform (isoform 1) contains both the coiled-coiled domain and the phosphorylation domain. Isoforms 2 and 3 lack the coiled-coiled domain but still contain the phosphorylation domain, whereas the shortest isoform (isoform 4) lacks both the coiled-coiled and the phosphorylation domain. By expressing p120ctn isoforms lacking or containing these interaction domains, cells may regulate the number of proteins p120ctn is able to interact with. The majority of serine/threonine phosphorylation sites are located within the phosphorylation domain upstream of the fourth start codon and are therefore missing in p120ctn isoform 4. At least two serine/threonine phosphorylation sites reside within the coiled-coiled domain and are therefore only present in p120ctn isoform 1. Thus, differential expression of isoforms also changes the number of serine/threonine phosphorylation sites present in the p120ctn molecule. A cluster of three serine phosphorylation sites (serines 346, 349 and 352), however, as well as two serine/threonine sites within the C-terminus, are downstream of the fourth ATG start site and therefore present in all p120ctn isoforms.

As mentioned, at least two serine/threonine phosphorylation sites are situated within the coiled-coil domain of p120ctn. Serine phosphorylation has been shown to modulate coiled-coil assembly (Szilak et al., 1997) and has been suggested to regulate coiled-coil domain-mediated protein-protein interactions (Burkhard et al., 2001; Liang et al., 1999). Serine/threonine phosphorylation could therefore be a mechanism to regulate coiled-coil-mediated interactions of the N-terminus of p120ctn isoform 1 with other proteins.

Replacing the serine/threonine phosphorylation sites with unphosphorylatable alanine residues did not abrogate the upward shift of p120ctn on SDS-gels following calyculin A treatment. Additional phosphorylation sites, missed in the IMAC/MS analysis, may be required for inducing the shift. Alternatively, several phosphorylation sites may have an additive effect on the electrophoretic mobility of p120ctn and preventing the phosphorylation only of individual sites may have little effect on overall p120ctn gel mobility.

It is surprising that none of the phosphorylation sites affected known functions of p120ctn, such as E-cadherin-binding, induction of the branching phenotype or nuclear translocation. p120ctn mutants carrying either inactivating alanine or activating aspartate residues at the position of the phosphorylation sites behaved identically to wild-type p120ctn, suggesting that the identified phosphorylation sites are involved in other, so far unknown functions of p120ctn. Alternatively, some p120ctn serine/threonine
phosphorylation sites may be functionally redundant. Changes in the function of proteins often require phosphorylation of several near-by residues. For instance, efficient transcriptional activation by the serum response factor (SRF) requires phosphorylation of one of its components, Elk-1, on multiple phosphorylation sites by MAP kinase (Hill et al., 1993). Because the majority of serine phosphorylation sites are clustered within a short stretch of the phosphorylation domain, it is feasible that the same kinase could efficiently phosphorylate several serines in this domain. Functional redundancy of the phosphoserines would mirror the situation of the phosphotyrosine sites in the phosphorylation domain: the tyrosine phosphorylation sites are clustered, are targeted by the same kinase (Src) and appear to be individually redundant (Mariner et al., 2001). The investigation of serine/threonine phosphorylation-dependent changes in pl20ctn function may therefore require mutation of several serine/threonine phosphorylation sites.

In conclusion, the serine/threonine phosphorylation state of pl20ctn in E-cadherin-negative and -positive cell lines was investigated using a panel of kinase and phosphatase inhibitors, by phosphopeptide mapping and by IMAC/MS analysis. The dependency of pl20ctn serine/threonine phosphorylation on E-cadherin association was confirmed. In addition to the broad-specificity kinase inhibitor staurosporine, the kinase inhibitor H-89 was found to affect pl20ctn serine/threonine phosphorylation. Although H-89 is generally used as a PKA inhibitor, pl20ctn was not found to be a PKA substrate and other H-89-sensitive kinases may phosphorylate pl20ctn. Staurosporine and H-89 changed the serine/threonine phosphorylation pattern of pl20ctn in different patterns, suggesting that they inhibit different subsets of kinases. pl20ctn is therefore the target of more than one serine/threonine kinase. Calyculin A, a class PP-1 phosphatase inhibitor, induced hyperphosphorylation of pl20ctn and was used to increase the phosphorylation state of pl20ctn to allow phosphopeptide analysis by 2D-phosphopeptide mapping and IMAC/MS. Phosphopeptide mapping revealed the presence of a large number of serine/threonine phosphorylation sites. IMAC/MS analysis demonstrated the existence of at least 13 serine/threonine phosphorylation sites, 8 of which could be identified. Mutation of individual residues did not affect known pl20ctn functions, such as E-cadherin binding, nuclear translocation or induction of the branching phenotype. The serine/threonine phosphorylation sites may therefore be involved in other pl20ctn functions, or they may function additively or redundantly.
Chapter 5

5 p120ctn and HGF

5.1 Introduction

Treatment of epithelial Madin-Darby canine kidney (MDCK) cells with hepatocyte growth factor (HGF) is a model system to investigate the mechanisms of adherens junction disassembly. HGF, also known as scatter factor, is a secreted morphogen, mitogen and motogen that plays multiple roles in embryogenesis, wound healing and is implicated in the development of cancer (Birchmeier and Gherardi, 1998; Zamegar and Michalopoulos, 1995). HGF is secreted by cells of mesenchymal origin and acts through its receptor, the Met tyrosine kinase, which is expressed by many cell types including epithelial and endothelial cells. HGF initially induces centrifugal spreading of MDCK cells in colonies and subsequently stimulates cell-cell dissociation, allowing each cell to scatter or detach from colonies and migrate independently of other cells (Ridley et al., 1995; Stoker and Perryman, 1985). HGF has been shown to induce adherens junction disassembly by a pathway that involves the activation of both p42/p44 MAPK and PI 3-kinase by Ras (Potempa and Ridley, 1998). However, the mechanisms whereby HGF promotes the loss of adhesion at the level of the junctions and subsequent cell-cell detachment are still poorly understood. HGF induces a shift of the adherens junction proteins E-cadherin and β-catenin from the NP-40-insoluble to the NP-40-soluble fraction of MDCK cell lysates (Potempa and Ridley, 1998). Detergent insolubility is indicative of association with the actin cytoskeleton and adherens junctions (Nathke et al., 1994) and therefore, it can be concluded that HGF promotes the loss of these proteins from adherens junctions. Furthermore, E-cadherin has been proposed to be removed from the plasma membrane of HGF-treated MDCK cells by co-endocytosis with the HGF receptor Met (Kamei et al., 1999).

Increased tyrosine phosphorylation of E-cadherin complex members often correlates with loss of cell-cell adhesion (Behrens et al., 1993) (Ozawa and Kemler, 1998a) and tyrosine phosphorylation has therefore been implicated as a way to modulate cadherin-mediated adhesion (Daniel and Reynolds, 1997). HGF stimulation induces tyrosine phosphorylation of E-cadherin, β-catenin and p120ctn in a variety of cell lines (Shibamoto et al., 1995; Shibamoto et al., 1994) and could in this way decrease cadherin-mediated adhesion.

p120ctn is part of the core E-cadherin complex, it is an excellent tyrosine kinase substrate and it has been implicated as a regulator of E-cadherin-mediated adhesion (Anastasiadis
and Reynolds, 2000; Aono et al., 1999; Fujita et al., 2002; Ozawa and Kemler, 1998b; Ozawa and Ohkubo, 2001). Although this makes pl20ctn a candidate for transducing HGF signalling to the E-cadherin complex, its role during HGF-induced adherens junction disassembly has not been thoroughly studied. Therefore, the behaviour of pl20ctn during HGF-induced adherens junction disassembly, in particular the effect of HGF treatment on pl20ctn protein levels, its tyrosine phosphorylation status and its intracellular localization, was investigated.

