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Dissertação orientada pelo Prof. Doutor Alan Hall

e pelo Prof. Doutor Rui Manuel dos Santos Malhó

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ABSTRACT

Rho GTPases are signal transduction proteins that affect a wide variety of cellular processes through the interaction with effector proteins. The protein kinase C related kinases (PRK) are a family of Rho GTPase effectors composed of three members: PRK1, PRK2 and PRK3. PRKs have a catalytic domain homologous to protein kinase C and a regulatory domain that interacts with Rho GTPases. Depletion of PRK2 by siRNA in mammalian cells has been shown to affect cell cycle progression. The way PRK2 function is regulated during the cell cycle is, however, not well understood and is the main focus of the present work.

It was shown that PRK2 is phosphorylated in mitosis in a way that gives rise to a mobility shift when subjected to SDS-PAGE gel electrophoresis. Here we were able to show that PRK2 is phosphorylated when cells are in prometaphase and metaphase and is dephosphorylated as cells progress to cytokinesis. Using an in vitro kinase assay to compare the activities of mitotically phosphorylated PRK2 and interphase PRK2, we observed that PRK2 is slightly less active when phosphorylated. We have also identified two sites in PRK2 that are specifically phosphorylated in mitosis.

PRK2 localizes at the cleavage furrow and around the midbody during cytokinesis. The HR1 domain, which binds Rho and Rac, is required for PRK2 localization during cytokinesis. Rho is an important regulator of cytokinesis that shows a similar localization pattern to PRK2 and we propose that Rho mediates PRK2 localization during cytokinesis. While analyzing the localization of PRK2 during cytokinesis, we realized that PRK2 also localizes at sites of cell-cell contact that form between daughter cells and that the HR1 domain is also required for this localization. In addition, PRK2 localizes at apical junctions in polarized epithelial cells.

These results suggest a new role for PRK2 during epithelia morphogenesis. We propose a functional link between the localization of PRK2 at the cleavage furrow/midbody and at apical junctions. We propose that junctional/polarity proteins are recruited to the cleavage furrow or around the midbody during cytokinesis to facilitate junction formation between daughter cells.

RESUMO

As Rho GTPases são proteínas transdutoras de sinal que funcionam como interruptores moleculares uma vez que ciclam entre uma forma activa e inactiva dependendo se se encontram ligadas a GTP ou a GDP, respectivamente. Quando ligadas a GTP, as Rho GTPases adquirem uma conformação tal que lhes permite a interacção com proteínas efectoras. Cerca de 100 proteínas efectoras das Rho GTPases foram identificadas até ao momento e entre estas incluem-se membros das mais variadas classes de proteínas, como cinases, phosphatases, lipases e proteínas scaffold (Bishop and Hall, 2000; Jaffe and Hall, 2005).

Através da interacção com proteínas efectoras, as Rho GTPases regulam inúmeros processos celulares, incluíndo a organização dos citosqueletos de actina e de microtúbulos, o tráfico membranar, a expressão génica e o estabelecimento de polaridade intracelular. Todos estes processos são essenciais para o cumprimento da maioria das funções celulares. Desta forma as Rho GTPases são moléculas fundamentais na divisão, migração, sobrevivência e morfologia celular. Em células humanas existem 20 Rho GTPases entre as quais as as mais bem estudadas sao as Rac, as Rho e a Ccd42 (Heasman and Ridley, 2008).

As PRKs (Protein kinase C-related kinases) formam uma família de proteínas efectoras das Rho GTPases constituída por três membros: PRK1, PRK2 e PRK3. As PRKs são cinases de serinas e treoninas que possuem um domínio catalítico homólogo ao da proteína PKC (Protein Kinase C) e um domínio regulatório (HR1) que interage com as Rho GTPases. As PRKs possuem ainda um domínio homólogo ao domío C2 (“C2-like”) cuja função é ainda em geral desconhecida (Mukai, 2003). Foi demonstrado em células humanas, que a

redução dos níveis intracelulares de PRK2 compromete a progressão normal do ciclo celular. O modo como a PRK2 é regulada durante o ciclo celular é no entanto desconhecido e constitui o tema principal do presente trabalho (Schmidt et al., 2007).

Foi demonstrado previamente que a PRK2 é fosforilada durante a mitose de células HeLa. Esta fosforilação, de um ou mais resíduos em PRK2, pode ser visualizada por western-blot, uma vez que provoca uma redução na migração da proteína por electroforese (Schmidt et al., 2007). Presentemente é desconhecido qual a função desta fosforilação em PRK2. No presente trabalho, analisou-se detalhadamente este evento.

Através do uso de nocodazole, uma droga que quando adicionada em determinadas concentrações bloqueia as células em prometaphase, foi possível isolar células HeLa em prometafase e observar que toda a população de PRK2 se encontra fosforilada durante esta fase do ciclo celular. Ao libertar as células do bloqueio de nocodazole e ao seguir a fosforilação de PRK2 durante a mitose, verificou-se que PRK2 se encontra fosforilada em prometafase e metafase e que deixa de estar fosforilada à medida que as células entram em citocinese.

De forma a investigar se a fosforilação de PRK2 durante a mitose influencia a sua actividade cinásica, desenvolveu-se um sistema de fosforilação “in vitro” através do qual as actividades de PRK2 endógena proveniente de células em prometafase e em interfase, foram comparadas. Usando este sistema, verificou-se que a PRK2 se encontra menos activa quando fosforilada em prometafase do que quando não fosforilada em interfase. Este resultado sugere que estamos perante uma fosforilação inibitória. No futuro seria interessante analisar a actividade de PRK2 durante a citocinese, uma vez que foi demonstrado que a redução dos níveis celulares de PRK2, por RNA de interferência, compromete a citocinese de células HeLa (Schmidt et al., 2007).

Com o intuito de identificar quais os resíduos fosforilados em mitose que são responsáveis pela alteração da electroforese de PRK2, fez-se uso dos resultados obtidos num estudo de espectrofotometria de massa em larga escala (Dephoure et al., 2008). Neste estudo foram identificados alguns resíduos em PRK2 que se encontram fosforilados especificamente em mitose. Com base neste estudo e na conservação dos resíduos identificados entre PRK1, PRK2 e PRK3, quatro serinas e uma treonina foram escolhidas como candidatas, e mutadas para alaninas. A migração destes mutantes num gel de poliacrilamida, depois do tratamento das células com nocodazole, foi analisada. Desta análise foi possível demonstrar que uma ou ambas as serinas 301, 305 em PRK2 de ratinho (correspondendo às serinas 302 e 306 de PRK2 humano), são fosforiladas em mitose. Verificou-se também que fosforilações adicionais noutros resíduos em PRK2 terão de acontecer durante a mitose, uma vez que quando ambas as serinas acima mencionadas são substituídas por alaninas, a migração de PRK2 proveniente de células tratadas com nocodazole é acelerada, mas não se torna equivalente à migração de PRK2 proveniente de células em interfase. Outros locais de fosforilação terão portanto que ser ainda identificados.

Numa tentativa de tentar perceber se a fosforilação das serinas 301 e 305 é um evento essencial à progressão do ciclo celular, os mutantes acima descritos foram expressos em células. Desta análise, verificou-se que as células que expressam estes mutantes proliferam normalmente. Um aumento de células multinucleadas foi detectado, no entanto um fenótipo semelhante foi obtido quando a PRK2 não mutada se expressa, o que não permitiu concluir se a fosforilação nos resíduos identificados é necessária para a progressão normal da citocinese.

A localização de PRK2 também varia ao longo do ciclo celular. PRK2 localiza-se ao nível do citoplasma em interfase e nos primeiros estádios da mitose e ao nível do sulco de clivagem (“cleavage furrow”) e do corpo médio (“midbody”)

durante a citocinese. No presente trabalho mostra-se a localização de PRK2 endógena durante a citocinese por imunofluorescência e a localização de uma fusão de PRK2 com a proteína GFP em células vivas.

Com o intuito de identificar qual o domínio em PRK2 que é responsável pela sua localização durante a citocinese, a localização de vários mutantes de PRK2 em que determinados domínios foram sistematicamente eliminados, foi analisada. Desta análise foi possível demonstrar que o domínio HR1 é responsável pela localização de PRK2 durante a citocinese. Adicionalmente observou-se que a actividade cinásica de PRK2 não é necessária para a sua localização durante a citocinese, uma vez que um mutante de PRK2 que não possui o domínio catalítico, localiza de forma semelhante à da proteína não mutada. Visto que o domínio HR1 é responsável pela interacção de PRK2 com as Rho GTPases e que Rho se localiza de forma semelhante a PRK2 durante a citocinese, propõe-se que Rho seja responsável pela localização de PRK2 ao nível do sulco de clivagem e do corpo médio.

No decorrer do estudo da localização de PRK2 durante a citocinese de células HeLa, verificou-se que PRK2 também se localiza ao nível dos contactos inter-celulares que se formam entre as células filhas durante a divisão. Mostra-se aqui a localização da proteína endógena e sobre-expressada e a sua localização em relação à localização de Scribble, uma proteína necessária à formação de junções epiteliais que também localiza ao nível dos contactos inter-celulares que se formam entre as células filhas durante a divisão. Mostra-se também que o domínio HR1 de PRK2 é responsável por esta localização.

Uma vez que as células HeLa não são consideradas o melhor modelo para estudar a formação de junções epiteliais, a localização de PRK2 foi analisada nas células 16HBE e Caco-2 (duas linhas celulares que formam verdadeiras junções epiteliais). Usando estas células como modelo, foi possível verificar que

PRK2 localiza ao nível das junções apicais quando as células formam uma monocamada polarizada. No decorrer deste trabalho, um projecto paralelo do nosso grupo de investigação identificou PRK2 como uma proteína necessária à formação de junções apicais em células 16HBE, através do uso de RNA de interferência. Deste conjunto de resultados concluiu-se que a PRK2 é necessária à formação de junções epiteliais.

Por razões ainda não apuradas, a redução dos níveis intracelulares de PRK2 por RNA de interferência em células 16HBE, nao resulta na formação de células binucleadas, fenótipo que é obtido quando a mesma abordagem é efectuada nas células HeLa. Apesar de uma análise mais profunda desta observação ser necessária, este resultado sugere que PRK2 não é essencial à citocinese das células 16HBE. No entanto foi possível verificar que também em 16HBE, PRK2 localiza ao nível do sulco de clivagem e do corpo médio.

Publicações recentes apontam para o facto de que proteínas envolvidas no establecimento de junções epiteliais e na morfogénese epitelial são recrutadas para a zona do corpo médio durante a citocinese (Alford et al., 2009; Jaffe et al., 2008; Schluter et al., 2009). Esta poderia ser a razão pela qual PRK2 se localiza a este nível ainda que não contribua para a citocinese neste tipo de células. Com o objectivo de investigar mais detalhadamente a relação entre a citocinese e a formação de junções em tecidos epiteliais, a localização das proteínas ZO-1 e Scribble durante a citocinese foi analisada e verificou-se que ambas as proteínas se localizam ao nível do corpo médio. Este resultado sugere que a localização de proteínas envolvidas na formação de junções epiteliais ou no estabelecimento de polaridade intracelular é regulada durante o ciclo celular.

Com o objectivo de investigar se ZO-1 e Scribble, são, de forma semelhante a PRK2, também reguladas por fosforilação durante a mitose, a electroforese de ambas as proteína num gel de poliacrilamida foi analisada depois de um boqueio

das células em prometafase com nocodazazole. Verificou-se que a migração de ambas estas proteínas é especificamente alterada em prometafase, o que demonstra que estas proteínas são efectivamente reguladas por fosforilação durante a mitose.

O presente trabalho contribuiu com informação adicional à forma como PRK2, uma cinase efectora das Rho GTPases, é regulada durante a mitose. Foi também possível identificar uma nova função para PRK2 – a regulação da formação de junções epiteliais. Finalmente o presente trabalho apresenta observações que contribuem para a ideia recente de que a regulação de determinadas moléculas durante a citocinese é necessária para a formação de junções epiteliais. Propõe-se com o presente trabalho a existência de uma relação funcional entre a localização de PRK2 ao nível do sulco de clivagem e do corpo médio e ao nível das junções epiteliais. Propõe-se ainda que determinadas proteínas necessárias ao estabelecimento de junções epiteliais, sejam recrutadas para o sulco de clivagem ou corpo médio, de modo a facilitar a formação de junções epiteliais entre células filhas durante e depois da citocinese.

Finalmente apresenta-se um sistema de morfogénese epitelial em 3 dimensões – a formação de cistos de células Caco-2, como um modelo para investigar a formação de junções epiteliais durante e depois da citocinese e o papel de PRK2 neste processo.

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CHAPTER 1 - INTRODUCTION

1.1 Rho GTPases

Rho GTPases are signal transduction proteins that cycle between an active and an inactive state depending on whether they are bound to GTP or GDP, respectively. Rho GTPases act downstream of cell surface receptors and trigger multiple signaling pathways through their interaction with effector proteins. Over 100 Rho GTPase effector proteins have been identified and this includes regulators of the cytoskeleton, membrane trafficking, gene expression and enzymatic activities. Through the regulation of these many effectors, Rho GTPases control a wide range of essential cellular processes, such as cell proliferation and survival, morphology, polarity and movement (Figure 1.1).

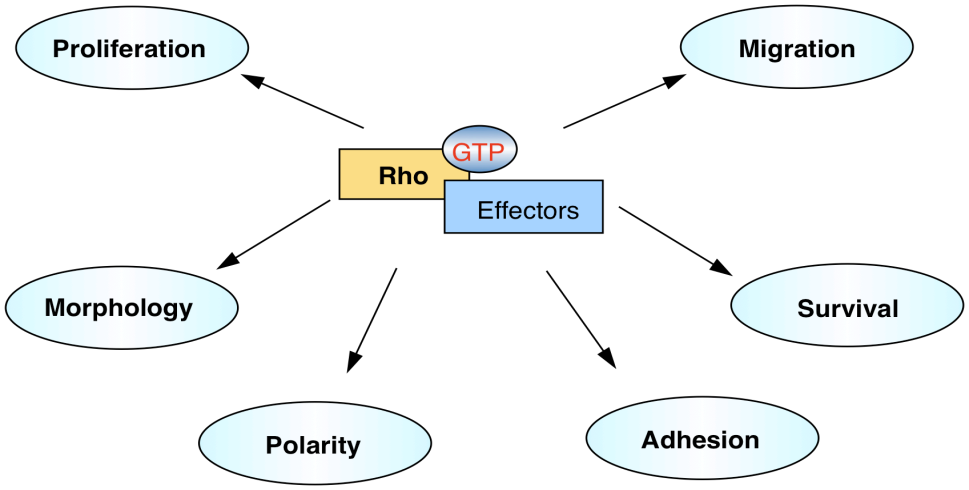


Figure 1.1 Rho GTPases control essential cellular processes. Through the interaction with effector proteins Rho GTPases regulate a wide range of cellular processes such as cell proliferation; morphology; polarity; adhesion to other cells, or to the substrate; survival and migration.

Rho GTPases constitute a family within the Ras superfamily of small GTPases that also includes the Ras, Rab, Arf, Ran and Miro families (Colicelli, 2004; Reis et al., 2009; Wennerberg et al., 2005) (see Figure 1.2-A). These groupings reflect both sequence and functional similarities. Traditionally, members of these families are linked preferentially to the control of a particular cellular function: Ras family members are closely linked to the control of cell proliferation and differentiation, Rabs and Arfs are master regulators of membrane trafficking, Ran is involved in nuclear transport and Miro-related proteins are associated with mitochondria. Rho GTPases are best known as key regulators of the actin cytoskeleton (Colicelli, 2004).

The Ras superfamily of proteins are characterized by the presence of conserved domains, called G boxes (G1-G5) at their N terminus that form the G domain. The G-domain is responsible for binding to GTP or GDP. The Rho family members share high homoloy in their G1 to G5 boxes and they also contain a distinct insert sequence, between the G4 and the G5 boxes, that is not found in other Ras superfamily members and is involved in binding to some effectors and regulators (Colicelli, 2004; Freeman et al., 1996). Another structural characteristic of Ras-related GTPases is the presence of a CAAX C-terminus motif in which C is a cysteine, A, an aliphatic amino acid residue and X any amino acid. The CAAX motif together with other residues at the hypervariable C-terminal region serve as a signal for post-translational modifications, including lipid modification which is essential for correct membrane localization (Wennerberg et al., 2005)(Wennerberg et al., 2005).

Rho GTPases are well conserved throughout the evolution and members of this family are found in yeast, plants, worms, flies and mammals. In humans, 20 Rho GTPases have been identified so far: three Rho isoforms (A, B and C); three Rac isoforms (1, 2 and 3); Rho G; Cdc42; TC10\RhoQ; TCL\RhoJ, Chp\RhoV, Wrch-1\RhoU; Rho H; Rho BTB1, Rho BTB2, Rnd1, Rnd2; Rnd3/Rho E; RIF\RhoF and

Rho D (Heasman and Ridley, 2008; Jaffe and Hall, 2005). Of all these members, by far the best studied are Rho, Rac and Cdc42 (see Figure 1.2).

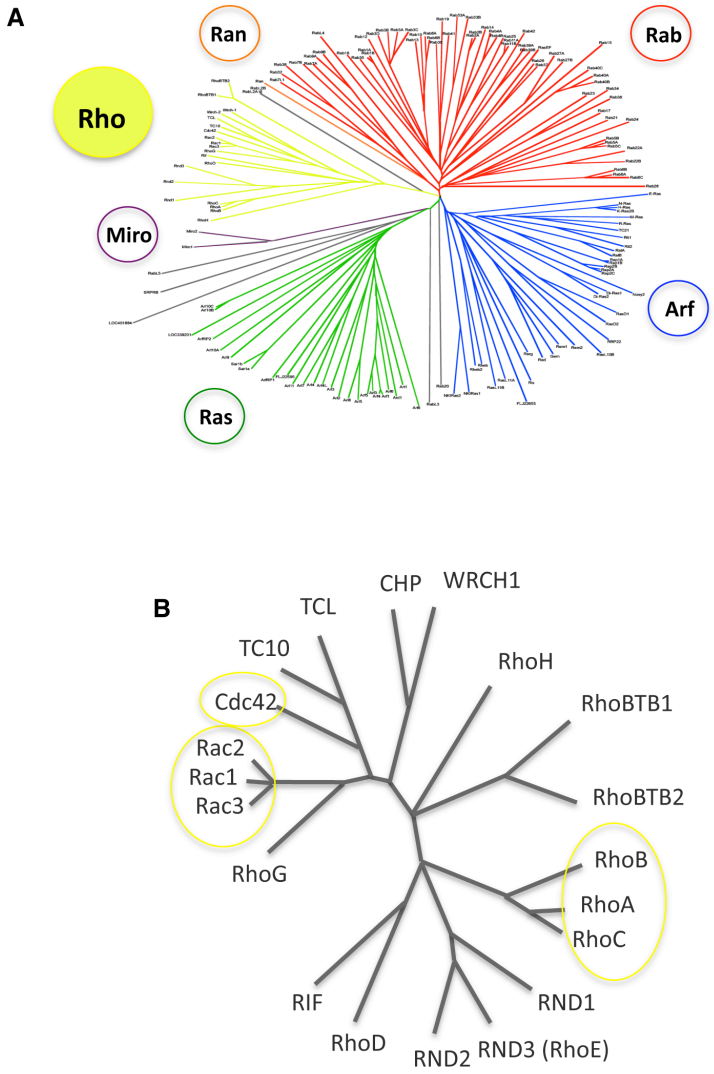


Figure 1.2 The Rho family of small GTPases. Rho GTPases form a family within the Ras superfamily of small GTPases that also include the Ran, Rab, Arf, Ras and Miro families (A). There are 20 human Rho GTPases, from which the by far best studied are Rho, Rac and Cdc42 (B). (Adapted from Wennerberg et al., 2005 (A) and Heasman and Ridley, 2008 (B)).

1.2 Regulation of Rho GTPases

Rho GTPases signal by activating effector proteins and their interaction with such effectors is regulated in several ways. First, there is the GTP versus GDP loading state, which is associated with conformational changes in the GTPases that regulate their ability to bind to the effectors. Secondly, it is thought that correct localization of both the GTPases and their effectors to distinct membranes is important and regulated. Finally, Rho GTPases can be phosphorylated and ubiquitinated, although the way these post-translational modifications affect Rho GTPase activity is not well understood (Jaffe and Hall, 2005).

1.2.1 The GTP/GDP cycle – GAPs, GEFs and GDIs

When bound to GTP, Rho GTPases acquire an active conformation that allows their direct physical interaction with effector proteins. In the GDP-bound state they are not able to interact with effectors and are considered to be inactive. Three classes of protein are involved in regulating the GTP/GDP cycle: GEFs, GAPs and GDIs. GEFs (Guanine Exchange Factors) promote the release of GDP allowing association with GTP, and are considered to be positive regulators of Rho family proteins. GTPases have an intrinsic GTP/GDP exchange activity, although this is slow. GEFs catalyze the dissociation of GDP allowing binding of GTP, which is present in cells at a much higher concentration than GDP (Rossman et al., 2005; Schmidt and Hall, 2002). GAPs (GTPase activating proteins) are negative regulators of Rho GTPases, they stimulate the otherwise intrinsic slow GTPase activity associated with most members of the Rho family (Bernards and Settleman, 2004). It is though that inhibition of GAPs may be a mechanism of Rho GTPase activation in response to various stimuli. Finally, there is a third class of protein that regulates Rho proteins, the GDIs (Guanine nucleotide dissociation proteins). GDIs are also negative regulators of Rho

GTPases and act in several ways: they bind to the GDP-bound form of Rho GTPases and inhibit spontaneous GDP dissociation, they prevent GTPases from interacting with membranes and they block GEF activity (DerMardirossian and Bokoch, 2005) (Figure 1.3).

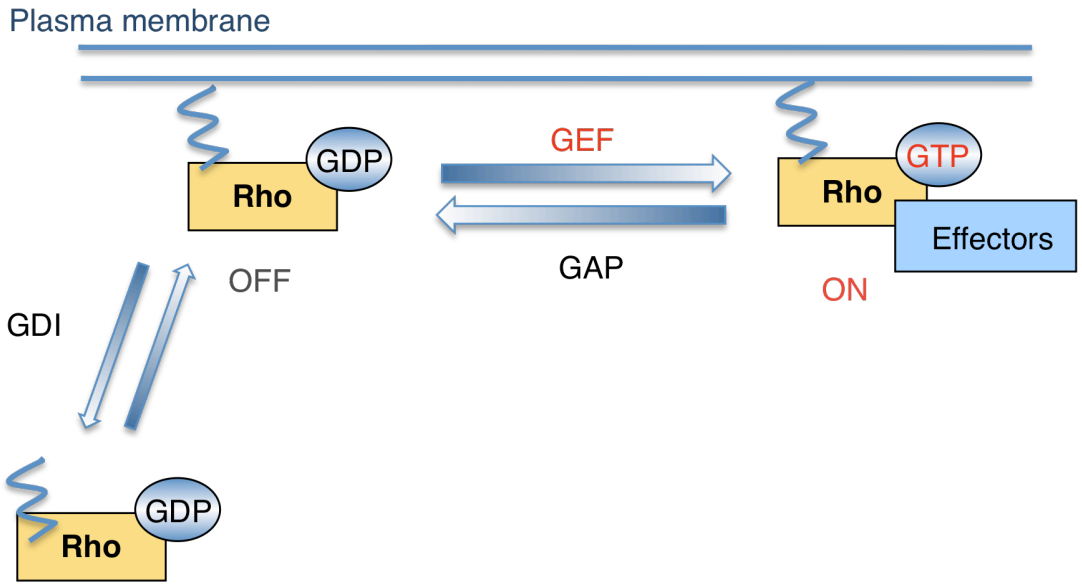


Figure 1.3 Regulation of Rho GTPases. Rho GTPases cycle between an active (GTP-bound) and an inactive (GDP-bound) state. When bound to GTP they interact with effector proteins. Three classes of proteins regulate this cycle: GEFs, GAP and GDIs. Rho GEFs promote the GTP-bound state of Rho GTPases and activate them, while Rho GAPs act by accelerating the intrinsic GTPase activity of the GTPases, leading to inactivation. Rho GDIs negatively regulate Rho GTPases as they associate with the lipid modifications and keep GTPases away from membranes.

Some members of the Rho family are “atypical” and are though not to have a GTP/GDP cycle, but to remain predominantly in the GTP-bound state. These proteins have amino acid substitutions in residues required for GTP hydrolysis or tight nucleotide binding. Atypical Rho GTPases are Chp and Wrch-1, RhoH, the Rnds, RhoBTB1 and RhoBTB2 and they are though not to be regulated by GEFs

or GAPs. Atypical Rho GTPases have been considerably less studied than other members of the family (Heasman and Ridley, 2008).

An interesting observation concerning Rho GTPase regulators and downstream effectors is that they clearly outnumber the Rho GTPases themselves. In humans there are 82 Rho GEFs, 67 Rho GAPs, 3 Rho GDIs, and, as already mentioned, more then 100 Rho effectors. Multiple GEFs and GAPs clearly regulate the same GTPase and each Rho GTPase is capable of activating multiple downstream effectors. This raises the question of how specificity is achieved. One possibility, is that GEFs could, through their location and/or through protein-protein interactions, influence which effectors are engaged by the GTPase upon activation (Schmidt and Hall, 2002).

1.2.2 Localization – lipid modification and GDIs

Rho GTPases are synthesized in the cytosol and then undergo post-translational lipid modifications at their CAAX motifs (Allal et al., 2000; Hori et al., 1991; Mohr et al., 1990). Expression studies using Rho GTPases tagged with the green fluorescence protein (GFP) have shown that these proteins localize at the cytoplasm, at the plasma membrane and on endomembranes. For example, RhoA and Rac1 are found in the cytosol and the plasma membrane, while RhoB localizes predominantly at the Golgi and on endosomes (Roberts et al., 2008). It is believed that the cytoplasmic pool is mainly GDP-bound and inactive, while the activated GTP-bound form of the Rho GTPases localizes predominantly at the membranes and this is regulated by Rho GDIs. Rho GDIs associate with the lipid modification of the GDP-bound Rho GTPases forming a cytosolic complex. This allows Rho proteins to be recycled from membranes. Although there are reports of GDIs being able to bind to GTP-bound Rho GTPases, it seems they interact preferentially with the GDP-bound forms, consequently Rho-GDP is found in the

cytosol and activated Rho-GTP is associated with membranes. The specific signals that regulate the Rho GDI:GTPase interaction are not well characterized, although protein and lipid binding, as well as phosphorylation of both GDI and the GTPase have been reported to be involved (DerMardirossian and Bokoch, 2005).

1.3 Rho GTPase effector proteins

Effectors are proteins that interact specifically with the GTP-bound form of the GTPases. So far more than 100 Rho GTPase effectors have been identified and these include protein and lipid kinases, phosphatases, lipases, oxidases and scaffold proteins. The binding sites on effectors are diverse. Rho GTPase specificity towards the effectors is also quite complex, some effectors seem to bind to a single member of the Rho family, while others bind to multiple Rho GTPases (Bishop and Hall, 2000).

1.3.1 Identification of the effectors

The search for proteins that preferentially associate with the GTP-bound form of Rho GTPases was carried out primarily through biochemical means, either using affinity chromatography or yeast two hybrid screens. The scaffold protein rhophilin, for example, one of the first Rho effectors to be identified, was pulled out from a yeast two-hybrid screen developed using as bait constitutively GTP-bound RhoA (Watanabe et al., 1996). Around the same time Protein Kinase N (PKN or PRK1) and also Rho kinase (ROCK), were isolated from bovine brain extracts using affinity chromatography, in which glutathione-S-transferase (GST) fused with GTP-γ-S-Rho (GTP-γ-S is a non-hydrolyzable GTP analog), or (as a

control) GDP-RhoA, were immobilized on glutathione sepharose columns. (Amano et al., 1996; Matsui et al., 1996).

1.3.2 Rho GTPase:effector interactions

No single recognizable binding domain characterizes all Rho GTPase effectors, although some do share conserved motifs. Several Rac and Cdc42 effector proteins harbor a conserved Rac/Cdc42 binding motif called CRIB (Cdc42\Rac-interactive binding). CRIB motifs are found in kinases such as activated Cdc42-associated tyrosine kinase (ACK), p-21 activated kinase (PAK) and some Map Kinase Kinase Kinases (MEKKs), as well as in scaffold proteins, such as the Wiskott-Aldrich-syndrome protein (WASP) (Bishop and Hall, 2000; Burbelo et al., 1995). Among the effectors for Rho, there are some that share a certain level of homology in their Rho-binding domains. The REM (Rho effector homology) domain, characterized by a poybasic region followed by a leucine-zypper-like motif, is found in Rho effectors such as PRK, rhophilin and rhotekin, while the RKH (ROCK-kinectin homology) motif, is found in ROCK, citron kinase and kinectin (Bishop and Hall, 2000). A crystal structure of the REM of PRK1 bound to Rho is available and it shows that this interaction is quite different from the way the CRIB peptides of ACK or WASP bind to Cdc42 (Maesaki et al., 1999).

Structural differences between the GTP- and GDP-bound states of Rho GTPases are not extensive and are mainly restricted to two segments called switch region I and II, which localize around Gbox 2. The conformational change that occurs after GTP hydrolysis can be best described as a loaded spring mechanism: in the GTP-bound form two amino acid residues, one in each switch region, bind to the GTP's γ-phosphate. When GTP hydrolysis occurs it allows the two switch regions to relax and adopt a different conformation (Vetter and Wittinghofer, 2001). Effector proteins interact preferentially with the switch regions and must

recognize the difference between the GTP- and GDP-bound forms of the GTPases.

Other regions, outside switch I, are also involved in Rho GTPase binding to certain effectors. For example two amino acids in loop 6 (just C-terminal to Switch II), Asp^{87} and Asp^{90} are important for Rho binding to both ROCK and PRK2 (Zong et al., 1999). The insert region, encompassing residues 124-135, which is present in the Rho family but not in members of the Ras family seems to be also important for effector binding. Point mutations in, or deletion of these residues affect NADPH oxidase activation by Rac, for example. Interestingly these mutations have no effect on another effector interactions such as PAK. In another study it has been shown that a region close to the C-terminus of Rac (amino acids 143-175) is important for both NADPH and PAK activation (Diekmann et al., 1995; Freeman et al., 1996). It is clear that different effectors interact with different regions of the GTPases and that this likely provides the specificity for GTPase/effector interactions.

Even within the switch regions, effectors interact with different residues. For example, a Tyr to Cys mutation at codon 42 in Rho blocks its interaction with PRK1, but not with ROCK, while a Phe to Leu mutation at codon 39 blocks Rho interaction with ROCK but not PRK1 (Sahai et al., 1998). As point mutations within the switch1 region of Rho, Rac and Cdc42 can differentially disrupt their interaction with various downstream targets, this strategy has been used to investigate which effector is responsible for distinct downstream signaling pathways.

1.3.3 Regulation of effector proteins by Rho GTPases

The most common mechanism for effector activation by Rho GTPases appears to be the disruption of intramolecular autoinhibitory interactions (see Figure 1.4-A). Many Rho GTPase effectors, in particular kinases, contain intramolecular autoinhibitory domains that are displaced upon interaction with the corresponding GTPase. Examples include type I PAK and ROCK. The partial crystal structure of PAK1 in an autoinhibited conformation has been determined and suggests that PAK1 exists as a homodimer in its basal inactive state. The protein is in a *trans*-inhibited conformation where, the N-terminal regulatory domain of one PAK1 molecule binds and inhibits the C-terminal catalytic domain of the other. The dimerization interface overlaps the Rac/Cdc42 binding domain and this structural data together with genetic and biochemical evidence support a model in which GTPase binding disrupts dimerization and leaves the kinase domain free to interact with its substrates (Bokoch, 2003; Lei et al., 2000; Parrini et al., 2002). For the ROCK isoforms there are no equivalent structural studies available, but the effect of overexpressing protein mutants lacking either the regulatory or the kinase domain support the idea that these proteins would adopt an autoinhibited configuration. The kinase domain of ROCK, located at the N-terminus, and the C-terminal Rho-binding domain can interact with each other in vitro. In vivo, mutants that lack the C-terminal region act as constitutively active forms, whereas kinase deficient mutants function as dominant negatives. In addition, it has been shown that, when overexpressed, ROCK containing mutations at the C-terminus that disrupt Rho binding, interacts with endogenous ROCK (Amano et al., 1997). These data suggest that ROCK is able to adopt an autoinhibited conformation and that Rho acts to disrupt this autoinhibition.

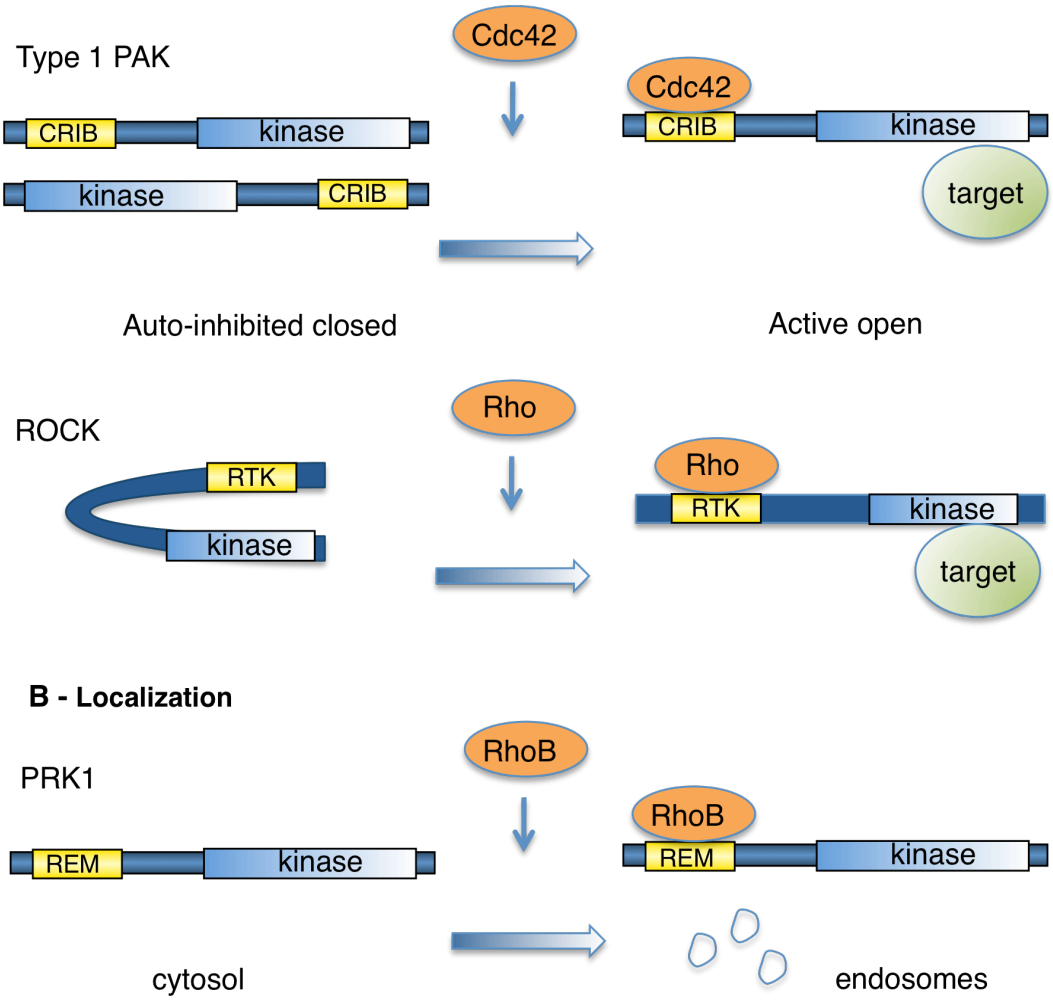


Figure 1.4 Mechanisms of activation of effectors by Rho GTPases. No single recognizable binding motif characterizes all Rho GTPase effectors although some do share conserved motifs such as the CRIB (Cdc42\Rac-interactive binding), the RKH (ROCK-kinectin homology) and the REM (Rho effector homology). The most common mechanism for effector activation by Rho GTPases appears to be the disruption of autoinhibitory configurations (A). This is believed to be the way Rac and Cdc42 activate type I PAK and Rho activates ROCK. PAK1 exists as a homodimer in which the N-terminal of one PAK1 binds and inhibits the C-terminal catalytic domain of the other. The dimerization interface overlaps with the Cdc42 and Rac binding domain (CRIB) and it is believed that GTPase binding disrupts dimerization and leaves the kinase domain free to interact with its substrates. ROCK and PRKs are also believed to be regulated in this way, however it is not known whether these proteins would also dimerize or whether the autoinhibition is intramolecular (A- second panel). Finally Rho GTPases can also affect effector localization. For example RhoB has been shown to mediate the translocation of PRK1 into endosomes (B).

