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Role of the Z-/turn motif and of the N-Terminus of PRK2 in the regulation of the interaction between protein kinase C-related protein kinase 2 (PRK2) and 3-phosphoinositide dependent protein kinase 1 (PDK1)

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1. Abbreviations

4EBP1	4E-binding protein 1
ACC-region	Antiparallel coiled-coil region
AGC	This name is related to the cAMP dependent protein kinas (PKA), cGMP dependent protein kinase and protein kinase C (PKC)
Ala	Alanine
Akt/PKB	Akt/Protein kinase B
AR	Androgen receptor
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
BAD	Bcl-XL/Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
cAMP	Cyclic adenosine monophosphate
$CaCl_2$	Calcium chloride
CAM-KK	Ca^{2+}/cal-modulin-dependent protein kinase
Cav-1	Caveolin-1
CCI-779	Temsirolimus
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
CE	Crude extract
CGMP	Cyclic guanosine monophosphate
CISK	Cytokine-independent survival kinase
CO_2	Carbon dioxide
CSF	Colony stimulating factor
Cys	Cysteine
CZ	Charges aa and Leu-zipper-like sequence
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor, also known as ErbB1
EGTA	Ethylene glycol tetraacetic acid
ErbB1	Erythroblastoma B1; also known as EGFR
ERK	Extracellular signal-regulated kinases
FGF	Fibroblast growth factor
FHL2	Four and a half LIM domains 2
FL	Full length
FKBP12	FK506-binding protein 12
FKHR	Forkhead (Drosophila) homolog (rhabdomyosarcoma)
FKHRLI	Forkhead (Drosophila) homolog (rhabdomyosarcoma) like 1
FOXO3a	Forkhead box O3A
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GRB2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
GST-CT-PRK2	C-terminal fragment of PRK2 conjugated to GST
GTP	Guanosine triphosphate
HBS	Hepes buffered saline
HEK293 cells	Human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER-2	EGF receptor 2, also known as ErbB2
HER-2/neu	Protooncogen for the receptor Her2
HM	Hydrophobic motif
His	Histidine
HR	Homology region
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IGF-1	Insulin-like growth factor 1

IGF-1R	Insulin-like growth factor-1 receptor
IkB	I-kappa-B kinase
Ile	Isoleucine
IRS-1	Insulin receptor substrate-1
JMJD2C	Jumonji domain containing 2C
KLK2	Kalikrenin-related peptidase 2
Leu	Leucine
Lys	Lysine
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2 oncogene
MEKK-1	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1
Met	Methionine
MSK	Mitogen-and stress-activated protein kinase
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NF	Neurofilament
Ni-NTA	Nickel-nitrilotriacetic acid
PAGE	Polyacrylamide gel electrophoresis
PAK	Protease activated kinase
PC-3 cells	$PTEN^{-/-}$ prostate cancer cells
PDGF	Platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology
Phe	Phenylalanine
PHLPP	PH domain and Leucine rich repeat Protein Phosphatase
PI3K	Phosphatidylinositol 3-kinase
PIF	PDK1-interacting fragment
PKA	cyclic AMP dependent protein kinase
PKB	Protein kinase B
PKC	Protein kinase C

PKG	cyclic GMP dependent protein kinase
PKN	Protein kinase N
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PRK	Protein kinase C-related kinase
Pro	Proline
PSA	Prostate specific antigene
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol (4) monophosphate
PtdIns(3,4)P_2	Phosphatidylinositol (3,4) bisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol (4,5) bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5) trisphosphate
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PTKs	Protein-tyrosine kinases
PTP-BL	Protein tyrosine phosphatase-basophil like
RAD001	Everolimus
RBD	Rho binding domain of ROCK-1
ROCK	Rho-associated coiled-coil containing protein kinase
ROK	RhoA-binding kinase
RSK	p90 ribosomal S6 kinase
RTK	Receptor tyrosine kinases
S6K	p70 ribosomal S6 kinase
Ser	Serine
SH	Src homology (Src as it is short for sarcoma)
SHPTP2	Src Homology 2 Protein Tyrosine Phosphatase
SDS	Sodium dodecyl sulfate
SGK	Serum- and glucocorticoid-inducible protein kinase
SH2	Src homology 2
SHIP	Src homology 2 domain-containing inositol 5-phosphatase
Src family kinases	Family of proto-oncogenic tyrosine kinases
Syp	Synaptophysin
TAU-5	Transactivation unit 5
TEMED	N,N,N’,N’-Tetramethylethylendiamin
Thr	Threonine

TNF	Tumor necrosis factor
Tris-HCl	Tris-hydrochloric acid
Trp	Tryptophan
TSC-2	Tuberous sclerosis complex 2
Tyr	Tyrosine
Val	Valine
Wt	Wild type

2. Abstract

2.1. English version

The members of the AGC group of kinases often have three phosphorylation sites regulating their activity: The activation loop, the Z-/turn motif and the hydrophobic motif (HM) phosphorylation site.