5.2 Nuclear localization of pl20ctn in response to HGF treatment

5.2.1 HGF-induced scattering of epithelial cells coincides with the release of β-catenin and pl20ctn from adherens junctions and nuclear translocation of pl20ctn

To demonstrate the suitability of the scatter assay as a model for junctional disassembly, MDCK cells were seeded at low density and allowed to grow until they formed colonies containing 20 to 50 cells (Figure 5.1 A). Within the first hour of HGF treatment, cells spread considerably but retained their cell-cell contacts. After 4 h of stimulation, cells started to detach from each other and by 16 h, cells had often completely separated and had assumed a fibroblast-like morphology. To investigate the effect of HGF treatment on the intracellular localization of pl20ctn, cells were fixed at different time points and pl20ctn visualized by staining with the ppl20 antibody (Figure 5.1 B). In untreated cells, pl20ctn localized almost exclusively to the plasma membrane at sites of cell-cell contact. After 1 h of HGF treatment, junctional pl20ctn staining was reduced. Surprisingly, a fraction of pl20ctn showed clear nuclear localization. This nuclear localization was still visible after 4 h of stimulation, whereas junctional localization of pl20ctn was further reduced at this point. After 1 or 4 h of stimulation, there was only low cytoplasmic pl20ctn staining but in some cells there appeared to be some perinuclear staining. However, careful analysis of a series of confocal slices taken at different depths into the cells revealed that a proportion of pl20ctn released from junctions had redistributed across the apical plasma membrane. In well-spread, poorly polarized cells the membrane region above the nucleus protruded by several μm from the surrounding apical membrane. Confocal images collected at the position of the nucleus consequently also sliced through areas of the apical membrane in the vicinity of the nucleus (Figure 5.2). The "perinuclear" staining is therefore in fact created by plasma membrane-associated pl20ctn and not due to pl20ctn localizing to perinuclear organelles, such as the
endoplasmic reticulum or the Golgi apparatus. After 16 h the loss of cell-cell contacts coincided with a nearly complete removal of p120ctn from cell-cell interactions. The majority of p120ctn appeared to be distributed diffusely throughout the cytoplasm and the nuclear or perinuclear localization was not as pronounced as at earlier time points. The loss of β-catenin from sites of cell-cell contacts followed a similar time course (Figure 5.1 C). However, although there was some cytoplasmic and perinuclear (Golgi-associated) β-catenin staining after 4 h of HGF treatment, β-catenin did not translocate into the nucleus. It therefore appears that both p120ctn and β-catenin are lost from sites of cell-cell contact during HGF treatment, but that only p120ctn transiently localizes to the nucleus.

The prostate cancer cell line DU145 responds to HGF stimulation with a scattering response similar to MDCK cells (Miura et al., 2001). It was therefore investigated whether p120ctn would also translocate to the nucleus in these cells in response to HGF treatment. Similar to MDCK cells, p120ctn accumulated in the nucleus of DU145 cells between 1 and 4 h after HGF stimulation, whereas nuclear localization of p120ctn was less obvious after 16 h of stimulation (Figure 5.3). This demonstrates that HGF-induced nuclear accumulation of p120ctn is an effect not restricted to MDCK cells but could be a general phenomenon occurring in epithelial or epithelially-derived cells.

Nuclear accumulation of p120ctn was even more pronounced in HGF-stimulated cells expressing p120ctn-GFP (Figure 5.4). In unstimulated cells expressing low or moderate levels of the exogenous protein, p120ctn-GFP localized almost exclusively to sites of cell-cell contact. Upon stimulation with HGF for 1 h, most of the overexpressed protein localized to the nucleus with only low amounts remaining at the plasma membrane. At high expression levels, p120ctn-GFP was frequently found in the nuclei of unstimulated and HGF-treated cells. These cells, however, also showed high levels of cytoplasmic p120ctn-GFP. Only cells that displayed low cytoplasmic, but high nuclear levels of p120ctn-GFP were considered to show nuclear translocation specifically in response to HGF stimulation.

To quantitate the increase in nuclear p120ctn in response to HGF, a nuclear fractionation experiment was performed. Analysis of the nuclear extracts generated from untreated and HGF-treated cells by western blotting showed an increase in nuclear p120ctn after 4 h of HGF stimulation (Figure 5.5). After 16 h of stimulation, the amount of p120ctn in the nuclear fraction had dropped to levels comparable to unstimulated cells, underlining the
transient nature of the HGF-induced nuclear translocation of p120ctn. Reprobing of the blot for E-cadherin showed small amounts of E-cadherin in the nuclear fractions, indicating a low level contamination of the nuclear fractions with membrane proteins. When cells are only incompletely lysed, parts of the plasma membrane may sediment with the nuclei and contaminate the nuclear fraction. In addition, the endoplasmic reticulum (ER) and the nuclear envelope form a continuous membranous system. When the nuclei are collected, parts of the ER may cosediment and proteins being trafficked through the ER may provide an additional source of contamination. However, in contrast to p120ctn, E-cadherin levels in the nuclear fractions remained constant. Therefore, the increase of p120ctn in the nuclear fraction after 4 h of HGF treatment cannot be attributed to an increased amount of contaminating extranuclear proteins in this fraction but reflects a specific enrichment of p120ctn.
Figure 5.1  β-catenin and p120ctn are lost from adherens junctions during HGF-induced scattering, but only p120ctn translocates into the nucleus

A: MDCK cells were seeded at low density and allowed to grow until they had formed colonies containing 20 to 50 cells. Cells were left untreated or stimulated with 10 ng/ml HGF for 1, 4 or 16 h and ultimately fixed. Phase contrast images were collected using a cooled CCD camera mounted on an axiophot microscope and a x40 objective (Zeiss).  

B and C: MDCK cells treated as in A were stained with antibodies to p120ctn (B) or β-catenin (C) and confocal images were collected at the plane of adherens junctions. Bars 50 μm.
Figure 5.2 Pseudo-perinuclear p120ctn staining at the intersection of the plasma membrane with the confocal plane

Schematic representation depicting the origin of the apparently perinuclear p120ctn staining in HGF-treated MDCK cells. A subpopulation of p120ctn released from adherens junctions in response to HGF treatment redistributes over the apical membrane and creates a pseudo-perinuclear staining where the apical membrane crosses the confocal plane. The green colour indicates p120ctn staining. A: Side view. B: Top view.
DU-145 cells were seeded at low density and allowed to grow until they had formed colonies containing 20 to 50 cells. Subsequently, cells were left untreated or stimulated with 10 ng/ml HGF for 1, 4 or 16 h, fixed and then stained with antibodies to p120ctn and TRITC-phalloidin to visualize filamentous actin. Confocal images were collected at the plane of the maximum diameter of the nuclei. Bars μm.
MDCK cells were grown until they formed colonies containing approximately 50 cells and then transfected with the p120ctn-GFP construct using Fugene 6 as the transfection reagent. After expression for 24 h, cells were stimulated with 10 ng/ml HGF for 4 h and then fixed. p120ctn-GFP was visualized by direct fluorescence. Images were collected from cells expressing low or moderate amounts of exogenous protein, indicated by low cytoplasmic p120ctn-GFP levels. Bar 5 μm.
Figure 5.5 p120ctn transiently increases in the nuclear fractionation of HGF-treated MDCK cells

A: MDCK cells were seeded at 5 x 10^5 cells per 15 cm dish and grown for 3 days. By that time, cells had reached approximately 40% confluency. Cells were then stimulated with 10 ng/ml HGF for 4 or 16 h or left untreated. Cells were then harvested by trypsinization and nuclear fractions were prepared by swelling the cells in a hypotonic buffer, followed by mechanical disruption of plasma membranes by passing the cell suspension repeatedly through a narrow gauge needle. After spinning out remaining intact cells at 100 g (whole cell fraction), the nuclei were collected by centrifugation at 12000 g. The supernatant was collected as the postnuclear fraction. The pelleted nuclei were resuspended in a high salt buffer, followed by spinning out of nuclear debris. The supernatant was dialysed against a lower salt buffer to yield the nuclear fraction. Protein concentration of all fractions were determined using the Bradford protein assay and samples containing equal amounts of protein were separated by SDS-PAGE and analysed by western blotting for p120ctn (L = whole cell fraction, PN = postnuclear fraction, N = nuclear fraction). As a control for the purity of the nuclear fractions, the blot was reprobed for E-cadherin. B: Densitometric quantitation of the relative amount of p120ctn in the nuclear fractions. The amounts of p120ctn in the nuclear fractions are indicated in arbitrary units with the amount in the nuclear fraction of unstimulated cells set to 1. Average results of two independent experiments are shown.
5.2.2 Nuclear export of p120ctn does not counteract HGF-induced nuclear import

In addition to the HGF-induced nuclear translocation of p120ctn described in the previous section, nuclear localization of p120ctn can be frequently observed in fibroblasts or in E-cadherin-negative carcinoma cancer cell lines (van Hengel et al., 1999). Because proteins larger than 60 kDa are not able to enter or leave the nucleus by passive diffusion (Gorlich and Kutay, 1999), there must be a specific nuclear transport mechanism to enable p120ctn proteins, whose masses range between approximately 100 and 120 kDa, to shuttle in and out of the nucleus. The nuclear export of p120ctn isoforms containing exon B has been shown to be inhibited by Leptomycin B (LMB) (van Hengel et al., 1999), a highly specific inhibitor of exportin/Crm1-dependent nuclear export (Kudo et al., 1998). The exportin/Crm1 machinery facilitates nuclear export of proteins containing a leucine-rich nuclear export signal (NES) (Ossareh-Nazari et al., 2001), and p120ctn exon B encodes such a NES (van Hengel et al., 1999).