The relief of autoinhibitory configurations by Rho GTPase is also a common mechanism of activation of targets which are not kinases, for example the scaffold-like proteins that are involved in actin polymerization, such as mammalian diaphanous (mDia), WASP and N-WASP (Bishop and Hall, 2000).

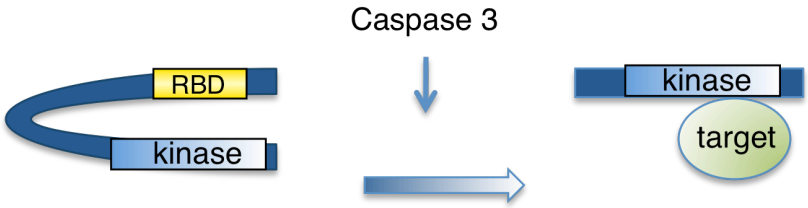
Another way in which Rho GTPases can regulate the activity of their effectors is through localization, either to a particular sub-cellular compartment, or to a specific protein complex. This is believed to be the way that Rho GTPases regulate some adaptor proteins, but also some kinases. For example, binding of Cdc42-GTP to Type II PAKs (also called non-conventional PAK), does not stimulate kinase activity, but rather results in the translocation of the kinase to the Golgi apparatus (Abo et al., 1998) (Figure 1.4 - B).

1.3.4 GTPase-independent regulation of effector proteins

Rho GTPase effectors can be regulated independently of Rho GTPases. For example, PAK2 is cleaved by caspase-3 during apoptosis to generate a constitutively active kinase. Activation of PAK2 in this way is thought to regulate morphological changes seen in apoptotic cells (Rudel and Bokoch, 1997). Effector localization may be also regulated independently of GTPases. Most Rho GTPase effectors contain additional functional motifs, including other protein-protein or protein-lipid interaction domains. PRK2 and PRK3, WASP and PAKs all contain proline-rich motifs which interact with SH3 domain-containing proteins such as Nck. Nck is an adaptor protein that also contains an SH2 domain which as been implicated in recruiting PAK1 to the plasma membrane. Interestingly, myristoylation of the SH3-containing motif of Nck or myristoylation of PAK1, are both sufficient to activate the kinase in the absence of Cdc42. Another group of SH3 domain-containing proteins that is important in localizing PAK is the

Cdc42/Rac GEF, PIX (α-PIX and β-PIX). In this way, a GEF (PIX), a GTPase (Rac) and an effector (PAK) form a signaling complex (Zhao and Manser, 2005). Protein-lipid interactions are important in regulating some effectors. The kinase activities of both PRK1 and ROCK, for example, are stimulated by lipids such as unsaturated fatty acids (Feng et al., 1999; Kitagawa et al., 1995). Finally, several Rho GTPase effectors, in particular kinases are regulated by phosphorylation events (Zhao and Manser, 2005) (see Figure 1.5) .

A – Cleavage



B – Interaction with proteins and lipids and post-translational modifications

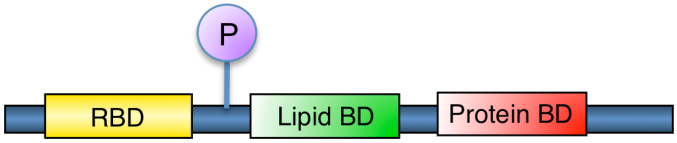


Figure 1.5 GTPase-independent mechanisms of effector regulation. Rho GTPase effectors can be regulated by Rho-GTPase independent ways. For example PAK2 is cleaved by caspase-3 during apoptosis to generate a constitutively active kinase (A). Furthermore, most Rho GTPase effectors contain other functional domains in addition to the Rho Binding domain, such as lipid or protein binding domains that are involved in the regulation of their activity and/or localization (B). Finally, effectors are also regulated by post-translational modifications such as phosphorylation events (B). RBD (Rho binding domain), BD (binding domain).

1.4 Cellular functions of Rho GTPases

1.4.1 Experimental manipulation

Early indications of the cellular function of Rho GTPases came from overexpression studies using constitutively active and dominant negative versions of the proteins, as well as from the use of bacterial toxins that block Rho GTPase activity. Amino acid substitutions of valine for glycine at codon 14, or leucine for glutamine at codon 63 in Rho (RhoV14 and RhoL63 mutants), render the protein constitutively active by preventing intrinsic and GAP-mediated GTP hydrolysis (Bishop and Hall, 2000). Similar corresponding substitutions have been made in Rac (RacV12, RacL61) and Cdc42 (Cdc42V12, Cdc42L61). A substitution of asparagine for threonine at codon 19 creates a dominant negative version of Rho (RhoN19) that binds very strongly to GEFS, but is not able to interact with effectors. Similar substitutions have been made in Rac (RacN17) and Cdc42 (Cdc42N17). These mutants are thought to compete with endogenous Rho by sequestering cellular GEFs. Overall, these dominant negative proteins appear to be rather specific, although a potential problem is that many GEFs can interact with more than one Rho family proteins. (Bishop and Hall, 2000; Feig, 1999).

Bacterial proteins such as toxin B or the C3 transferase exoenzyme, from *Clostridium dificile* and *Clostridium botulinum*, respectively, have also been used to analyze the cellular functions of Rho GTPases. Both toxins act by interfering with effector binding. Toxin B inactivates most, if not all Rho family GTPases, while the C3 transferase exoenzyme is specific for RhoA, RhoB and RhoC (Genth et al., 2003; Just et al., 1995).

1.4.2 Rho GTPases regulate the actin cytoskeleton

The first cellular function to be attributed to Rho GTPases was to mediate the effect of growth factor receptors on the actin cytoskeleton (see Figure 1.6). In pioneering studies, the microinjection of recombinant Rho (constitutively active) into Swiss 3T3 fibroblasts was seen to promote the formation of contractile actin-myosin filaments (stress fibers) and focal adhesions (regions where stress fibers are attached to the plasma membrane and where the cell adheres most tightly to the substrate) (Ridley and Hall, 1992). At the same time the microinjection of Rac (constitutively active) produced actin-rich, peripheral protrusions (lamellipodia) (Ridley et al., 1992). It was also shown that endogenous Rho and Rac could be activated by the addition of extracellular ligands. For example lysophosphatidic acid (LPA) activates Rho, while platelet derived growth factor (PDGF), or insulin, activates Rac. It was concluded that these Rho family GTPases, act as molecular switches to control signaling transduction pathways that link membrane receptors to the actin cytoskeleton. Later, similar experiments with Cdc42, further supported the conclusion that Rho GTPases are important regulators of the actin cytoskeleton. Overexpression of Cdc42 (wild type or constitutively active) promotes the formation of actin-rich finger-like membrane extensions termed filopodia (Nobes and Hall, 1995).

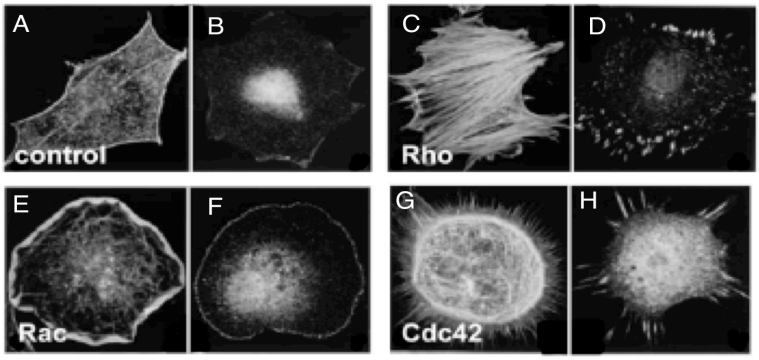


Figure 1.6 Rho, Rac and Cdc42 promote the assembly and organization of the actin cytoskeleton. Quiescent, serum-starved Swiss 3T3 fibroblasts contain very few stress fibers (A) or vinculin-containing integrin adhesion complexes (B). Activation of Rho leads to stress fiber (contractile actin myosin filaments) (C) and focal adhesion formation (D). Microinjection of constitutively active Rac induces lamellipodia (actin-rich peripheral protrusions) (E) and associated adhesion complexes (F). Activation of Cdc42, leads to formation of filopodia (actin rich finger like membrane extensions) (G) and the associated adhesion complexes (H). In (A), (C), (E), and (G), actin filaments were visualized with rhodamine phalloidin; in (B), (D), (F), and (H), the adhesion complexes were visualized with an antibody to vinculin (Hall, 1998).

Since these first reports many studies have supported and extended these conclusions in other mammalian cell types, but also in model organisms such as yeast, *Drosophila* and *C. elegan*s. Rho GTPases were shown to be essential for the control of most, if not all cellular behaviors that require the assembly and reorganization of actin filaments (Etienne-Manneville and Hall, 2001; Hall, 1998). Examples of such processes are: 1) Cell shape determination. Axonal extension for example, a process driven by actin polymerization requires both Rac and Cdc42 (Luo, 2000). Another example of a role for Rho GTPases in establishing and maintaining cell shape is during epithelia morphogenesis as will be discussed later. 2) Cell movement. Macrophages move in response to chemotactic factors (macrophage chemotaxis) and this requires polymerization of actin and the formation of lamellipodia and filopodia protrusions. A dominant negative form of Rac or Cdc42 was shown to block lamellipodia and filopodia, respectively, during macrophage chemotaxis (Allen et al., 1997). 3) Cell division.

Cytokinesis, the physical separation of a dividing cell into two daughter cells at the end of mitosis is driven by the contraction of a ring formed by actin and myosin. Rho is an important regulator of actin polymerization and actin-myosin contraction and is required for cytokinesis in animal cells (Piekny et al., 2005) (see Figure 1.7).

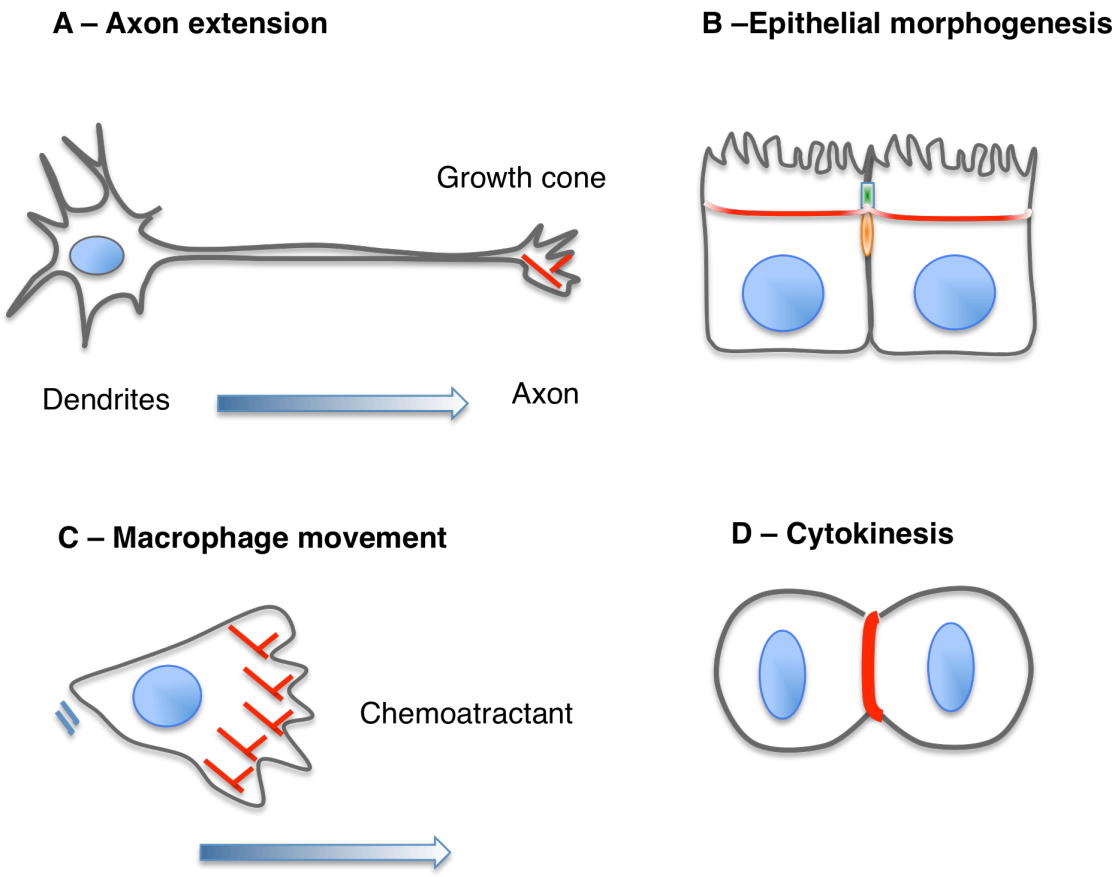


Figure 1.7 Cellular processes regulated by Rho GTPases and the actin cytoskeleton. Rho GTPases are essential for the control of most, if not all cellular processes that require the assembly and reorganization of the actin cytoskeleton. Examples of these processes are: Axon extension (A), epithelial morphogenesis (B), macrophage chemotaxis (C) and cytokinesis (D). Filopodia and lamellipodia protrusions within the growth cone drive axonal extension (A). Actin polymerization and contraction is required for the formation of polarized epithelial cells (figure shows actin circumferential belt that forms in polarized epithelial cells) (B). Macrophage movement in response to chemotactic factors requires the formation of filopodia and lamellipodia at the front of the cell. Finally, cytokinesis is driven by the contraction of an actin myosin ring (D). Actin, red; nucleus, blue; tight junctions green; adherens junctions orange.

1.4.2.1 Actin polymerization – Formins, WASP and WAVE

Actin polymerization in eukaryotic cells occurs through the activity of two major types of actin polymerization factor, the Arp2/3 complex and the formins. They can both nucleate actin filament formation and promote polymerization, but use very different mechanisms that generate different actin structures. It is generally accepted that Rac promotes the protrusion of lamellipodia at the plasma membrane by activating Arp2/3, whereas Rho regulates stress fibers formation by activating formins. Cdc42 is thought to activate both Arp2/3 and formins to generate filopodia. The Diaphanous family of formins is direct Rho GTPase effectors, while polymerization mediated by the Arp2/3 complex is regulated by members of the Wiscott-Aldrich syndrome protein (WASP) family (see Figure 1.8).

Formins

The mammalian diaphanous related formin (mDia1-3) are RhoA effector proteins (Watanabe et al., 1997) and are responsible for actin polymerization in mammalian cells. They have a characteristic formin homology domain 2 (FH2) which is necessary and sufficient to nucleate an actin filament *in vitro* (Zigmond, 2004). They also have a characteristic autoinhibitory domain and a Rho binding domain that interact with each other. Activation of mDia1 by Rho GTPases occurs through the relief of this autoinhibitory interaction. (Li and Higgs, 2003). Typically formins nucleate from actin monomers and create linear filaments such as those found in stress fibers, or the contractile ring at cytokinesis. Formin-induced actin structures can also undergo contraction when associated with myosin II (Zigmond, 2004).

WASP, N-WASP and WAVE

The WASP-family of proteins consists of WASP, neuronal wasp (N-WASP) and the WASP-family verprolin homologous protein (WAVE), also known as Scar. WASP was originally identified as a protein altered in patients with Wiskott–Aldrich Syndrome, a genetic immunodeficiency disorder characterized by reduced motility of lymphoid immune cells. WASP and N-WASP bind *in vitro* to Cdc42, while WAVE acts downstream of Rac, but it does not interact directly with the GTPase. WASP and WAVE also interact with Arp2/3 subunits through a C-terminal domain. *In vitro*, purified WASP and WAVE stimulate the actin filament nucleation activity of the Arp2/3 complex and actin polymerization can be reconstituted using purified Cdc42, N-WASP, Arp2/3 and acidic phospholipids. In vivo, overexpression of WASP or N-WASP induces ectopic actin polymerization that is inhibited by dominant negative mutants of Cdc42, but not Rac or Rho. The model for direct WASP activation by Cdc42 is again the relief of an autoinhibitory interaction, although Cdc42 might be also involved in regulating WASP indirectly through the action of another effector called Toca (transducer of Cdc42-depedent actin assembly) (Jaffe and Hall, 2005; Millard et al., 2004; Mullins, 2000).

1.4.2.2 Actin-myosin contraction – ROCK

Actin myosin contraction is mediated by the sliding of myosin II along actin filaments. Two substrates for ROCK are likely to be key players in regulating this process: the myosin light chain subunit (MLC) of myosin and the myosin binding subunit of MLC phosphatase. Myosin is an ATPase and uses its ATPase activity to move along actin filaments, an event that is stimulated upon myosin II binding to actin. Interaction of myosin II with actin is regulated by phosphorylation of the regulatory myosin light chain subunit, mainly on Ser19. ROCK phosphorylates MLC at Ser19 and therefore promotes the assembly and contraction of the actin-

myosin filaments. In addition ROCK also phosphorylates and inactivates MLC phosphatase, which further increases the MLC phosphorylation and contractility (Riento and Ridley, 2003).

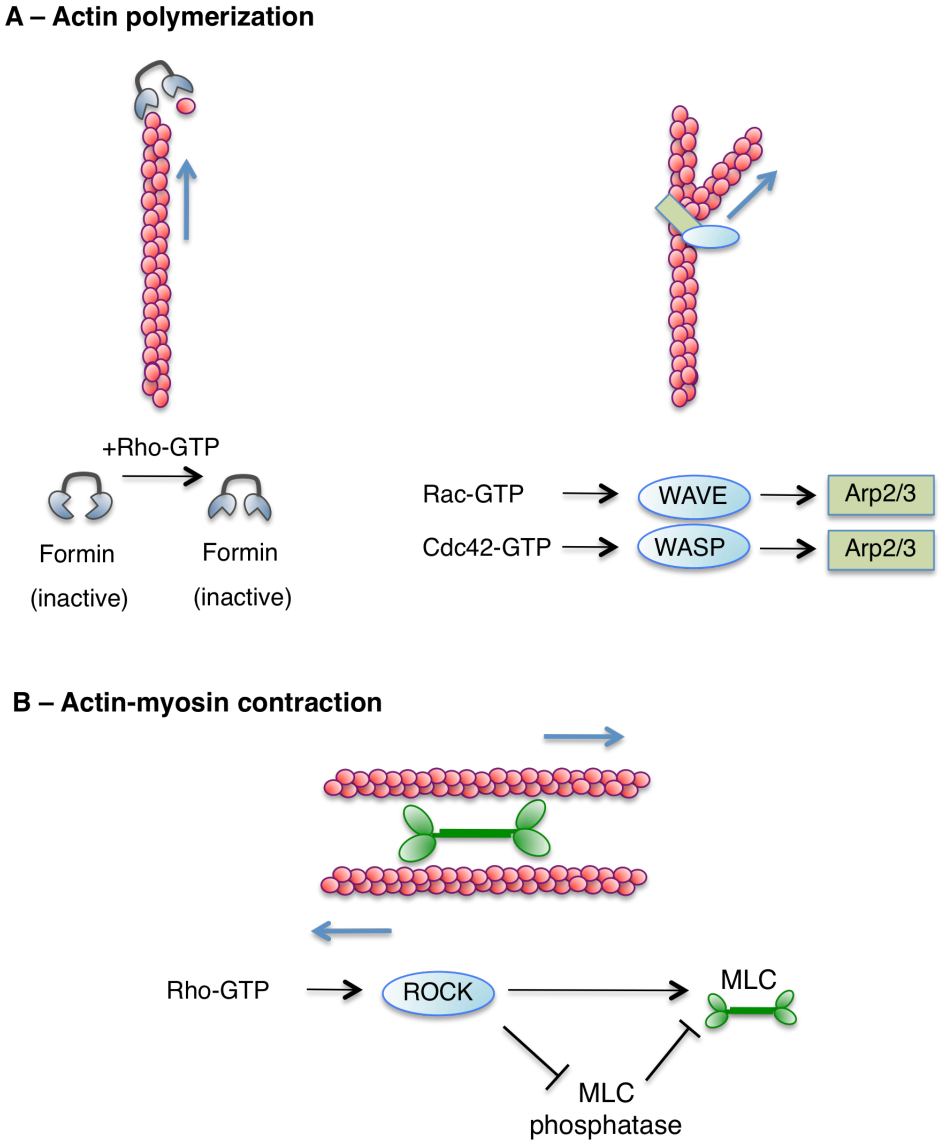


Figure 1.8 Regulation of actin filament assembly and contraction by Rho GTPases. Actin polymerization in eukaryotic cells occurs through the activity of two major types of actin polymerization factors: formins and the Arp2/3 complex (A). Rho binds directly to formins and activates them to promote linear elongation of actin filaments (A-left). Rac and Cdc42 activate Arp2/3 via WAVE and WASP, respectively, to initiate a branched filament network (A-right). Finally, Rho regulates actin-myosin contraction by activating ROCK, which both directly phosphorylates the myosin light chain subunit of myosin, or phosphorylates and inhibits myosin phosphatase (B). (Adapted from Jaffe and Hall, 2005).

1.4.3 Rho GTPases regulate more than the actin cytoskeleton

Given the large number of effectors of Rho GTPases (~100), it is not surprising that these proteins regulate other cellular activities, in addition to actin filaments assembly. They have been shown to participate in the establishment of cell polarity, the regulation of microtubule dynamics, gene transcription, G1 cell cycle progression, vesicular transport pathways and a variety of enzymatic activities ranging from NADPH oxidase activity in phagocytic cells to glucan synthase in yeast. (Etienne-Manneville and Hall, 2001; Heasman and Ridley, 2008; Jaffe and Hall, 2005). (Figure 1.9)

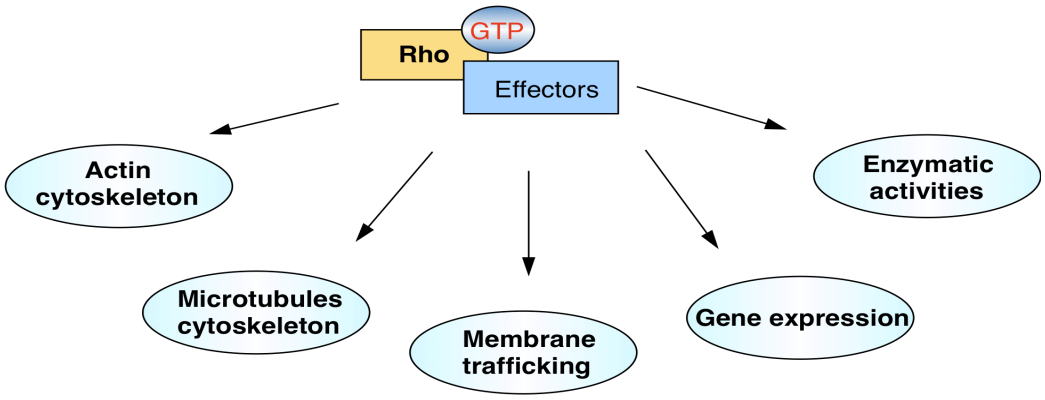


Figure 1.9 Fundamental cellular processes regulated by Rho GTPases and their effectors. Through the interaction with effectors Rho GTPases regulate several essential cellular processes in addition to actin cytoskeleton reorganization. Examples of these are the organization of the microtubules cytoskeleton, membrane trafficking, gene expression and enzymatic activities.

1.5. Rho GTPases and the cell cycle

The eukaryotic cell cycle consists of a DNA replication phase (S) and a nuclear/cell division phase (M), separated by two GAP phases (G1 and G2). Sequential series of biochemical steps regulate the ordered progression through the distinct cell cycle phases and numerous molecular checkpoints ensure that one is completed before the next starts. The main regulators of the cell cycle are the cyclin-dependent kinases (Cdks). Cdks are regulated by the association with cyclins and by phosphorylation and dephosphorylation events. In mammals there are four cyclins (A, B, D and E) and their levels inside the cell vary between different cell cycle stages. cyclinD/Cdk4 and cyclinD/Cdk6 control G1 progression, cyclin E/Cdk2 is required both before and after the G1/S transition, and cyclinA/Cdk1 and cyclinB/Cdk1 complexes regulate mitosis.

Rho GTPases play roles at several stages during cell cycle progression. It is well established that they influence the expression of cyclinD1 during G1 to S phase progression and the microtubule and actin cytoskeleton during mitosis. In particular, Rho regulates cytokinesis (see Figure 1.10).

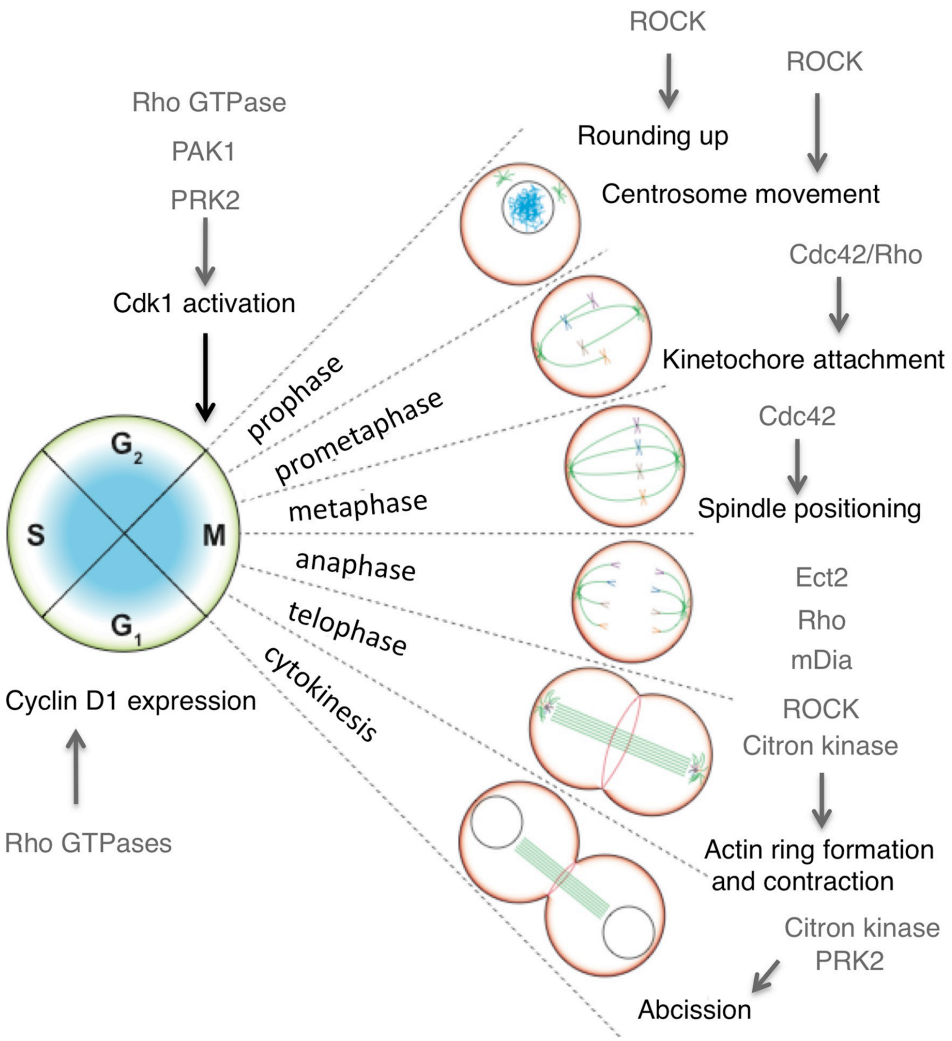


Figure 1.10 Rho GTPases and the cell cycle. Rho GTPases and effectors regulate several events during cell cycle progression. These include: the expression of cyclin D1 during G1 to S transition; Cdk1 activation at G2 to M progression; cell rounding up and centrosome movement during prometaphase; kinetochore attachment and spindle positioning during prometaphase and metaphase, respectively, and actin ring formation and contraction, as well as abcission, during cytokinesis. Some examples of the players in each process are referenced. Microtubules. green; actin, red; condensed chromosome, blue. (Adapted from Jaffe and Hall, 2005).

1.5.1 G2/M progression

During the G2/M transition cells undergo dramatic morphological and biochemical changes in preparation for cell division. The most prominent changes are chromosome condensation, centrosome maturation and separation, and cell rounding. Cdk1 in complex with its activating cyclins, cyclin A and cyclin B, is the main activator of the mitotic events. Essential for initiating mitosis is the dephosphorylation and activation of Cdk1 at tyr15 an thr14 by members of the Cdc25 family of protein phosphatases, which are themselves activated by phosphorylation (Karlsson-Rosenthal and Millar, 2006). Several kinases can phosphorylate the Cdc25 family of phosphatases, including Cdk1 itself, in a positive feedback loop, AuroraA, Polo-like kinase, and Chk-1. It is believed that Cdk1 is first activated at the centrosome through Aurora-A (Hirota *et al.*, 2003). Several molecules have been implicated in the centrosomal activation of Aurora-A, notably the Rac and Cdc42 effector PAK1 (Zhao *et al.*, 2005).

Inactivation of Rho GTPases by toxin B treatment has been shown to delay mitotic entry by 2 hours. Furthermore, PAK1 activation at the centrosome was also delayed. These results suggest that Rho GTPases function at the G2/M transition of mammalian cells by mediating multiple signaling pathways converging on the centrosomal activation of Aurora-A (Ando et al., 2007). A second Rho GTPase effector that has been implicated in G2/M transition is PRK2. Depletion of PRK2 by RNAi in HeLa S3 cells induces a similar phenotype to toxin B, namely a 2 to 4-hour delay in G2/M progression (Schmidt et al., 2007).

1.5.2 Mitosis

Mitosis consists of the separation of the sister chromatids and the formation of two daughter cells. This is precisely regulated, both spatial and temporally, by the microtubule and actin cytoskeleton. Rho GTPases have been implicated in the regulation of numerous mitotic events. The rounding up of cells at the onset of mitosis, for example, involves cortical retraction and is likely to be driven by Rho and ROCK (Maddox and Burridge, 2003).

Before nuclear envelope breakdown (prophase), centrosomes move to each pole of the cell. This process requires the activity of the astral microtubules, but also cortical myosin II and ROCK (Rosenblatt et al., 2004). Once the centrosomes are properly localized, nuclear envelope breakdown occurs and the spindle microtubules invade the nucleus space. Attachment of the spindle microtubules to kinetochores has been reported to be regulated by Rho GTPases, at least in some cell lines. Cdc42 appears to regulate this event in both HeLa cells and *Xenopus* egg extracts. Interestingly in Rat2 cells spindle attachment is mediated by Rho (not Cdc42) (Narumiya and Yasuda, 2006).

Finally, Rho GTPases can regulate the positioning of the mitotic spindle. The alignment of chromosomes during metaphase is driven primarily by the spindle microtubules, which emanate from the centrosomes, while elongation and shortening of this set of microtubules defines spindle positioning. Asymmetric positioning of the spindle gives rise to daughter cells of different sizes and with different cell fates. This process has been analyzed extensively in the first cell division of the *C. elegans* zygote and both Rho and Cdc42 play essential roles in spindle positioning (Schonegg and Hyman, 2006). Cdc42 regulates the Par6/Par3/aPKC complex, which in turn affects other polarity-related proteins.

1.5.3 Cytokinesis

One of the best-characterized cell cycle functions of Rho GTPases is the regulation of cytokinesis, the concluding step of cell division by which the cytoplasm is separated to form two daughter cells. At the end of telophase, a plasma membrane-anchored actin-myosin ring forms between the prospective daughter cells. Cytokinesis begins with ring contraction leading to the formation of a cleavage furrow, which ingresses until only a narrow tubular intracellular bridge connects the dividing cells. This region is designated the midbody and is composed of bundled microtubules derived from the central spindle and many other proteins required for membrane fission (abcission) (Eggert et al., 2006).

A requirement for Rho during cytokinesis was first described in sand dollar and *Xenopus* eggs, using the C3 transferase inhibitor. C3 microinjected during nuclear division of sand dollar eggs, interfered with the localization of actin filaments at the equatorial cortical region of the eggs and subsequent cleavage furrow formation. C3 exoenzyme had no apparent effect on nuclear division, and multinucleated embryos developed from the microinjected eggs (Mabuchi et al., 1993). A similar effect of Rho inhibition either by C3 transferase treatment or siRNA was seen in most animal cell types analysed (Jantsch-Plunger et al., 2000; Kamijo et al., 2006; Yuce et al., 2005).

1.5.3.1 Rho signaling

Additional insight into the role of Rho in cytokinesis comes from the discovery of Rho pathway components. Ect2, a GEF for Rho appears to be involved in cytokinesis in most cell types analyzed. The *Drosophila* and *C. elegans* Ect2 orthologues: Pebble and LET-21, respectively, are required for cytokinesis in these organisms and depletion of Ect2 prevents cytokinesis in many human cell

types (Kim et al., 2005; Morita et al., 2005; Prokopenko et al., 1999; Tatsumoto et al., 2003). A Rac GAP MgcRacGAP is also known to be involved in cytokinesis. Despite its name, this was originally thought to be a Gap for Rho, although more recent data suggests that it is acting to inhibit Rac at cytokinesis (Canman et al., 2008). Finally at least four effector proteins – mDia1, ROCK, citron kinase and PRK2 are believed to act downstream of Rho to promote the assembly and contraction of the actin-myosin ring (mDia1, ROCK, citron kinase) and also abscission (citron kinase and PRK2).

The diaphanous family of formins is believed to be the main actin nucleator at the contractile ring. In *Drosophila,* mutations in the *diaphanous* gene resulted in the formation of multinucleated cells and in mammalian cells, dominant negative forms of mDia1 and microinjection of neutralizing antibodies block cytokinesis (Castrillon and Wasserman, 1994; Tominaga et al., 2000). As mDia1 is known to be a Rho effector protein, it is believed to be regulated by Rho during the assembly of the actin ring, although no direct link has been demonstrated.

Contraction of the cytokinetic ring is driven by myosin II activity. Two Rho effectors seem to be involved in regulating actin-myosin contraction – ROCK and citron kinase. Data supporting a role for ROCK in cleavage furrow ingression comes from studies in *Drosophila*, *C. elegans* and human cells, in which inhibition of ROCK activity either leads to a delay or failure in cytokinesis. (Piekny et al., 2005). In human cells, ROCK is seen to accumulate at the cleavage furrow during cytokinesis (Kosako et al., 1999) and a dominant negative form of the kinase increases the percentage of cells with multiple nuclei (Yasui et al., 1998). The use of ROCK inhibitors was shown to inhibit phosphorylation of myosin II at the cleavage furrow, however cells did not fail cytokinesis but were only delayed (Goto et al., 2000; Madaule et al., 1998). siRNA depletion of ROCKI and ROCK II in HeLa cells was also not sufficient to produce a multinucleated phenotype, though the possibility of a delay was not investigated (Kamijo et al.,

2006). It is likely that ROCK is affecting myosin II activity at the contratile ring although other kinases may also be involved in this process, as the phenotype after myosin II inhibition is usually stronger than ROCK inhibition. Another possibility is that residual ROCK activity or genetic redundancy is preventing the analysis of a complete failure to phosphorylate myosin II (Piekny et al., 2005).

Citron kinase also plays an important role in cytokinesis in many cell types. Loss of function of citron kinase in *Drosophila* gives rise to the production of multinucleated cells in several tissues analyzed (Shandala et al., 2004). Mouse embryos homozygous for a mutation in the *citron* gene have multinucleate testis and brain cells (Di Cunto et al., 2000). Finally depletion of citron kinase by RNAi in HeLa cells also causes cytokinesis failure (Kamijo et al., 2006). Genetic interactions in *Drosophila,* between citron kinase and Rho1 or *Pbl*, suggest that citron kinase is part of the Rho signaling pathway at cytokinesis (D'Avino et al., 2004; Shandala et al., 2004). In addition, citron kinase localizes to the cleavage furrow and midbody during cytokinesis in a way that seems to be Rho-dependent, as human cells treated with C3 transferase no longer show citron kinase localization at the cleavage furrow and in Drosophila *Pbl* mutants (the Ect2 Drosophila orthologue) citron kinase localization at cytokinesis is also perturbed (Eda et al., 2001; Shandala et al., 2004). Although some disorganized cleavage furrow formation and ingression have been detected in cells depleted of citron kinase (D'Avino et al., 2004), it appears that the stronger effect is seen at the last step of cytokinesis, i.e. abscission (D'Avino et al., 2004; Echard et al., 2004; Naim et al., 2004). The studies involving citron kinase in cytokinesis suggest that Rho acts at late stages of cytokinesis in addition to a role in the formation and contraction of the actin-myosin ring. (Piekny and Glotzer, 2008).