At least 23 protein kinases of this group are activated through phosphorylation of their activation loop by their upstream kinase, the phosphoinositide-dependent protein kinase 1 (PDK1). Phosphorylation of the HM in a subset of AGC-kinases such as the p90 ribosomal S6 kinase (RSK), the p70 ribosomal S6 kinase (S6K), the Serum- and glucocorticoid-inducible protein kinase (SGK), leads to the docking of the phosphoHM to the HM/PIF-binding pocket and the bordering phosphate binding site on PDK1. The docking of the substrate then allows its phosphorylation at the activation loop by PDK1.

The protein kinase C related protein kinase 2 (PRK2) is a member of the AGC-group of kinases. However, in contrast to other members of this family, PRK2 possesses an acidic residue in the position of the HM phosphorylation site mimicking a phosphorylation at this site. Therefore, the docking of PRK2 to PDK1 cannot be regulated by HM phosphorylation. The present thesis had the objective to evaluate the mechanisms that control PRK2 interaction with PDK1.

Since PRK2 is phosphorylated at the Z-/turn motif, we wanted to characterize the influence of this phosphorylation on the interaction of PRK2 with PDK1 in this study. In this sense, we mutated the Z-/turn motif phosphorylation site within the C-terminal segment of PRK2 and the residues forming part of the PRK2 Z-/turn motif phosphate-binding site and examined the effects on the activity and on the interaction with PDK1. We could show that the mutation of the Z-/turn motif phosphorylation site to Alanine (Ala) or the destruction of the Z-/turn motif phosphate binding site by mutating it to Glutamic acid (Glu), led to a similar increase in the binding of PRK2 to PDK1, suggesting that this phosphorylation could negatively regulate the interaction with PDK1.

Additionally, for further characterization of the role of the N-terminus, we observed that ΔN-PRK2 had greatly increased ability to interact with PDK1. This result suggested that the N-terminal segment of PRK2 inhibits the interaction of PRK2 with PDK1. During these studies our biochemical assays suggested that the autoinhibitory regulation of PRK2 activity by its N-terminal segment may not be mediated by an intra-molecular but by an inter-molecular interaction. In this sense, using co-transfection and pull-down assays, we also provide evidence that the N-terminal region of PRK2 participates in an inter-molecular

interaction with another PRK2 molecule. We then tested the effect of the FL-PRK2 TST/AAA inactive mutant on the activity of FL-PRK2 or ΔN-PRK2, and found that FL-PRK2, but not ΔN-PRK2 activity, was inhibited in trans by the inactive PRK2 protein. In this context, our results indicate that PRK2 molecules are able to form oligomers via their N-terminal extensions and that this interaction causes an inter-molecular inhibition.

Furthermore, it is known that PRK2 is a Rho effector target. Admittedly, PRK2 is described to be activated in a nucleotide-dependent or -independent way. However, in our study, Rho, Rho-GDP and Rho-GTP did not affect the in vitro activity of PRK2.

As the C-terminus of PRK2 interacts with a very high affinity with PDK1, we further mutated other non-conserved motifs within the C-terminus of PRK2 and studied if they could also play a role in the high affinity interaction with PDK1. Here, we could show that a novel hydrophobic patch affects the interaction with PDK1 and a novel acidic patch was important for the stability of PRK2 itself.

Altogether the present study sheds light on the molecular mechanism that regulates the interaction between PRK2 and its upstream kinase PDK1. In addition, our results show for the first time that the inhibition of PRK2 is related to an inter-molecular interaction mediated by the N-terminal domain. This was surprising because the mechanism of inhibition of all closely related protein kinases (PKCs) is thought to be mediated by an intra-molecular interaction.