Treatment of MDCK cells with LMB for 4 h caused a strong accumulation of p120ctn in the nucleus (Figure 5.6), suggesting that exon B is included in the majority of p120ctn isoforms expressed in these cells and that this exon mediates the nuclear export of these isoforms. For these isoforms to accumulate in the nucleus, they have to shuttle continuously between the nucleus and the cytoplasm. A substantial fraction of p120ctn, however, remained associated with the plasma membrane at sites of cell-cell contact, presumably by binding E-cadherin. In contrast to MDCK cells, LMB treatment of E-cadherin-negative MDA-MB-231 cells caused near complete localization of p120ctn into the nucleus (Figure 5.6). Simultaneous HGF and LMB treatment of MDCK cells did not increase the amount of p120ctn accumulating in the nucleus compared to cells treated with HGF alone (Figure 5.6), suggesting that exportin/Crm1-dependent nuclear export does not counteract HGF-induced nuclear import.
Figure 5.6 Nuclear export of p120ctn is leptomycin B-dependent

MDCK cells (upper panel) or MDA-MB-231 cells (lower panel) were grown until they reached approximately 50% confluency and then incubated with 5 ng/ml leptomycin B (LMB), 5 ng/ml leptomycin B and 10 ng/ml HGF (LMB + HGF) or left untreated (con). p120ctn was visualized by immunofluorescence staining. F-actin was stained using TRITC-phalloidin. Images were collected at the plane of the cell nuclei using a confocal microscope. Bars 20 μm.
5.2.3 Effect of HGF treatment on tyrosine phosphorylation and protein levels of p120ctn in MDCK cells

In MDCK cells transformed with the oncogene v-Src, several members of the E-cadherin complex, including E-cadherin, β-catenin (Behrens et al., 1993) and p120ctn (Reynolds et al., 1994), become extensively tyrosine phosphorylated, and this increase in tyrosine phosphorylation coincides with the disruption of cadherin junctions (Behrens et al., 1993). Likewise, incubation of MDCK cells with pervanadate, which inhibits tyrosine phosphatases (Hecht and Zick, 1992), increases tyrosine phosphorylation of E-cadherin, β-catenin and plakoglobin/γ-catenin and causes loss of E-cadherin-mediated adhesion (Ozawa and Kemler, 1998a). Consequently, tyrosine phosphorylation of E-cadherin complex proteins has been generally suggested to lead to weakening or disassembly of adherens junctions (Daniel and Reynolds, 1997).

The relevance of p120ctn tyrosine phosphorylation for the function of the E-cadherin complex is still unclear. Tyrosine phosphorylation of p120ctn increases its affinity for E-cadherin (Roura et al., 1999), whereas phosphorylation of p120ctn tyrosine 217 has been suggested to reduce E-cadherin adhesion activity in v-Src-transfected L-cells (Ozawa and Ohkubo, 2001). HGF-induced junctional disassembly coincides with the induction of tyrosine phosphorylation of p120ctn in the carcinoma cell line HT29 (Shibamoto et al., 1995), indicating that HGF may affect E-cadherin adhesion by inducing p120ctn tyrosine phosphorylation.

The effect of HGF stimulation on p120ctn tyrosine phosphorylation in MDCK cells was investigated. p120ctn was immunoprecipitated from untreated and HGF-stimulated MDCK cells and separated by SDS-PAGE. The tyrosine phosphorylation state of p120ctn was analysed by western blotting using an anti-phosphotyrosine antibody. Contrary to reports showing that p120ctn is virtually unphosphorylated on tyrosine residues in MDCK cells (Reynolds et al., 1994), p120ctn showed clear basal tyrosine phosphorylation in the MDCK cell strain used (Figure 5.7). Tyrosine phosphorylation of p120ctn was slightly increased between 20 min and 4 h after HGF stimulation, but had returned to basal levels by 16 h of stimulation. The increase in tyrosine phosphorylation occurred in a period during which adherens junctions are dynamically rearranged (Figure 5.1). During the time course of HGF treatment, the protein levels of p120ctn remained constant. HGF therefore does not appear to promote the loss of p120ctn from adherens junctions by inducing degradation of p120ctn.
Figure 5.7  HGF treatment slightly increases tyrosine phosphorylation of p120ctn in MDCK cells

MDCK cells were grown in 10cm tissue culture dishes until they had reached 40% confluency. Cells were then stimulated with 10 ng/ml HGF for the indicated times or left untreated. After cell lysis in NP-40 lysis buffer, p120ctn was precipitated using the pp120ctn antibody. Immunocomplexes were separated on a 6%-polyacrylamide gel and then transferred to PVDF membrane. Blots were blocked in 5% BSA and then probed for phosphotyrosines using the PY99 anti-phosphotyrosine antibody. Blots were subsequently stripped and reprobed for p120ctn using the pp120ctn antibody.
Section 5.3 Discussion

p120ctn localizes to the nucleus in fibroblasts and a number of E-cadherin-deficient carcinoma cell lines (van Hengel et al., 1999). Besides p120ctn, nuclear localization has been described for a number of ARM repeat proteins, such as β-catenin/armadillo (Ben-Ze'ev and Geiger, 1998; Henderson and Fagotto, 2002), plakoglobin/γ-catenin (Simcha et al., 1998), APC (Bienz, 2002) and the p120ctn family members ARVCF (Mariner et al., 2000), p0071 (Hatzfeld et al., 2003) and plakophilin 1 (Schmidt et al., 1997), 2 (Klymkowsky, 1999; Mertens et al., 1996) and 3 (Bonne et al., 1999). Nuclear localization could thus emerge as a general feature of ARM repeat proteins. Interestingly, proteins of the karyopherin family of nuclear import and export receptors, which include α- and β-importin and exportin/Crm1, are ARM repeat protein themselves (Chook and Blobel, 2001), further indicating a functional link between ARM repeat structures and nuclear translocation.

Little is known about the mechanisms that regulate cytoplasmic versus nuclear localization of p120ctn family proteins. In p120ctn, exon B encodes a leucine-rich NES and isoforms containing exon B are thought to be actively exported from the nucleus (van Hengel et al., 1999). Nuclear export of p120ctn is LMB-sensitive and therefore exportin/Crm1-dependent (Kudo et al., 1998; van Hengel et al., 1999). In addition, proteins of the p120ctn family contain a basic motif inserted into ARM repeat 6 (Anastasiadis and Reynolds, 2000) which has been suggested to function as a NLS (Lu et al., 2002). In MDCK and Cos-7 cells, p120ctn mutants missing the basic motif clearly showed decreased nuclear localization (Figure 3.19), supporting the idea that the basic motif is involved in mediating the nuclear import of p120ctn. However, other reports map the sequences responsible for nuclear import of p120ctn (Aho et al., 2002) and ARVCF (Mariner et al., 2000) to their N-termini, suggesting that this basic motif is not required for their nuclear translocation.

Whatever the mechanism of nuclear transport, nuclear localization of p120ctn has so far only been described to occur in E-cadherin-negative cells (van Hengel et al., 1999), suggesting that p120ctn is only able to enter the nucleus when it is not sequestered at the plasma membrane by E-cadherin. The finding that endogenous p120ctn transiently translocates to the nucleus in HGF-treated MDCK and DU-145 cells is the first example of a physiological stimulus inducing p120ctn nuclear translocation and the first observation of nuclear localization of p120ctn in an E-cadherin-positive cell line.
However, the loss of E-cadherin from the plasma membrane may still be required for nuclear translocation of p120ctn in response to HGF: The loss of p120ctn from adherens junctions (Figure 5.1) coincides with the reported removal of E-cadherin from the plasma membrane in HGF-treated MDCK cells by co-endocytosis with the HGF receptor Met (Kamei et al., 1999). Removal of E-cadherin from the plasma membrane eliminates the binding sites for p120ctn at the plasma membrane. As a consequence, p120ctn may be released into the cytoplasm, enabling it to be transported into the nucleus. In HGF-treated cells, p120ctn continued to localize to the plasma membrane at sites where cell-cell adhesion, most likely E-cadherin-mediated, had been maintained (Figure 5.1), supporting the idea that loss of E-cadherin from the plasma membrane is a prerequisite for p120ctn nuclear translocation.