1.5.3.2 Spatial and temporal regulation of Rho activity

It is generally accepted that the establishment of a localized pool of active Rho triggers cytokinesis (Somers and Saint, 2003). Rho accumulates at the furrow site at the end of anaphase as a narrow cortical band overlying the mitotic spindle. This is believed to correspond mainly to active Rho (Piekny et al., 2005). Both central spindle and astral microtubules seem to be essential for the localization of the cleavage furrow and elegant experiments have shown that spindle displacement leads to repositioning of Rho localization (Bement et al., 2005; Piekny et al., 2005). Ect2 and MgcRacGap localize at the mitotic spindle (in a narrow line at the equatorial plane) and are believed to be essential for restricting localization and activation of Rho and the triggering of cytokinesis. Both proteins, when knocked down by siRNA or depleted of function in genetic models give rise to a multinucleated phenotype in a wide variety of cell types (Eggert et al., 2006; Piekny et al., 2005). Additionally, when both these proteins are depleted of function, Rho fails to localize properly (Yuce et al., 2005).

Ect2 localization at the mitotic spindle is in turn a crucial event for the initiation of cytokinesis. Ect2 requires binding to MgcRacGap for activity and this appears to be temporarily regulated. Ect2 is phosphorylated in metaphase in a way that inhibits its binding to MgcRacGap and at mitotic exit, the degradation of cyclin B and inactivation of Cdk-1 stops this inhibitory signal and allow Ect2 to be recruited to the right place. This phosphorylation event is also believed to create a docking site for the polo-like kinase 1 (PLK-1) which in turn promotes the recruitment of Ect2 to the central spindle (Niiya et al., 2006; Petronczki et al., 2007).

1.6. Rho GTPases and epithelial morphogenesis

Epithelial morphogenesis is the process through which a functional epithelium forms. Rho GTPases have been implicated in the control of several steps during epithelial morphogenesis, including the assembly of adherens junctions and tight junctions, the establishment of polarity and the maintenance of the epithelium's integrity (see Figure 1.11).

Different cell types and tissues have different functions within an organism and this often requires the acquisition of particular cell shapes. The main function of the epithelial tissue is to establish a barrier between the host and the external environment. For this, epithelial cells have a polarized morphology and associate with each other at specialized cell-cell contact sites. There are two main types of epithelial cell junctions: adherens junction and tight junctions. Adherens junctions provide mechanical strength to the epithelial cell contacts, while tight junctions form a physical barrier that control the selective permeability of the epithelial layer to ions and small molecules. Tight junctions also demarcate the boundary between the apical and basolateral membrane domains and prevent the mixing of proteins and lipids between these two domains.

The separation of components between the apical and basolateral membranes is essential for epithelial cell function, and is usually referred to, as apical-basal epithelial cell polarity. Apical-basal polarity is particularly critical for the function of transporting epithelia, such as those found in the kidney and gastrointestinal tract. These types of epithelia establish permeability barriers between distinct compartments and directional transport along the apical-basal axis enables the maintenance of ionic homeostasis (Van Aelst and Symons, 2002).

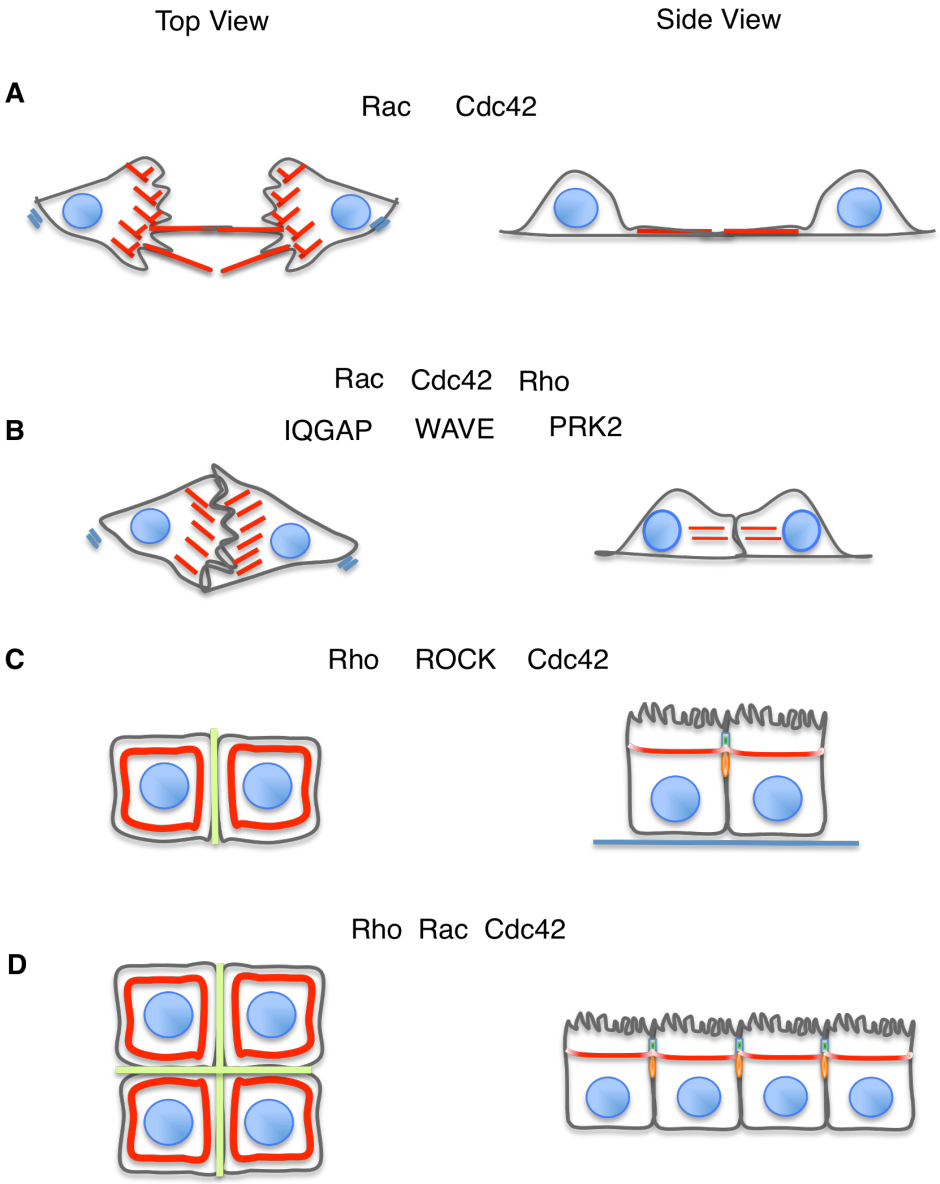


Figure 1.11 Rho GTPases and epithelial morphogenesis. Epithelial morphogenesis is the process through which a functional epithelium forms. Rho GTPases regulate several events during epithelial morphogenesis, including the formation of lamellipodia and filopodia that mediate the first cell-cell contacts (A), the stabilization of the cell contacts through the polymerization of actin filaments (B), the maturation of the junctions along the apical –basal axis which is believed to require actin myosin contractility and polarity (C) and finally, the maintenance of the integrity of the junctions and of the epithelium, which requires regulated membrane trafficking events and the transduction of signals from the extracellular matrix, both processes are regulated by Rho GTPases (D). Some examples of the players in each process are referenced. Actin, red; nucleus blue; tight junctions, green; adherens junctions, orange; apical junctions (tight junctions + adherens junctions), yellow.

1.6.1 Adherens junctions

Adherens junctions are composed of transmembrane cadherins. The extracellular domain of cadherins participates in Ca^{2+}-dependent homophilic interactions to create a link between adjacent cells in a tissue. The cytoplasmic side promotes the stability of this link by interacting with intracellular proteins, such as α-catenin and β-catenin and the actin cytoskeleton. Rho, Rac and Cdc42 have each been implicated in adherens junction assembly (Braga, 2000).

The organization of epithelial cells to form a polarized monolayer in tissue culture, has been used extensively to characterize junction assembly. Upon addition of Ca^{2+}, cadherins become competent for homophilic binding to neighboring cells and this adhesive interaction leads to cadherin clustering at cell–cell contact sites in a process that remains poorly understood, but that requires the actin cytoskeleton. The first step in the formation of initial contact sites can be observed when cells collide, for example during wound healing (Van Aelst and Symons, 2002). Observations made with mouse keratinocytes in culture, but also with epidermal cells during *C. elegans* development, and ectodermal cells during *Drosophila* dorsal closure; have revealed that filopodia and lamellipodia emerge from cells and penetrate into the neighboring cells (Jacinto et al., 2001; Raich et al., 1999; Vasioukhin et al., 2000). This seems to provide a driving force to generate intimate cell-cell contacts and Cdc42 and Rac are likely to be involved in the process. It was also observed that once the contacts are established, Rac and Cdc42 are recruited to the adhesive sites, where their major role might be to stabilize the junction through the assembly of actin filaments (Braga, 2000; Takaishi et al., 1997). High-resolution live-cell imaging and the use of biosensors, together with molecular inhibitors, have provided important data and suggest that the process might be more complex than first thought. It was observed that Rac1 and also RhoA activities are restricted to zones at the edges of the expanding cell-cell contact, but that rounds

of activation and down-regulation of these GTPases are required in the initiation, expansion and completion of cell-cell adhesion (Yamada and Nelson, 2007).

1.6.2 Effectors involved in adherens junction formation

Arp2/3 localizes to sites of cell-cell contacts when the contacts extend, and inhibition of Arp2/3 prevents E-cadherin contact formation (Kovacs et al., 2002; Verma et al., 2004). The Arp2/3 complex is likely to be acting downstream of Rac, and a role for the WAVE family of proteins in reorganizing the actin cytoskeleton during adherens junction formation, has also been described in Madin-Darby canine kidney (MDCK) cells (Yamazaki et al., 2007).

Another Rac effector that has been linked to cadherin mediated cell-cell contact formation is IQGAP, a scaffold protein that can also bind to Cdc42. IQGAP has been implicated in actin assembly at junctions, but it may also sequester β-catenin and it is believed that Rac and Cdc42 can disrupt this complex allowing β-catenin to participate in junction assembly (Kuroda et al., 1999; Noritake et al., 2004).

The establishment of cadherin-mediated cell-cell contacts is likely to also involve Rho. Inhibition of Rho by C3 transferase treatment prevents cadherins from accumulating at newly formed and mature junctions. This phenotype can be rescued by the introduction of a mutant Rho molecule that is insensitive to the C3 toxin (I41L63Rho) (Braga et al., 1997; Takaishi et al., 1997). The effect of the C3 transferase on cadherins has been attributed to a disruption of actin-myosin contractility through inhibition of ROCK and formins, but there is evidence that other activities might also be involved (Braga, 2000; Braga et al., 1997).

Finally, another event downstream of Rho, that has been shown to contribute to adherens junction regulation, is the phosphorylation of the Fyn tyrosine kinase. Constitutively active Rho stimulates the Fyn-mediated phosphorylation of catenins on tyrosine residues and this is believed to promote adherens junction formation. The Rho effector PRK2 has been linked to Fyn activation, since overexpression of PRK2, like activated Rho, stimulates Fyn activity (Calautti et al., 2002).

1.6.3 Maturation of junctions

As junctions mature, cells elongate along the apical-basal axis and assembling tight junctions become restricted to the apical side of the lateral membrane. Tight junction assembly marks the establishment of a fully polarized epithelial phenotype with distinct apical and basolateral membrane compartments. Profound alterations of the actin cytoskeleton also occur to form the characteristic perijunctional actin ring found in polarized epithelial cells. These changes in cell shape and junctional maturation require myosin II activity and actomyosin contractility (Zhang et al., 2005). Myosin II is recruited to junctions in a Rho- and ROCK dependent manner (Yamazaki et al., 2008).

1.6.4 Tight Junctions

Tight junctions are multimolecular complexes that form specialized membrane microdomains. They are composed of: 1) integral membrane proteins, such as occludins, claudins and junctional adhesion proteins (JAMs) and 2) cytoplasmic proteins, such as: zonula occludens protein (ZO-1). ZO-1 contains several protein-protein interaction motifs and may act as a molecular scaffold bringing

together other tight junction proteins, including, claudins, occludin, ZO-2, ZO-3, cingulin, JAM and actin (Zahraoui et al., 2000).

Two in vitro assays have been used to analyze the molecular mechanisms underlying tight junction formation. These are: 1) immunofluorescence to visualize tight junction proteins and 2) paracellular permeability to access functional activity.

Several studies have shown that Rho is essential for the proper functional assembly of tight junctions. Inhibition of Rho by C3 transferase displaces ZO-1 from junctions and increases the paracellular permeability of polarized human epithelial cells (Nusrat et al., 1995), while expression of dominant negative Rho in MDCK cells abolishes the fence function of tight junctions, although no dramatic changes in their organization or composition was detected (Jou et al., 1998). The more pronounced effect of C3 transferase on tight junctions probably reflects the weaker inhibitory effect of the dominant negative mutant. The expression of constitutively active Rho, even at relatively low levels perturbs the function of tight junctions (Jou et al. 1998). This observation may suggest that that Rho GTPases need to cycle to function correctly in tight junction assembly (Symons and Settleman 2000). However, it could be that the constitutively active form activates other effectors inappropriately.

Dominant negative and constitutively active forms of Rac and Cdc42 also affect tight junction formation (Van Aelst and Symons, 2002). Cdc42 is though to control tight junction formation through the Par6/aPKC/Par3 complex. This evolutionary conserved complex plays a role in a large number of functions that involve cell polarity and Par6 is a direct target of Cdc42. Cdc42 has been shown to induce aPKC activation through interaction with Par6 (Yamanaka et al., 2001). The Par6/aPKC/Par3 complex localizes apically with tight junctions, but unlike

Cdc42 seems to be only involved in tight junction formation and not adherens junction formation.

1.6.5 Establishment of polarity

Other protein complexes that are associated with the establishment of polarity contribute to epithelial morphogenesis, notably the Crumbs/PALS1/PAJ and the Dlg/Lgl/Scribble complexes. Crumbs/PALS1/PAJ localize at tight junctions, while Dlg/Lgl/Scribble are basolateral. A general idea of how polarity is achieved and maintained is that the Crumbs and Par complexes determine the apical domain of epithelial cells by excluding the basolateral determinants. Similarly, the Scribble complex antagonizes Crumbs and Par complexes at the lateral membrane. This mutual exclusion between polarity complexes is thought to be the basis of the establishment and maintenance of apical-basal polarity in epithelia (Shin et al., 2006).

1.6.6 Maintenance of the epithelial integrity

Rho GTPases may contribute to epithelial cell morphogenesis through other activities independently of junction assembly, such as membrane trafficking or the transduction of signals from the extracellular matrix. Membrane trafficking events are very important for the maintenance of adherens junctions and tight junctions, and cadherins and claudins are constantly being recycled through endocytosis even in a fully mature monolayer. In addition, polarized membrane trafficking ensures the maintenance of discrete apical and basolateral membrane compartments. The disruption of Cdc42 signaling in MDCK monolayers results in the inhibition of vesicular transport specifically to basolateral surfaces, leading to a selective depolarization of basolateral membrane proteins (Kroschewski et al.,

1999).

Tissue morphogenesis depends on the coupling of epithelial apical-basolateral polarity with the extracellular environment. Epithelia are formed with their apical domains facing the lumen of the organ and the basal surface contacting the basal lamina. Epithelial cell growth in three-dimensional matrices (3D) has provided insights into how this orientation is achieved. It seems that the interaction with the extracellular matrix provides signals for the proper orientation of the apical-basal axis and Rac has been identified as a critical player in mediating the transduction of these signals (O'Brien et al., 2001).

Cdc42 plays an important role in controlling epithelial cell polarity. In MDCK cells Cdc42 has been reported to facilitate the polarized trafficking of a vacuolar compartment containing apical markers (Martin-Belmonte et al., 2007). In a second study, using Caco-2 cells, Cdc42 is required to position the apical surface with respect to the growing three-dimensional structure. This is achieved by controlling the orientation of the mitotic spindle (Jaffe et al., 2008; Lechler, 2008).

1.7 The protein kinase C-related protein kinases

The human protein-kinase C-related kinases (PRKs) form a family of Rho GTPase effectors composed of three isoforms, PRK1, PRK2 and PRK3, which are the product of three independent genes. The domain structure of the PRK isoforms is shown schematically in Figure 1.12. PRKs contain a C-terminal serine-threonine kinase domain that shows high sequence homology with the kinase domain of the PKC family (PRKs are included in the PKC superfamily of kinases). At the N-terminus they contain three homologous stretches of approximately 70 amino acids, each containing a region of relatively rich in

charge residues, followed by a leucine zipper-like sequence. This region, called REM (Rho effector homology) or, HR1 domain (homologous region 1), is responsible for binding to Rho GTPases and has some homology to Rho binding regions in other effectors such as rhophilin and rhotekin. The HR1 domain contains three subdomains: HR1a, HR1b and HR1c.

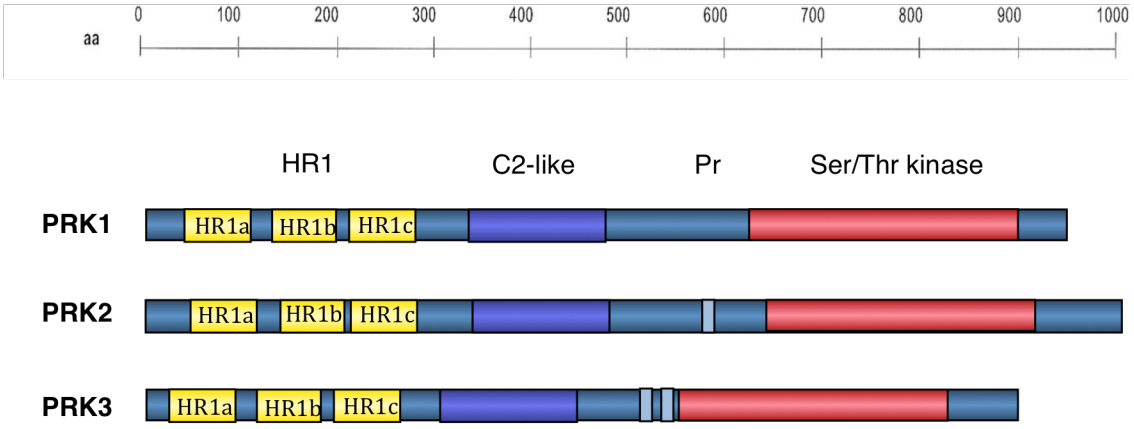


Figure 1.12 The PRK family of Rho GTPase effectors. The human protein-kinase C-related kinases (PRKs) form a family of Rho GTPase effectors composed of three isoforms, PRK1, PRK2 and PRK3, which are the product of three independent genes. PRKs contain a C-terminal serine-threonine kinase domain that shows high sequence homology with the kinase domain of the PKC family. At the N-terminus they contain three homologous stretches of approximately 70 amino acids each: HR1a, HR1b and HR1c, that form the HR1 domain. The HR1 domain is known to bind Rho GTPases. Between the HR1 and the catalytic domain, PRKs contain a C2-like domain of around 130 amino acids. Finally, PRK2 and PRK3 contain one and two proline rich regions, respectively, in between the C2-like and the kinase domain.

Between the HR1 domain and the catalytic domain, PRKs contain a C2-like domain of around 130 amino acids (Palmer et al., 1995b). This domain has homology to the C2 domain of PKC members, but its tertiary structure and function are unknown. C2 domains are believed to confer Ca^{2+} and phospholipids binding, which is required for classical PKCs activity, although the C2-like domain of PRK lacks conserved aspartate residues required for Ca^{2+} binding (Mellor and Parker, 1998). It is also unclear whether these domains are capable of binding to

phospholipids even though it has been shown that PRKs are activated by phospholipids. The C2-like domain in PRK appears to contain an autoinhibitory region that is sensitive to arachidonic acid, one of the activators of PRK (Mukai, 2003; Yoshinaga et al., 1999). C2 domains might also be involved in protein-protein interaction (Benes et al., 2005; Cho, 2001; Raftopoulou et al., 2004; Rizo and Sudhof, 1998).

Finally, PRK2 and PRK3 but not PRK1, contain proline rich regions in between the C2-like and the kinase domain. One proline rich region is found in PRK2 and two in PRK3. These Pro-rich regions contain the minimal consensus sequence for Src homology 3 (SH3) domain recognition. In fact some Nck and Grb4, two adaptor proteins that also contain SH2 domains and bind to phosphorylated tyrosines in growth factor receptor tyrosine kinases, have been shown to interact with PRK2. Also GRAF, a GAP for Rho and Cdc42 is able to interact with PRK3 (Mukai, 2003).

1.7.1 Tissue distribution and subcellular localization

PRK1 and PRK2 mRNAs are expressed widely in mammalian tissue and cell lines. PRK3 mRNA, in contrast, was almost undetectable in normal human adult tissue, but has been reported in various human cancer cell lines (Mukai, 2003).. PRK1 and PRK2 have been shown to localize mainly in the cytosolic fraction in various cultured cells. PRK1 has also been detected in cell fractions corresponding to endosomes and cytoskeletal components in human epithelial kidney cells (HEK 293) and PRK2 accumulates at the cleavage furrow during cytokinesis in HeLaS3 cells (Mellor et al., 1998; Schmidt et al., 2007). Stresses such as heat shock or arsenite treatment and serum deprivation have been seen to induce nuclear translocation of PRK1 (Mukai et al., 1996). Finally PRK3 has been detected in the cell nucleus and the perinuclear region, presumably

corresponding to the Golgi apparatus, but not in the cytoplasm (Oishi et al., 1999).

1.7.2 Interaction with Rho GTPases

The interaction of PRK1 and PRK2 with Rho GTPases has been analysed extensively (see table 1.1). Although having 56% homologous HR1 domains, PRK1 and PRK2 appear to differ in the way they bind Rho GTPases. In 1996 two independent groups identified PRK1 as a Rho GTPase effector that was able to bind Rho, but not Rac, in a GTP-dependent manner. A region in PRK1 responsible for Rho binding was identified which is now known to include the HR1a domain and part of the HR1b domain (Amano et al., 1996; Watanabe et al., 1996). Further studies revealed that the PRK1 HR1 sub-domains bind Rho differently. Both HR1a and HR1b bind GTP-Rho while no binding was detected with HR1c. However, it was observed that HR1a binding was GTP-dependent whereas HR1b could bind to both GTP- and GDP-bound forms of Rho (Flynn et al., 1998). Another group used a scintillation proximity assay and showed that PRK1 was also able to bind to Rac through the HR1b and HR1a domains and that Rho binds to the HR1a domain more strongly than to HR1b (Owen et al., 2003).

		Rho		Rac		
		GTP	GDP	GTP	GDP	References
PRK1	FL	++	-	-	-	Amano et al., 1996 Watanabe et al., 1996
	HR1	++				Flynn et al., 1998
	HR1a	++	-			Flynn et al., 1998
		++		++		Owen et al., 2003
	HR1b	++	++			Flynn et al., 1998
		+		++		Owen et al., 2003
	HR1c	-	-			Flynn et al., 1998
PRK2	FL	++	++	++	-	Vincent and Settleman, 1998
		++	+	-	-	Quilliam et al., 1998
Drosophila Pkn	FL	++	-	++	-	Lu and Settlemann, 1998
	HR1a	++	-	++	-	Betson and Settlemann, 1998
	HR1b	++	-	-	-	
	HR1c	++	-	-	-	

Table 1.1 Interaction of PRKs with Rho GTPases. A summary of the data concerning the interactions of PRK1, PRK2 and *Drososphila* Pkn, with Rho and Rac is shown. For PRK1 and *Drosophila* Pkn, the ability of the different HR1 sub-domains to interact with Rho GTPases has been analysed. Majority of studies have used affinity chromatography or overlay assays to analyse the interaction between the proteins, and have used GTP-γ-S-Rho and GTP-γ-S-Rac, as the GTP-bound GTPases. One study has used a scintillation proximity assay and used L61 Rho, and L63 Rac as the GTP-bound GTPases (Owen et al 2003). ++, means the proteins interact; +, means that a weak interaction was detected; -, means no interaction was found; blank, means the interactions were not analysed.

The ability of PRK2 to bind Rho GTPases has also been analyzed but there are contradictory reports relating to GTPase binding. In one report, PRK2 binds equally well to GTP- and GDP-bound Rho. Additionally, PRK2 is also able to bind to Rac, while PRK1 is not (or it binds very weakly), and binding to Rac seems to be GTP-dependent (Vincent and Settleman, 1997). In another report PRK2 binds preferentially to Rho-GTP, although some weak binding is also seen with Rho-GDP (Quilliam et al., 1996).

Drosophila Pkn which has 60% overall identity to both human PRK1 and PRK2 and contains all three HR1 sub-domains also interacts with the Drosophila Rho1 and Rac1, apparently in an exclusively GTP-dependent manner (Lu and Settleman, 1999). In this case, binding studies with deletion mutants demonstrated that the HR1a domain is required for Pkn binding to Rac1 while the three sub-domains seems to make interactions with Rho1 (Betson and Settleman, 2007).

1.7.3 Regulation of activity

PRK1 and PRK2 kinase activities are enhanced by binding to Rho GTPases or to fatty acids such as arachidonic acid, or by removal of the N-terminal regulatory domain by caspase 3 cleavage during apoptosis. Before stimulation, PRKs are believed to be in a closed autoinhibited conformation, as suggested by the caspase 3 cleavage that generates a constitutively active fragment. Fatty acids activate PRKs by relieving this autoinhibited conformation. The mechanism through which Rho GTPases activate PRKs is still unclear but it might relate to the phosphorylation of the kinase's activation loop by the PDK-1 family of kinases. (Figure 1.13)

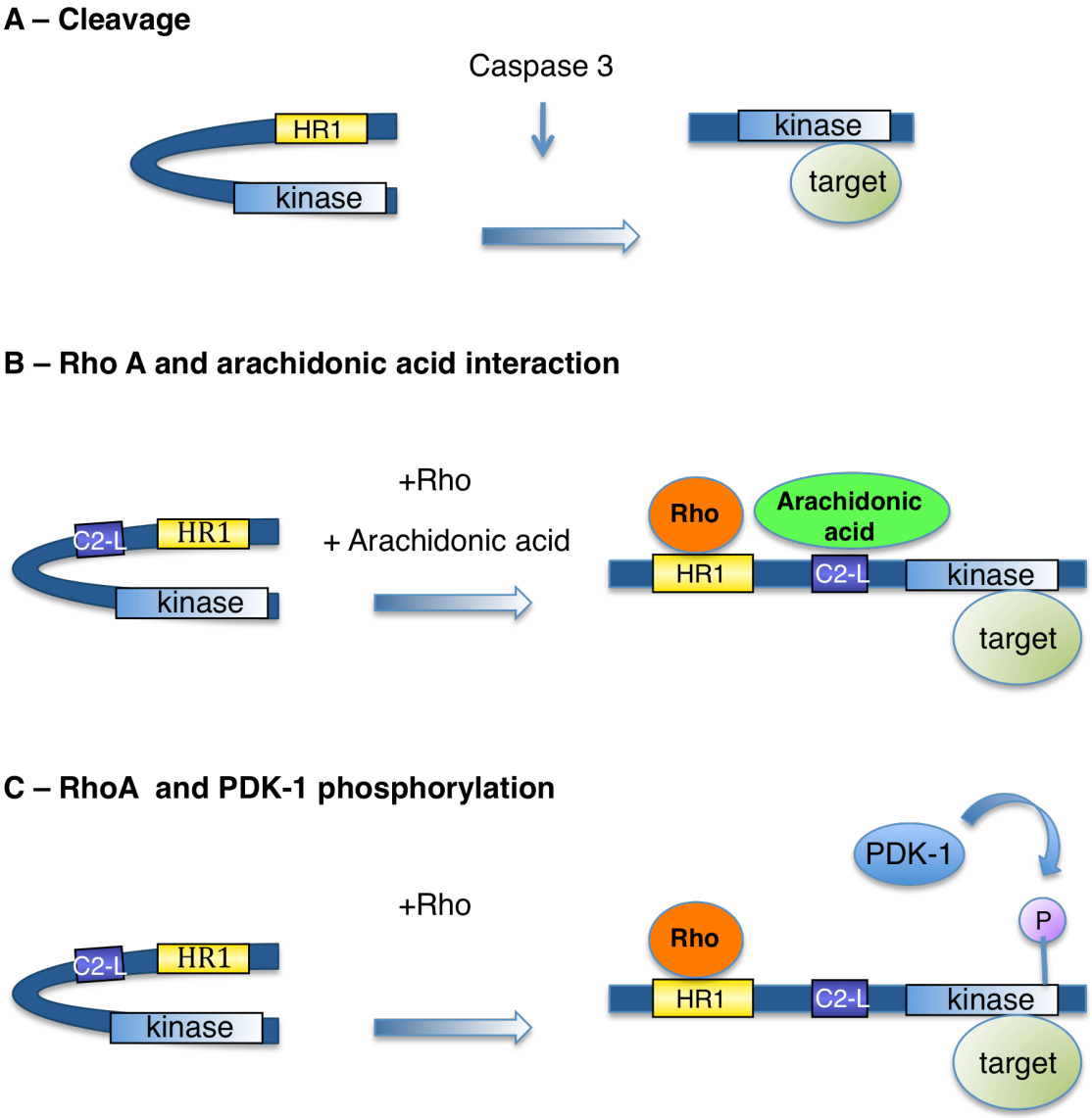


Figure 1.13 Regulation of PRK activity. The N-terminal region of PRK1 can interact directly with the C-terminal region and PRKs are believed to adopt a basal autoinhibited conformation. Removal of the N-terminal regulatory domain by caspase 3 cleavage, during apoptosis, generates a constitutively active fragment (A). Rho GTPases and Arachidonic acid have also been shown to activate PRKs and the mechanism suggested has been the release of the autoinhibited conformation. There are also data supporting that interaction with Rho GTPases induces activation loop phosphorylation of PRK by PDK-1.

Recombinant PRK1 protein has kinase activity and can phosphorylate itself preferentially on serine residues (Watanabe et al., 1996). PRKs can also phosphorylate PKC substrates such as myelin basic protein (MBP) and serine-containing synthetic peptides based on the PKC pseudosubstrate site. The in vitro kinase activity of PRKs has been mainly analysed with respect to their ability to autophosphorylate or to phosphorylate the substrates just mentioned (Mukai, 2003). PRK1 kinase activity towards the PKC-δ pseudosubstrate peptide was found to be significantly enhanced by unsaturated fatty acids such as arachidonic acid, linoleic acid and oleic acid (Kitagawa et al., 1995; Mukai et al., 1994; Mukai et al., 1996). The kinase activity of PRK2 and PRK3 was less sensitive to arachidonic acid than that of PRK1 (Yoshinaga et al., 1999). PRK1 kinase activity has also been shown to be enhanced by several phospholipids but not by diacylglycerol or Ca^{2+}, as happens with other PKC family members (Mukai et al., 1994; Palmer et al., 1995a; Peng et al., 1996).

1.7.3.1 Regulation of activity by Rho GTPases

Two independent studies have shown that GTP-γ-S-GST-RhoA stimulates PRK1 autophosphorylation in vitro by two- to five- fold, whereas GDP-GST-RhoA or GTP-γ-S-GST-Rac had no effect on kinase activity. A similar pattern of behavior was observed for PRK1 phosphorylation of the PKC pseudosubstrate peptide. Additionally Rho-dependent activation and phosphorylation of PRK1 was examined in cells. Myc-tagged PRK1 was expressed in Cos7 cells together with wild type Rho or V14Rho. The kinase activity of PRK1 immunoprecipitated from these cells was enhanced by ~1.5-fold when co-expressed with wild type Rho and > 2-fold when co-expressed with the constitutively active mutant. Finally, treatment of cells with lysophosphatidic acid (LPA), which stimulates Rho, was not seen to lead to an increase in PRK1 activity, although the authors reported a 2-fold increase in PRK1 phosphorylation upon LPA treatment. C3 transferase

treatment inhibited this phosphorylation, from which the authors conclude that PRK1 might be phosphorylated by a Rho-dependent mechanism (Amano et al., 1996; Watanabe et al., 1996).

Both Rho and Rac were shown to increase PRK2 autokinase activity in vitro by several-fold although Rho was reported to activate PRK2 in a GTP-independent manner. In vivo, expression of a kinase-deficient form of PRK2 disrupted stress fibers formation suggesting that PRK2 act as an effector for Rho in this pathway (Vincent and Settleman, 1997). In addition, the Drosophila Pkn can be activated by Rho1 and Rac1 (Lu and Settleman, 1999). Putting all these results together it seems that PRK1 and PRK2 are activated in a Rho-GTPase dependent mechanism.

1.7.3.2 Disrupting auto-inhibition

The first indication that PRKs were able to adopt an autoinhibited conformation came from limited proteolysis studies. Trypsin pretreatment of PRK1 produces a 43-kDa fragment that has strong in vitro kinase activity towards the PKC-δ pseudosubstrate peptide. In order to generate a fragment of that size, the cleavage site must lie near residue 600. This result suggests that the N-terminal could restrict PRK1 kinase activity (Mukai et al., 1994). Later, yeast two hybrid assays and in vitro binding assays using GST fusion proteins have shown that the N-terminal region of PRK1 can interact directly with the C-terminal region. In addition and similar to other PKC family members, PRK1 has a potent pseudosubstrate-like sequence centered around Ile^{46} (residues 39 to 53) of the HR1a region. A synthetic peptide of this sequence in which Ile^{46} was replaced by a Ser, to function as a phosphate acceptor, is efficiently phosphorylated by PRK1. In addition a synthetic peptide of the non-mutated site competitively inhibits the phosphorylation of the pseudosubstrate site of PKCδ. These data

suggest that residues 39 to 53 act as an autoinhibitory sequence of PRK1 and as Rho was known to bind (Kitagawa et al., 1996). At that time it was already known that Rho binds to the N-terminal region of PRK1 and also that it was able to stimulate PRK1 kinase activity (Amano et al., 1996; Watanabe et al., 1996). Therefore it was an attractive model that Rho binding to PRK1 could release the pseudosubstrate site from the catalytic domain and lead to the activation of the kinase. However, Rho is not a very strong activator of PRK and a later study demonstrated that the HR1 domain is not required for the autoinhibition as a truncated mutant lacking the entire HR1 domain (amino acids 1-455) had a low basal kinase activity, similar to that of wild type PRK1. An essential autoinhibitory domain was mapped to a region comprising amino acids 455 to 511, which encompasses the C2-like domain. A fragment containing this region was able to inhibit the kinase activity towards the PKCδ in a competitive manner, suggesting it might function as an autoinhibitory domain. The ability of this peptide to interfere with PRK1 kinase activity was less dramatic when in the presence of arachidonic acid, suggesting arachidonic acid might regulate PRK1 through the interaction with the C2-like domain (Yoshinaga et al., 1999). To further support this possibility it was observed that full length PRK1 in vitro kinase activity towards the PKC-δ pseudosubstrate peptide was shown to be highly dependent on arachidonic acid while the N-terminal lacking PRK1 fragment obtained after tryptic digestion was not (Mukai et al., 1994). The mechanism through which Rho GTPase binding induces conformational changes in PRK that lead to its activation is still to be clarified.

1.7.3.3 Activation loop phosphorylation – the role of Rho GTPases

One possible model for Rho GTPase regulation of PRK activity is through the induction of activation loop phosphorylation by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Flynn et al., 2000; Mukai, 2003). PDK-1 is responsible

for activation loop phosphorylation of several AGC superfamily kinases, including the PKC family members. PRKs possess a putative activation loop phosphorylation site that fits the consensus of all characterized PDK1 substrates (Thr774 for PRK1 and Thr816 for PRK2) (Parekh et al., 2000). It has been proposed that activated Rho binding to PRK1 could induce a conformational change that allows activation loop phosphorylation by PDK-1 (Hodgkinson and Sale, 2002). In vitro and in vivo experiments (using phospho-specific polyclonal antibodies) have shown that PDK-1 is able to phosphorylate both human PRK1 and PRK2 at the activation loop sites and that phosphorylation at the activation loop is essential for activity (Flynn et al., 2000). It is also clear that PDK-1 is able to interact with PRKs (Balendran et al., 2000; Dettori et al., 2009; Flynn et al., 2000). However, at least for PRK2 it is not clear whether activation loop phosphorylation constitute a critical point of activation (Mukai, 2003). Contradictory results have also been reported concerning whether Rho is involved in mediating activation loop phosphorylation. Flynn *et al*. showed that in Hek293 cells activation loop phosphorylation increases upon overexpression of activated forms of RhoA or RhoB (Flynn et al., 2000). In this study the authors also showed that PRK1 associates with PDK-1 in a Rho-dependent manner. However, another group reported that co-expression of activated Rho had no effect on PDK-1 phosphorylation of the activation loop site in PRK2 when PRK2 was co-expressed with the activated form of Rho (Balendran et al., 2000). In this last study it is also reported that the activation loop site was already phosphorylated to a high stoichiometry when PRK2 was overexpressed in unstimulated Hek293 cells.