HGF induces tyrosine phosphorylation of p120ctn in the carcinoma cell line HT29 (Shibamoto et al., 1994). Because tyrosine phosphorylation of E-cadherin complex members has been suggested to promote loss of cadherin-mediated adhesion (Behrens et al., 1993; Daniel and Reynolds, 1997; Ozawa and Kemler, 1998a), HGF may promote adherens junction disassembly by phosphorylating p120ctn. HGF treatment slightly increased p120ctn tyrosine phosphorylation between 20 min and 4 h of stimulation. The increase in tyrosine phosphorylation therefore occurred during a phase marked by dynamic junctional rearrangement, suggesting that tyrosine phosphorylation correlates with weakening of adherens junctions. However, p120ctn showed high levels of basal tyrosine phosphorylation in MDCK cells used, demonstrating that tyrosine phosphorylated p120ctn is able to be part of functional E-cadherin complexes.

Treatment of MDCK cells with LMB caused p120ctn to accumulate in the nucleus, demonstrating that the nuclear export of p120ctn is exportin/Crm1-dependent. It also shows that there is continuous shuttling of p120ctn between the plasma membrane and the nucleus in the absence of HGF stimulation. Because no nuclear localization of p120ctn could be observed in the absence of LMB, the amount of p120ctn that is part of this shuttling pool must be small. The nuclear translocation of p120ctn in LMB-treated MDCK cells was not complete. A significant fraction of p120ctn remained associated with the plasma membrane. Apparently, in MDCK cells there is a population of cadherin-associated p120ctn that is prevented from entering the nucleus. Posttranslational modifications of p120ctn resulting from E-cadherin-binding, such as serine/threonine phosphorylation (see Section 4.3), could block its nuclear import and thereby remove this
population of p120ctn from the bulk of p120ctn shuttling between the plasma membrane and the nucleus. Alternatively, the non-nuclear p120ctn pool could be consist of p120ctn isoforms lacking exon B or containing as-yet-unidentified LMB-insensitive NES or of isoforms that lack, also still nuclear localization sequences.

LMB treatment of E-cadherin-negative MDA-MB-231 cells, on the other hand, caused near complete accumulation of p120ctn in the nucleus, supporting the idea that binding of p120ctn to E-cadherin counteracts its nuclear import. It is also possible that MDA-MB-231 express p120ctn isoforms that translocate to the nucleus more efficiently than the isoforms expressed in MDCK cells.

In MDCK cells treated with LMB and HGF simultaneously, nuclear p120ctn levels were not elevated compared to cells treated with HGF alone, suggesting that the nuclear export of p120ctn does not counteract its HGF-induced nuclear import. Rather, p120ctn molecules entering the nucleus in response to HGF seem to be efficiently retained in the nucleus and do not recirculate to the cytoplasm or the plasma membrane. Alternatively, HGF may primarily stimulate nuclear import of p120ctn rather than inhibiting its export.

The significance of the nuclear translocation of p120ctn in general, and in particular in response to HGF, remains to be elucidated. In a yeast-two-hybrid screen, p120ctn was found to bind to the transcription factor Kaiso (Daniel and Reynolds, 1999). Kaiso belongs to the POZ/ZF (Pox virus and zinc finger) family, many members of which are sequence-specific transcriptional repressors (Albagli et al., 1995; Bardwell and Treisman, 1994). The genes controlled by Kaiso are still unknown. However, Kaiso has recently been shown to bind methyl-CpG dinucleotides (Daniel et al., 2002; Prokhortchouk et al., 2001), as well the specific consensus sequence TCCTGCNA (Daniel et al., 2002). Two copies of such a motif are present in the promoter of the gene for matrilysin/MMP7, a metalloproteinase (MMP). MMPs are secreted or membrane-bound proteases that degrade the extracellular matrix (ECM), cleave membrane proteins and release cytokines and play a central role in many biological processes, such as embryogenesis, wound healing, angiogenesis and tissue remodelling during development (Visse and Nagase, 2003). In addition, MMPs have been associated with cancer-cell invasion and metastasis (Egeblad and Werb, 2002; Stemlicht and Werb, 2001). For metastasis to occur, epithelial cells must cross the epithelial basement membrane, invade the surrounding stroma, then enter blood vessels and finally extravasate and establish secondary colonies. All of these steps require the crossing of ECM barriers, a process that is thought to be promoted by
aberrant MMP activity (Sternlicht and Werb, 2001). MMP7, a collagenase upregulated in a number of cancers (Crawford et al., 2002; Ishikawa et al., 1996; Masaki et al., 2001; Miwa et al., 2002; Newell et al., 2002) and has been shown to promote invasion (Yamamoto et al., 1995).

Assuming that the MMP7 gene is under the control of Kaiso/p120ctn, this suggests a model for the involvement of p120ctn in promoting HGF-dependent invasion (Figure 5.8). HGF stimulation induces adherens junction disassembly, thereby weakening cell-cell adhesion and enabling the cells to become motile. p120ctn is released from the E-cadherin complex and translocates into the nucleus, where it induces the transcription of the MMP7 gene via Kaiso. In turn, secreted MMP7 promotes degradation of the extracellular matrix and allows cells to invade the surrounding stroma. Interestingly, MMP7 also cleaves E-cadherin at the cell surface (McGuire et al., 2003; Noe et al., 2001). Shedding of the extracellular domain E-cadherin from the cell surface has been proposed to stimulate invasion (Banks et al., 1995; Griffiths et al., 1996; Katayama et al., 1994). MMP7 could therefore further promote invasion by removing remaining functional E-cadherin molecules from the cell surface, which in turn may release additional p120ctn molecules into the cytoplasm. Increasing the cytoplasmic concentration of p120ctn in E-cadherin-negative cells promotes cell migration via the regulation of Rho GTPases (Grosheva et al., 2001). The HGF-induced release of p120ctn from adherens junctions into the cytoplasm could therefore further promote cell migration. However, most of the p120ctn released from adherens junctions in the early phase of HGF stimulation (1 to 4 h) appeared to localize in the nucleus rather than in the cytoplasm. Only after 16 h of stimulation, p120ctn could be clearly detected in the cytoplasm. In addition to its role in adherens junction disassembly, p120ctn may promote HGF-induced cell migration in two further steps: First by activating the transcription of MMP7 in the nucleus and later in the cytoplasm by increasing cell motility through the regulation of Rho GTPases.
In intact epithelium, p120ctn localizes to adherens junctions and promotes E-cadherin-mediated cell-cell adhesion. Upon HGF stimulation, p120ctn becomes tyrosine phosphorylated and is lost from the E-cadherin complex, thereby promoting junctional disassembly. As its cytoplasmic concentration increases, p120ctn translocates to the nucleus where via the interaction with the transcription factor Kaiso it may induce gene transcription, for example of the potential Kaiso target MMP7. MMP7 could then promote HGF-induced scattering by helping the cells to degrade the surrounding extracellular matrix, ultimately leading to invasion and metastasis.
6 p120ctn and ZO-1

6.1 Introduction
p120ctn localizes to different cellular compartments: to adherens junctions (Reynolds et al., 1994; Reynolds et al., 1996), the microtubule network and the centrosome (Chapter 3), the cytoplasm or the nucleus (van Hengel et al., 1999 and Chapter 5). Except for the recruitment to intercellular junctions, which relies on binding of p120ctn to the juxtamembrane region of cadherins (Daniel and Reynolds, 1995; Reynolds et al., 1996), the protein-protein interactions that target p120ctn to the different intracellular locations are unknown. In order to identify binding partners that may regulate the intracellular localization of p120ctn, in particular the localization along microtubules, pulldown experiments were performed using GST-fusion proteins containing different domains of p120ctn. Coprecipitating proteins were subsequently analysed by mass spectrometry.

6.2 Identification of p120ctn interaction partners by mass spectrometry
The association with microtubules required sequences both within the N-terminus and ARM repeats 3, 4 and 6 of p120ctn (see Section 3.6.1). GST-fusion proteins containing either the N-terminus (GST-N-term) or ARM repeats 4, 5 and 6 (GST-ARM-456) precipitated α-tubulin (see Section 3.6.2), further implicating these domains in mediating p120ctn/microtubule association. In an initial attempt to identify proteins that bind to the N-terminus or to ARM repeats 4, 5 and 6 and that may facilitate the interaction of p120ctn with microtubules, GST-N-term and GST-ARM-456 fusion proteins were expressed in Cos-7 cells and precipitated on glutathione-sepharose beads. To control for non-specific interactions, unmodified GST protein was expressed and precipitated in parallel. GST-fusion proteins and coprecipitating proteins were resolved on a SDS-polyacrylamide gel and subsequently visualized by silver staining (Figure 6.1). Both fusion proteins precipitated several proteins that were also precipitated by GST alone (indicated by asterisks). These proteins were considered p120ctn-non-specific and excluded from the analysis. Because preliminary western blot analysis had revealed a number of degradation products of the p120ctn fusion proteins (compare also Figure 3.18), bands running at lower molecular weights than the fusion proteins were ignored. The remaining bands in the GST-ARM-456 and GST-N-term precipitations were judged to be specific for the respective construct and 9 prominent bands, including the bands containing the
Figure 6.1 Silver stain analysis of proteins coprecipitating with GST-fusion proteins containing different p120ctn domains

GST-fusion proteins containing p120ctn ARM repeats 4, 5 and 6 (GST-ARM-456) or the p120ctn N-terminus (GST-N-term) or GST alone were expressed in Cos-7 cells. After cell lysis, GST proteins complexes were purified on glutathione-sepharose beads. Protein complexes were separated on a 10% SDS-polyacrylamide gel. Proteins were visualized by silverstaining. (A) The bands corresponding to GST or the GST-fusion proteins are indicated. Asterisks indicate bands corresponding to proteins precipitating non-specifically. (B) The same gel as in (A) showing the bands that were excised from the gel for mass spectrometric analysis. Bands 4 and 9 correspond to the fusion proteins and were submitted as a positive control.
fusion proteins as controls, were excised from the gel and submitted for protein identification by mass spectrometry.

Mass spectrometric protein identification was performed by Dr Rainer Cramer and Malcom Saxton, LICR-UCL. Except for two samples corresponding to the bands running at the highest molecular weight (bands 5 and 6, Figure 6.1), protein identifications were obtained for all samples submitted (Table 6.1). Several of the identified proteins coprecipitating with the fusion proteins belonged to the heat shock family of proteins. In addition, several highly abundant proteins, such as cysteinyl-tRNA-synthetase and β-actin, were identified. No obvious candidate proteins that could mediate the interaction of p120ctn with microtubules, such as motor proteins or MAPs, were identified. However, the GST-fusion protein containing the N-terminus of p120ctn precipitated the tight junction protein ZO-1, pointing to a novel interaction between p120ctn and ZO-1.
### Table 6.1 Identification of protein coprecipitating with GST-fusion proteins containing different p120ctn domains by mass spectrometry

Mass spectrometric identification of proteins coprecipitating with GST-fusion proteins containing p120ctn ARM repeats 4, 5 and 6 (bands 1-3) or the p120ctn N-terminus (bands 5-8). The gel band mass was calculated from molecular weight markers whereas the protein mass was calculated from NCBI protein database sequences. No protein identities were obtained for bands 5 and 6.

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<td>Heat shock 70 protein 8</td>
<td>~70000</td>
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6.3 ZO-1 interacts with the N-terminus of p120ctn

To confirm the interaction of ZO-1 with the N-terminus of p120ctn by western blotting, the membrane used in Figure 3.18 to show the interaction of GST-N-term and GST-ARM-456 with $\alpha$-tubulin was stripped and reprobed for ZO-1 (Figure 6.2). ZO-1 antibodies reacted with a protein corresponding to the molecular weight of ZO-1 in GST-N-term, but not GST-ARM-456 or GST precipitations, confirming the specific interaction of the p120ctn N-terminus with ZO-1. In addition, this showed that although both GST-N-term and GST-ARM-456 precipitated $\alpha$-tubulin, the two fusion proteins precipitate different protein complexes.
Figure 6.2  Confirmation of the Interaction of ZO-1 with the N-terminus of p120ctn by western blotting

GST-fusion proteins containing p120ctn ARM repeats 4, 5 and 6 (GST-ARM-456) or the p120ctn N-terminus (GST-N-term) or GST were expressed in Cos-7 cells and analysed by western blotting as described in Section 3.6.2. The membrane presented in Figure 3.18 was stripped and reprobed for ZO-1.
6.4 ZO-1 interacts with p120ctn via its N-terminus

ZO-1 is a protein with an apparent molecular weight of 220 kDa and its known domains include three N-terminal PDZ (PSD-95/Discs-large/ZO-1) domains, a basic domain between PDZ domains 1 and 2, an SH3 domain, a non-functional guanylate kinase domain, and an acidic domain as well as a C-terminal polyproline domain (Figure 6.3). To characterize which domains of ZO-1 are needed for the interaction with the N-terminus of p120ctn, different ZO-1 deletion and truncation constructs carrying an N-terminal HA (hemagglutinin)-tag (Figure 6.3, constructs kindly provided by Dr Maria Balda, UCL) were co-expressed with the GST-fusion protein containing the N-terminus of p120ctn in Cos-7 cells (Figure 6.4, upper panel). No protein expression was obtained from a construct encoding full length ZO-1 (HA-ZO-1), whereas a construct carrying a deletion of the SH3 domain (HA-ZO-1ΔSH3) was only weakly expressed. In contrast, constructs containing the N-terminal PDZ domains (HA-PDZ1-3) or the N-terminus till the end of the guanylate kinase domain (HA-NT-GUK) were expressed at high levels, although a fraction of the protein product of these construct appeared to be degraded. All expressing ZO-1 fragments associated with GST-N-term (Figure 6.4, lower panel). Therefore, the N-terminus of ZO-1, containing the three PDZ domains and the basic domain, is sufficient to mediate the interaction with p120ctn.

The specificity of the interaction of the N-termini of ZO-1 and p120ctn was confirmed in a more detailed coprecipitation experiment. Construct HA-PDZ1-3 was expressed alone or together with GST or the GST-fusion protein GST-N-term in Cos-7 cells. Cell lysates were then incubated with glutathione-sepharose beads and the associated proteins were analysed by western blotting using anti-GST and anti-HA antibodies (Figure 6.5). HA-PDZ1-3 coprecipitated with GST-N-term but not with GST alone. In addition, HA-PDZ1-3 did not bind non-specifically to glutathione-sepharose beads. The specific interaction between the N-terminus of p120ctn with the N-terminus of ZO-1 was therefore confirmed. However, despite the clear interaction of GST-N-term fusion with endogenous ZO-1 (Figure 6.2) or with the HA-tagged N-terminus of ZO-1 (Figure 6.4 and Figure 6.5), it was not possible to coimmunoprecipitate endogenous p120ctn and ZO-1 from Cos-7 or MDCK cells.
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**Figure 6.3  Domain structure of different HA-ZO-1 constructs**

Overview of the domains included in several ZO-1 constructs. All constructs carry an N-terminal HA-tag. Note: the HA-tag is not the commonly used HA-tag, but has the sequence MQDLPGNDNSTAG (Daro et al., 1996). Abbreviations: HA: Hemagglutinin-tag, PDZ: PSD-95/Discs-large/ZO-1 domain, SH3: Src homology 3 domain, GK: guanylate kinase domain, (+): basic domain, PPP: polyproline domain.
Figure 6.4  The N-terminus of ZO-1 is sufficient to mediate the interaction with p120ctn

HA-tagged ZO-1 deletion and truncation constructs were co-expressed with the p120ctn N-terminus GST-fusion construct in Cos-7 cells. Upper panel: western blot analysis of cell lysates using anti-HA antibodies showed that constructs HA-PDZ1-3 and HA-NT-GUK were expressed at high levels but partially degraded. Construct HA-NT-ASH3 was expressed at low levels, whereas no full-length HA-ZO-1 protein could be detected. The anti-HA-antibody non-specifically reacted with several proteins in the cell lysate. Lower panel: protein complexes containing the GST-N-term fusion protein were precipitated using glutathione–sepharose beads. Protein complexes were separated on a SDS-polyacrylamide gel and transferred to PVDF membrane. Coprecipitating HA-ZO-1 mutants were detected using the anti-HA antibody. The antibody cross-reacted with the GST-fusion protein.
Figure 6.5  The N-termini of p120ctn and ZO-1 specifically interact

The HA-tagged construct containing the three PDZ domains of ZO-1 (HA-PDZ1-3) was expressed on its own or together with GST or a construct containing the N-terminus of p120ctn (GST-N-term) in Cos-7 cells. Protein expression was confirmed by western blot analysis of cell lysates using anti-GST and anti-HA antibodies (left panel). Lysates were incubated with glutathione-sepharose beads and GST- and HA-tagged proteins were visualized by western blotting (right panel).
6.5 The interaction between the N-terminal regions of p120ctn and ZO-1 is preserved in the presence of several tyrosine or serine/threonine kinase and phosphatase inhibitors