1.7.3.4 Regulation of PRK2 intracellular localization by RhoB

In addition to the possible allosteric regulation of kinase activity, small GTPases may also affect the cellular localization of PRK. Overexpression of RhoB in

fibroblasts has been shown to recruit PRK1 into vesicular compartments. Rho B, unlike RhoA or RhoC, localizes on endosomes. Overexpressed PRK1 localizes diffusely in the cytoplasm, however when co-transfected with RhoB, it translocates to endosomes. The HR1 domain of PRK1 was shown to be responsible for the RhoB-mediated PRK1 localization to endosomes. Co-expression of the HR1 domain with RhoB resulted in translocation of the HR1 polypeptide to the endosomal compartment, while a PRK1 truncation mutant that lacks the HR1 domain failed to localize to this same compartment. Co-immunoprecipitation studies were also able to demonstrate that PRK1 can interact with the activated mutant of RhoB in a GTP-dependent manner, similar to what happens with RhoA, suggesting that PRK1 can be an effector for RhoB. Finally, cell fractionation studies showed that endogenous PRK1 is enriched at endosomes in HEK 293 cells even without overexpression of RhoB (Mellor et al., 1998).

1.7.4 Cellular functions of PRKs

The biological functions of the PRK family of proteins are unclear. Some potential targets have been proposed based on *in vitro* kinase assays, especially for PRK1, but in the majority of cases, it is not known whether they constitute physiologically relevant substrates. There have been a numbers of reports linking PRKs to a variety of cellular processes, but again, the mechanisms through which PRK might act in those processes are still to be identified. Finally, although some binding studies have shown that PRK1 and PRK2, as well as *Drosophila* Pkn, are able to bind to both Rho and Rac, the majority of in vivo studies assumed that Rho is the major player.

1.7.4.1 Possible targets

No physiological targets have been identified for PRK family members, however PRK1 has been shown to phosphorylate *in vitro* some intermediate filament proteins, such as vimentin and glial fibrillary acidic protein (GFAP) (Matsuzawa et al., 1997) . PRK1 has also been shown to phosphorylate CPI-17, a protein that is specifically expressed in smooth muscle cells and might be involved in smooth muscle contraction (Hamaguchi et al., 2000). Finally PRK1 can efficiently phosphorylate Tau, a neuronal microtubule associated protein that promotes microtubule assembly and has been linked to Alzeimer's disease (Taniguchi et al., 2001). PRK1 has also been shown to bind α-actinin (an actin cross linking protein) but not to efficiently phosphorylate it. Apart from cytoskeleton-related proteins PRK family members have been shown to phosphorylate *in vitro* other proteins, as diverse as the Cdc25 family of phosphatases that are involved in mitosis, histone H3 involved in transcriptional regulation, the hepatitis C virus RNA polymerase and the human papillomavirus E6 oncoprotein (Gao et al., 2000; Kim et al., 2004; Metzger et al., 2008; Schmidt et al., 2007).

1.7.4.2 Actin cytoskeleton organization

Some studies have linked PRKs to the regulation of the actin cytoskeleton. The microinjection of a kinase deficient form of PRK2 was reported to disrupt actin stress fibers in NIH3T3 cells. This effect was not seen when just the HR1 domain of PRK2 was expressed at high levels, consistent with a role for PRK in regulating actin stress fiber formation and not just a non-specific effect of blocking Rho activity (Vincent and Settleman, 1997). No PRK family member has been reported to phosphorylate myosin or myosin phosphatase.

A role for PRK1 has been proposed in insulin-induced actin cytoskeleton reorganization downstream of PDK1 (Dong et al., 2000). In this case, PRK1 overexpression induced actin stress fiber depolymerization and enhanced cortical actin in 3T3-L1 and Rat1 fibroblasts. Interestingly in a different study the same authors showed that PRK2 is not able to cause the same effect in mouse embryonic fibroblasts (MEFs) (Lim et al., 2004). The effect of PRK1 overexpression on the cytoskeleton is similar to what is observed upon PDK1 overexpresion or insulin treatment and the authors suggest that PDK-1 and PRK1 might be working in the same pathway (Dong et al., 2000).

Finally, PRK1 might be involved in cortical actin formation during the migration of PTEN-knockout MEFs. Loss of PTEN in these cells enhances migration and correlates with the accumulation of cortical actin. Overexpression of kinase dead PRK1 (but surprisingly, not kinase dead PRK2) was observed to inhibit cortical actin accumulation in PTEN-/- cells. Despite this no effect on cell migration was observed (Lim et al., 2004).

1.7.4.3 Drosophila dorsal closure

Drosophila Pkn shows 60% overall identity to human PRK1 and PRK2. Pkn is believed to bind both Rho1 and Rac1 in a GTP-dependent manner (but not Cdc42) and interaction with the active forms of these GTPases has been shown to stimulate Pkn kinase activity by several fold (Lu and Settleman, 1999). Pkn loss of function affects cell shape changes seen during dorsal closure, a morphogenic process in early *Drosophila* development. Dorsal closure is the movement of the lateral and ventral epidermis that takes place to enclose the *Drosophila* embryo. This process involves profound cell shape changes that are associated with rearrangements in the actin cytoskeleton and require the maintenance of cell-cell contacts. In the first phase of dorsal closure the dorsal-

most cells of the lateral epidermis elongate along the dorsal–ventral axis; this is associated with the accumulation of actin and non-muscle myosin at the leading edge of the epithelial sheet (Jacinto et al., 2002). Although the precise role for Pkn in dorsal closure is unknown it is likely that Pkn is transducing a Rho-dependent signal (but not Rac), in the leading edge cells. Pkn is enriched in those cells and loss of function of Rho-1 exhibits a very similar dorsal-open phenotype to the one observed for Pkn (Lu and Settleman, 1999). Rescue experiments with Pkn mutants further suggest that Pkn may be acting downstream of Rho (i.e. not Rac) during this step of the Drosophila embryo development (Betson and Settleman, 2007).

1.7.4.4 Cell-cell adhesion

There has been one report that PRK2 affects keratinocyte cell-cell adhesion through activation of Fyn tyrosine kinase. The results reported in this work are: 1) active Rho stimulates adherens junction formation through the Fyn-mediated tyrosine phosphorylation of α- and β- catenins, 2) overexpression of PRK2 enhances the recruitment of E-cadherin to cell-cell adhesions and tyrosine phosphorylation of α and β- catenins, 3) the incubation of Fyn tyrosine kinase with the kinase domain of PRK (but not with a kinase dead mutant) increased Fyn tyrosine kinase activity *in vitro,* 4) PRK2 kinase activity (measured by autophosphorylation) increases with keratinocyte differentiation, 5) expression of constitutively active Rho (RhoV14), but not a constitutively active Rho mutant carrying a mutation that impairs its ability to bind to PRKs (RhoV14-Y42C), promotes the recruitment of E-cadherin to cell-cell junctions. The authors suggest Rho GTPases activate Fyn tyrosine kinase through PRK2 (Calautti et al., 2002).

1.7.4.5 G2/M transition

Entry into mitosis is regulated by the Cdk1/CyclinB complex. Two members of the Cdc25 family of protein phosphatases, Cdc25B and Cdc25C activate the Cdk1/CyclinB complex by dephosphorylation of Tyr15 and Thr14 in Cdk1. In mammalian cells, Cdc25B is expressed slightly earlier than Cdc25C and is thought be the initial activator of the Cdk1/CyclinB. After being activated, in a positive feed-back loop the Cdk1 further promote its own dephosphorylation by phosphorylating and activating Cdc25B and Cdc25C (Karlsson-Rosenthal and Millar, 2006).

PRK2 has been linked to the transition from G2 to mitosis. In HeLa S3 cells RNAi depletion of PRK2 induced a 2- to 4-hour delay in cells progressing from G2 into mitosis, as seen by several mitotic markers. PRK2 siRNA caused a 2 to 4-hour delay in Cdk1 tyr15 dephosphorylation and also in Cdc25B phosphorylation, suggesting that PRK2 is acting upstream of Cdc25B in regulating G2/M transition. In agreement with it was reported that that PRK2 is able to phosphorylate Cdc25B in vitro (Schmidt et al., 2007). A similar delay in mitotic entry has been seen after treatment of HeLa S3 cells with toxin B suggesting that Rho GTPases are involved in this process and could be acting through PRK2 (Ando et al., 2007).

Three other reports, however, one in *Xenopus* eggs and the two other in human cells, suggested that PRK2 is a negative regulator of this step of the cell cycle by inactivating Cdc25C. Exogenous addition of the active form of PRK1 (kinase domain) was shown to delay the entry into mitosis of *Xenopus* egg cycling extracts. In vitro experiments indicated that PRK1 phosphorylates and inhibits Cdc25C, suggesting that delayed mitotic timing in the egg extracts is caused by inhibition of Cdc25C (Misaki et al., 2001). The same authors showed that activation of PRK1 caused a delay in G2/M progression in HeLa cells and that

PRK1 is involved in phosphorylating and inhibiting Cdc25C after arsenite treatment (Isagawa et al., 2005). In vascular smooth cells, it was showed that TGF-ß exposure (which induces differentiation) induces a two-hour delay in G2/M transition through PRK1 activation. The TGF-ß-induced G2/M delay was abolished using the small molecule inhibitors HA 1077 and Y-27632, which inhibit PRKs or one PRK1-specific siRNA. The authors suggest this effect could be due to PRK1 inhibition of Cdc25C (Su et al., 2007).

1.7.4.6 Cytokinesis

PRK2 depletion by RNAi was reported to produce a binucleate phenotype in HeLa S3 cells. Time-lapse microscopy analysis of depleted cells revealed that the formation of the binucleate phenotype was due to a late cytokinesis defect (i.e. abcission). The cells showed normal ingression of the cleavage furrow, but then failed to undergo abcission and the furrow retracted giving rise to binucleate cells. Consistent with a role in abcission, PRK2 localizes at the cleavage furrow and the midbody during cytokinesis.

Abscission is a complex process that requires delivery of membrane vesicles and membrane fusion events. Two proteins that interact with PRK and have been linked to cytokinesis are: PTP-BL, a tyrosine phosphatase that localizes to the cleavage furrow and midbody, and CG-NAP, a scaffold protein that localizes to the Golgi apparatus, the centrosome and the midbody.

Finally, PRK2 has been shown to be phosphorylated during mitosis, as seen by the presence of a slower migrating band after SDS-PAGE gel electrophoresis. This phosphorylation event seems to occur downstream of Cdk1 (Schmidt et al., 2007), but it is not known whether or how this phosphorylation affects PRK2 activity or function.

1.7.4.7 Other possible functions

PRKs have been linked to other cellular processes such as membrane trafficking, transcriptional regulation, gene expression, apoptosis, glucose transport and tumorigenesis (Gampel et al., 1999; Leenders et al., 2004; Marinissen et al., 2001; Metzger et al., 2003; Mukai, 2003; Schmidt et al., 2007; Takahashi et al., 2003).

1.8 Conclusion

Rho GTPases are signal transduction proteins that signal by activating effector proteins. Work with cultured cell lines and model organisms have implied Rho GTPase signaling in a wide variety of cellular events including cell cycle progression and tissue morphogenesis.

Previous work in the Hall lab has led to the identification of the Rho GTPase effector PRK2 as a regulator of G2/M transition and cytokinesis. Out of this work, two interesting observations concerning the regulation of PRK2 stroke our attention and we have decided to further analyze them. The first observation was that PRK2 is specifically phosphorylated at mitosis and the second that it localizes at the cleavage furrow and around the midbody during cytokinesis.

In the course of analyzing PRK2 localization at cytokinesis we noticed that PRK2 also localizes at sites of cell-cell contact that form between the two daughter cells that are dividing. This result led us to analyze PRK2 in the context of epithelial morphogenesis.

CHAPTER 2 – MATERIAL AND METHODS

Unless otherwise stated all reagents were obtained from Sigma.

2.1 Molecular Biology

2.1.1 DNA constructs

The DNA constructs used in this study are listed in table 2.1.

Plasmid name	Description and source
pRK5myc::mPRK2	Myc-tagged (N-terminus) mouse PRK2. From H. Mellor (University of Bristol, UK)
pEGFP::PRK2	GFP-tagged (N-terminus) human PRK2. From Peter J. Parker and described here: (Torbett et al., 2003)
pRK5myc::PRK2NT	Myc-tagged (N-terminus) amino acids 1-597 of human PRK2. From Anja Schmidt (Hall lab).
pRK5myc::PRK2CT	Myc-tagged (N-terminus) amino acids 597-984 of human PRK2. From Anja Schmidt (Hall lab).
pRK5myc::PRK2HR1	Myc-tagged (N-terminus) human PRK2, containing a STOP codon at position 326 in order to generate a PRK2 fragment that contains aminoacids 1-325. Point mutations were generated by PCR-based site directed mutagenesis using as a template pRK5myc::PRK2NT and the following primers: forward: 5'-ctatccaaaccagcatgactaacaggtactttg-3' reverse: 5'-caaagtacctgttagtcatgctggtttggatag-3'

Table 2.1 DNA constructs used in this study. List of the DNA constructs used in this study. The source of the constructs or the cloning strategy used is referenced when applicable. Restrictions sites and mutations are indicated in red. All constructs were verified by sequence analysis [Sloan-Kettering Institute (SKI) DNA sequencing facility. (continues next page)

Plasmid name	Description and source
pRK5myc::PRK2ΔHR1	Myc-tagged (N-terminus) aminoacids 296-984 of human PRK2. A DNA fragment was generated by PCR using pEGFP::PRK2 as a template and the following primers: forward: 5'-gacctg**ggatcc**ctttcacttgttgctgcatcacc-3' reverse: 5'-cattgctgattggtgttaa**gaattc**gacctg-3' The fragment was digested with BamH1 and EcoR1 restriction enzymes and subcloned into BamH1/EcoR1 digested pRK5myc plasmid.
pRK5myc::mPRK2 S301A/S305A	Myc-tagged (N-terminus) mouse PRK2 containing three point mutations to substitute serines 301 and 305 for alanines. Point mutations were generated by PCR-based site directed mutagenesis using pRK5myc::mPRK2 as a template and the following primers: forward: 5'- ttcactggttgcg**g**caccaaccctg**gc**tccacggcagag-3' reverse: 5'-ctctgccgtgga**gc**cagggttggtg**c**cgcaaccagtgaa-3'
pRK5myc::mPRK2 S530A/T532A/S534A	Myc-tagged (N-terminus) mouse PRK2 containing four point mutations to substitute serine 530, threonine 532 and serine 534 for alanines. Point mutations were generated by PCR-based site directed mutagenesis using pRK5myc::mPRK2 as a template and the following primers: forward: 5'-cagtaaatcat**g**ctggc**g**cattc**gc**ccctcagactcctg-3' reverse: 5'-caggagtctgaggg**gc**gaatg**c**gccag**c**atgatttactg-3'
pRK5myc::mPRK2 S301D/S305D	Myc-tagged (N-terminus) mouse PRK2 containing three point mutations to substitute serines 301 and 305 for aspartates. Point mutations were generated by PCR-based site directed mutagenesis using pRK5myc::mPRK2 as a template and the following primers: forward: 5'-ttcactggttgcg**gat**ccaaccctg**gat**ccacggcagag-3' reverse: 5'-ctctgccgtgga**tc**cagggttgg**atc**cgcaaccagtgaa-3'

2.1.2 Site directed mutagenesis

Site directed mutagenesis was performed using single step PCR and the primers listed in table 2.1. PCR reactions were carried out in a reaction volume of 50 μl, using 10 ng of plasmid DNA template, 125 ng of each primer (see table 2.1), 0.25 mM dNTPs, 1 μl (2.5 units) PFU turbo DNA polymerase and the provided polymerase buffer (Stratagene). Reactions were carried out in an Eppendorf Mastercycler using the following parameters: 95°C for 30 secs followed by 16 cycles of [95°C for 30 secs, 55 °C for 1 min, 68°C for 2 mins/Kb plasmid]. Upon completion, the PCR mixture was treated with 1 μl (20 units) of Dpn1 (New England Biolabs) to digest the template DNA. 5 μl of the reaction were then used to transform $CaCl_2$-competent DH5α *E. coli*.

2.1.3 Polymerase chain reaction (PCR)

PCR to amplify the DNA fragment for generating pRK5myc::PRK2ΔHR1 was carried out in a reaction volume of 50 μl, using 50ng of pEGFP::PRK2, 25 pmol of each primer (see table 2.1), 125 μM dNTPs, 2mM MgSO4, 1 μl (5 U) of Platinum Taq DNA polymerase Hight Fidelity and 5 μl of the the DNA polymerase buffer provided (Invitrogen). Reactions were carried out in an Eppendorf Mastercycler, using the following parameters: 95°C for 2 mins, followed by 25 cycles of [94°C for 1 min, 52°C for 2 mins, 72°C for 3 mins, followed by 72°C for 10 mins. PCR products were partially purified using the QIAquick PCR Purification Kit (QIAGEN), following the manufacturer's instructions.

2.1.4 Restriction digests and ligation

pRK5myc plasmid (5μg) and the PCR products from 2.1.3 were digested in a final volume of 50 μl using 1μl of each restriction enzyme (see Table 2.1) (New England Biolabs) and the provided buffers. Reactions were incubated at 37°C. Digested DNA fragments were purified by running them in 1% agarose gels,

excising the appropriate bands and extracting the DNA using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. Ligations were carried out using a 1:8 molar ratio of vector:insert in a final volume of 10μl, using 1 μl (400 units) of T4 ligase in the provided buffer (New England Biolabs). Reactions were incubated at RT for 1 hr and the entire volume used to transform $CaCl_2$- competent DH5α *E. coli.*

2.1.5 DNA gel electrophoresis

1% agarose gels were prepared by dissolving 1 g of agarose in 100 ml of TAE (40 mM Tris Acetate. 1mM EDTA pH 8.0). Ethidium bromide (1μg /ml) was added prior to polymerization. 6X DNA loading buffer (30% glycerol v/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v) was added to DNA samples to achieve a final concentration of 1X DNA loading buffer. Samples were run at 100 V for approximately 1 hour. DNA bands were visualized using a UV transiluminator.

2.1.6 Preparation of $CaCl_2$-competent *E.coli*

A single colony of DH5α was used to inoculate 5 ml of LB (SKI media facility) and the culture was incubated overnight at 37°C with vigorous shaking. The following morning 2.5 ml of the overnight culture were used to inoculate 250 ml of LB. The culture was incubated at 37°C with vigorous shaking until it reached an OD_{600} of 0.7. After this, the culture was chilled on ice for 30 mins. *E.coli* were centrifuged at 4500 rpm at 4°C for 12 mins. Pelleted *E.coli* was resuspended gently in 80 ml of ice-cold TB (10 mM Pipes, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH6.7) and left on ice for 15 min. *E. coli* was then pelleted again by centrifugation at 4500 rpm at 4°C for 12 mins. Pelleted *E.coli* were resuspended in 20 ml of ice cold TB. After resuspension, 0.7 ml of DMSO were added to the suspension while swirling to mix. *E.coli* were placed on ice for 5 mins. After the 5 mins, an

additional 0.7 ml of DMSO (final concentration 7%) was added to the culture which was incubated on ice for an additional 5 mins. Aliquots of 200 μl were prepared, snap frozen stored and at -80°C.

2.1.7 Transformation of $CaCl_2$-competent *E.coli*

$CaCl_2$-competent DH5α *E.coli* were thawed on ice and, per transformation, 50 μl were mixed with the appropriate amount of DNA and incubated on ice for 30 mins. Samples were incubated at 42°C for 2 mins to induce DNA uptake, followed by addition of 1 ml LB (SKI media facility) and incubation at 37°C for 1 hour. Samples were centrifuged at 4000 rpm for 3 mins and the pelleted *E.coli* were resuspended in 50-100 μl of LB. Transformants were streacked on to LB-agar plates (MSKCC media facility) containing either ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

2.1.8 Purification of DNA

Plasmid DNA was purified using either QIAprep Spin miniprep Kit or QIAfilter Plasmid Maxi Kit (both from QIAGEN), following the manufacturer's instructions.

2.2 Cell Biology

2.2.1 Cell lines and culture conditions

All tissue culture plasticware was purchased from Nunc.

2.2.1.1 HeLa S3

HeLa S3 cells (Hall lab) were grown in DME HG + sodium pyruvate (Dulbecco's modified Eagle's medium + 4.5 g/L of D-glucose + 1 mM Sodium Pyruvate – SKI

media facility), supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, lot number 104021) and a mixture of penicillin (100 U/ml) and streptomycin (100 mg/ml) antibiotics (Gibco). Cells were grown in a humidified incubator at 37°C in 5% CO_2. Cells were passaged when ~75% confluent, every 2-3 days.

2.2.1.2 MDA-MB-231

MDA-MB-231 cells, abbreviated here to MDA-231, were obtained from ATCC and grown in Leibovitz's L-15 medium (Gibco 11415), supplemented with 10% FBS (Omega Scientific, lot number 104021) and a mixture of penicillin (100 U/ml) and streptomycin (100 mg/ml) antibiotics (Gibco). Cells were grown in a humidified incubator at 37°C in 0% CO_2. Cells were passaged when ~75% confluent, every 3-4 days.

2.2.1.3 Caco-2

Caco-2 cells (Hall lab) were cultured in DME HG + sodium pyruvate supplemented with 10% FBS (Omega Scientific, lot number 104021) and a mixture of penicillin (100 U/ml) and streptomycin (100 mg/ml) antibiotics (Gibco). Cells were grown in a humidified incubator at 37°C in 5% CO_2. Cells were passaged when ~75% confluent, every 3-4 days.

2.2.1.4 U373

U373 cells were obtained from Cancer Research UK Institute and grown in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated FBS (Omega Scientific, lot number 104021), a mixture of penicillin (100 U/ml) and streptomycin (100 mg/ml) antibiotics (Gibco), 20 mM Hepes (Gibco) and 1x MEM non-essential aminoacids (Gibco). Cells were grown in a humidified incubator at 37°C in 5% CO_2. Cells were passaged when ~75% confluent, every 2-3 days.

2.2.1.5 16HBE14o-

16HBE14o- cells, abbreviated here to 16HBE cells, were generously provided by the Gruenert lab (California Pacific Medical Center). 16 HBE cells were grown in MEM + GlutaMAX + Earle's salts (Gibco), supplemented with 10% FBS (BenchMark lot number A27A00X) and a mixture of of penicillin (100 U/ml) and streptomycin (100 mg/ml) antibiotics (Gibco). Cells were grown in a humidified incubator at 37°C in 5% CO_2. Cells were passaged when ~75% confluent, every 3-4 days.

2.2.2 DNA transfection of HeLa S3 cells

DNA transfections were performed using Lipofectamine 2000 (Invitrogen). For biochemistry studies, $1x10^5$ HeLa S3 cells were seeded on 6-well culture plates and let adhere overnight. Cells were thoroughly resuspended before seeding to avoid aggregation. The day after, cells were transfected in antibiotic-free media with 1 μg plasmid DNA, using 3 μl (6 μg) of lipofectamine 2000 transfection reagent and following the manufacturers instructions. Cells were incubated with the transfection mix for 6-18 hours, before the media was changed to fresh growth media.

For immunofluorescence studies $0.2x10^5$ cells were seeded on glass coverslips placed on 24-well culture plates (Nunc) and let adhere overnight. Cells were thoroughly resuspended before seeding to avoid aggregation. The day after, cells were transfected in antibiotic-free media with either 0.2 or 0.02 (pRK5myc::PRK2 NT) μg plasmid DNA, using 0.6 μl 1.2 μg of lipofectamine 2000 transfection reagent and following the manufacturers instructions. Cells were incubated with the transfection mix for 6-18 hours, before the media was changed to fresh growth media.

2.2.3 siRNA transfection of Caco-2 cells

Caco-2 cells were transfected with siGenome non-targeting siRNA (siControl) (Dharmacon, D-001206-13) or PKN2 siGenome SMARTpool (Dharmacon, M-001206-03) using Dharmafect1 (Dharmacon). $0.5x10^5$ Caco-2 cells were seeded on 6-well culture plates and let adhere overnight. Cells were thoroughly resuspended before seeding to avoid aggregation. The day after, cells were transfected in antibiotic-free media using 100 pmol of siRNA and 5 μl of Dharmafect1, following the manufacturers instructions. Cells were incubated with the transfection mix for 6 hours before the media was changed to fresh growth media.

2.2.4 Caco-2 cyst formation assay

To produce cysts RNAi-transfected Caco-2 cells were trypsinized and resuspended (10^4 cells/ml) in media containing 2% Matrigel (BD). 400 μl of suspension was plated in each well of an 8-well chamber slide (BD) precoated with 30 μl of Matrigel. After 6 days in culture cells were analysed by phase microscopy before and 12 hours after the addition of 0.1 μg /ml of cholera toxin.

2.2.5 Cell synchronization

2.2.5.1 Nocodazole arrest

Either $2x10^5$ or $6x10^5$ cells were seeded on 6-well or 100 mm culture plates and let adhere overnight. The day after, cells were incubated in the presence of 100 ng/ml of nocodazole. After 22 hours either mitotically-arrested cells or mitotically-arrested and interphase cells were collected.

2.2.5.2 Single thymidine block and release

Cells were incubated in the presence of 2mM thymidine for 18 hours. Cells were washed 3 times with PBS and released into fresh medium for 11 hours.

2.2.5.3 Thymidine/nocodazole arrest and release

$5x10^5$ cells were seeded in a 100 mm culture dish and let adhere overnight. The next day cells were incubated in the presence of 2mM of thymidine for 18 hours. Cells were washed 3 times with PBS and released into fresh medium for 6 hours after which they were incubated in the presence of 100 ng/ml of nocodazole. After 6 hours, mitotically arrested (and therefore loosely attached cells) were collected and washed 2 times with fresh media (washes were performed by centrifugation at 900 rpm for 3 mins followed by resuspension). After washing cells were resuspended in 10 ml of fresh media. 2 ml of the suspension were plated on top of poly-L-lysine-coated 35 mm dishes and 0.5ml on top of poly-L-lysine-coated coverslips placed on 24-well culture plates. At specific time points cells plated on 35 mm dishes cells were collected for western bolt analysis, while cells plated on the glass coverslips were fixed and analysed by immunofluorescence.

2.3 Microscopy

2.3.1 Preparation of coverslips

13 mm glass coverslips (Fisher Scientifics) were treated with nitric acid for 15 mins with gentle agitation. Coverslips were washed from the nitric acid under a flowing water tap for 20 mins. Coverslips were rinsed once with deionized water and once with Ethanol to remove water. Coverslips were spread on clean paper-

towel and left to air dry. Once dry, coverslips were sterilized by baking for 30 mins at 180°C.

2.3.2 Fixation and immunofluorescence staining

Cells plated on glass coverslips were washed twice with PBS and fixed with -20°C methanol for 10 min at -20°C. Following fixation coverslips were washed twice with PBS and either stained or stored at 4°C until they were stained. Immunostaining was performed with the primary antibodies indicated in the Results chapter and listed in table 2.2. Staining with primary antibodies was performed at room temperature for 1 hour using antibodies diluted in PBS in the concentrations indicated in table 2.2. After primary antibody staining, the coverslips were washed 3 times with PBS. Coverslips were incubated with 5 μg/ml Alexa 488- and Alexa 568-conjugated secondary antibodies (Invitrogen), for 1 hour at room temperature. Coverslips were then washed 3 times with PBS and incubated with 1μg/ml of Hoechst (1 μg/ml) in PBS for 10 minutes at room temperature, to visualize the DNA. Following immunostaining coverslips were mounted on glass microscope slides (Fisher Scientific) using Dako fluorescent mounting medium (DakoCytomation). Mounting solution was allowed to set by incubating coverslips at 37°C for 30 mins or overnight at room temperature.

2.3.3 Immunofluorescence microscopy

Stained cells were visualized using a AxioImager.A1 (Carl Zeiss, Inc.) microscope equipped with EC Plan-neofluor 40X, 0.5 NA, lenses (Carl Zeiss, Inc.). Images were captured using Hamamatzu ORCA-ER digital camera and AxioVision computer software. Captured images were saved as tiff files to allow further analysis in other software programs. In most cases, images were adjust for

brightness and contrast, to improve image quality and cropped, to increase image size. Images of Caco2 cysts were captured using an Axiovert 200 microscope (Carl Zeiss, Inc.) equipped with plan Neofluor 10x 0.3 NA, lenses (Carl Zeiss, Inc).

2.3.4 Time-lapse microscopy

Time-lapse microscopy was carried out on an Axiovert 200M microscope (Carl Zeiss, Inc.) equipped with Plan-Apocromat 63X, 1.4 oil NA lenses (Carl Zeiss, Inc.), in a CO_2 and temperature-controlled chamber, using an Hamamatzu ORCA-ER digital camera and AxioVision computer software. Captured images were saved as tiff files to allow further analysis in other software programs. Images were taken every 30 seconds. Images were adjust for brightness and contrast to improve image quality and cropped and enlarged to facilitate visualization.

2.4 Protein biochemistry

2.4.1 Preparation of cell lysates

To prepare total cell extracts from attached cells, cells plated on 6-well cell culture plates were placed on ice, washed twice with ice-cold PBS (SKI media core facility) and lysed by adding 200 μl of ice-cold 1% NP-40 lysis buffer [10mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM EDTA pH 8.0, 1% (v/v) NP-40 (Igepal CA630); supplemented with Complete protease inhibitor cocktail tablet (Roche), 30 mM NaF, 1mM Na_3VO_4 and 12 mM β-glycerophosphate] to the cells, followed by scrapping using a cell scraper (Corning) and incubation on ice for 10 mins.

For collecting total cell extracts from mitotic cells, loosely attached, nocodazole-arrested cells, were collected from 6-well cell culture plates, centrifuged at 900 rpm for 3 mins at room temperature, washed twice with ice-cold PBS by resuspension followed by centrifugation and placed on ice. Cells were finally resuspended in 200 μl of ice-cold 1% NP-40 lysis buffer and incubated on ice for 10 mins.

All cell extracts were mixed with 50 μl of 5x concentrated SDS protein sample buffer [50 mM Tris-HCl pH 6.8, 2% SDS (w/v), 0.1 M DTT, 10% (v/v) glycerol, 0.1% (v/v) bromephenol blue], boiled at 100°C for 5 mins to denature proteins and sonicated in a sonicating waterbath (Branson) for 1 min to shear DNA. Total cell extracts were stored at -20°C until they were used.

2.4.2 PRK2 Immunoprecipitation

To immunoprecipitate endogenous PRK2 from interphase and mitotic cells, $6x10^5$ HeLa S3 cells were plated in 4 100 mm dishes and let adhere overnight. Two of the dishes were treated with 100 ng/ml of nocodazole to arrest cells in prometaphase. 22 hours later interphase cells, from untreated dishes and suspended mitotic cells, from nocodazole-treated dishes, were washed two times with PBS and lysed in 1 ml of 0.1% NP-40 lysis buffer [10mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM EDTA pH 8.0, 0.1% (v/v) NP-40 (Igepal CA630); supplemented with Complete protease inhibitor cocktail tablet (Roche), 30 mM NaF, 1mM Na_3VO_4 and 12 mM β-glycerophosphate] either by scraping or by resuspension, respectively. The cell extracts obtained were incubated on ice for 10 mins and centrifuged at 14000 rpm for 15 mins at 4°C. After centrifugation, the soluble fractions from both samples were incubated with 100 μl of 50% slurry Protein G- Sepharose beads (Sigma P3296) for 30 mins at 4°C on a rotating wheel (pre-clear). After pre-clear, beads were pelleted by centrifugation at 13000

rpm for 30 s at room temperature, the soluble fractions were collected and 400 µl from each sample was incubated with 2 µl anti-PRK2 or 9E10 anti-myc (control) antibodies and 100 µl of Protein G-Sepharose beads for 2 hours at 4°C on a rotating wheel. Following immunoprecipitation, beads were washed 3 times on 0.1% NP-40 lysis buffer and once on 2x kinase buffer before kinase reaction (see section 2.4.4).

2.4.3 PRK2 kinase assay

To measure the *in vitro* kinase activity of PRK2, the HTScan PRK1 Kinase Assay Kit was used (Cell Signaling, 7615). Either recombinant Human Protein Kinase N2 (Invitrogen, PV3879) or different amounts of Protein G-Sepharose beads coming from PRK2 immunoprecipitations, were incubated in 50 µl of kinase buffer (25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1mM $NaVO_4$, 5 mM β-glycerophosphate, 2 mM DTT) containing 75 pmol of CREB (Ser133) Biotinylated Peptide (Cell Signaling, 1331) and 2 nmol of ATP (Cell Signaling, 9804), and the adequate concentration of the y 27632 inhibitor, when required, for different amount of times at room temperature. Reactions were stopped by adding 50 µl of STOP buffer (50 mM EDTA pH 8). For the reaction with immunoprecipitated PRK2, tubes were shaked every 2 minutes to resuspend beads. At the end of the reaction, beads were pelleted by centrifugation at 13000 rpm for 30 s at room temperature, 75 µl of kinase reaction product was collected from which 25 were loaded into streptavidin coated plates (StreptaWell from Roche) for ELISA analysis. 5 µl of 6x concentrated protein sample buffer was added to the pelleted beads on 25 µl of kinase reaction and the samples were boiled at 100°C for 5 mins to dissociate PRK2 from the sepharose beads and perform western blot analysis in order to quantify the amount of PRK2 present in each reaction. When comparing the kinase activity of PRK2 immunoprecipitated from nocodazole-

arrested and interphase cells, the activities were normalized by dividing the values obtained on the ELISA by the immunoreactive density of PRK2 estimated using a densitometer and Image J software.

2.4.4 Colorimetric ELISA assay

For the ELISA assay, 75 μl of deionized water and 25 μl of the kinase reaction product were loaded onto streptavidin-coated wells (96 well plate – StreptaWell, from Roche) and incubated from 1 hour at room temperature in a platform shacker (Innova 2000) set for 100 rpm. Each well was washed 3 times for 5 mins with PBS-T (PBS containing 0.1% Tween 20) before being incubated with 100 μl of 1% BSA in PBS-T, containing a 1:1000 dilution of the primary antibody provided with the kit [phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb, Cell Signaling, 9624] for 2 hours at room temperature with rocking. Wells were washed 3 times with 200 μl of PBS-T for 5 mins and incubated with 100 μl of 1% BSA in PBS-T containing 0.25 μg/ml of HRP-conjugated Polyclonal Goat Anti-Rabbit Immunoglobulins (Dako), for 30 mins at room temperature. Samples were washed 5 times with 200 μl of PBS-T for 5 mins with shaking and incubated with 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma), for 15 mins at room temperature. The colorimetric reaction was stopped by adding 100 μl of ELISA Stop Solution (1N HCL). After mixing, by shaking for 1 min in a platform shacker (Innova 2000) set for 100 rpm, the absorbance of the final solution was read at 450 nm in a spectrophotometer (SmartSpec plus, Biorad).

2.4.5 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were prepared in protein sample buffer and loaded onto 1.5 mm thick polyacrylamide gels. Separating gels were prepared by diluting a stock solution of 30% acrylamide/0.8% bis-acrylamide (National Diagnostics) to a final

concentration of 6% (w/v) acrylamide in 750 mM Tris-HCl pH 8.8, 0.1% SDS (w/v), and adding 0.05% (w/v) ammonium persulfate (Amersham Biosciences) and 0.0005% (v/v) tetramethylethylenediamine (TEMED, Fisher Scientific) to polymerize. Stacking gels were prepared diluting a stock solution of 30% acrylamide/0.8% bis-acrylamide (National Diagnostics) to a final concentration of 4% (w/v) acrylamide in 125 mM Tris-HCl pH 8.8, 0.1% SDS (w/v), and adding 0.1% (w/v) ammonium persulfate (Amersham Biosciences) and 0.001% (v/v) tetramethylethylenediamine (TEMED, Fisher Scientific) to polymerize. A full-range rainbow protein marker (Amersham) was included to allow determination of protein size. Proteins were resolved by running gels at 120 V for approximately 90 mins in a Bio-Rad minigel apparatus, in a running buffer (200 mM glycine, 25 mM Tris base and 0.05% (w/v) SDS).