The majority of p120ctn tyrosine (Mariner et al., 2001) and serine/threonine phosphorylation sites (Section 4.5.3 and (Thompson et al., in press) are located in the N-terminal region of p120ctn. To test whether the phosphorylation of N-terminal serine/threonine or tyrosine residues affects the interaction with the ZO-1 N-terminus, Cos-7 cells co-expressing GST-N-term and HA-PDZ1-3 were incubated with the phosphatase inhibitor calyculin A to increase the level of serine/threonine phosphorylation on GST-N-term (see Section 4.5.2). Alternatively, cells were treated with the Src kinase inhibitor PP2 to decrease Src-dependent tyrosine phosphorylation, or with pervanadate to inhibit tyrosine phosphatases and thereby increase tyrosine phosphorylation of GST-N-term. The interaction of HA-PDZ1-3 with GST-N-term was then analysed by western blotting (Figure 6.6). Treatment with the different inhibitors changed the electrophoretic mobility of GST-N-term (Figure 6.6, bottom panel), demonstrating the effectiveness of the inhibitors in altering its phosphorylation state. The electrophoretic mobility of HA-PDZ1-3 was also affected by inhibitor treatment, indicating the presence of serine/threonine and tyrosine phosphorylation sites within the N-terminus of ZO-1. However, similar levels of ZO-1 precipitated with GST-N-term purified from PP2-, pervanadate- or untreated cells, suggesting that tyrosine-phosphorylation of the p120ctn and ZO-1 N-termini does not affect their interaction. Likewise, calyculin A treatment had no influence of the amount of HA-PDZ1-3 coprecipitating with GST-N-term, indicating that serine/threonine phosphorylation is also not regulating the interaction between the N-termini of p120ctn and ZO-1. However, it is possible that serine/threonine phosphorylation positively regulates the interaction between p120ctn and ZO-1 and that therefore only dephosphorylation of the respective phosphorylation site(s), for instance as a result of staurosporine treatment, would disrupt the interaction.

6.6 Mutant p120ctn does not recruit ZO-1 to microtubules

As demonstrated in the previous sections of this chapter, the N-terminal regions of p120ctn and ZO-1 interact. However, because it was not possible to coprecipitate endogenous proteins, it is not clear whether endogenous ZO-1 and p120ctn interact in
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**p120ctn and ZO-1**

Figure 6.6 Interaction of the N-terminal domains of p120ctn and ZO-1 in the presence of kinase and phosphatase inhibitors

Cos-7 cells were transfected with vectors HA-PDZ1-3 and pEF-Bos-GST or pEF-Bos-GST-N-term. After expression for 24 h, cells were treated with 100 nM calyculin A for 30 min (caly), 1 μM PP2 for 1 h (PP2) or 50 μM pervanadate for 1 h (Van). After cell lysis, equal expression of HA-PDZ1-3 proteins was checked by western blotting using anti-HA-antibodies (top panel). Lysates were incubated with glutathione-sepharose beads and precipitated protein complexes were analysed by western blotting using anti-HA (middle panel) or anti-GST (bottom panel) antibodies.
cells. In epithelial and endothelial cells ZO-1 is a component of tight junctions (Gonzalez-Mariscal et al., 2000) and consequently localizes to sites of cell-cell contact. p120ctn and ZO-1 showed a similar localization pattern in MDCK cells making an interaction of p120ctn and ZO-1 feasible in these cells (Figure 6.7, upper panel). However, the apparent colocalization of p120ctn and ZO-1 is also the result of the proximity of tight and adherens junction and therefore offers no proof that p120ctn and ZO-1 are part of the same protein complex. However, AAA-p120ctn localizes to microtubules (Section 3.2.3), whereas ZO-1 has never been reported to localize along microtubules. If mutant p120ctn were able to recruit ZO-1 to microtubules, this would offer direct proof for an interaction between p120ctn and ZO-1 proteins in cells. However, in MDCK cells showing microtubule association of AAA-p120ctn, ZO-1 continued to localize exclusively to sites of cell-cell contact (Figure 6.7, lower panel), demonstrating that ZO-1 and p120ctn do not interact under these conditions.
Figure 6.7  Mutant p120ctn does not recruit ZO-1 to microtubules

MDCK were fixed and stained for endogenous p120ctn and ZO-1 (upper panel), or transfected with AAA-p120ctn-VSV (AAA), fixed and stained with antibodies for ZO-1 and the VSV epitope (lower panel). Because of the proximity of adherens and tight junctions, endogenous p120ctn (p120ctn) and endogenous ZO-1 both localize at sites of cell-cell contact in MDCK cells (upper panel). The microtubule-targeted AAA-p120ctn mutant (AAA) does not recruit ZO-1 from intercellular junctions to microtubules (lower panel), suggesting that the two proteins do not interact under this conditions. Bar 5 μm.
6.7 Discussion

Mass spectrometric analysis of proteins coprecipitating with GST-fusion proteins containing the p120ctn N-terminus or ARM repeats 4, 5 and 6 showed that several of these proteins belong to the heat-shock protein (HSP) family. HSPs are best known for their role as molecular chaperones, in which they assist newly synthesized or denatured proteins to attain their functional conformation (Craig et al., 1994; Ellis, 1987; Ellis and van der Vies, 1991; Schlesinger, 1990). Fusion proteins containing only individual domains of a protein are often unable to fold properly. HSPs recognize these partially folded fusion proteins and are therefore frequently found to co-purify with them. Thus, the association with HSPs could indicate folding problems of the GST-fusion proteins.

The GST-fusion protein containing ARM repeats 4, 5 and 6 precipitated predominantly HSPs. It has been questioned whether individual ARM domains are stable on their own (Peifer et al., 1994). Because of the extensive contact between neighbouring ARM repeats mediated by hydrophobic interactions (Andrade et al., 2001), proper ARM repeat folding probably requires the presence of several adjacent repeats. The GST-ARM-456 fusion protein contains just three adjacent ARM repeats, which may not allow the fusion protein to attain proper ARM repeat domain folding and which would explain the association with HSPs.

On the other hand, HSPs are emerging as important regulators of the cytoskeleton, including the microtubule network (Liang and MacRae, 1997). For instance, several members of the HSP family associate with microtubules during interphase and mitosis (Weller, 1988) and have been suggested to regulate tubulin polymerisation (Ahmad et al., 1990). HSP-70 binds directly to tubulins (Sanchez et al., 1994) and is involved in regulating tubulin folding (Lewis et al., 1996). In addition, HSPs localize to the mitotic spindle and centrosomes and have been proposed to regulate spindle assembly and disassembly (Agueli et al., 2001; Brown et al., 1996; Rattner, 1991; Wigley et al., 1999). Therefore, HSPs could have a role in mediating the association of p120ctn with microtubules and the centrosome (see Chapter 3).

The GST-fusion protein containing the N-terminus of p120ctn specifically interacted with the tight junction protein ZO-1. ZO-1 is best characterized for its role in tight junction function. Tight junctions separate the apical from the basolateral membrane and restrict the passage of ions and molecules across the epithelial monolayer (Cereijido et al., 1993). Transmembrane proteins of the claudin family, as well as occludin, constitute the tight
juction strands (Tsukita and Furuse, 1999), whereas ZO-1 (and the related proteins ZO-2 and -3) acts as a linker between the proteins that constitute the strands and the actin-based cytoskeleton (Gonzalez-Mariscal et al., 2000).

Whereas ZO-1 localizes to tight junctions in polarized epithelial cells, in epithelial cells where adherens and tight junction formation has been inhibited due to the lack of extracellular calcium ("calcium switch"), it colocalizes with the adherens junction proteins α-, β- and γ-catenin and E-cadherin in intracellular granular clusters (Itoh et al., 1993; Yonemura et al., 1995). Upon restoration of normal calcium levels, ZO-1 is redistributed to the plasma membrane, where it transiently colocalizes with immature adherens junctions, before it localizes to tight junctions as the cells polarize completely (Rajasekaran et al., 1996). Subsequently, the N-terminal region of ZO-1 was shown to bind directly to α-catenin (Itoh et al., 1997) and it has been suggested that during the early phase of junctional formation ZO-1 is recruited to the plasma membrane by the cadherin/catenin system and subsequently relocates to tight junctions as the cells fully polarize (Gonzalez-Mariscal et al., 2000).

Via E-cadherin and β-catenin or γ-catenin, p120ctn is also in a complex with α-catenin. It is however unlikely that the observed interaction of p120ctn and ZO-1 is mediated via α-catenin. Firstly, the GST-fusion protein GST-N-term that binds ZO-1 lacks the ARM repeat domain necessary for E-cadherin binding. GST-N-term is therefore unable to associate with E-cadherin/catenin complexes. In addition, the interaction between the N-terminal region of p120ctn and ZO-1 was observed in the E-cadherin-negative cell line Cos-7. Therefore, the interaction of p120ctn and ZO-1 appears to be an additional, α-catenin-independent way whereby ZO-1 could associate with adherens junctions.

In addition to tight and adherens junctions, ZO-1 also associates with gap junctions (Giepmans and Moolenaar, 1998; Kausalya et al., 2001; Toyofuku et al., 1998). Gap junctions are aggregates of channels formed from hexamers of proteins of the connexin family and permit the direct exchange of ions and small molecules between cells (Nicholson and Bruzzone, 1997; Paul, 1995; White et al., 1995). By directly binding to connexin-45 (Kausalya et al., 2001) and connexin-43 (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998), ZO-1 has been suggested to play a role in the recruitment and organization of gap junctions. Interestingly, p120ctn has recently been shown to colocalize with connexin-43 gap junctions in neural crest cells and connexin-43
deficiency altered the subcellular distribution of p120ctn in these cells (Xu et al., 2001). p120ctn and ZO-1 may therefore also interact at connexin-43-positive gap junctions.

The N-terminus of ZO-1, containing three PDZ domains and a basic motif, was sufficient to mediate the interaction with p120ctn. PDZ domains are protein-protein interaction domains, which usually recognize specific ~5 amino acids motifs that occur at the C-terminus of target proteins or structurally related internal motifs (Harris and Lim, 2001). However, the interaction between ZO-1 and p120ctn is unlikely to be mediated via the ZO-1 PDZ domains. Firstly, the N-terminal region of p120ctn was sufficient to mediate the interaction with ZO-1, ruling out the requirement for a C-terminal PDZ-binding motif. In addition, although the other members of the p120ctn family of proteins contain C-terminal PDZ-binding motifs (Anastasiadis and Reynolds, 2000), p120ctn itself lacks such a motif. Internal PDZ-motif-mediated PDZ interactions have only been described to occur in the dimer formation between PDZ domains, i.e. the PDZ domain of one protein recognizes a PDZ-binding motif within the PDZ domain of another protein (Harris and Lim, 2001). p120ctn does not contain a PDZ domain and therefore is unlikely to possess an internal recognition motif. Thus, the interaction between p120ctn and ZO-1 is unlikely to occur by direct binding of the PDZ domains of ZO-1 to the N-terminus of p120ctn. The interaction between p120ctn and ZO-1 may also be indirect and could involve the binding of the ZO-1 PDZ domains to a bridging protein.

The Src-induced tyrosine phosphorylation sites (Mariner et al., 2001) and the majority of serine/threonine phosphorylation sites of p120ctn (Thompson et al., in press) map to the N-terminal region of p120ctn. Phosphorylation may be a way of regulating the interaction between p120ctn and ZO-1. However, neither hypo- or hyperphosphorylation of the Src-dependent sites nor increased serine/threonine phosphorylation had an influence on the ZO-/p120ctn interaction.

In conclusion, the interaction between the adherens junction protein p120ctn and the tight junction protein ZO-1 suggests a novel interplay between the two types of junctions, but when this interaction occurs, the mechanisms by which it is regulated and its relevance for junctional function remain to be investigated.
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Concluding remarks

p120ctn is an ARM repeat domain protein involved in a wide variety of cellular functions (Anastasiadis and Reynolds, 2000). At the plasma membrane, p120ctn binds to the cytoplasmic domain of cadherins and is involved in modulating the adhesive properties of cadherin adhesion complexes. In contrast, in the cytoplasm it regulates the actin cytoskeleton and promotes cell migration via Rho family GTPases (Anastasiadis and Reynolds, 2001). In addition, p120ctn frequently shows nuclear localization and a recently discovered interaction with the transcription factor Kaiso suggests a role for p120ctn in regulating transcription.

The finding that specific mutations in the ARM repeat domain of p120ctn promote its association with microtubules reveals an additional role for p120ctn connected with the microtubule network. The association of p120ctn with microtubules leads to their remodeling into thick, curvy bundles. Thus, p120ctn is able to alter cell morphology in at least two ways: by reorganizing the microtubule network as well as the actin cytoskeleton. However, the effects of p120ctn on the microtubule network and the actin cytoskeleton appear to be mechanistically linked via Rho GTPases. p120ctn regulates the actin cytoskeleton by inhibiting RhoA and activating Rac1 and Cdc42. Microtubule-associated p120ctn, however, is unable to activate Rac1. In addition, a basic motif within the p120ctn ARM repeat domain is required both for the inhibition of RhoA and for the binding to microtubules. Association of p120ctn with microtubules may lead to RhoA displacement from p120ctn and termination of the inhibitory effect on RhoA. It therefore appears that binding of p120ctn to microtubules is inversely related to its ability to regulate Rho GTPases, and consequently the actin cytoskeleton. Future research should be directed at elucidating the precise molecular mechanisms by which microtubule association prevents p120ctn from regulating Rho GTPases. For example, in vitro binding assays should clarify whether RhoA and microtubules compete with each other for binding to p120ctn. In addition, it should be investigated whether binding of p120ctn to microtubules affects the p120ctn/Vav2 interaction (Chauvet et al., 2003; Noren et al., 2000) and the nucleotide exchange activity of Vav2 towards Rac1 and Cdc42.

The association with mutant p120ctn stabilizes microtubules, as seen by an increase in acetylation. Microtubules in cellular extensions induced by overexpression of wild-type p120ctn also show a high degree of acetylation, suggesting that p120ctn may specifically bind to and stabilize microtubules within these extensions. p120ctn-induced cellular
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extensions strikingly resemble neurite extensions formed by differentiating neurons. Interestingly, neurite outgrowth, like p120ctn-induced branching, requires the activation of Rac and Cdc42 and the inhibition of RhoA (Kozma et al., 1997; Yuan et al., 2003), as well as microtubule polymerization (Nobes and Hall, 1999; Tanaka et al., 1995) and stabilization (Falconer et al., 1989; Ferreira and Caceres, 1989). The combined abilities of p120ctn to induce a dendritic phenotype in fibroblast and neuronal cell lines, to stabilize microtubules and to regulate Rho proteins suggest a role for p120ctn in promoting neuronal outgrowth. Consistent with this idea, p120ctn is highly expressed in the developing rat brain and localizes to growth cones and dendritic spines in cultured hippocampal neurons. Furthermore, NPRAP/δ-catenin, p120ctn’s closely related neuronal family member, induces a p120ctn-like branching phenotype in fibroblasts and enhances dendritic morphogenesis in primary hippocampal neurons (Kim et al., 2002; Lu et al., 2002), suggesting that it regulates Rho proteins in a similar way to p120ctn. The branching phenotype observed in p120ctn- or NPRAP/δ-catenin-overexpressing fibroblast may therefore reflect a physiological function for p120ctn and NPRAP/δ-catenin in neurons, where they would promote neurite outgrowth by stabilizing microtubules and regulating Rho GTPases.

Interestingly, overexpression of a dominant-negative N-cadherin construct, consisting of the transmembrane domain and the JMD (which contains the p120ctn binding site), inhibits N-cadherin-promoted neurite outgrowth (Riehl et al., 1996) and it has therefore been suggested that the JMD of N-cadherin is required for neurite outgrowth. However, the dominant-negative construct is also likely to sequester p120ctn at the plasma membrane. The interaction of p120ctn with E-cadherin and the regulation of GTPases are thought to be mutually exclusive events and the same may be true for N-cadherin. Hence, the N-cadherin JMD may in fact have an inhibitory effect on neurite outgrowth by preventing p120ctn from regulating Rho GTPases and associating with microtubules in the cytoplasm. Investigating the effect of overexpression of N-cadherin molecules carrying p120ctn-uncoupling mutations within the JMD on neurite outgrowth may clarify the role of p120ctn and the JMD in N-cadherin-promoted neurite outgrowth. If the JMD has a positive role in neurite outgrowth, for instance by recruiting p120ctn which then positively modulates N-cadherin function, the p120ctn-uncoupled N-cadherin construct should fail to promote neurite outgrowth. On the other hand, if the JMD negatively
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regulates neurite outgrowth by sequestering p120ctn, the p120ctn-uncoupled construct should have no inhibitory effect on neurite outgrowth.