2.4.6 Western blotting

Proteins were transferred from a polyacrylamide gel to methanol-activated PVDF membranes (0.45 μM pore size, Millipore) using a Bio-Rad minitransfer apparatus in a transfer buffer (125mM, 25mM Tris base, 0.1% (w/v) SDS and 10% (v/v) methanol) at 40 V for 2 hours at 4°C. After transfer, membranes were blocked by incubation with 5% milk in TBS-T (50 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1% Tween20) for 1 hour at room temperature with shacking. The membrane was washed from the milk, once with TBS-T and incubated overnight at 4°C with a dilution of the primary antibody indicated on table 2.2, in TBS-T. The next day membranes were washed 3 times with TBS-T for 10 mins at room temperature with shacking and incubated with a 1:5000 dilution of HRP-conjugated secondary antibodies (Dako) in 5% milk in TBS-T for 1 hour at room temperature. Membranes were washed 3 times with TBS-T for 10 mins at room temperature with shacking. Bands were visualized using ECL Western Blotting

Detection Reagents (Amersham), following the manufacturer's instructions and visualized by exposing Fuji medical X-ray film (Crystalgen).

For quantification of the western-blot bands, films were scanned in a Calibrated Densitometer (GS-800, Bio-rad) and exported with a resolution of 300 dpi as grey scale tiff files. Intensity of the bands were quantified using Image J software.

2.5 Antibodies used in this study

The antibodies used in this study are listed in table 2.2 in order of appearance in chapters 3-5.

Antibody	**Host**	**Clone**	**Source**	**Cat#**	**Stock** (μg/ml)	**IF** (μg/ml)	**WB** (μg/ml)
PRK2	mouse	22	BD Transduction	610795	250	2.5	0.25
α-tubulin	rat	YL1/2	Serotec	MCA77S	n/a	1:100	1:1000
myc	mouse	9E10	CRUK	n/a	1200	6	1.2
Scrib	goat	Poly	Santa Cruz	Sc-11049	200	2	0.2
ZO-1	rabbit	Poly	Zymed	61-7300	250	2.5	0.25
PKCζ	rabbit	Poly	Santa Cruz	Sc-216	200	2	0.2

Table 2.2 Antibodies used in this study. List of the antibodies used in this study, either for immunofluoresce microscopy (IF) or western blot analysis (WB), in order of appearance in chapters 3-5. The concentrations used are referenced for the majority of the cases. When the stock concentration is not available (n/a), the working dilution is given. Poly, means polyclonal.

CHAPTER 3 - RESULTS

Analysis of PRK2 phosphorylation during mitosis

3.1 Introduction

The regulation of protein function by phosphorylation and dephosphorylation is a key mechanism of intracellular signaling in eukaryotic organisms, in particular during cell cycle progression. Several kinases are phosphorylated in mitosis downstream of the Cdk-1/cyclinB1 complex and other mitotic kinases (Nigg, 2001). Phosphorylation events can directly regulate the activity of kinases, their ability to bind to other proteins, or their localization inside the cell.

PRK2 was found to be phosphorylated during mitosis in HeLa S3 cells and this phosphorylation event leads to a slower migrating species when subjected to SDS PAGE gel electrophoresis (Schmidt et al., 2007). This chapter describes further analysis of PRK2 phosphorylation during the cell cycle.

3.2 Results

3.2.1 Phosphorylation of PRK2 in mitotic cells

A double thymidine block followed by release has been used to synchronize HeLa S3 cells. Eight to twelve hours after thymidine release, two bands recognized by a specific PRK2 antibody were seen on a western blot and the slower migrating form was shown to correspond to a phosphorylated form of PRK2 (Schmidt et al., 2007). To confirm that this phosphorylation event is specific to mitotic cells and to address the question of whether all PRK2, or only a

fraction is phosphorylated in mitosis, HeLa S3 cells were arrested in prometaphase using nocodazole. After 22 hours of nocodazole treatment, the majority of the cells were round (as would be expected for a prometaphase arrest) and were easily detached from the plate by shake off. As can be seen in Figure 3.1, only one PRK2 band can be detected in the nocodazole-arrested cell population and this corresponds to the phosphorylated form. This result confirms that PRK2 is specifically phosphorylated in mitosis. In addition, it suggests that probably all PRK2 present in cells at this stage of the cell cycle is in this phosphorylated state. As nocodazole treatment induces high levels of phosphorylated PRK2, it will be used as an approach to further investigate the significance of the mitosis-specific phosphorylation event.

Figure 3.1 Phosphorylation of PRK2 upon nocodazole treatment. HeLa S3 cells were cultured in media with (+) or without (-) 100 ng/ml of nocodazole for 22 hours (A) Total cell extracts were ran on an SDS-PAGE gel and PRK2 detected on a western blot using an anti PRK2 antibody. For the cells that have been treated with nocodazole, only the detached mitotically-arrested cells were collected.

3.2.2 Phosphorylation of PRK2 during mitosis in different cell lines

Before analyzing the role of the mitotic phosphorylation of PRK2 in more detail, the generality of the event was investigated using a panel of human cell lines. MDA-231 (breast carcinoma), Caco-2 (colorectal cancer), 16HBE (lung epithelia) and the U373 (glioblastoma) cell lines, were treated with nocodazole and examined for the presence of a mobility shift. As can be seen in Figure 3.2,

PRK2 is expressed in the five cell lines analyzed and a slower migrating form of PRK2 can be detected in all cell lines upon nocodazole treatment. This suggests that regulation of PRK2 by phosphorylation during mitosis is a general event in human cells. In the 16HBE and Caco-2 cell lines the shake off technique was not able to detach the mitotic cells from the plate and all cells in the plate had to be collected. This might be the reason for the presence of a faster migrating band in the nocodazole-treated population in both cases. A faster migrating band for PRK2 can be also detected in the MDA-231 cell line, although in this case only the round cells were collected. The reason for this is not clear, but it is possible that some interphase cells that would have been less adherent were collected together with the mitotic population.

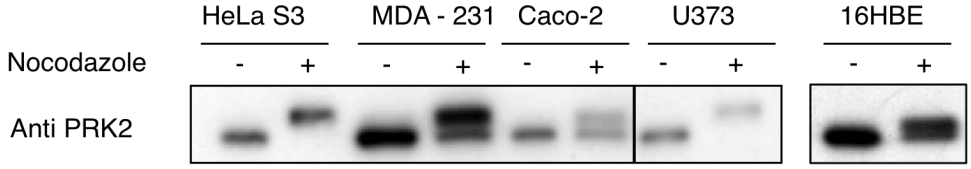


Figure 3.2 Phosphorylation of PRK2 during mitosis in different cell lines. The cell lines indicated were cultured in media with (+) or without (-) 100 ng/ml nocodazole for 22 hours. In the cases where mitotically-arrested cells were easily detached by shake off, only the supernatant, containing floating cells, was collected (HeLa S3, MDA-231 and U373). For the other cell lines, as the mitotic cells remained attached to the plate, all the cells on the plate were lysed (Caco-2 and 16HBE). Total cell extracts from treated and untreated cell populations were electrophoresed on an SDS-PAGE gel and proteins detected on a western blot using an anti PRK2 antibody.

3.2.3 Timing of PRK2 phosphorylation during mitosis

Nocodazole treatment arrests cells in prometaphase and it is not known whether PRK2 is also phosphorylated in other mitotic stages. To examine the timing of PRK2 phosphorylation during mitosis in more detail, HeLa S3 cells were arrested in prometaphase by a single thymidine block followed by nocodazole treatment, mitotically rounded cells were isolated and analyzed for the mobility of PRK2 following release from the nocodazole arrest. As can be seen in Figure 3.3, slower migrating PRK2 is present in prometaphase and metaphase cells, but it disappears as cells progress to cytokinesis. 90 minutes after release from the nocodazole arrest, the majority of the cells are in cytokinesis and almost no slower migrating PRK2 is detected. This result suggests that PRK2 is dephosphorylated as cells enter cytokinesis. However, since some faster migrating PRK2 could still be detected at this time point, the possibility that some phosphorylated PRK2 is present during cytokinesis can not be excluded.

3.2.4 An ELISA-based kinase assay to measure PRK2 activity

To examine whether mitotic phosphorylation affects PRK2 kinase activity, the *in vitro* activities of endogenous PRK2, immunoprecipitated from unsynchronized or nocodazole-arrested cells, were compared. For this, an enzyme-linked immunosorbent assay (ELISA)-based kinase assay was used - the HTScan PRK1 kinase assay from “cell signaling”. This kinase assay uses a biotinylated peptide substrate optimal for PKC family members, which include PRKs, and a phospho-serine/threonine antibody for detection of the phosphorylated form of the substrate peptide. As the substrate peptide is biotinylated, it can be immobilized on streptavidin plates and the level of phosphorylation measured by ELISA. A schematic representation of this assay is shown in Figure 3.4.

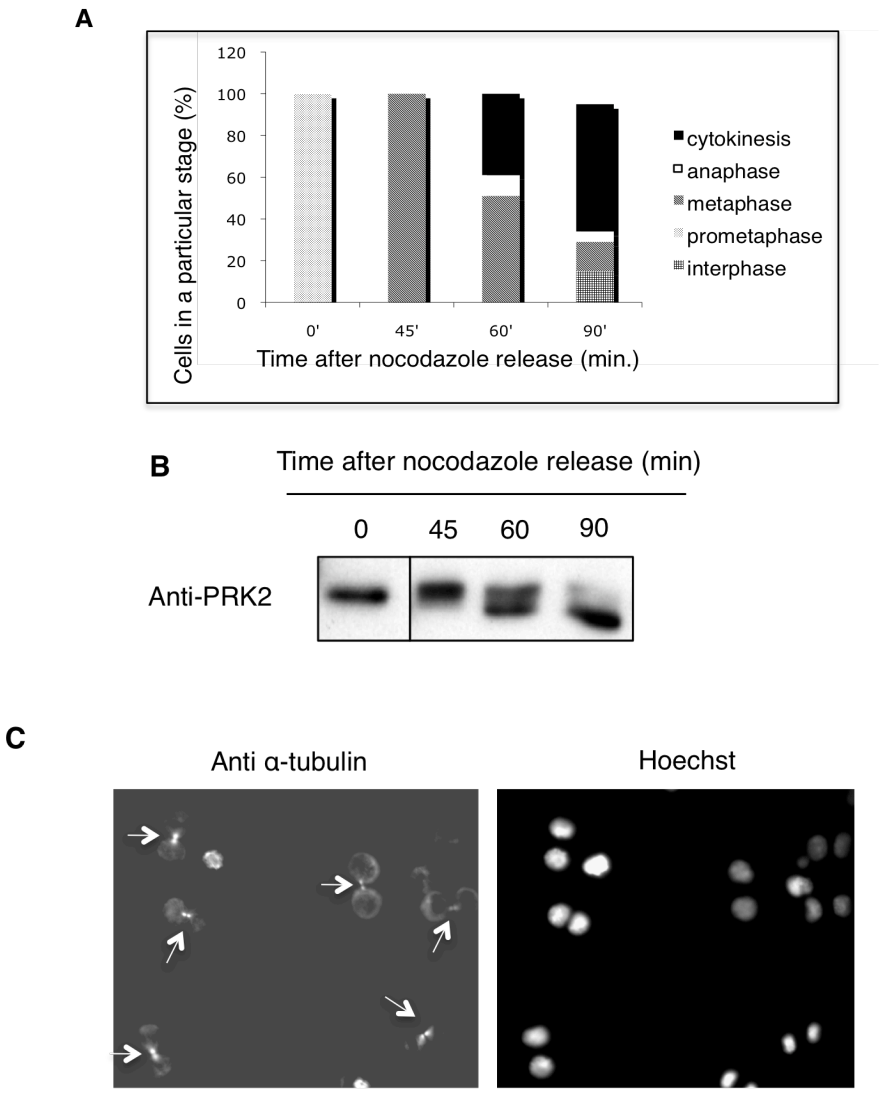


Figure 3.3 Phosphorylation of PRK2 during mitosis. HeLa S3 cells were released from a nocodazole arrest and plated on poly-L-lysine-coated culture dishes or coverslips. At the indicated times, cells were either fixed for determination of the mitotic stage by immunofluorescence using an anti α-tubulin antibody and Hoechst staining (A), or harvested for western blot analysis using an anti PRK2 antibody (B). For each time point, 100 randomly-picked cells were scored for a mitotic stage based on microtubules and nucleus organization. A representative image of cells at 90 minutes after release from nocodazole is shown (C). Arrows in C indicate midbodies, microtubule remnants during and after abscission.

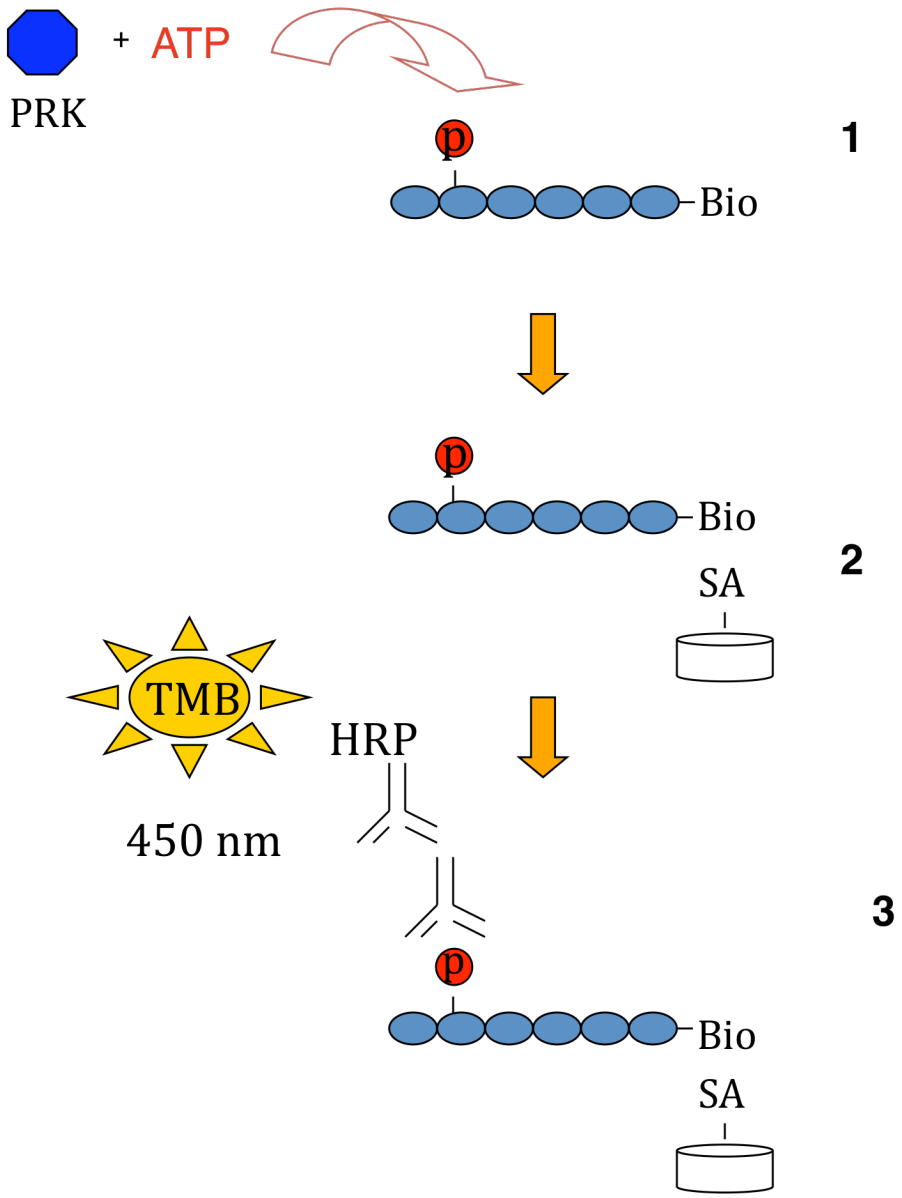


Figure 3.4 HTScan PRK kinase assay using TMB as a read out. Schematic representation of the assay. 1) The biotinylated substrate is incubated with the kinase and ATP; 2) the product of the reaction is immobilized on a streptavidin coated-plate (SA); 3) an anti phosphorylated substrate-specific primary antibody is used and an ELISA is performed using a horseradish peroxidase–conjugated secondary antibody and a colorimetric assay with TMB (3,3', 5,5'-tetramethylbenzidine) as a peroxidase substrate. Absorbance is read at 450 nm.

Recombinant PRK2 is able to phosphorylate the biotinylated peptide (Figure 3.5). To optimize the reaction PRK2 was immunoprecipitated from cycling HeLa cells and a time course and dose-dependent curve of the kinase activity were performed (Figure 3.6).

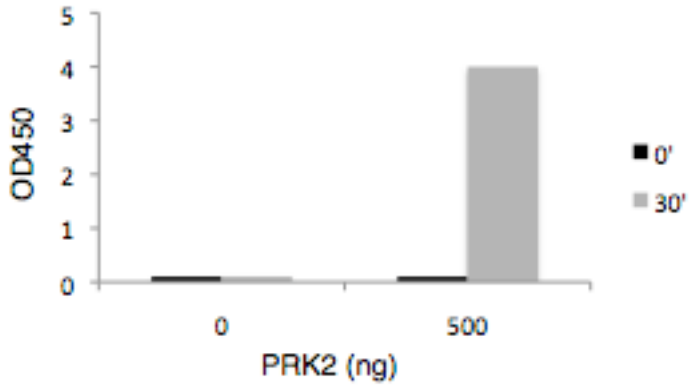
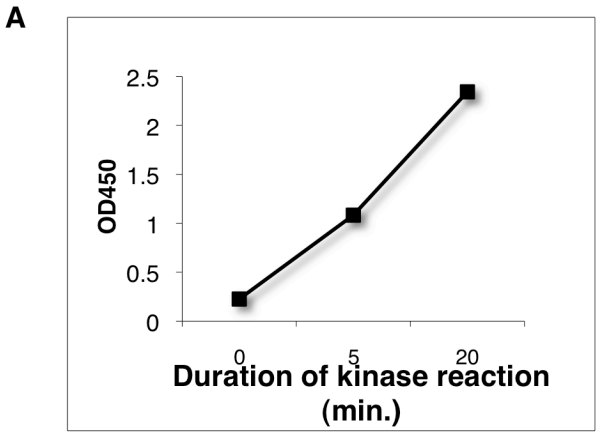
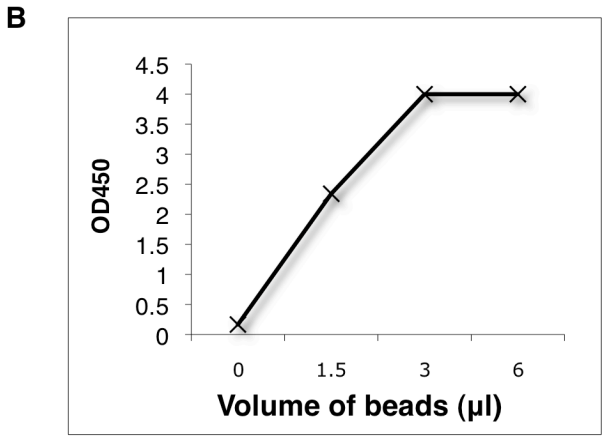


Figure 3.5 Phosphorylation of the biotinylated peptide by recombinant PRK2. 0 or 500 ng of recombinant PRK2 were added with 75 pmol of substrate to 50 µl of kinase reaction, which was performed at room temperature (RT) for the length of time indicated. The reaction products were loaded onto streptavidin-coated plates and analyzed by a colorimetric ELISA assay.





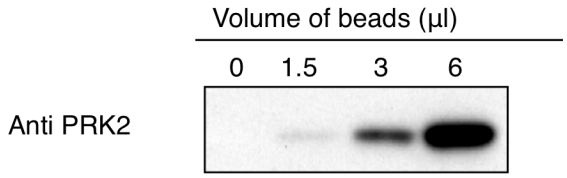


Figure 3.6 Time course and dose-dependence curve of immunoprecipitated PRK2 kinase assay. PRK2 was immunoprecipitated from unsynchronized HeLa S3 cells using a specific anti PRK2 antibody and Protein G-sepharose beads. After immunoprecipitation, 1.5 µl of beads and 75 pmol of substrate were added to 50 µl of kinase reaction, which was performed at RT for the length of time indicated. At the end of the reaction beads were centrifuged and an equal amount of the reaction product was loaded onto a streptavidin plate and a colorimetric ELISA assay was performed as indicated in Figure 3.4 (A). Dose curve of PRK2 kinase activity using increasing amounts of beads and 75 pmol of substrate in 50 µl of reaction. The reaction was performed at RT for 20 min after which the procedure was as in (A) (B – upper panel). In addition after centrifugation, the beads from each of the conditions indicated were ressuspended in protein sample buffer, boiled at 100°C for 5', and then centrifuged. The supernatants were loaded on an SDS PAGE gel and analyzed by western blot using an anti PRK2 antibody as shown (B-bottom panel).

3.2.5 Effect of the mitotic phosphorylation on PRK2 kinase activity

To compare the activities of mitotic and interphase PRK2, PRK2 was immunoprecipitated from unsynchronized and nocodazole-arrested cells and analysed for kinase activity. The phosphorylation(s) responsible for the mobility shift on PRK2 persisted all through the length of the kinase assay, as confirmed by western blot analysis at the end of the reaction (western blots in Figures 3.7-A and -B). Other kinases, apart from PRKs, are able to phosphorylate the substrate peptide used, therefore, there is the possibility that one of these kinases could have been co-immunoprecipitated with PRK2 and be responsible for the activity measured. To control for this possibility, the Y 27632 compound was used at concentrations that are known to inhibit PRK2 (Davies et al., 2000). These concentrations of the inhibitor were seen to successfully inhibit the reaction, supporting the idea that the activity measured corresponds to PRK2 (Figure 3.7). Out of three experiments, mitotically phosphorylated PRK2 was consistently seen to be 60% less active than PRK2 immunoprecipitated from unsynchronized cells. This difference in activities is not very dramatic, but suggests that mitotic phosphorylation could be having an inhibitory effect on PRK2 (Figure 3.7).

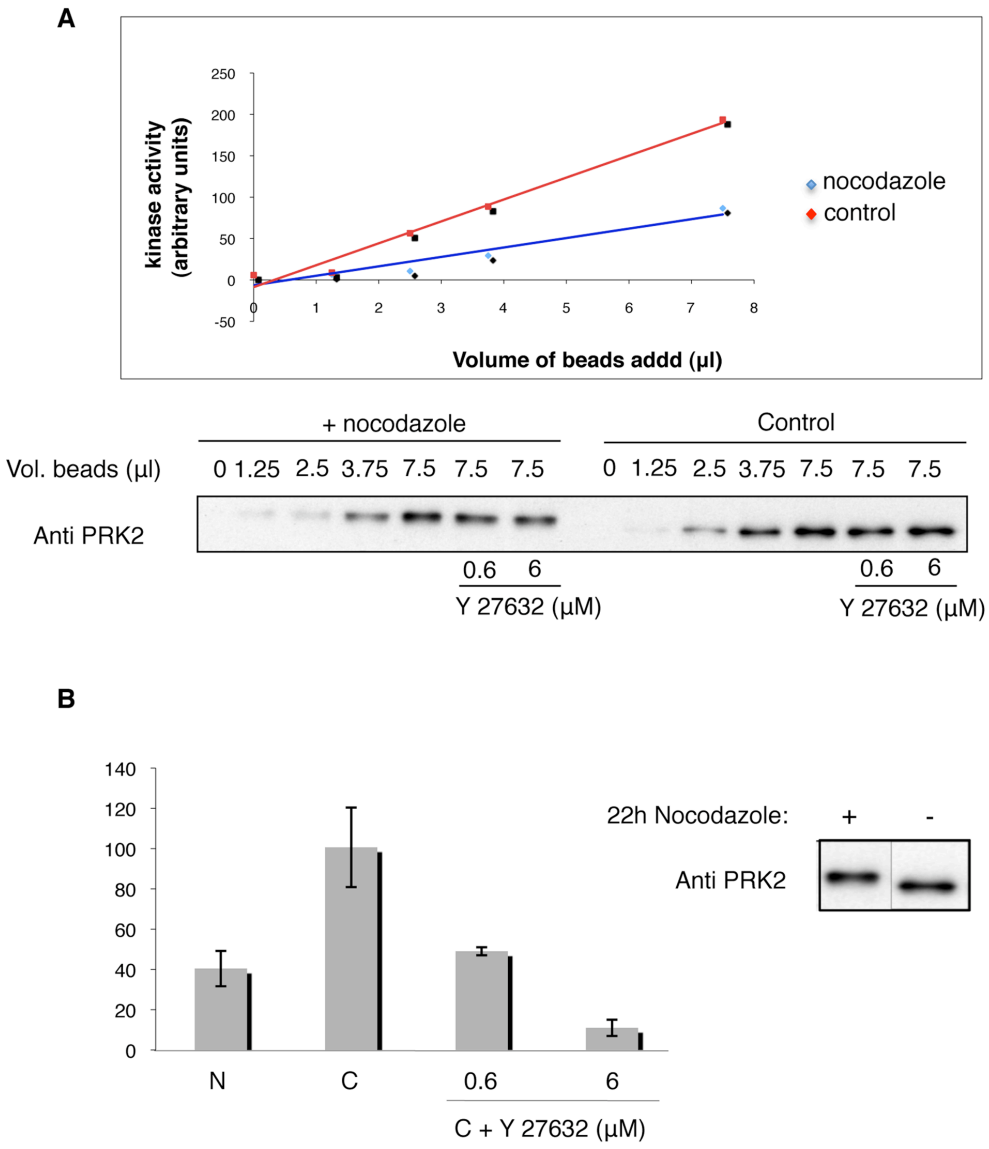


Figure 3.7 Kinase activity of mitotically phosphorylated PRK2. Endogenous PRK2 was immunoprecipitated from unsynchronized (control) and nocodazole-arrested HeLa S3 cells, using an anti PRK2 antibody and protein G-sepharose beads. After immunoprecipitation, increasing volumes of beads and 75 pmol of substrate were added into 50 µl of kinase reaction. After 20 min, the beads were centrifuged, the supernatants containing the product of the kinase reaction were collected and the level of phosphorylation of the substrate was analysed by ELISA as indicated in Figure 3.4 (A). At the same time, the beads were ressuspended in protein sample buffer, boiled at 100°C for 5 min and centrifuged. The supernatants were run on an SDS-PAGE gel and analysed by western blot using an anti PRK2 antibody (A-bottom panel). The values obtained for the ELISA were corrected for the levels of PRK2 present in each reaction as measured based on the intensity of the western blot bands using a densitometer and ImageJ software (A). The average of the kinase activities measured when using 7.5 µl of beads from each condition, in three distinct experiments, are represented, together with the kinase activity obtained when the indicated concentration of the Y 27632 was added into the control reaction (B).

3.2.6 Mapping the mitosis-specific PRK2 phosphorylation site(s)

To define the region and specific residues in PRK2 that are phosphorylated during mitosis and responsible for a shift in electrophoretic mobility, myc-tagged full-length mouse PRK2 (myc-mPRK2) and the PRK2 deletion mutants indicated in Figure 3.8, were transiently expressed in HeLa S3 cells and analysed for their mobility on western blots, using an anti myc antibody. As can be seen in Figure 3.8, overexpressed full-length myc-mPRK2 was phosphorylated during mitosis, although not 100%, as a faster migrating form can also be detected. From the analysis of the mutants it can be concluded that PRK2 is phosphorylated on residues located between residues 296-984, as a mutant lacking this region (PRK2 ΔHR1) still undergoes a mobility shift. Unfortunately both PRK2 NT (1-597) and PRK2 CT (597-984) did not undergo a motility shift and it was, therefore, not possible to further narrow down the region of the phosphorylation event, in this way.

3.2.7 Identification of the phosphorylation site(s)

Mitosis-specific phosphorylation sites in PRK2 have been found in a recent large-scale mass spectrometry study (Dephoure et al., 2008). The number of independent observations of each phosphopeptide is noted in the publication and two sites on PRK2 were found to be particularly abundant: S302 and S306. In addition, another three sites found in this study S531, T533 and S535 are highly conserved in PRK1 and PRK2 (see Figure 3.9). For these reasons, the correspondent sites were mutated to alanines in myc-mPRK2. Two independent mutants were constructed: pRK5myc::mPRK2 S301A/S305A and pRK5myc::mPRK2 S530A/T532A/S534A (see Figure 3.9 and note that mouse PRK2 has one aminoacid less than human PRK2, therefore the numbers are change). Mutations in S530, T531 and S534 did not inhibit the mobility shift of

myc-mPRK2 after nocodazole treatment. However substitution of serines 301 and 305 for alanines (S301A/S305A) significantly reduced the phosphorylation of the protein (Figure 3.9-B). This result confirms that either serine 301 or 305 in mouse PRK2, corresponding to serine 302 and 306 in human PRK2, are phosphorylated in mitosis. The residual mobility shift seen when both these serines were mutated into alanines, suggests that other sites contribute to the mobility shift.

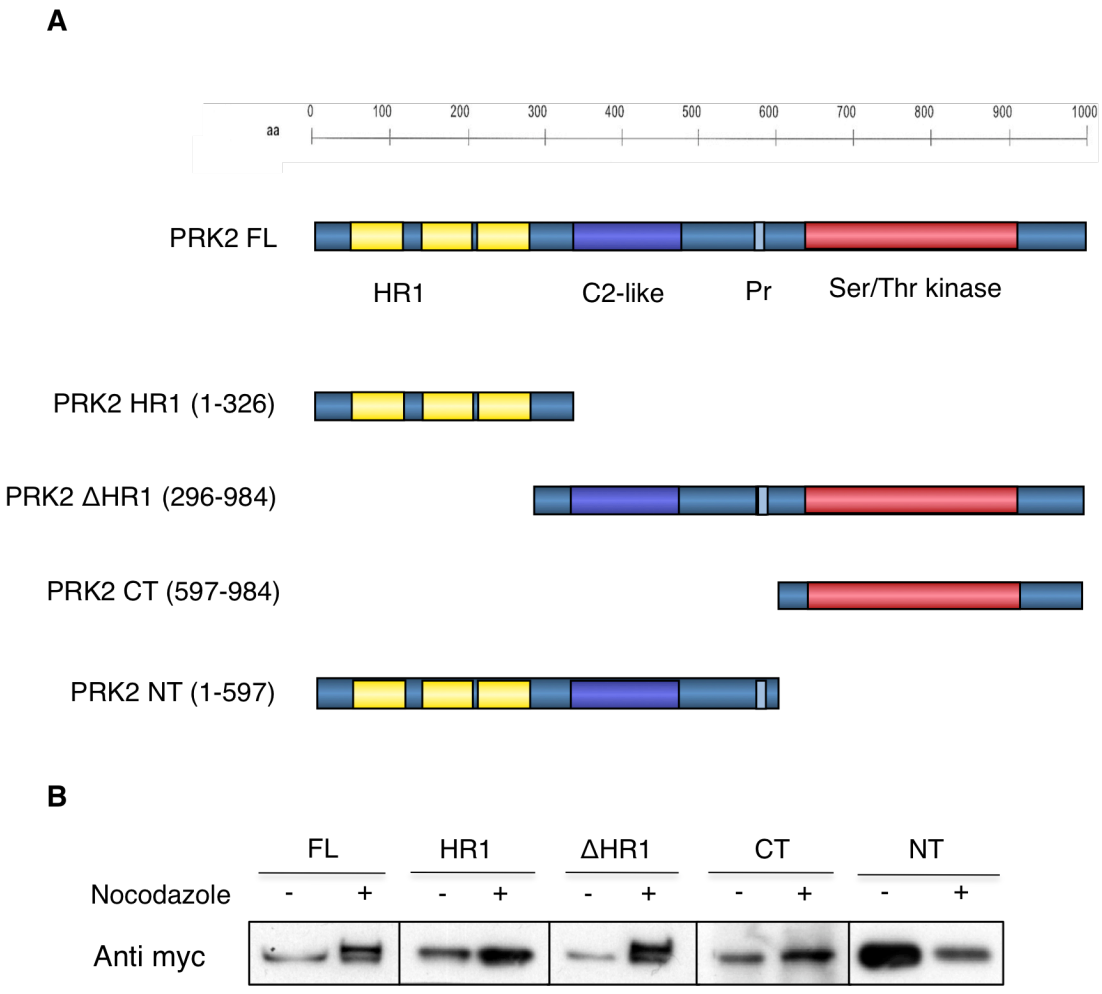


Figure 3.8 Mitotic mobility shift of PRK2 deletion mutants. A schematic representation of full-length human PRK2 and of the PRK2 deletion mutants analysed is shown (A). HeLa S3 cells were transiently transfected with myc-tagged versions of the PRK2 constructs indicated. 24 hours after transfection cells were treated with (+) or without (-)100 ng/ml nocodazole for 22 hours.

Total cell extracts from unsynchronized (-) and nocodazole-arrested cells (+) were collected and analysed by western blot using an anti myc antibody.

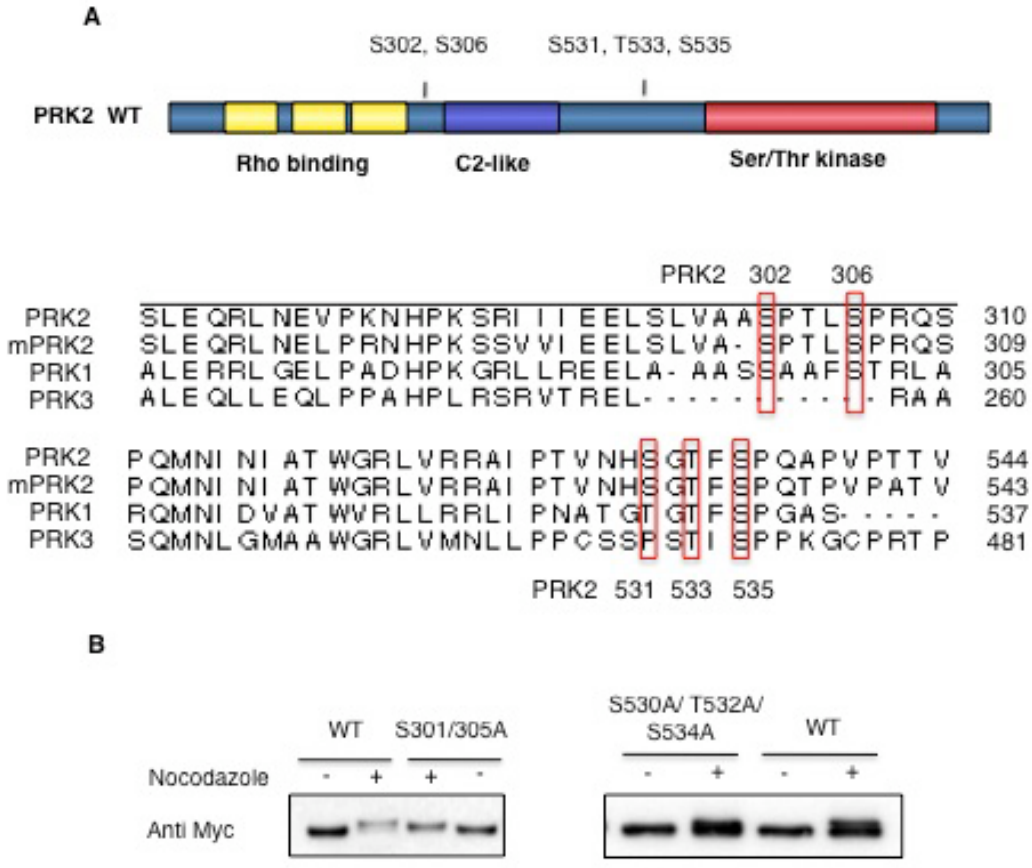


Figure 3.9 Mutational analysis to identify the PRK2 phosphorylation site(s) responsible for the mobility shift. A schematic representation of wild type human PRK2 (PRK2 WT) and the localization of the residues that were mutated is shown (upper panel), together with an alignment of, PRK2, mouse PRK2 (mPRK2), PRK1 and PRK3 showing the conservation of the residues. The numbers indicated correspond to human PRK2 (A). HeLa S3 cells were transiently transfected with myc-tagged versions of wild type mouse PRK2 (WT) and mouse PRK2 constructs carrying the aminoacid substitutions indicated. 24 hours after transfection cells were treated with (+) or without (-)100 ng/ml nocodazole for 22 hours. Total cell extracts from unsynchronized (-) and nocodazole-arrested cells (+) were collected and analysed by western blot using an anti myc antibody.