Whereas the evidence presented above argues for a role of p120ctn in promoting the formation of cellular extensions in neuronal cells, the ability of p120ctn to induce a dendritic morphology may also be important for fibroblast function. Although fibroblasts cultured on 2D substrata usually display a flattened morphology, they develop an elaborate network of dendritic extensions when cultured in floating 3D collagen matrices (Grinnell, 2003; Grinnell et al., 2003 and Figure 7.1). Such 3D matrices provide a more physiological environment than standard “tissue culture” plastic and the dendritic morphology of fibroblasts grown in these matrices may therefore represent the in vivo situation of fibroblasts embedded within the ECM. It would be interesting to investigate whether fibroblasts in which the expression of p120ctn has been suppressed by RNAi are still able to form dendritic extensions in 3D matrices.
Figure 7.1  Fibroblasts cultured in floating 3D collagen matrices develop a dendritic morphology

The morphology of fibroblasts grown on a 2D collagen substrate or in a 3D floating collagen matrix was visualized by phalloidin staining for F-actin. Adapted from Grinnell et al, 2003.
The mutant p120ctn also localizes to the mitotic spindle and both mutant and endogenous p120ctn accumulate at the centrosomes during mitosis, suggesting a novel role for p120ctn during cell division. The accumulation of p120ctn at the centrosome or the pericentrosomal region suggests that p120ctn may regulate microtubule organization around the centrosome. Alternatively, p120ctn may be involved in orienting the mitotic spindle in relation to the cell cortex. In Drosophila melanogaster, the ARM repeat domain proteins APC2 and armadillo help to anchor the mitotic spindle to cortical actin (Allan and Nathke, 2001; McCartney et al., 2001). During mitosis mutant p120ctn showed some punctate localization around the cell cortex and could similarly be involved in anchoring astral spindle microtubules to the cortex. To further study the role of p120ctn during cell division, it would be interesting to assess the effect of p120ctn depletion by RNAi on the shape and orientation of mitotic spindles and on cell cycle progression.

Using IMAC/MS a number of novel p120ctn serine/threonine phosphorylation sites were identified. The majority of these serine/threonine phosphorylation sites are situated within the N-terminal domain of p120ctn and several of them map to a region also known to contain the majority of the Src-induced tyrosine phosphorylation sites (Mariner et al., 2001). Simultaneous serine/threonine and Src-induced tyrosine phosphorylation would therefore create a highly negatively charged region just upstream of the first ARM repeat. This region may provide multiple docking sites for proteins containing phosphoserine or phosphotyrosine-binding domains. Alternatively, accumulating phosphorylation of serine/threonine and/or tyrosine residues within this region may induce conformational changes in p120ctn, thereby altering protein-protein interactions.

p120ctn is only weakly phosphorylated on serine/threonine residues when it localizes to the cytoplasm but its phosphorylation is strongly induced upon recruitment to the plasma membrane by the E-cadherin complex (Thoreson et al., 2000). This specific increase in serine/threonine phosphorylation upon E-cadherin binding suggests a role for these phosphorylation events in modulating E-cadherin function. However, serine/threonine phosphorylation appears not to be a prerequisite for p120ctn binding to E-cadherin because (1) N-terminally truncated p120ctn molecules missing the majority of phosphorylation sites are still able to interact with E-cadherin (Aono et al., 1999) and (2) staurosporine treatment did not affect the interaction of E-cadherin with p120ctn despite decreasing the level of p120ctn serine/threonine phosphorylation. Consistent with this
finding, p120ctn mutants carrying individual serine to alanine mutations at the position of the novel phosphorylation sites were all able to bind E-cadherin. Furthermore, neither inactivating alanine nor activating aspartate mutations of individual phosphorylation sites affected the intracellular localization of p120ctn or blocked its ability to induce the "branching phenotype", and so the functions of these phosphorylation sites remains unknown.

Induction of serine/threonine phosphorylation of p120ctn coincides with a decrease in p120ctn electrophoretic mobility. Such changes in electrophoretic mobility or "shifts" are thought to be caused by conformational changes that impede the protein's migration through the polyacrylamide mesh. Individual mutations of the newly-identified phosphorylation sites had no influence on p120ctn electrophoretic mobility. The shift-inducing phosphorylation sites may have been missed during the IMAC/MS analysis. It is more likely, however, that several phosphorylation sites act together to induce a conformational change in p120ctn responsible for the shifting of p120ctn bands on SDS-polyacrylamide gels. Elucidating the physiological relevance of p120ctn serine/threonine phosphorylation may therefore require the combined mutation of several phosphorylation sites. In addition, the generation of phospho-specific antibodies would provide a useful tool for analyzing the phosphorylation level of the individual sites in a variety of cellular contexts. Ultimately, the identification of the respective kinase(s) will be crucial for precisely understanding the role of these phosphorylation sites in the function of p120ctn.

Nuclear localization of p120ctn has been reported in a number of fibroblast and cancer cell lines (van Hengel et al., 1999). These cell lines appear to have in common that they express no or only low levels of E-cadherin. Nuclear localization of p120ctn can be efficiently counteracted by reintroducing E-cadherin, suggesting that nuclear localization of p120ctn is usually prevented in the presence of E-cadherin. The observation that HGF stimulation induces nuclear translocation of p120ctn in MDCK cells is the first example of nuclear localization of p120ctn in an E-cadherin-positive cell line. Specific mechanisms must exist to enable p120ctn to translocate to the nucleus under these conditions. Such mechanisms may involve expression of p120ctn isoforms carrying sequences that promote nuclear localization, interaction of p120ctn with proteins that facilitate nuclear shuttling and/or changes in the phosphorylation state of p120ctn. Phospho-specific antibodies raised against the novel serine/threonine phosphorylation
sites could be used to determine the phosphorylation profile of p120ctn in the nuclear versus the cytoplasmic fractions.

The function of p120ctn in the nucleus is unknown, but the discovery that p120ctn associates with the transcription factor Kaiso (Daniel and Reynolds, 1999) suggests a role for nuclear p120ctn in regulating gene transcription. So far, no Kaiso target genes have been identified. However, the promoter of the Matrilysin/MMP7 gene contains putative Kaiso consensus sequences (Daniel et al., 2002). Interestingly, in prostate cancer cells HGF treatment promotes cell migration by mediating the release of active MMP7 from the cell surface, resulting in the extracellular cleavage of E-cadherin (Davies et al., 2001). p120ctn could be part of a feedback loop, signaling HGF-induced disassembly of E-cadherin complexes to the nucleus and, by stimulating MMP7 transcription, in turn contribute to the further reduction of E-cadherin-mediated adhesion. It would now be interesting to investigate whether p120ctn is able to stimulate MMP7 expression at the mRNA or protein level and whether MMP7 is indeed a Kaiso target. Such experiments should be complemented by microarray studies which could provide a more comprehensive analysis of p120ctn/Kaiso-regulated changes in transcription.

Via its N-terminal domain, p120ctn is able to interact with the tight junction protein ZO-1. Previously, ZO-1 has been shown to localize to adherens junctions by binding to α-catenin (Itoh et al., 1997; Rajasekaran et al., 1996). The interaction with p120ctn may present an alternative (or complementary) mechanism by which ZO-1 localizes to adherens junctions. Alternatively, ZO-1 may recruit p120ctn to tight junctions or gap junctions. Further studies should attempt to show whether ZO-1 and p120ctn interact directly, when and where they interact under physiological conditions and how this interaction is regulated.

In conclusion, the studies presented in this thesis demonstrate a novel association of p120ctn with microtubules and the centrosome, describe the translocation of p120ctn into the nucleus in response to HGF treatment and show a novel interaction of p120ctn with the tight junction protein ZO-1. In addition, a number of serine/threonine phosphorylation sites were identified, which should facilitate further studies aimed at understanding the role of serine/threonine phosphorylation for the function of p120ctn.
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229
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## Abbreviations

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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>APC</td>
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<td>ARM</td>
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<td>ARVCF</td>
<td>armadillo repeat gene deleted in velo cardio facial syndrome</td>
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<td>filamentous actin</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin-receptor substrate</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule-associated protein</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
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<td>NES</td>
<td>nuclear export sequence</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<td>NLS</td>
<td>nuclear localization sequence</td>
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<td>NP-40</td>
<td>Nonidet P-40</td>
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<td>p120ctn</td>
<td>p120 catenin</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<td>PBD</td>
<td>p21-activated kinase binding domain</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PCV</td>
<td>packed cell volume</td>
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<td>PDA</td>
<td>piperazine diacrylamide</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-L-lysine</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>ROCK</td>
<td>Rho-kinase</td>
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<td>SDS-PAGE</td>
<td>SDS-PolyAcrylamide Gel Electrophoresis</td>
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<td>Abbreviation</td>
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<td>SH2</td>
<td>Src-homology domain 2</td>
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<td>SRF</td>
<td>serum response factor</td>
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<td>TBS</td>
<td>tris-buffered saline</td>
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<td>TEMED</td>
<td>N, N, N', N'- tetramethylethylenediamine</td>
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<td>Tiam-1</td>
<td>T-lymphoma invasion and metastasis</td>
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<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>TRITC</td>
<td>tetramethylrhodamine isothiocyanate</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZO</td>
<td>zonula occludens</td>
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