3.2.8 Effect of overexpression of the phosphorylation mutants on cells

To address whether the phosphorylation of serines 302/306 (human) play any functional role during mitosis/cytokinesis, cells were transfected with myc-mPRK2 S301A/S305A and with a myc-mPRK2 mutant in which both serines were substituted for aspartates (S301D/S305D), to create a potential phosphomimetic mutant. Four days after transfection, cells were analysed under the microscope for any defect in proliferation. The percentage of cells expressing the PRK2 mutants was similar to control, which suggests that cells expressing these mutants proliferate normally. A relatively high number of multinucleation was observed in the cells expressing the mutants (Figure 3.10), but a similar phenotype was also observed for cells expressing wild type PRK2, as previously reported (Schmidt et al., 2007). No conclusion can therefore be reached, on whether the mutations introduced in PRK2 interfere with cytokinesis.

3.2.9 Localization of the phosphorylation mutants during cytokinesis

Phosphorylation of PRK2 could also regulate its localization. PRK2 has been shown to localize in the cytoplasm during interphase, prometaphase and metaphase. At telophase and cytokinesis, PRK2 localizes at the cleavage furrow and midbody, respectively. To investigate whether phosphorylation at serines 302 and 306 (mouse), or both, regulate PRK2 localization, myc-mPRK2 S301D/S305D and a myc-mPRK2 S301A/S305D, were overexpressed in cells and their localization at cytokinesis was analysed by immunofluorescence using an anti myc antibody. Localization of myc-mPRK2 at the cleavage furrow and around the midbody during cytokinesis was quantified in the transfected cells. As it is shown in Figure 3.11, ~90% of cells in cytokinesis that express wild type myc-mPRK2 show the characteristic localization of PRK2, ~70% of the cells

expressing the S301/S305 and ~80% of cells expressing the S301D/S305D mutants show identical localization.

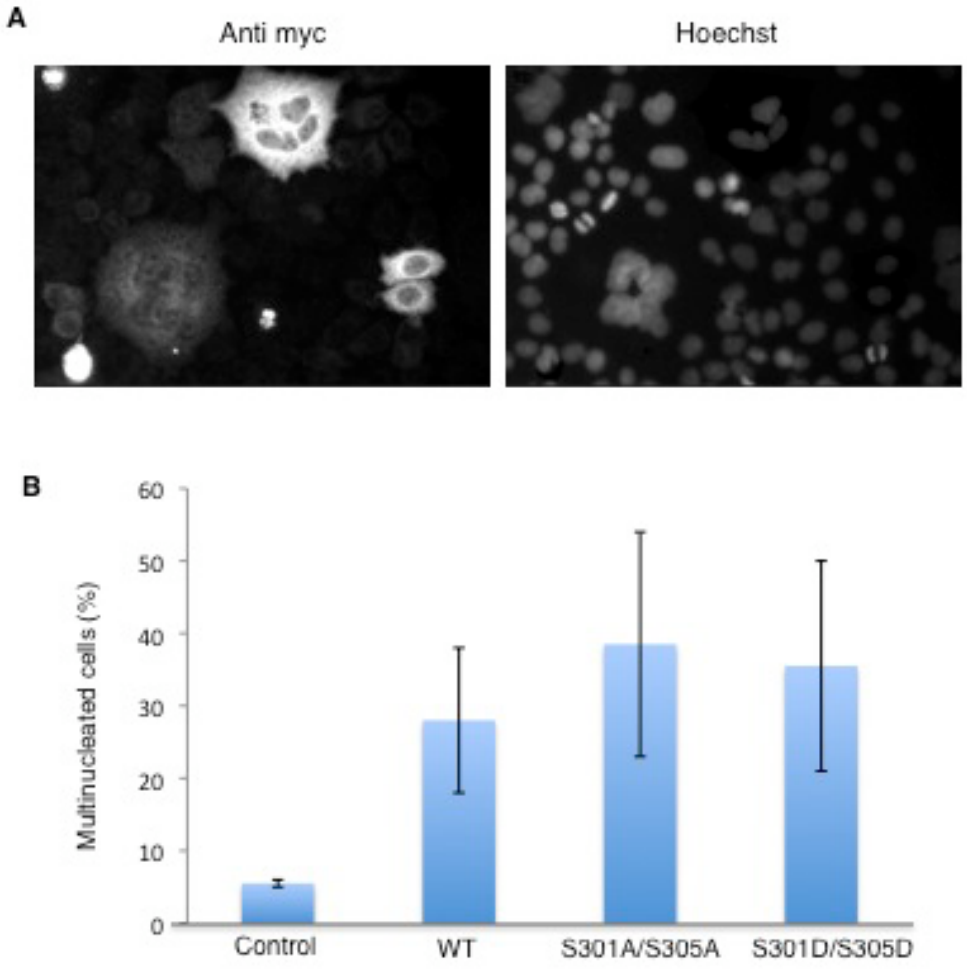


Figure 3.10 Effect of overexpression of the phosphorylated mutants in the proliferation of the cells. HeLa S3 cells were transfected with either control, or myc-tagged versions of wilt type mPRK2 and mPRK2 carrying the mutations indicated. After 4 days, cells were fixed and stained with anti myc and anti α-tubulin antibodies, as well as Hoechst. The figure is a representative image of cells shows cells expressing myc-mPRK2 S301A/S305A (A). The percentage of transfected cells that were multinucleated is indicated for each case. The quantifications shown are an average of two independent experiments in which 100 transfected cells were analysed (B).

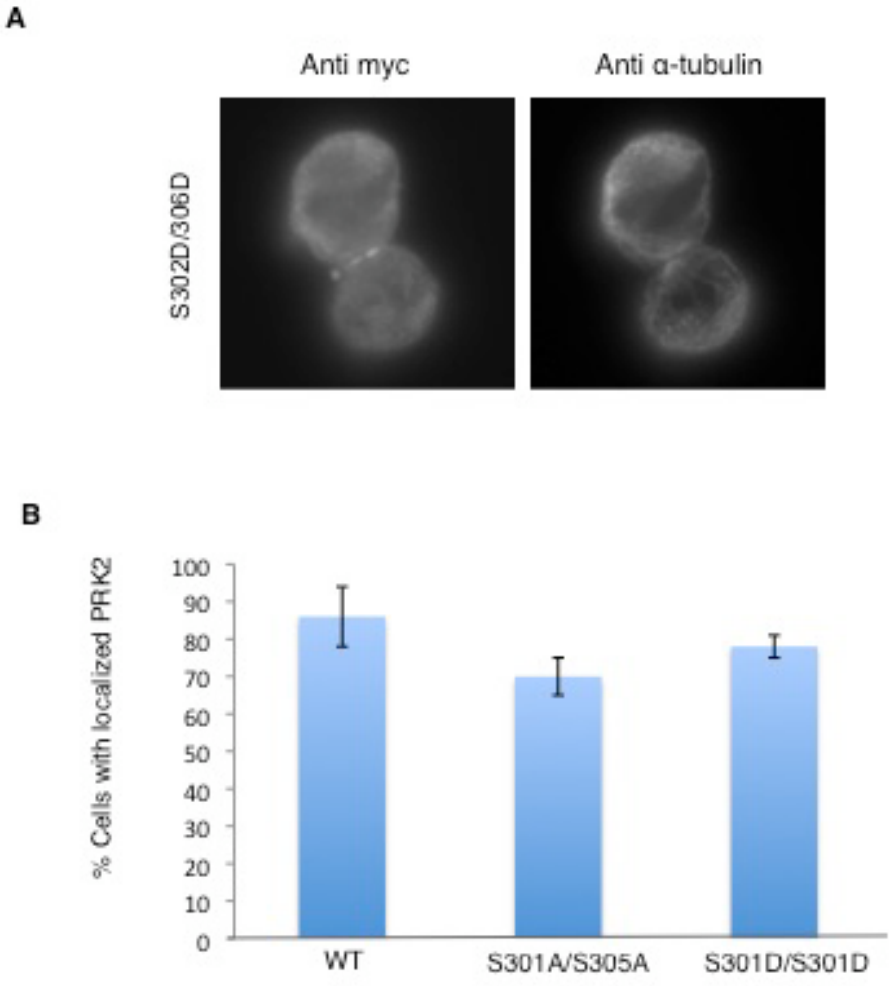


Figure 3.11 Localization of the phosphorylation mutants in cytokinesis. HeLa S3 cells were transfected with wild type myc-mPRK2 vectors (WT) or myc-mPRK2 vectors carrying the mutations indicated. To increase the percentage of cells in cytokinesis, one day after transfection cells were synchronized in mitosis after a single thymidine block (18h), followed by release (11 hours). After this, cells were fixed and stained with anti-myc and anti-α-tubulin antibodies. Expressing cells that were undergoing cytokinesis, as judged by the organization of the microtubule cytoskeleton, were scored for the presence of myc-mPRK2 at the cleavage furrow or around the midbody. The figure shows a cell expressing myc-mPRK2 S301D/S305D (A). The quantifications shown are an average of two independent experiments in which 20 transfected cells were analysed (B).

3.3 Discussion

The Rho GTPase effector PRK2 has been shown to be phosphorylated during mitosis in HeLa S3 cells in a way that leads to a mobility shift when subjected to SDS PAGE gel electrophoresis. How this phosphorylation event affects PRK2 activity and function is not known. Here we were able to confirm that almost all PRK2 is specifically phosphorylated in mitotic HeLaS3 cells by using nocodazole to arrest cells in prometaphase. In addition we were able to extend this observation to other human cell types. PRK2 was seen to be expressed in five cell lines analysed and a mobility shift could be detected in all these cell lines upon nocodazole treatment.

Analysis of the PRK2 mobility shift after release from prometaphase arrest, showed that PRK2 is phosphorylated during prometaphase and metaphase but is dephosphorylated as cells progress to cytokinesis. 90 minutes after nocodazole wash, the majority of the cells are in cytokinesis and almost no slower migrating PRK2 is detected (Figure 3.3). However, since some faster migrating PRK2 could still be detected at this time point, the possibility that some phosphorylated PRK2 is present during cytokinesis can not be excluded. The timing of PRK2 phosphorylation observed correlates with the timing of Cdk1/cyclinB activity during mitosis (Nigg, 2001) and is consistent with the suggestion that PRK2 phosphorylation occurs downstream of Cdk1 (Schmidt et al., 2007).

No link has been reported, so far between PRK2 and the regulation of mitotic events that occur at prometaphase, metaphase or anaphase, although, PRK2 has been reported to be involved in G2/M transition and in cytokinesis. The phosphorylation event could be related to these events. In HeLa S3 cells, PRK2 has been implicated in positively regulating Cdk1 activity at G2/M transition through the phosphorylation of Cdc25B. A phosphorylation event on PRK2 downstream of Cdk1 in prometaphase could be part of a positive feed-back loop

and the role for PRK2 at this stage would be to propagate Cdk1 activation (Schmidt et al., 2007). In contrast to this idea, there have been two reports suggesting that PRK1 can inhibit Cdk1 activity in response to certain stimuli such as arsenite or TGFß (see introduction) (Isagawa et al., 2005; Su et al., 2007). However, in one of these studies, which was done in vascular smooth muscle cells, no PRK1 mobility shift was detected at G2/M. The mechanism of PRK regulation during the cell cycle in these cells might be different from the one analysed here. Another possibility is that PRK2 is activated at G2/M transition, inactivated during mitosis through this phosphorylation and activated again in cytokinesis by dephosphorylation.

To try to address the question of whether mitotic phosphorylation affects PRK2 kinase activity, an ELISA-based *in vitro* kinase assay was used to compare the activities of immunoprecipitated PRK2 from prometaphase and cycling cells. Mitotically phosphorylated PRK2 was 60% less active than PRK2 immunoprecipitated from cycling cells. This difference in activites is not very dramatic although it's similar to what has been reported for PRK1 and PRK2 in other contexts. For example, the co-expression of V14 RhoA with PRK1 was shown to stimulate PRK1 kinase activity towards the PKCδ pseudosubstrate by 2.2 fold (Amano et al., 1996; Watanabe et al., 1996). Another study, reported that hyperosmotic stress induces GFP-PRK1 activation by ~2 fold (Torbett et al., 2003). A 2-fold increase in auto-kinase activity was also reported for endogenous PRK2 immunoprecipitated from keratinocytes after a Ca2+ switch, in a report that proposes a role for PRK2 in keratinocyte differentiation (Calautti et al., 2002). These data suggest that small changes in PRK kinase activities might be physiologically relevant.

Others have attempted to analyse PRK1 activity during the cell cycle in smooth muscle cells using the phospho-Thr774 antibody, which recognizes the PDK-1 phosphorylation site that is believed to be required for PRK1 activity (Flynn et al.,

2000). Phosphorylation of Thr774 after release of the cells from a G1 block induced by hydroxyurea, resulted in a ~1.5 fold increase in activity, when majority of the cells were at the G2/M phase (Su et al., 2007). This result suggests an increase in PRK1 activity at G2/M, although since cells were not fully synchronized it is possible that activities at G2 and M are different. In addition, as already mentioned, no mobility shift in PRK1 was detected in this study when cells were synchronized at G2/M, which suggests that the mechanism of PRK regulation in these cells might be different. The state of activation loop phosphorylation was not analysed in the present study as it is known that phosphorylation at the activation loop does induce a mobility shift on PRK2 (Flynn et al., 2000).

Cell cycle events are strictly regulated in time by numerous checkpoints in order that one event doesn't starts before the previous is finished. As PRK2 has been linked to cytokinesis in HeLaS3 cells, one possible role for mitotic phosphorylation is to prevent premature function of PRK2. Consistent with this hypothesis is the observation that PRK2 is dephosphorylated during cytokinesis.

In an attempt to identify the phosphorylation site(s) that lead to the PRK2 gel mobility shift, PRK2 deletion mutants were expressed in HeLaS3 cells and their mobility after nocodazole treatment analyzed. From this analysis the residues located between amino acid 1 to 295 were excluded as possible phosphorylated sites, as a mutant lacking this region (PRK2 ΔHR1) still undergoes a mobility shift after nocodazole treatment. However, PRK2 NT (1-597) and PRK2 CT (597-984) did not undergo a mobility shift. Several reasons could be responsible for this observation, such as that the protein domains missing in either mutant are required for the interaction of PRK2 with the kinase that is phosphorylating it, or with other proteins that are important for the phosphorylation to happen. In any case, due to this fact, it was not possible to further narrow down the region of the phosphorylation event in this way.

It was interesting to notice that the Rho binding domain (HR1) is not required for the mitotic phosphorylation of PRK2. This suggests that Rho GTPases are not required for this phosphorylation and would be consistent with a possible inhibitory role found for the phosphorylation event. However, as the HR1 domain in PRK, act as an auto-inhibitory domain that keeps the protein in a closed conformation, it is possible that the PRK2 ΔHR1 mutant mimics the effect of Rho GTPase binding and allows phosphorylation. In fact, *Schmidt et al.* showed that inhibition of Rho GTPase activity by toxin B blocked PRK2 phosphorylation at G2/M. However, it is not known if this effect is through a direct interaction between Rho GTPases and PRK2 or through an indirect effect on cell cycle progression. Further experiments, for example point mutations on PRK2 to inhibit Rho binding, would be required to address whether Rho GTPase binding to PRK2 is required for the mitotic phosphorylation event to take place.

A large-scale mass spectrometry study identified mitosis-specific phosphorylation sites in PRK1, PRK2 and PRK3 after nocodazole treatment of HeLaS3 cells (Dephoure et al., 2008). Based on these results, five serines were mutated into alanines in mouse PRK2 and the mobility of the mutants on SDS PAGE electrophoresis after nocodazole treatment was analysed. In this way, serine 301/ serine 305 (human serine 302, 306), were shown to contribute to the mobility shift observed in PRK2 during mitosis. This data confirms the result published from the mass spectrometry study in which these two serines were found to be the most abundant phospho-sites detected in PRK2 coming from nocodazole-arrested HeLaS3 cells. Both serine 301 and 305 are followed by a proline residue on PRK2. Cdk1 is a highly proline-directed kinase and readily phosphorylates S/TP sites in a number of mitotic substrates. This result supports the idea that Cdk1 is the kinase responsible for the mitotic phosphorylation that gives rise to a mobility shift in PRKs, although there are other proline directed kinases that could be acting downstream of Cdk1. Interestingly, although serines 302 and 306 appear to be conserved in PRK1, the surrounding residues are not,

in particular, the proline residue is not (see Figure 3.9). As PRK1 has also been shown to shift during mitosis it would be interesting to know whether these serines are also responsible for the mobility shift in PRK1. Serines 302 and 306 are not conserved in PRK3 (Figure 3.9), but because of the lack of validated specific antibodies for PRK3 it has not been possible to evaluate whether PRK3 is also phosphorylated during mitosis.

Some residual mobility shift is found when serines 301 and 305 are mutated into alanines, suggesting that other sites contribute to the shift. 10 other phospho-sites in PRK2 were found to be upregulated in nocodazole arrested HeLa cells in the same mass spectrometry study, although the frequency with which these sites were found was not very high. Based on conservancy of these potential sites between PRK1 and PRK2, the S530, T532 and S534 residues on mouse PRK2 were mutated into alanines, but these mutations did not interfere with the nocodazole-induced mobility shift of PRK2. Other sites contributing to the mobility shift seen in PRK2 in mitosis are still to be identified.

In order to address whether phosphorylation of serine 301 and/or serine 305 has an effect on cell cycle progression of HeLa cells, the PRK2 S301A/S305A mutant and the PRK2 S301D/S305D mutant (in which both serines were substituted for aspartic acid to create a potential phosphomimetic mutant), were transfected into cells and the cells were analysed for any defect in cell cycle progression. No effect on cell number was detected compared with control, suggesting that cells carrying the mutated forms of PRK2 proliferate normally. Some cells expressing the mutants were binucleate, although as a binucleate phenotype is also observed in cells transfected with wild-type PRK2 (control), no major conclusion can be drawn from this observation.

In addition to regulating kinase activity, mitotic phosphorylation could potentially affect PRK2 localization. As will be described into more detail in Chapter 4, PRK2

localizes at the cleavage furrow during cytokinesis, while it is mainly cytoplasmic in the other mitotic stages as well as in G1 and S phase. Cdk1 activity is known to prevent cytokinesis and recent reports have shown that Cdk1 phosphorylation of proteins involved in cytokinesis, during metaphase, prevents their localization at the mitotic spindle or association with other proteins involved in cytokinesis (Hummer and Mayer, 2009; Neef et al., 2007). In order to address the question of whether mitotic phosphorylation affects PRK2 localization, the localization of both non-phosphorylatable and phosphomimetic mutants was analysed during mitosis. No premature localization of the non-phosphorylatable PRK2 mutant at the equatorial cortex was detected and the potential phosphomimetic – S302D/S306D mutant was still seen to localize at the cleavage furrow, similarly to wild-type PRK2 (see Figure 3.11). In conclusion, no clear physiological role for the phosphorylation event could be identified. It would be interesting to find the additional sites that are responsible for the mobility shift and analyse them in terms of an effect on PRK2 localization during mitosis.

CHAPTER 4 - RESULTS

Mapping the domain responsible for PRK2 localization during cytokinesis

4.1. Introduction

The precise localization of proteins in cells is essential for their biological function (Tsunoda et al., 1997). PRK2 has been shown to localize at the cleavage furrow and the midbody during cytokinesis, however the mechanism through which this is regulated is not known. Several protein:protein or protein:lipid interactions could be responsible for the PRK2 localization during cytokinesis. For example, the PRK2 HR1 domain binds Rho, which is a main regulator of cytokinesis that localizes at the cleavage furrow and midbody. In fact, Rho localization during cytokinesis is regulated by the GEF Ect2 and depletion of Ect2 by siRNA has been shown to disrupt PRK2 localization at the midbody, suggesting that Rho may be responsible for the localization of PRK2 during cytokinesis (Schmidt et al., 2007). However whether direct binding of PRK2 to Rho is involved, has not been directly investigated. Other mechanisms could be responsible for localizing PRK2 during cytokinesis. In addition to the HR1 domain, PRK2 also contains a C2-like domain, a C-terminal kinase domain and one proline-rich region. The N-terminus of PRK1 (including the HR1 and the C-2 domain) binds CG-NAP, a scaffold protein localized to the Golgi apparatus, the centrosome and the midbody (Takahashi et al., 1999). The C-terminus of PRK2 binds the tyrosine phosphatase PTP-BL, a PDZ-domain containing protein, which localizes to the cleavage furrow and midbody and has been implicated in cytokinesis (Gross et al., 2001; Herrmann et al., 2003). C2 domains are involved in protein:lipid interactions and also protein:protein interactions and, even though the function of the PRK2 C2-like domain is not known, it could be involved in recruiting PRK2 to

the cleavage furrow and/or midbody during cytokinesis (Benes et al., 2005; Cho, 2001; Raftopoulou et al., 2004; Rizo and Sudhof, 1998(Mukai, 2003). Finally, the proline-rich region in PRK2 could also be responsible for PRK2 localization. This chapter describes experiments to better characterize PRK2 localization during cytokinesis and identify the domain responsible.

4.2. Results

4.2.1 Localization of endogenous PRK2 during cytokinesis

Endogenous PRK2 localizes at the cleavage furrow during telophase and early cytokinesis and at the midbody during late cytokinesis (Figure 4.1). PRK2 localization at telophase and cytokinesis is very similar to what has been described for the localization of active RhoA (Nishimura and Yonemura, 2006; Yuce et al., 2005).

4.2.2 Localization of GFP-PRK2 at telophase and cytokinesis in living cells

To visualize the localization of PRK2 during cytokinesis in living cells, HeLa S3 cells were transfected with GFP-PRK2 and time-lapse imaging of these cells was performed. As can be seen in Figure 4.2, GFP-PRK2 can be detected at the equatorial cortex before furrow formation and is highly concentrated at the cleavage furrow during telophase and early cytokinesis and around the midbody at late cytokinesis. This localization is similar to what was seen for endogenous PRK2 using specific antibodies on fixed cells.

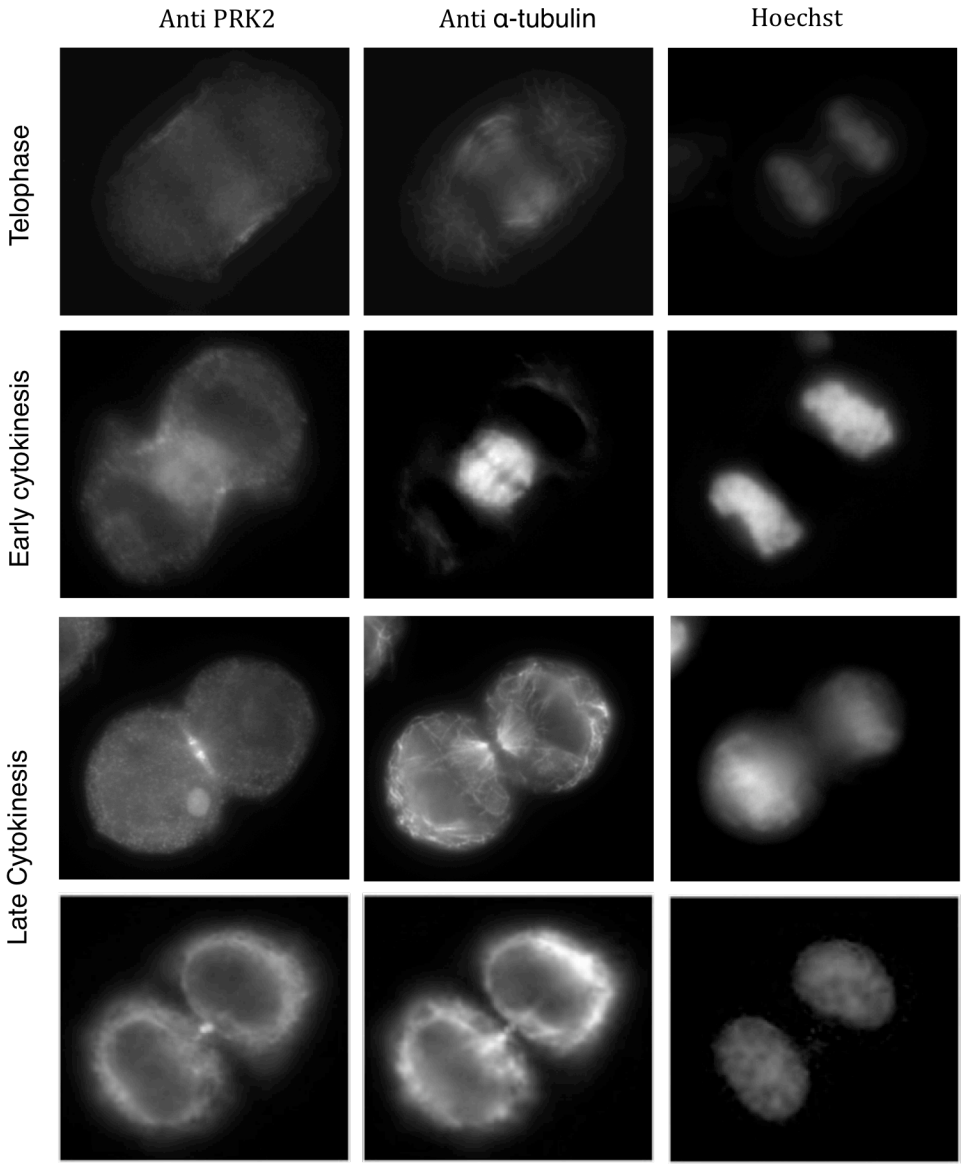


Figure 4.1 Localization of endogenous PRK2 during telophase and cytokinesis. HeLa S3 cells were fixed and stained with anti PRK2 and anti α-tubulin antibodies, as well as with Hoechst. Cells at telophase and at different stages of cytokinesis are shown

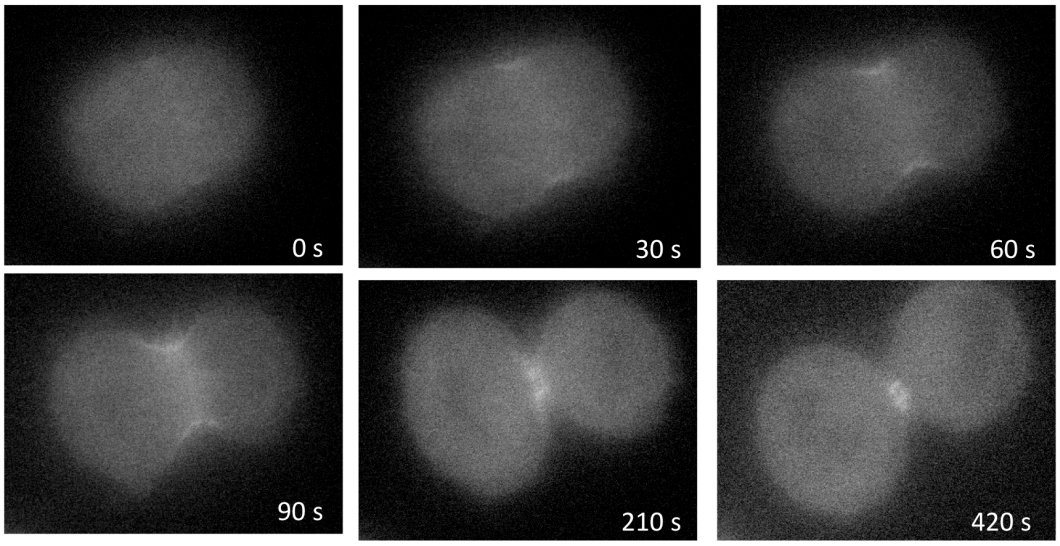


Figure 4.2 Localization of PRK2 in living cells. A HeLa S3 cell transiently expressing GFP-PRK2 at successive time points during telophase (0s) and cytokinesis (30-420s).

4.2.3 The C-terminal domain is not required for PRK2 localization during cytokinesis

To identify the domain that is required for the localization of PRK2 during cytokinesis, myc-tagged full-length PRK2 and PRK2 deletion mutants were transfected into HeLa S3 cells. The cells were analysed by immunofluorescence using an anti myc antibody and an anti α-tubulin antibody, to visualize microtubules and identify cells in cytokinesis. In order to increase the percentage of cells that are at this stage of the cell cycle, a single thymidine block followed by an 11-hour release was performed one day after transfecting the cells. The schematic representation of full-length PRK2 and each of the mutants analysed, is shown in Figure 4.3. Myc-tagged full-length PRK2 (PRK2 FL) localized similarly to endogenous PRK2 and GFP-PRK2 (Figure 4.4).

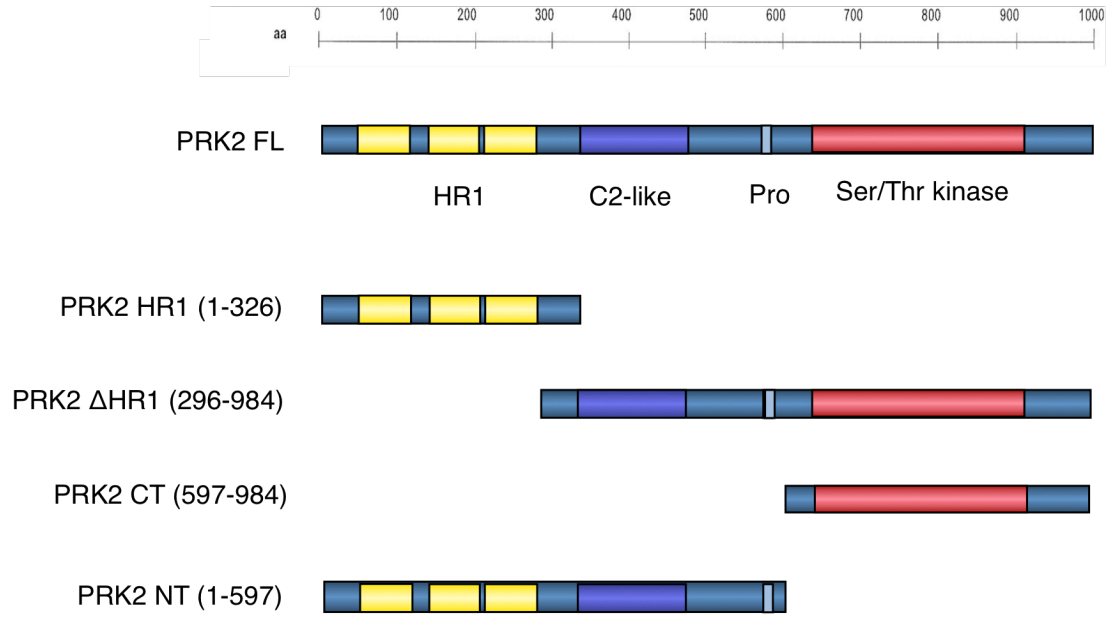


Figure 4.3 Schematic representation of full-length PRK2 and of the PRK2 deletion mutants for which localization was analysed.

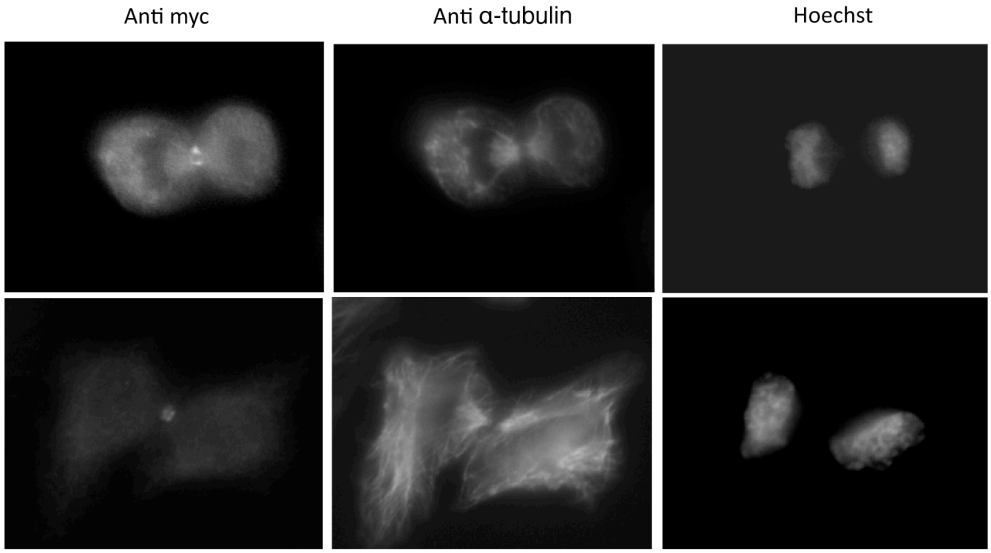


Figure 4.4 Localization of myc-tagged full-lenght PRK2 during cytokinesis. HeLa S3 cells were transiently transfected with myc-tagged full-lenght PRK2. The day after transfection, cells were incubated in the presence of thymidine for 18 hours, then released to fresh media for 11 hours and fixed. After fixation, cells were stained with anti myc and anti α-tubulin antibodies, as well as with Hoechst. The images show exogenous PRK2 localizing around the midbody during cytokinesis.

After confirming that myc-tagged PRK2 localized similarly to endogenous PRK2, the localization of the following two deletion mutants was analysed: PRK2 N-terminus (PRK2 NT - residues 1-597), which contained both the HR1 domain and the C2-like domain, but lacked the kinase domain, and the PRK2 C-terminus (PRK2 CT - residues 597-984), which contained the kinase domain. When compared with PRK2 FL or the PRK2 CT mutant, the percentage of cells expressing the PRK2 NT mutant was dramatically reduced. The few expressing cells that could be observed showed a strong change in morphology and adhesion to the substrate, which could be inter-related phenotypes (see Figure 4.5).

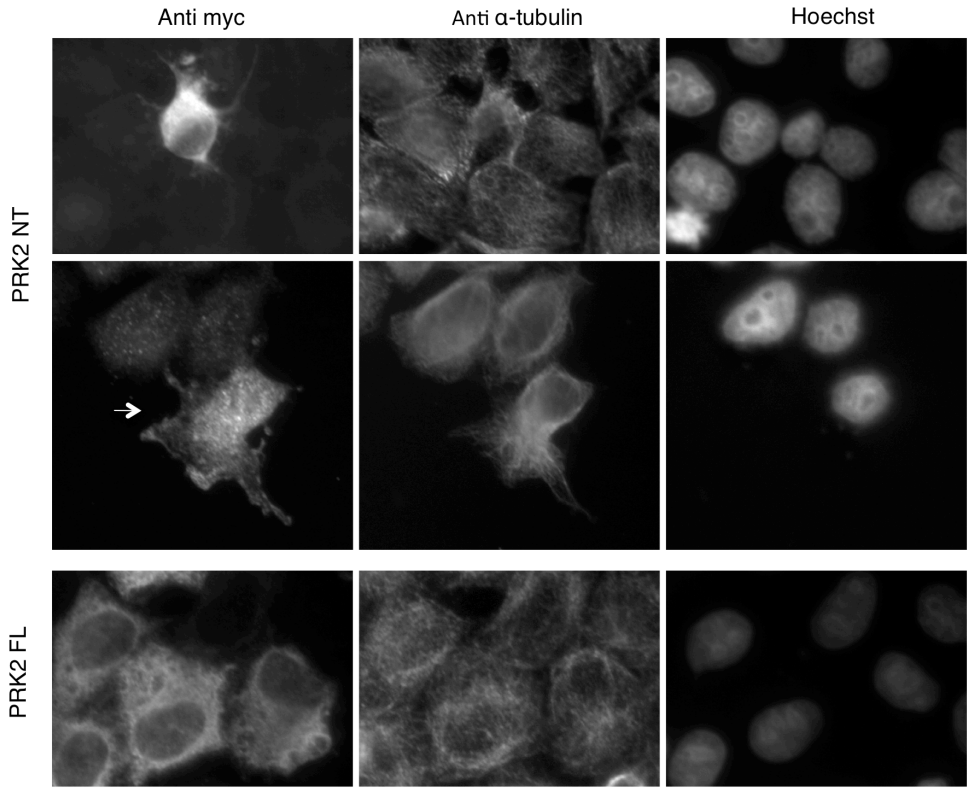


Figure 4.5 Effect on cell morphology of PRK2 N-terminus expression. HeLa S3 cells were transiently transfected with myc-tagged PRK2 NT or myc-tagged PRK2 FL, as indicated. For PRK2 NT second panel 1/10th of the DNA used in normal conditions was transfected (see material and methods for further description). The day after transfection cells where fixed and stained with anti myc and anti α-tubulin antibodies, as well as with Hoechst. Representative images of the morphology of the cells are shown. Arrow shows a cell with low levels of expression.

Lack of adhesion could be the reason why only a small percentage of cells expressing the mutant were present on the coverslips, but it is also possible that the expression of PRK2 NT causes cells to die. This phenotype could not be overcome when using low amounts of DNA (Figure 4.5, middle panel).

Due to the small number of cells that could be found expressing the PRK2 NT, only 10 cells that were both expressing the PRK2 NT mutant and at cytokinesis could be analysed. In 7 of those cells, the mutant was seen to localize at the midbody region (Figure 4.6 – A, upper panel). In the same experiments, no localization at the midbody or the cleavage furrow was seen for cells expressing PRK2 CT (0/60 cells analysed – Figure 4.6-A lower panel and B). PRK2 FL localized at the midbody in 70% of the cells analysed (42/60 – Figure 4.6-B).

These results suggest that the C-terminal domain is not required for the localization of PRK2 during cytokinesis. The dramatic change in morphology seen in cells expressing the PRK2 NT mutant, is likely due to an interference with signaling downstream of Rho, as Rho is known to be required for the maintenance of cell morphology and adhesion to the substrate (Paterson et al., 1990). However, it is interesting to note that the cleavage furrow still ingresses in these cells, a process that is also Rho-dependent (Figure 4.6-A upper panel).

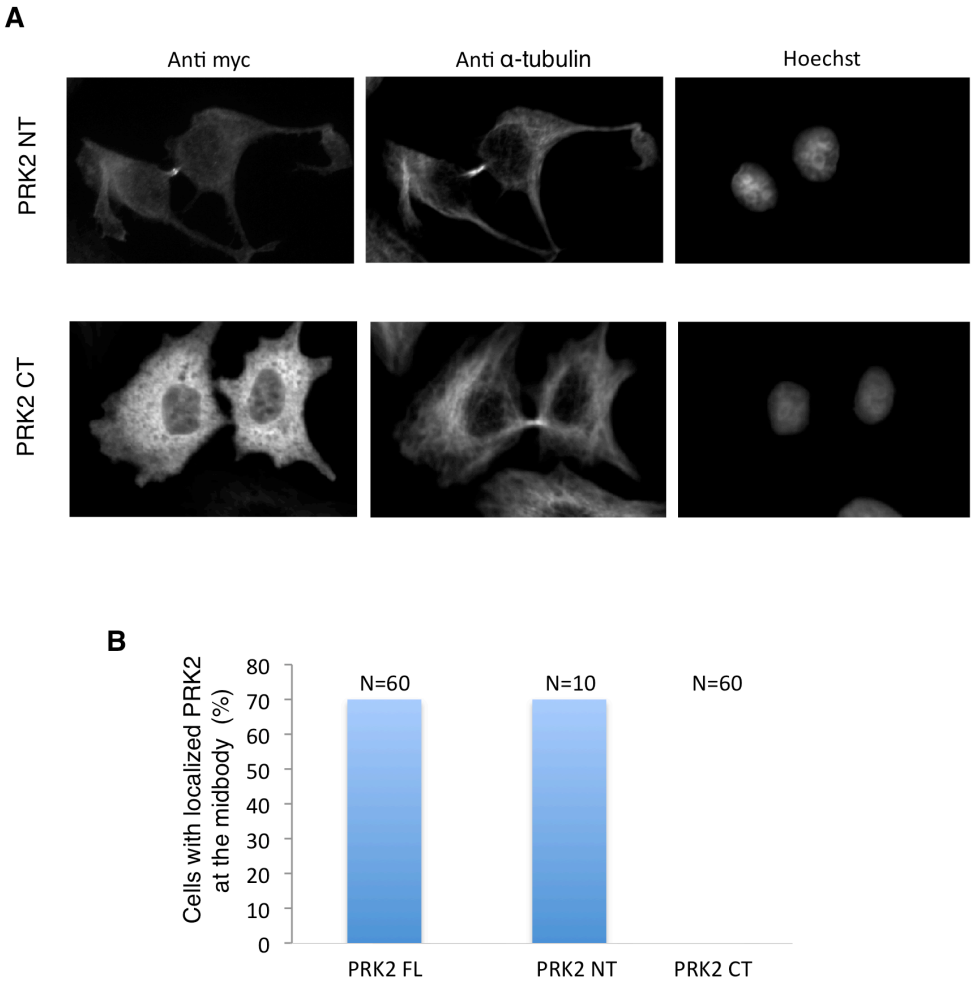


Figure 4.6 Localization of the PRK2 N-terminus and PRK2 C-terminus during cytokinesis. HeLa S3 cells were transfected with myc-tagged full-lenght PRK2 and the PRK2 deletion mutants indicated. The day after transfection, cells were incubated in the presence of thymidine for 18 hours, then released into fresh media for 11 hours and fixed. After fixation, cells were stained with anti myc and anti α-tubulin antibodies, as well as with Hoechst. The pictures are representative images of expressing cells at cytokinesis (A). Expressing cells at cytokinesis, as judged by the organization of the microtubule cytoskeleton, were scored for the presence of myc staining at the cleavage furrow or at the midbody. The graph shows three independent experiments in which cells at cytokinesis, expressing PRK2FL, PRK2 CT (60 cells, each) and PRK2 NT (10 cells) were analysed (see Figure 4.4 for representative images of PRK2 FL localization) (B).

4.2.4 The HR1 domain is responsible for PRK2 localization during cytokinesis

To further map the domain that is responsible for the localization of PRK2, the following deletion mutants were constructed: a mutant containing only the PRK2 HR1 domain (PRK2 HR1 - residues 1-326), and a mutant lacking the HR1 domain (PRK2 ΔHR1 - residues 296-984). These mutants were expressed in HeLa S3 cells and analysed for their localization during cytokinesis (see Figure 4.3, for a schematic representation of the mutants). Expression of the PRK2 HR1 mutant had a similar effect on cells as the PRK2 NT mutant and only few cells that were both expressing and at cytokinesis could be found. As can be seen in Figure 4.6, cells expressing both of the mutants were able to undergo contraction of the cleavage furrow and to form a midbody.

The PRK2 HR1 mutant was seen to localize at the midbody or and the cleavage furrow in 8/8 cells analysed in three independent experiments, while the PRK2 ΔHR1 was seen to localize at this region in 10% of the cells analysed (6/60) (Figure 4.6). This result suggests that the HR1 domain is involved in PRK2 localization during cytokinesis. The fact that the expression of the PRK2 HR1 domain had the same effect on cell morphology as the expression of the PRK2 NT domain, further supports the idea that this phenotype is caused by an interference with Rho signaling. However, as it happens with cells expressing the NT mutant, the cleavage furrow is still able to ingress (as seen by the presence of a midbody structure) even though the PRK2 HR1 localizes where active Rho is believed to be (Figure 4.6).

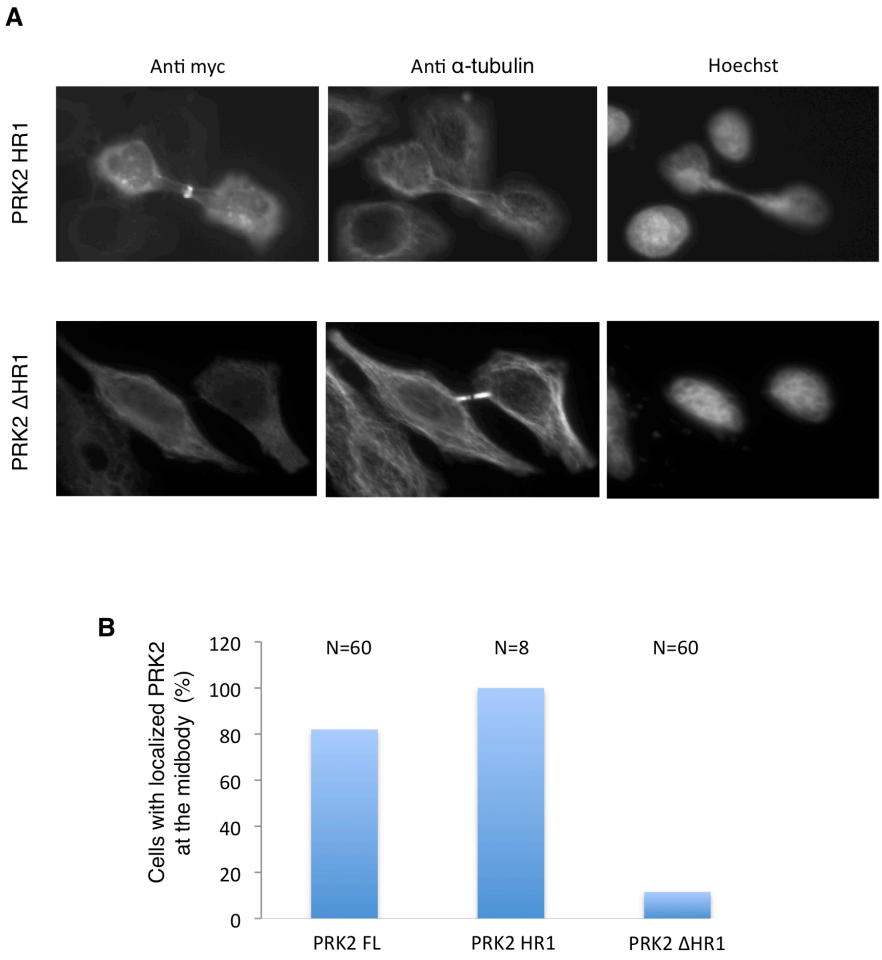


Figure 4.7 Localization of the PRK2 RBD and PRK2 ΔRBD deletion mutants during cytokinesis. HeLa S3 cells were transfected with myc-tagged full-lenght PRK2 and the PRK2 deletion mutants indicated. The day after transfection, cells were incubated in the presence of thymidine for 18 hours, then released into fresh media for 11 hours and fixed. After fixation, cells were stained with anti myc and anti α-tubulin antibodies, as well as with Hoechst. The pictures are representative images of expressing cells at cytokinesis (A). Expressing cells at cytokinesis, as judged by the organization of the microtubule cytoskeleton, were scored for the presence of myc-staining at the cleavage furrow or at the midbody. The graph shows three independent experiments in which cells at cytokinesis expressing PRK2FL, PRK2 ΔHR1 (60 cells, each) and PRK2 RBD (8 cells) were analysed (see Figure 4.4 for representative images of PRK2 FL localization) (B).

4.3 Discussion

PRK2 has been shown to localize at the cleavage furrow and midbody during cytokinesis in HeLa S3 cells. Here we were able to confirm this localization either by staining fixed HeLa cells with an anti PRK2 antibody or by analyzing the localization of overexpressed tagged versions of PRK2. Analysis of GFP-PRK2 localization in living cells shows that some PRK2 accumulates at the equatorial cortex in anaphase where the cleavage furrow is going to form. PRK2 stays at the cleavage furrow while the furrow ingresses and when the furrow has fully ingressed it localizes around the midbody. PRK2 localization during cytokinesis is very similar to what has been described for RhoA and proteins that associate with the contractile ring, such as anillin (Nishimura and Yonemura, 2006; Piekny and Glotzer, 2008; Yuce et al., 2005).

PRK2 binds Rho through its HR1 domain. The Rho-GEF Ect2 is believed to regulate RhoA localization during cytokinesis (Yuce et al., 2005). Localization of PRK2 at the midbody was shown to be Ect2-dependent, in a previous study (Schmidt et al., 2007). However, it is not known if Rho is directly involved in localizing PRK2 during cytokinesis. Here we addressed this question by analyzing the localization of PRK2 deletion mutants lacking specific domains. PRK2 has three main protein domains: a kinase domain, localized at the C-terminus, an N-terminus HR1 domain and a C2-like domain that is located in between the HR1 domain and the kinase domain. In addition, PRK2 also has one proline-rich region between the C2-like and the kinase domain. We were first able to show that the C-terminal domain of PRK2 is not responsible for its localization at cytokinesis, as a mutant lacking this domain (PRK2 NT 1-597), was still able to localize at the midbody, when overexpressed in cells, wile the C-terminal domain alone was not. In addition to showing that the C-terminus is dispensable for

PRK2 localization, this result also shows that kinase activity is not required for PRK2 localization.

The PRK2 NT mutant contains the HR1, the C2-like domain and the proline-rich region. As any of these regions could be signaling for PRK2 localization at the midbody, two other PRK2 deletion mutants were analysed, one containing only HR1 domain (PRK2 HR1 1-326) and the other lacking this domain (PRK2 ΔHR1 296-984). Although PRK2 HR1 (1-326) induced a strong morphological change in the cells, it could be seen to localize at the midbody in every cell analysed, while the PRK2 ΔHR1 mutant only localized in 10% of the cells. This result suggests that HR1 is the main domain responsible for PRK2 localization during cytokinesis. However an additional minor contribution from the C2-like and/or Pro-rich domain domains can not be excluded.

Rho binding domains of Rho effector proteins, including *Drosophila* Pkn, have been used as probes to detect active Rho in cells (Bement et al., 2005; Simoes et al., 2006). It is possible that localization of the PRK2 HR1 domain in the context analysed here is simply revealing the localization of active RhoA, which is known to be at the contractile ring. However, as the mutant lacking the HR1 does not localize properly in the majority of cells, we conclude that the HR1 domain is responsible for PRK2 localization. This result, together with the published data that Ect2 siRNA inhibits localization of PRK2 at the midbody, suggests that Rho regulates PRK2 localization during cytokinesis. It is possible that the PRK2 HR1 domain interacts with other proteins in addition to Rho and it would be interesting to further confirm this result by analyzing, for example the localization of a PRK2 mutant that is not able to bind Rho. PRK2 has been shown to be also able to bind Rac through its HR1 domain. A role for Rac in negatively regulating cytokinesis has been recently suggested by siRNA studies undergone in *C. elegans embryos (Canman et al., 2008).* siRNA of Rac in this study didn't affect cytokinesis in control embryos and siRNA of PRK2 blocks cytokinesis in HeLa cells, therefore a role for PRK2 in this process is more likely to be positive than negative, meaning

that Rho is more probable to be the GTPase involved in the regulation of PRK2 localization during cytokinesis. Finally, the N-terminal domain of PRK1 (1-474) binds CG-NAP, a scaffold protein that has been seen to localize at the midbody region. Even though CG-NAP localization is mainly associated with microtubules and not with cleavage furrow, this protein could play a role in regulating PRK2 localization, as it is known that signals from the spindle microtubules regulate Rho localization, for example.

Rho GTPases have been shown to regulate the localization of effector proteins in other contexts. For example, Cdc42-GTP regulates the localization of PAK-4 at the Golgi (Abo et al., 1998). For PRK1 in particular, RhoB, which has been shown to localize at endosomes, can translocate PRK1 from the cytoplasm to endosomes, when both proteins are co-expressed in cells. The HR1 domain was shown to be responsible for this translocation (Mellor et al., 1998). This data support the evidence presented in the present study, that Rho regulates the localization of its effector PRK2 during cytokinesis. It would be interesting to which of the three Rho isoforms is involved.

It would also be interesting to investigate what is the precise mechanism through which Rho translocates PRK2 to the cleavage furrow. Even though there are contradictory reports concerning the requirement of GTP-loading for the Rho:PRK2 interaction, Rho GTPase effectors in general bind preferentially to the GTP-bound form of GTPases. It is also generally accepted that the GDP-bound inactive form of Rho GTPases is present in the cytoplasm and bound to GDIs. Binding to GDIs might inhibit PRK2 binding to Rho in the cytoplasm (even if no GTP-loading is required on Rho for the PRK2 interaction). When Rho-GTP translocates from the cytoplasm to the equatorial region at telophase, PRK2 which is mainly cytoplasmic, would be able to bind to Rho and be recruited to the same region. The role for Rho in localizing citron kinase at the cleavage furrow has been analysed. C3-treatment, to inhibit Rho, disrupted citron kinase

localization at the cortex in anaphase cells and revealed a citron kinase localization at the midzone mitotic spindle, where citron kinase is not seen in control conditions (Eda et al., 2001). This result suggests that during anaphase citron kinase moves to the cortex via midzone spindles in a Rho-dependent manner, but this might be a very quick event that can not be detectable in normal conditions. It would be interesting to analyze if C3 treatment causes the same effect on PRK2 localization.

Finally, an interesting observation in this set of experiments was that the expression of either PRK2 NT or PRK2 HR1, had a strong effect on the morphology of the cells and their ability to adhere to the substrate (which could be two inter-related behaviors). Overexpression of wild-type PRK2 on the other hand, had only a very small effect on cell morphology in just a few cells. One explanation for this is that both mutants bind Rho and interfere with Rho signaling, which regulates cell morphology and cell adhesion (Etienne-Manneville and Hall, 2002). The phenotype seen is similar to the one described for inhibition of Rho using, for example, C3-transferase (Paterson et al., 1990). The PRK2 HR1 domain is believed to have a pseudosubtrate site that would bind to the kinase domain and keep the protein in a closed conformation. This could be the reason why when overexpressed, full length PRK2 does not interact with Rho and leads to loss of adhesion. This could suggest that another signal is required to open up the conformation of PRK2 before it is capable of interacting with Rho.

Another interesting feature is that although the HR1 domain appears to inhibit Rho for the definition of cell morphology and adhesion, it does not appear to inhibit Rho for the contraction of the ring during cytokinesis. One explanation for this could be that the definition of cell morphology and the establishment of cell adhesion to the substrate are cellular events that are more sensitive to an interference with Rho signaling.

CHAPTER 5 - RESULTS

PRK2 localizes at sites of cell-cell contact and is involved in epithelial junction formation

5.1 Introduction

In order to promote the barrier function of an epithelium, epithelial cells associate with each other at specialized cell-cell contact sites called junctions. There are two main types of epithelial cell junctions: adherens junctions and tight junctions. Adherens junctions are mainly involved in cell-cell adhesion, while tight junctions form a physical barrier that controls the selective permeability of the epithelial layer to ions and small molecules. Both adherens and tight junctions are composed of transmembrane and cytosolic proteins. In adherens junctions, cadherins bind to cadherins on neighboring cells via their extracellular domain and to catenins via their cytosolic domains. In tight junctions, the major transmembrane proteins are the occludins, claudins and JAMs and the main cytosolic proteins, ZO-1 and ZO-2 (Ebnet, 2008).

Epithelial junction formation starts with the establishment of cell-cell contacts, which promote the recruitment of the cadherin/catenin complex to the membranes. Some tight junction proteins are also recruited at this time. This nascent junctional complex forms spotlike structures, or puncta. As junctions mature, these puncta fuse together and form beltlike adherens junctions and tight junctions, in a process that requires both the reorganization of the cortical actin cytoskeleton and the establishment of cell polarity (Vasioukhin et al., 2000).

Three evolutionary conserved protein complexes regulate apical-basal polarity: Par-3-aPKC-PAR-6, Crumbs3-PALS1-PAT and Scribble-Dlg-Lgl (Nelson, 2003). Polarity begins with recruitment to cell-cell adhesions of Par3 and Par6, which initiate the formation of tight junctions. With the recruitment of these Par proteins and the formation of tight junctions, the apical membrane also forms. Spread of the apical membrane is impeded by the activity of the Lgl/Scribble complex, which is recruited to a position below the PAR complex/cadherin junction. The Lgl/Scribble complex antagonizes functions of the PAR complex and, later, Crumbs complexes, thereby maintaining lateral membrane identity below the adherens junctions. The Crumbs complex is recruited apically to the PAR3 complex/cadherin junction and antagonizes the activity of the Lgl/Scribble complex which blocks the spreading of lateral membrane and maintains apical membrane integrity above the adherens junctions (Nelson, 2003). In polarized mammalian epithelial cells, tight junctions localize at the apical end of the lateral membrane at the boundary between the apical and basolateral membranes. Adherens junctions localize just underneath tight junctions.

The formation and maintenance of epithelial junctions and of apical-basal polarity, is regulated by several molecular mechanisms, such as actin cytoskeleton organization, membrane trafficking and post-translational modifications, including phosphorylations of the junctional/polarity complexes. Rho GTPases and their effector proteins play essential roles in all these processes and have been implicated in the regulation of several events during epithelial morphogenesis (Van Aelst and Symons, 2002). This chapter describes the identification of PRK2 as a regulator of epithelial junction formation and a possible link between cytokinesis and junction formation

5.2 Results

5.2.1 PRK2 localizes at sites of cell-cell contact between daughter cells during cytokinesis

While analyzing PRK2 localization during cytokinesis in HeLa S3 cells, we noticed that in addition to accumulating at the cleavage furrow and midbody, PRK2 also accumulates at sites of cell-cell contact that form between the two cells that are dividing (Figure 5.1-A). Scribble, a polarity protein that is involved in junction formation in epithelial cells, also localizes at these sites of cell-cell contact, although not completely overlapping with PRK2 (Figure 5.1-B).

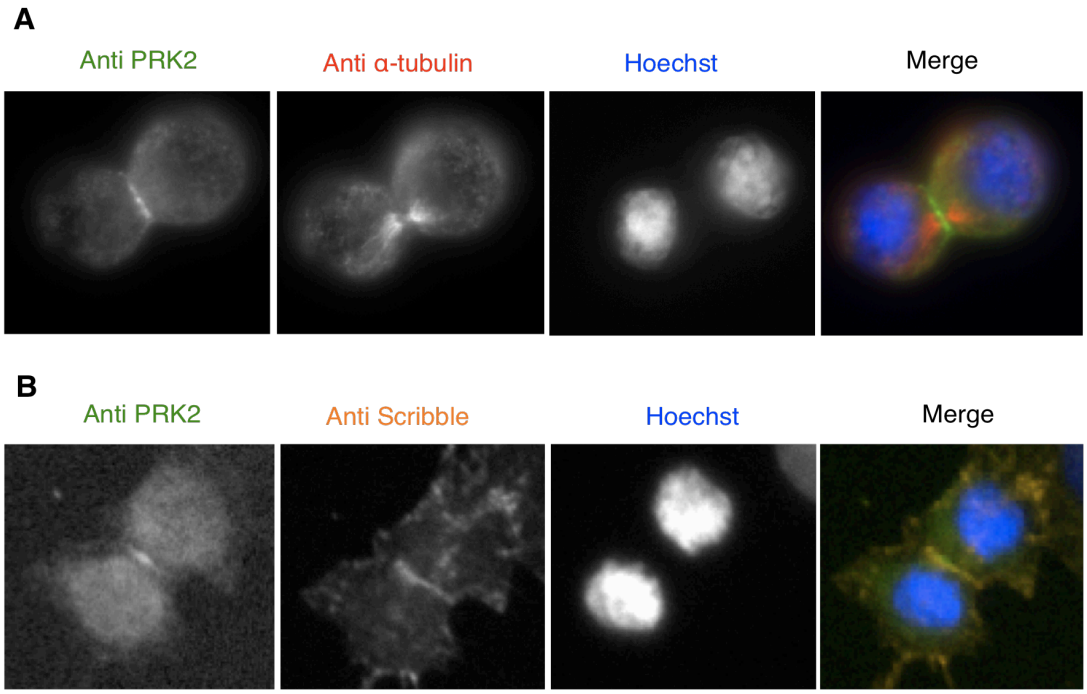


Figure 5.1 Localization of endogenous PRK2 at sites of cell-cell contact between daughter cells during cytokinesis. HeLa S3 cells were fixed and stained with anti PRK2 and anti α-tubulin (A), and with anti PRK2 and anti Scribble (B) antibodies, as well as with Hoechst. Images show localization of PRK2 at sites of cell-cell contact between daughter cells during cytokinesis.

Interestingly, while Scribble localizes at cell-cell contacts in most cells in cytokinesis during the establishment of contacts, PRK2 could only be detected at this location in some cells. This suggests that PRK2 localization at cell-cell contacts that form between daughter cells during cytokinesis is transient. The PRK2 localization observed using an anti PRK2 antibody is likely to be specific as overexpressed myc-tagged FL-PRK2 localizes similarly (Figure 5.2).

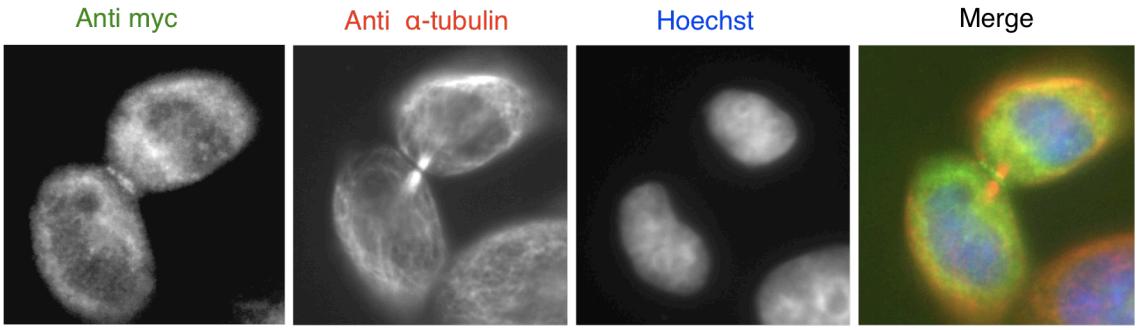


Figure 5.2 Localization of myc-tagged PRK2 at sites of cell-cell contact between daughter cells during cytokinesis. HeLa S3 cells were transfected with myc-tagged full-lenght PRK2. After 48 hours, cells were fixed and stained with anti myc and anti α-tubulin antibodies, as well as with Hoechst. Images show localization of PRK2 at sites of cell-cell contact between daughter cells during cytokinesis.

5.2.2 PRK2 localizes at cell-cell contacts in highly confluent HeLa S3 cells

Scribble is seen at sites of cell-cell contact between interphase HeLa S3 cells even when cells are grown at low confluency, while in these conditions, PRK2 localization at cell contacts is almost undetectable (Figure 5.3-A). However, if HeLa S3 cells are left to grow to high confluency, PRK2 is clearly seen to localize at sites of cell-cell contacts between interphase cells (Figure 5.3-B). This result suggested that PRK2 could be involved in regulating epithelial junction formation.

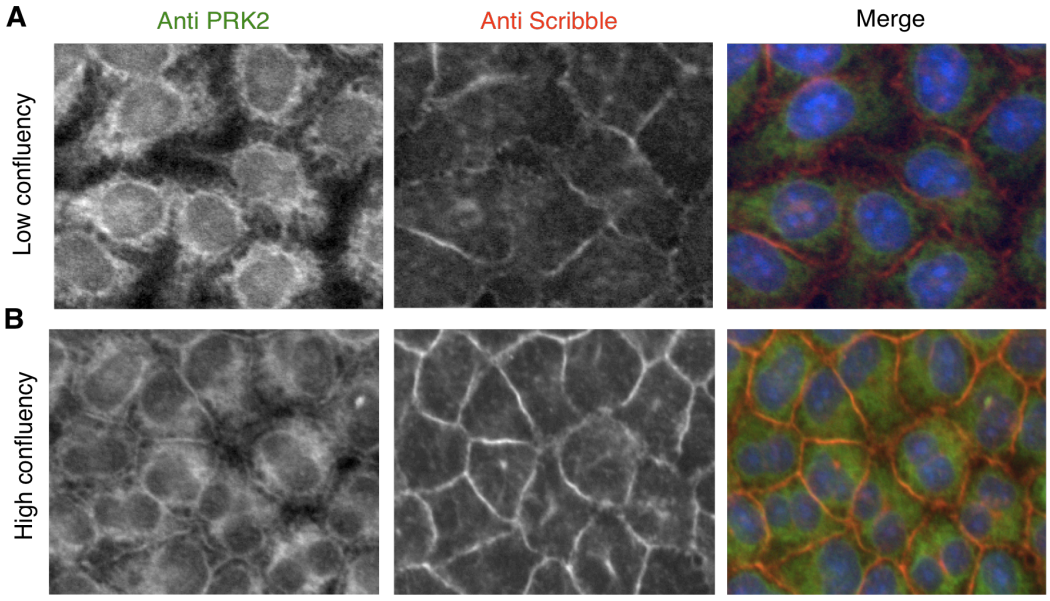


Figure 5.3 Localization of endogenous PRK2 at sites of cell-cell contact between interphase HeLa S3 cells. HeLa S3 cells were fixed at either low (A) or high confluency (B) and stained with anti PRK2 and anti Scribble antibodies, as well as with Hoechst (blue on merge). Representative images are shown.

5.2.3 PRK2 localizes at cell junctions in confluent Caco-2 and 16HBE cells and is required for epithelial junction formation

Although an epithelial cell line, HeLa cells are known to lack the expression of typical junctional proteins, such as E-cadherin, and are not considered a good model to study junction assembly and maintenance. For this reason, the localization of PRK2 in Caco-2 cells, a human intestinal epithelial cell line that has been used to study junction formation, was analysed. As can be seen in Figure 5.4, PRK2 localizes at cell-cell contacts in Caco-2 cells that were grown to confluency. This localization overlaps with both ZO-1 (a tight junction protein) and apically localized Scribble (adherens junction/basolateral). However, unlike Scribble, PRK2 is not present at the lateral membranes (wide band of fluorescence that is marked as an arrow on Figure 5.4).

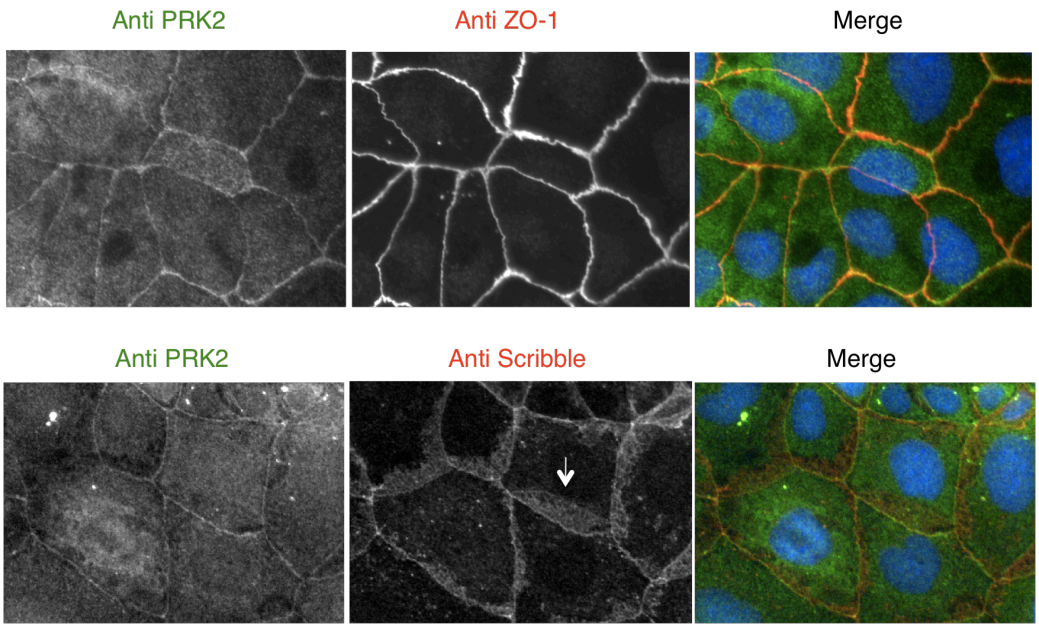


Figure 5.4 Localization of endogenous PRK2 with ZO-1 and Scribble in Caco-2 cells. Caco-2 cells were grown to confluency and then fixed. After fixation, cells were stained with anti PRK2 and anti Zo-1 antibodies as well as with Hoechst (blue on merge). Arrow shows Scribble basolateral localization.

At the same time that these experiments were being performed, Sean Wallace, another PhD student in the Hall lab, identified PRK2, in an siRNA based screen, as required for tight junction formation and adherens junction maturation in a human bronchial epithelia cell line (16HBE). PRK2 depletion in these cells, disrupted tight junction protein localization (ZO-1) (Figure 5. 5 – courtesy of Sean Wallace). In addition, PRK2 depletion also affected the maturation of adherens junctions, which are still seen at cell-cell contacts but to a lesser extent, and the organization of junctional F-actin (not shown). Endogenous PRK2 also localizes at junctions in 16HBE cells and this localization is likely to be specific as a myc-tagged PRK2 construct localizes similarly (Figure 5.6 and 5.7). These results suggest that PRK2 is involved in both adherens and tight junction formation.

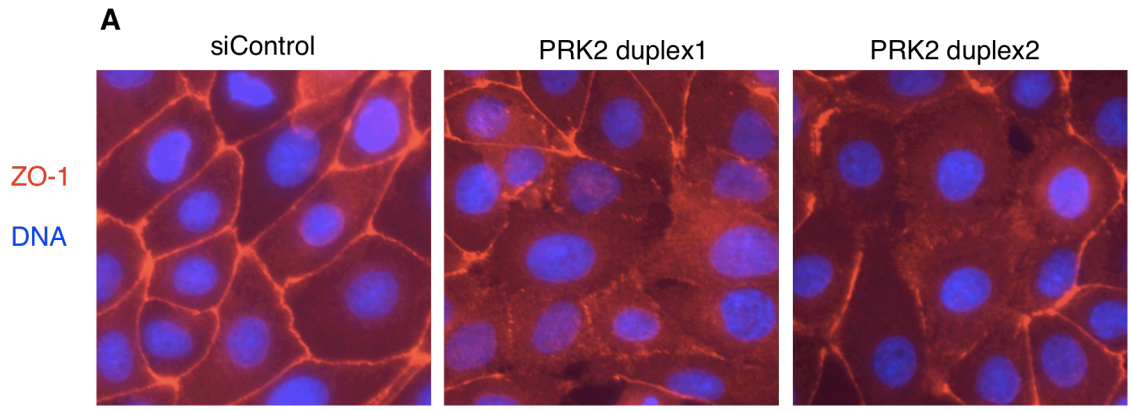


Figure 5.5 PRK2 is required for tight junction formation. 16HBE cells were seeded at low density and allowed to adhere overnight before being transfected with siGlo (control), or two distinct PRK2 siRNA duplexes. 72 hours post-transfection cells were fixed and stained with anti ZO-1 antibody (red) and Hoechst (blue). Representative images of what was observed are shown (A). (Courtesy of Sean Wallace)

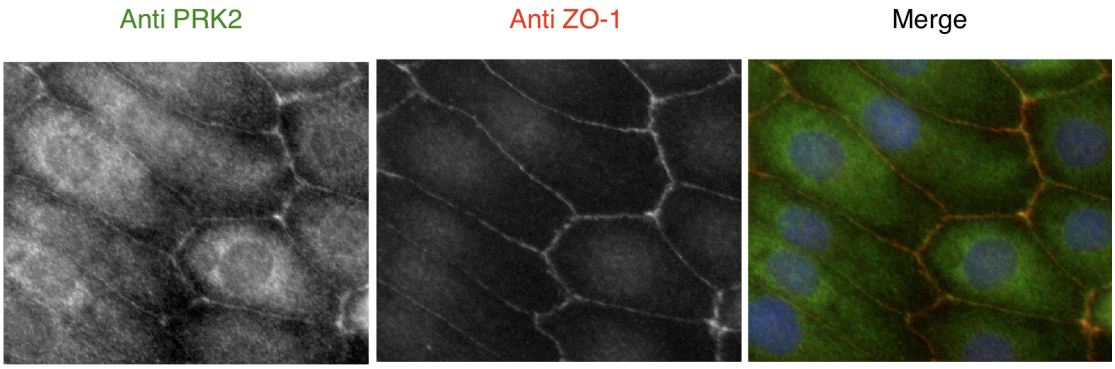


Figure 5.6 Localization of endogenous PRK2 with ZO-1 in 16HBE cells. 16HBE cells were grown to confluency and then fixed. After fixation, cells were stained with anti PRK2 and anti-Zo-1 antibodies as well as with Hoechst (blue on merge).

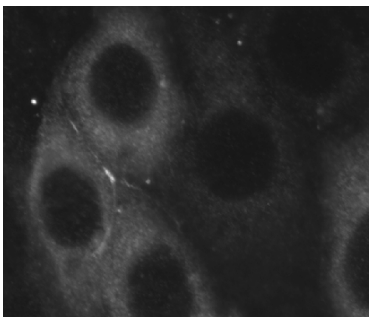


Figure 5.7 Localization of myc-tagged full-lenght PRK2 at cell-cell junctions in 16HBE cells. 16HBE cells were transfected with myc-tagged full-length PRK2 while at low density. 72 hours post-transfection cells were fixed and stained with an anti myc antibody as well as with Hoechst. Myc-PRK2 localization at cell-cell junctions is shown.

5.2.4 The HR1 domain is required for PRK2 localization at sites of cell-cell contact that form between daughter cells during cytokinesis

As Rho GTPases are known to be involved in epithelial junction formation, we decided to address the question of whether Rho GTPases are involved in localizing PRK2 at sites of cell-cell contact by mapping the domain responsible for PRK2 localization. For this, myc-tagged full-length PRK2 and PRK2 deletion mutants were transfected into 16HBE cells and the cells analysed by immunofluorescence using an anti myc antibody. The PRK2 deletion mutants analysed here are the same as those analysed in chapter 4 (see Figure 4.3 for a schematic representation). As reported earlier using HeLa cells, expression of the N-terminal domain (PRK2 NT) and PRK2 HR1 domain (PRK2 RBD) had a dramatic effect on the cell morphology and adhesion to the substrate of 16HBE cells (see Figure 5.8). This affected junction formation and it was not possible to address PRK2 localization in this way.

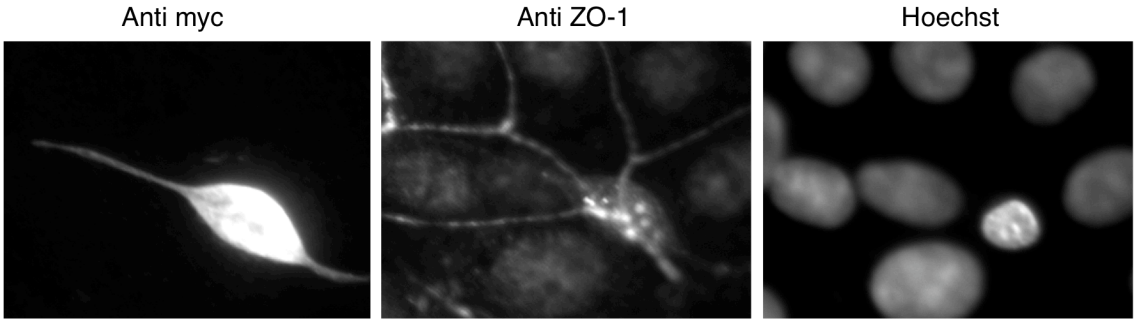
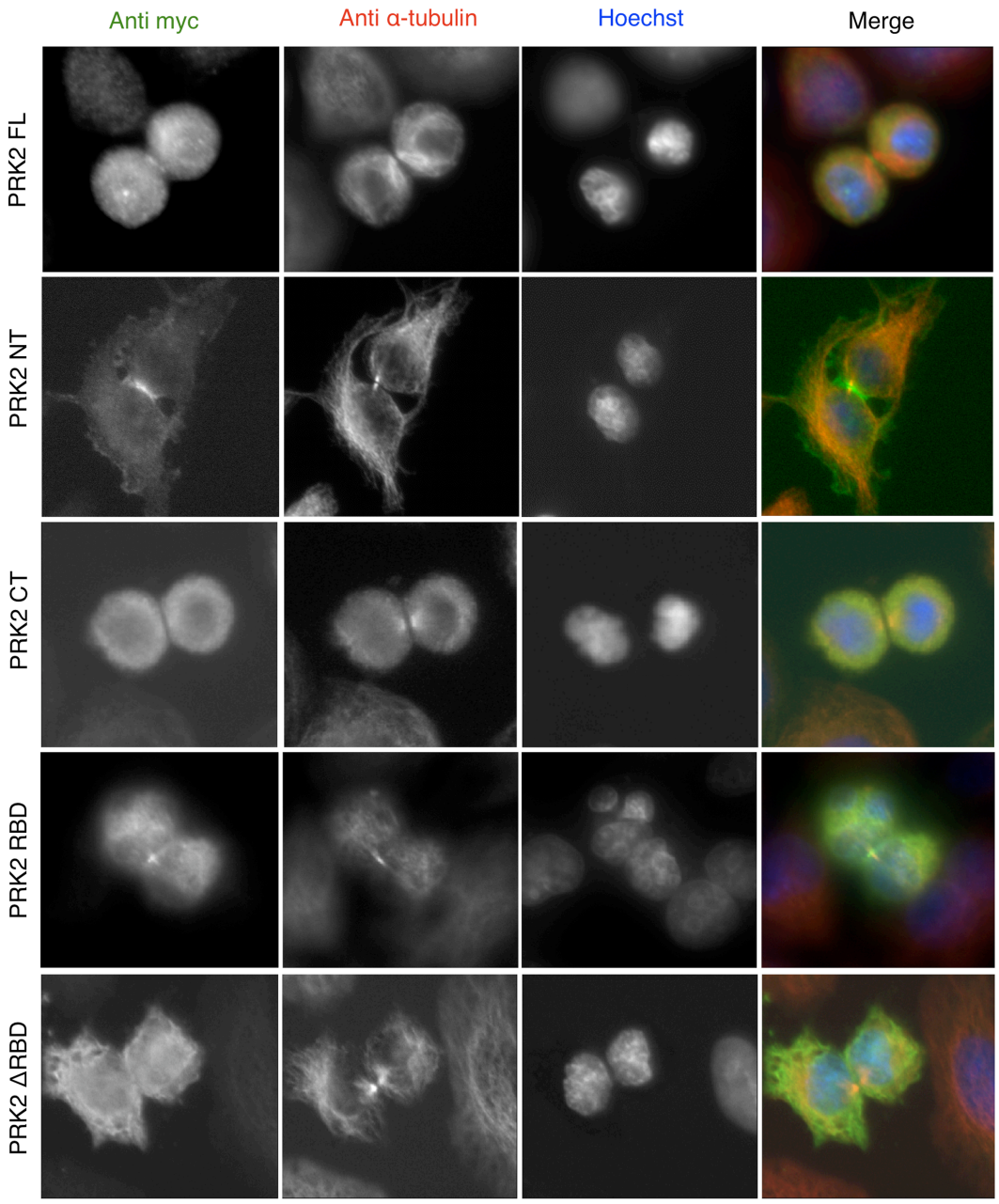


Figure 5.8 Overexpression of PRK2 NT disrupts 16HBE cell morphology and adhesion. 16HBE cells were transfected with myc-tagged full lenght PRK2 while at low density. 72 hours post-transfection cells were fixed and stained with anti myc and anti ZO-1 antibodies as well as with Hoechst. A representative image of the morphology of a transfected cell is shown.

For this reason HeLa cells were used instead, and the localization of the PRK2 mutants at the sites of cell-cell contact that form during cytokinesis, analysed. In these cells, despite the morphology phenotype, the localization of the PRK2 NT at cell-cell contacts between daughter cells during cytokinesis could still be visualized (Figure 5.9 – second panel). Interestingly, this localization was stronger than that observed after expressing full-length PRK2. The PRK2 HR1 domain was also seen to localize at the same location. For the reasons mentioned before (chapter4), not many examples of the localization of these two mutants could be found. However, no localization at cell contacts was seen for PRK2 CT or PRK2 ΔRBD mutants, in conditions in which PRK2 full-length localizes at cell-cell contacts in 30% of cells that where at cytokinesis and the two daughter cells where touching each other (Figure 5.9). These results suggest that the HR1 domain is responsible for PRK2 localization at the sites of cell-cell contact that forms between daughter cells during cytokinesis.



(**Figure 5.9** - Continue next page)

Figure 5.9 Localization of PRK2 deletion mutants at sites of cell-cell contact between daughter HeLa S3 cells. HeLa S3 cells were transfected with the DNA constructs indicated. The day after transfection, cells were synchronized by incubation with thymidine for 18 hours, followed by release into fresh media for 11 hours. After fixation, cells were stained with anti myc and anti α-tubulin antibodies, as well as with Hoechst. The images show the localization of the constructs at sites of cell-cell contact between daughter cells. The presence of a midbody indicates that cells are in the late stage or have just completed cytokinesis (A). Expressing cells at cytokinesis, that were making contacts, were scored for the presence of myc-staining at the cell-cell contact. The graph shows the average of three independent experiments in which 20 cells at cytokinesis, expressing PRK2FL, PRK2 CT and PRK2 ΔHR1 were analysed (B). (continue next page)

B

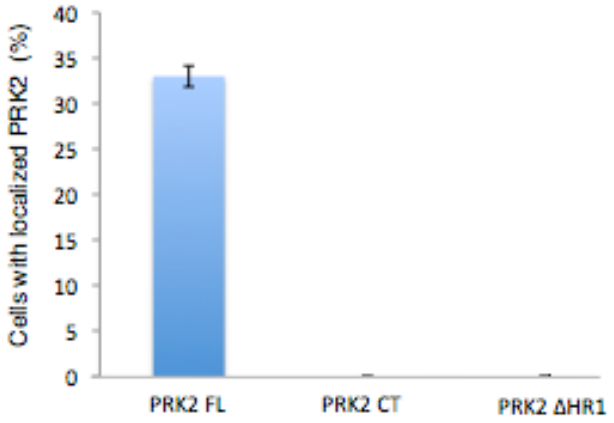


Figure 5.9 (continuation) Expressing cells at cytokinesis, that were making contacts, were scored for the presence of myc-staining at the cell-cell contact. The graph shows the average of three independent experiments in which 20 cells at cytokinesis, expressing PRK2FL, PRK2 CT and PRK2 ΔHR1 were analysed (B).

5.2.5 Scribble and ZO-1 accumulate around the midbody and are phosphorylated in mitosis in epithelial cell lines

As shown above, PRK2 localizes at both the cleavage furrow, around the midbody and at cell contacts during cytokinesis in HeLa S3 cells and at apical junctions in 16HBE and Caco-2 cells. In addition PRK2 has been shown to have a role both in cytokinesis (Schmidt et al., 2007) and in junction formation (Sean Wallace, unpublished data). Surprisingly, no cytokinesis failure was detected in 16HBE cells upon depletion of PRK2 by siRNA. The reason for this is unknown, it could be that the role for PRK2 in cytokinesis is cell-type specific or that the levels of PRK2 knock down in 16HBE cells were not high enough to cause a binucleate phenotype.

The formation of junctions during cell division is not very well documented in the literature, although it is assumed that epithelial cells need to maintain the integrity of their junctions while dividing and in agreement with this, junctional proteins are detected around mitotic cells (Kojima et al., 2001). However, during cytokinesis, cells need to form new cell-cell contacts as cells divide. PRK2 could be detected around the midbody and at sites of cell-cell contacts between daughter cells in 16HBE cells (see Figure 5.10). In order to investigate a possible link between junction formation and cytokinesis, the localization of other junctional proteins (Scribble and ZO-1) during cytokinesis was analyzed. Interestingly Scribble and ZO-1 could also be seen to accumulate around midbodies (Figure 5.10). To our knowledge, neither of these proteins has been implicated in cytokinesis, per se, therefore, one possibility for why they would accumulate in the midbody region during cytokinesis is that they participate in junction assembly.

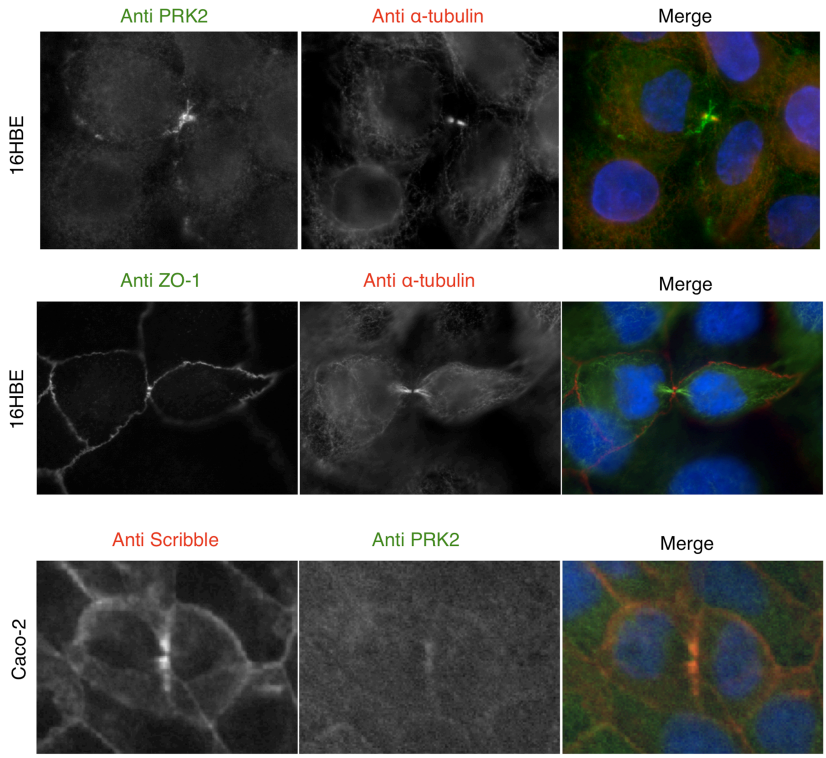


Figure 5.10 Localization of junctional proteins in late cytokinesis. 16HBE and Caco-2 cells were grown to confluency, fixed and stained with the antibodies indicated. Representative images of cells in late cytokinesis are shown.

As localization of junctional proteins at the midbody could be cell cycle regulated we analysed whether Scribble and ZO-1 are phosphorylated during mitosis. For this, HeLa S3 cells were synchronized in prometaphase by nocodazole treatment and the mobility of Scribble and ZO-1 after SDS-PAGE gel electrophoresis was analysed by western blot. As can be seen in Figure 5.11, similarly to what happens with PRK2, both proteins undergo a mobility shift after nocodazole treatment suggesting they are phosphorylated at mitosis. The migration of aPKC (another polarity protein) is used as a loading control, as no mobility shift could be detected in this case. The fact that aPKC does not undergo a mobility shift upon nocodazole treatment does not mean that it is not phosphorylated at mitosis, as not every phosphorylation event gives rise to a mobility shift on SDS-PAGE electrophoresis.

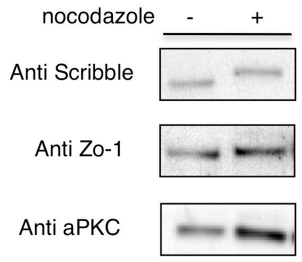


Figure 5.11 Phosphorylation of junctional proteins in mitosis. HeLa S3 cells were cultured in media with (+) or without (-) 100 ng/ml of nocodazole for 22 hours to arrest cells in prometaphase. Total cell extracts were electrophoresed on an SDS-PAGE gel and Scribble, ZO-1 and aPKC were detected on a western blot using specific antibodies.

5.2.6 PRK2 siRNA interferes with the architecture of Caco-2 cysts

Recently, a three-dimensional cyst formation system to study epithelial morphogenesis has been developed in the Hall lab using Caco-2 cells (Jaffe et al., 2008). When cultured in matrigel, these cells are able to develop into cysts

system is that the formation of the lumen is tightly linked to cytokinesis: apical markers localize around the midbody and the ingression of the cleavage is asymmetric, as judged by the apical localization of midbodies. A model has been proposed in which apical markers, such as aPKC would be deposited at the apical site during cytokinesis. This model supports a strong relationship between cytokinesis and epithelial tissue morphogenesis. In addition, in these system cell-cell contacts form between daughter cells after cell division and not cell-cell collision of interphase cells. For these reasons we decided to look at the effect of PRK2 RNAi in the development of Caco-2 cysts (in collaboration with Aron Jaffe, Hall lab). Control and PRK2 siRNAs were transfected into Caco-2 cells. 24 hours later, single cell suspensions with equal number of cells for each condition, were plated into matrigel and grown for 6 days. After 6 days, lumen formation was induced by the addition of cholera toxin (which dramatically induces lumen formation in this system). As can be seen in Figure 5.12, PRK2 siRNA leads to the formation of fewer, smaller structures that were not able to form a normal lumen after addition of cholera toxin. This result suggests that PRK2 may be required for the formation of well-organized epithelial cysts. The Caco-2 3D system will be a good model to further analyze the role of PRK2 in epithelial morphogenesis.

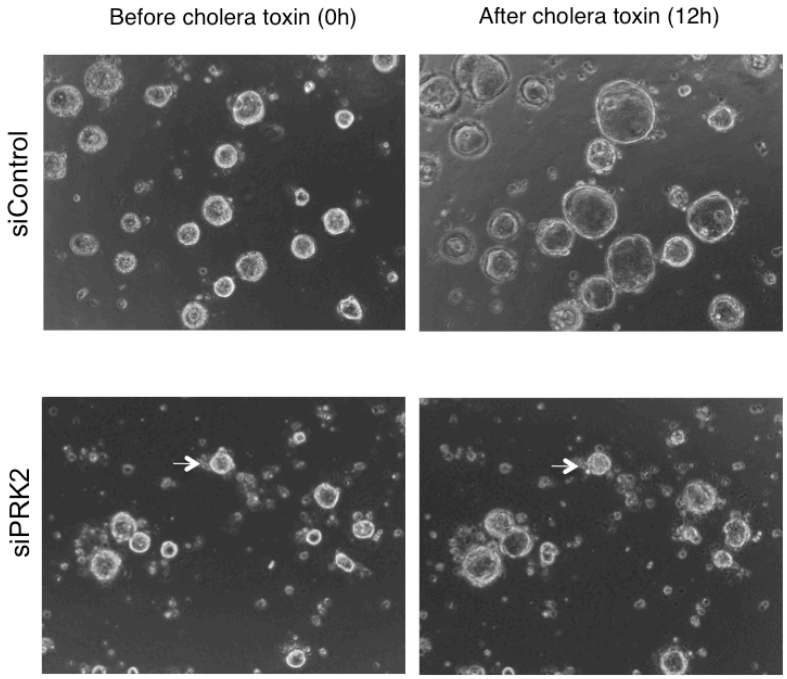


Figure 5.12 Effect of PRK2 siRNA treatment on Caco-2 cyst formation. Caco-2 cells were transfected with control or PRK2 siRNA. 24 hours after transfection, cells were plated in three-dimensions and treated with cholera toxin at day 6 to induce luminal swelling. Phase images from cysts before (0h) and after treatment (12 hours) are shown. Arrow indicates a cyst that didn't form a lumen.

5.3 Discussion

While analyzing PRK2 localization during cytokinesis in HeLa S3 cells, we noticed that PRK2 accumulates at sites of cell-cell contact that form between the two daughter cells. In addition PRK2 localizes at cell-cell contacts between interphase cells, when these are grown to high confluency. These results suggested that PRK2 could be involved in epithelial junction formation.

As HeLa cells are not a good model to study junction formation, the localization of PRK2 in Caco-2 cells, was analysed (see Figure 5.4). We were able to see that, in these cells, PRK2 localizes apically with tight junction proteins such as ZO-1 or with apically located Scribble (which localizes at adherens junctions and basolaterally (Dow et al., 2003)). PRK2 was not seen to colocalize laterally with Scribble. This localization, suggests that PRK2 localizes at apical junctions. In the conditions used here (cells plated on glass) it is not possible to fully separate adherens junctions and tight junctions. To distinguish whether PRK2 localizes at tight junction or adherens junctions, the localization of PRK2 should be analysed in cells grown on filters, which is known to increase polarity and allow a better separation between these two compartments.

At the same time that these experiments were being performed, Sean Wallace, another PhD student in the Hall lab, was able to show that PRK2 is required for tight junction formation and adherens junction maturation in 16HBE cells (human bronchial epithelia), as depletion of PRK2 by siRNA disrupts the localization of tight junction proteins (such as ZO-1) and the maturation of adherens junctions. We were also able to observe that both endogenous and ectopically expressed PRK2 localizes at junctions in 16HBE cells (Figure 5.6 and 5.7).

Rho GTPases are known regulators of adherens and tight junction formation and several Rho GTPase effectors have been implicated in the regulation of epithelial

morphogenesis (Van Aelst and Symons, 2002). PRK2, itself, has been suggested to have a role in mediating cadherin-based cell-cell contacts in keratinocytes (Calautti et al., 1998; Calautti et al., 2002). In this study, PRK2 kinase activity was seen to increase with junction formation after a Ca^{2+} switch and overexpression of PRK2 promoted the formation of cell-cell contacts. The authors proposed that PRK2 might act downstream of Rho during adherens junction formation, as overexpression of RhoV14 (constitutively active Rho) but not RhoV14-Y42C, a mutant that is defective for PRK1 binding, appeared to strengthen the junctions (Calautti et al., 1998). This result suggests that a member of the PRK family is acting downstream of Rho in this pathway. However the possibility that other Rho effectors are involved cannot be excluded, as the ability of the RhoV14-Y42C mutant to bind every possible Rho effector has not been analysed (Sahai et al., 1998). It is suggested in this study, that PRK2 promotes cell adhesion through the activation of Fyn tyrosine kinase, although it is not directly demonstrated whether Fyn acts as a substrate for PRK2. In 16HBE cells the major phenotype detected is in the maturation and not formation of adherens junctions, as E-cadherin is still seen at cell-cell contacts after PRK2 knock down (Sean Wallace, unpublished data), therefore a different mechanism of action is likely to be occurring.

Several events could be responsible for the effect on junctions observed in 16HBE cells after depletion of PRK2. For example, actin has a central role in epithelial junction formation and maturation, and PRK1 and PRK2 have been suggested to regulate actin reorganization (Dong et al., 2000; Vincent and Settleman, 1997). PRK1 has also been shown to be able to localize at vesicular compartments and vesicular trafficking is essential in the regulation of epithelial morphogenesis (Mellor et al., 1998). Finally PRK2 might directly phosphorylate junctional/polarity proteins.

It is likely that Rho GTPases (either Rho or Rac) regulate PRK2 during epithelial junction formation. In order to analyse whether PRK2 localization at cell junctions is Rho-dependent, we attempted to localize PRK2 deletion mutants in 16HBE cells. However, similarly to what happened in HeLa cells, the overexpression of the PRK2 NT mutant caused a severe disruption in cell morphology and adhesion to the substrate, which interfered with cell junction formation. For this reason it was not possible to address this question in 16HBE cells. In HeLa cells, however, despite the similar effects on cell morphology, localization of the PRK2 NT domain at cell-cell contacts between daughter cells during cytokinesis could be visualized. A similar localization was seen for the PRK2 HR1 domain. No localization at sites of cell-cell contacts during cytokinesis was found for the PRK2 CT and PRK2 ΔHR1 mutants, suggesting that the PRK2 HR1 domain is required for PRK2 localization at sites of cell-cell contacts that form between daughter cells during cytokinesis.

It is possible that the mechanism responsible for localizing PRK2 at cell-cell contacts between daughter cells during cytokinesis and between interphase cells after collision or a Ca^{2+} switch, is the same. However it could also be different. At this moment we don't know if the HR1 domain is also involved in localizing PRK2 at cell-cell junctions that form between interphase cells.

As discussed in Chapter 4, HR1 domains may interact with other proteins in addition to Rho GTPases, however it is a strong possibility that Rho GTPases mediate the localization of PRK2 at these sites of cell-cell contact that form between daughter cells during cytokinesis. It would be interesting to analyse the ability of PRK2 mutants that are not able to bind Rho and/or Rac to localize at these cell-cell contacts or at cell-cell junctions.

The formation of cell-cell contacts between daughter cells after division has not been much studied. The majority of the studies that have been undertaken to

look at epithelial morphogenesis, have used as a model the formation of cell-cell junctions upon collision of interphase cells, as in a wound healing or a after a Ca^{2+} switch. Cell junction formation upon collision starts with the recruitment of E-cadherin and ß-catenin to the sites of cell-cell contacts. There are two possibilities for how contacts between daughter cells during cytokinesis could be formed: 1) during ingression of the furrow as cell membranes from the two daughter cells touch, there is the recruitment of junctional proteins, 2) junctional proteins are recruited to the cleavage furrow and are ready to form junctions as the furrow ingresses. It would be interesting to analyse in real time the formation of junctions between daughter cells during cytokinesis in HeLa and in confluent 16HBE or Caco-2 cells.

In HeLa S3 cells, PRK2 depletion leads to an increase in binucleate cells (Schmidt et al., 2007). Surprinsingly, in 16HBE cells, no increase in the percentage of binucleate cells was observed after PRK2 depletion, even though PRK2 localizes around the midbody and also at the cleavage furrow. The reason for this discrepancy is unknown**,** it is possible that the role for PRK2 in cytokinesis is cell-type specific or that the levels of PRK2 knock down were not high enough to cause a failure in cytokinesis in 16HBE cells.

Cytokinesis has been recently linked to the formation of an apical surface in a 3-dimensional system of epithelial morphogenesis that uses Caco-2 cells. In this system, aPKC, an apical marker, is seen to localize at midbodies and it has been proposed that, through a process of asymmetric ingression of the cleavage furrow (from the basal to the apical side), apical markers are deposited in the apical site during cytokinesis (Jaffe et al., 2008). A similar mechanism could be happening with junctional proteins that are recruited to the cleavage furrow in order to form junctions. We were able to observe that Scribble and Zo-1 are enriched around the midbody region in 16HBE and Caco-2 cells. To our knowledge, neither of these proteins has been implicated directly in cytokinesis.

Therefore, one possibility for why they accumulate at the midbody region during cytokinesis is that they participate in junction assembly between daughter cells**.** Others have analysed the localization of tight junction proteins during cytokinesis in SV-40 immortalized mouse hepatocytes (Kojima et al., 2001). In these cells both occludin and claudin-1, but not ZO-1, were seen to localize at the midbody. It is possible that localization of ZO-1 around the midbody region is cell-type specific. Two other recent studies have reported the localization of polarity proteins and the cleavage furrow and midbody. Crumbs, which localizes at tight junctions and apical membranes, has been shown to be recruited during cytokinesis in order to form an apical domain in MDCK 3D cultures (Schluter et al., 2009). Finally, aPKC was reported to be enriched in the furrow during cleavage in sea urchin embryos and apical-basal polarity was shown to be established at the first cleavage (Alford et al., 2009). It would be interesting to analyse the localization of other junctional/polarity proteins during cytokinesis in 16HBE or Caco-2 cells.

If junctional and polarity proteins are recruited to the cleavage furrow and/or midbody during cytokinesis, this might be regulated in a cell cycle-dependent way. In an attempt to investigate whether Scribble and ZO-1 would be regulated by phosphorylation during the cell cycle, HeLa S3 cells were arrested in prometaphase by nocodazole treatment and the mobility of the proteins after SDS-PAGE electrophoresis analysed. We were able to observe that, similarly to what happens to PRK2, the presence of a slower migrating band in the nocodazole population could be detected for both proteins, suggesting that these proteins are phosphorylated during mitosis. It would be interesting to analyse if this event affects the localization of these proteins around the midbody. aPKC mobility upon nocodazole treatment was also analysed and no mobility shift could be detected. This fact does not mean that aPKC is not phosphorylated during mitosis, as not every phosphorylation event gives rise to a mobility shift on SDS-PAGE electrophoresis. Phosphorylation of polarity/junctional proteins during

prometaphase could act as a positive or negative signal for their recruitment to the midbody region. As shown in Chapter 3 we have identified two serines on PRK2 that get phosphorylated during mitosis. We also show that replacement of this serines for alanines or aspartic acid residues have no effect on PRK2 localization at the midbody. However, we don't know if the aspartic acid residues mimic the phosphorylation event. In addition, mutation of the serines for alanines didn't completely block the mobility shift, meaning that other sites contribute to the shift. For these reasons we believe it is still possible that the mitotic phosphorylation could affect the localization of junctional/polarity proteins.

In conclusion, recent studies point to a link between cytokinesis and the establishment of apical-basal polarity in 3-dimensional cell-culture systems (Jaffe et al., 2008; Schluter et al., 2009). As the formation of a fully polarized Caco-2 cyst is linked to cytokinesis we thought it would be interesting to further analyse the effect of PRK2 depletion in this system. In addition Caco-2 cysts develop from individual cells and every cell-cell junction forms after cell division and not cell-cell collisions. We believe this would be a good model to study the formation of junctions during mitosis and the role of PRK2 in this process. In a preliminary experiment we were able to see that PRK2-targetting siRNAs have a strong effect in the size and architecture of Caco-2 cysts, and also in the formation of a lumen. Even though this phenotype needs to be confirmed and correlated with levels of PRK2 knock down, the Caco-2 cyst formation system seems to be a good model to further analyse the role(s) for PRK2 during epithelial morphogenesis.

CHAPTER 6 – FINAL DISCUSSION

Rho GTPases regulate a wide range of cellular processes through their interaction with effector proteins. A big number of Rho GTPase effectors are known and some have been extensively studied concerning their biological function and regulation. Here we have analysed PRK2, a serine threonine kinase that binds Rho and Rac; in the context of cell cycle progression and epithelial morphogenesis.

As with many other kinases, PRK family members are regulated by phosphorylation. Several phosphorylation sites have been identified in PRKs, including autophosphorylation sites and a PDK-1 site (Flynn et al., 2000; Peng et al., 1996). With the exception of the PDK-1 site that is believed to be required for activity, the significance of the majority of these phosphorylations is not known. Here we characterized a phosphorylation event that takes place specifically during mitosis. PRK2 is phosphorylated in mitosis in a way that gives rise to a mobility shift when run on SDS-PAGE gel electrophoresis. We were able to show that all PRK2 is phosphorylated in prometaphase and metaphase cells and gets dephosphorylated as cells progress to cytokinesis. In an attempt to understand how this phosphorylation regulates PRK2, we analysed its effect on PRK2 kinase activity and were able to see that PRK2 was less active when phosphorylated. This could be a mechanism of keeping PRK2 inactive before cytokinesis; however further experiments would be required to confirm this hypothesis.

We have identified two serines: Ser 301 and Ser 305 in mouse PRK2 corresponding to serines 302 and 306 in human PRK2, that are specifically phosphorylated in mitosis and contribute to the mobility shift. At this moment we don't known if both serines, and only one, contribute to the shift. We were not able to identify a clear cellular role for the phosphorylation at those sites and further experiments would be required to investigate whether phosphorylation at

Ser 301/302 and Ser 305/306 plays an important role in regulating PRK2 function. Additionally, other sites contributing to the mobility shift are still to be identified.

The most common mechanism of activation of effectors by Rho GTPases is the disruption of an auto-inhibited conformation. As PRKs contain an auto-inhibitory domain that overlaps with the Rho-binding domain, this mechanism has been suggested also for PRKs. However, additional signals are likely to be involved. We suggest here that in addition to allosteric modifications, Rho GTPases might be responsible for regulating PRK2 localization. We show that PRK2 localizes at two different locations during cytokinesis: at the cleavage furrow and midbody region, and at sites of cell-cell contacts between daughter cells. The PRK2 Rho-binding domain seems to be involved in the recruitment of the kinase to both locations and we propose that Rho GTPases, either Rho or Rac are mediating this recruitment. Rho GTPases have been shown to regulate the localization of other effectors, for example citron kinase at the cleavage furrow during cytokinesis or mDia at junctions (Carramusa et al., 2007; Eda et al., 2001).

Finally, we were able to show that PRK2 localizes at junctions in polarized epithelial cells and regulates epithelial junction formation. Depletion of PRK2 by siRNA inhibits cytokinesis in HeLa S3 cells; however, in 16HBE cells no cytokinesis defect was observed after depletion of PRK2, even though PRK2 localizes around the midbody in these cells. The recent observations that polarity proteins are recruited to the midbody region during cytokinesis and the fact that PRK2 localizes around the midbody and at junctions has led us to propose that these localizations might be linked. There are three explanations for why PRK2 would localize around the midbody in 16HBE cells. One is that it regulates cytokinesis, the second, that it is recruited in order to regulate junctions and the third that it regulates both junction formation and cytokinesis.

We were able to show that ZO-1 and Scribble are also enriched around the midbody during cytokinesis of polarized epithelial cells. In addition we have data suggesting that both these proteins are phosphorylated in mitosis. In order to maintain the barrier function of the epithelia, the integrity of the epithelial cell-cell junctions and epithelia apical/basal cell polarity needs to be sustained during mitosis and in particular during cytokinesis. We propose that junctional/polarity proteins are recruited to the cleavage furrow and/or around the midbody region to maintain the integrity of the junctions and/or polarity during cytokinesis. We hypothesize that the cell cycle machinery could be regulating this localization.

In conclusion we have gained new insights into how the activity and localization of a Rho GTPase effector is regulated during mitosis. In addition we have identified a new localization for PRK2: at cell-cell contacts between daughter cells during cytokinesis and at apical junctions in polarized epithelial cells. Finally, we propose that during cytokinesis of epithelial cells, junctional and polarity proteins might be recruited to the midbody region in order to maintain the integrity of the epithelium.

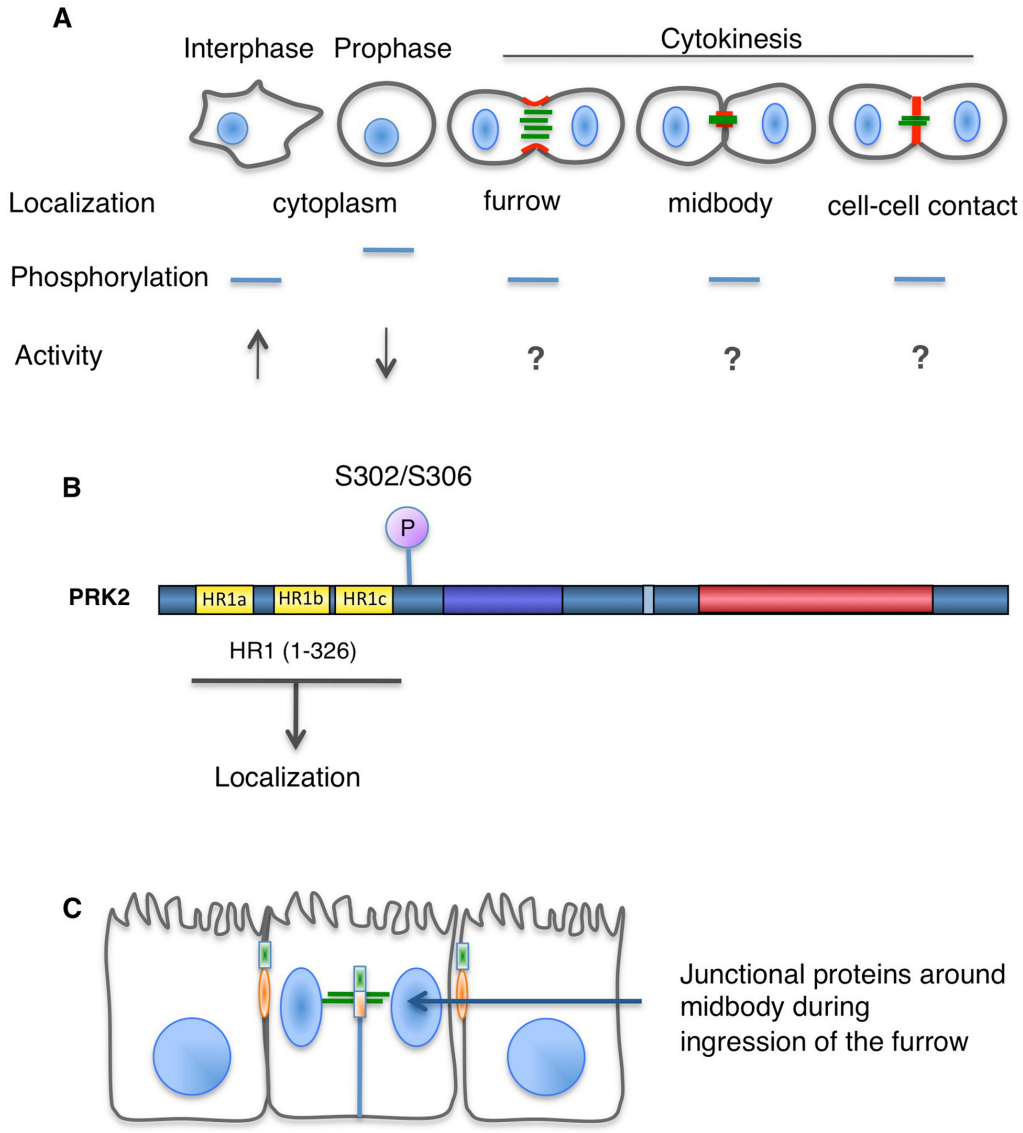


Figure 6.1 Summary. A schematic representation of the data presented in this dissertation is shown. We have found that PRK2 localizes at the cleavage furrow, around the midbody and at cell-cell contacts during cytokinesis. We have also found that PRK2 is phosphorylated during mitosis in a way that gives rise to a slow migrating band in an SDS-PAGE gel. PRK2 is phosphorylated in prometaphase and it gets dephosphorylated as cells progress to cytokinesis. Finally we have observed that PRK2 activity during prometaphase was lower than PRK2 activity in interphase cells (A). We identified one or two sites on PRK2 that are phosphorylated in prometaphase as Ser302 and Ser306. We have also shown that the HR1 domain is responsible for localizing PRK2 to the cleavage furrow, midbody and sites of cell-cell contact during cytokinesis (B). Finally we have shown that PRK2 localizes at junctions and regulates junction formation. We propose junctional markers are recruited to the midbody during cytokinesis to facilitate junction formation. The figure shows the model for the accumulation of junctional proteins during assymetric ingression of the cleavage furrow in polarized epithelial cells (C). Nucleus, blue; PRK2, red; midbody dark green; tight junctions light green; adherens junctions, orange.

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