2.2. German version/Deutsche Version

Die Mitglieder der AGC-Kinase-Familie teilen die Eigenschaft, 3 Phosphorylierungsstellen zu besitzen, welche ihre Aktivität regulieren: Die Activation loop-, die Z-/turn motif- und die hydrophobic motif (HM) Phosphorylierungsstelle.

Die meisten Proteinkinasen dieser Familie werden über die Phosphorylierung ihres Activation loops durch die ihnen vorgeschaltete Kinase 3-Phosphoinositide-dependent Protein Kinase 1 (PDK1) aktiviert.

Die Phosphorylierung des HM derjenigen AGC-Kinasen, die in diesem Bereich eine Phosphorylierungsstelle besitzen, führt zur Bindung des phosphorylierten HM (phosphoHM) an eine HM/PIF-Bindungstasche (HM/PIF-binding pocket) und an die angrenzende Phosphatbindungsstelle von PDK1.

Die Protein Kinase C Related Protein Kinase 2 (PRK2) sowie die Protein Kinase Cζ (PKCζ) sind ebenfalls Mitglieder dieser AGC-Kinase-Famile. Im Gegensatz zu anderen Mitgliedern der Familie wie beispielsweise die p90 Ribosomal S6 Kinase (RSK), die p70 Ribosomal S6 Kinase (S6K) oder die Serum- and Glucocorticoid-inducible Protein Kinase (SGK), besitzen diese beiden Kinasen jedoch einen sauren Aminosäurerest an der Position der HM Phosphorylierungsstelle, der durch die Ladung in diesem Bereich eine Phosphorylierung imitiert.

Da PRK2 und PKCζ am Z-/turn motif phosphoryliert werden, war es in dieser Arbeit unser Ziel, den Einfluss dieser Phosphorylierung auf die Interaktion von PRK2 mit PDK1 zu untersuchen. Daher mutierten wir die Z-/turn motif Phosphorylierungsstelle innerhalb des C-terminalen Segments von PRK2 und die Aminosäurereste, welche die PRK2 Z-/turn motif Phosphatbindungsstelle formen, und untersuchten den dadurch erzeugten Effekt auf die Aktivität und Interaktion mit PDK1. Wir konnten zeigen, dass die Mutation der Z-/turn motif Phosphorylierungsstelle zu Alanin (Ala) sowie die Destruktion dieser Stelle durch die Mutation zu Glutamat (Glu) jeweils zu einer ähnlichen Steigerung der Bindung von PRK2 an PDK1 führte. Dies ließ uns annehmen, dass diese Phosphorylierung die Interaktion mit PDK1 negativ regulieren könnte.

Um darüber hinaus die Rolle des N-Terminus genauer zu charakterisieren, beobachteten wir, dass ΔN-PRK2 eine wesentlich höhere Fähigkeit besaß, mit PDK1 zu interagieren. Dieses Ergebnis ließ vermuten, dass das N-terminale Segment von PRK2 die Interaktion von PRK2 mit PDK1 inhibiert. Weitere Untersuchungen des N-Terminus zeigten, dass die autoinhibitorische Regulation der PRK2 Aktivität durch das N-terminale Segment

nicht durch eine intra-molekulare, sondern durch eine inter-molekulare Interaktion reguliert wird.

In diesem Sinne zeigen wir, dass die N-terminale Region von PRK2 an einer inter-molekularen Interaktion mit einem anderen PRK2 Molekül beteiligt ist. Dazu testeten wir den Effekt der inaktiven Mutante FL-PRK2 TST/AAA auf die Aktivität von FL-PRK2, beziehungsweise auf die Aktivität von ΔN-PRK2. Es zeigte sich, dass die Aktivität von FL-PRK2, jedoch nicht die von ΔN-PRK2, durch die Zugabe inhibiert wird. In diesem Zusammenhang weisen unsere Ergebnisse darauf hin, dass PRK2-Moleküle über ihren N-Terminus untereinander Oligomere bilden könnten und dass diese Bindung eine inter-molekulare Inhibition verursacht.

Des Weiteren ist bekannt, dass PRK2 ein Rho Effektor ist. Allerdings wird kontrovers beschrieben, ob PRK2 nukleotid-abhängig oder –unabhängig aktiviert wird. In unserer Studie jedenfalls beeinflussten Rho, Rho-GDP und Rho-GTP die in vitro-Aktivität von PRK2 nicht.

Da der C-Terminus von PRK2 mit einer sehr hohen Affinität mit PDK1 interagiert, untersuchten wir des Weiteren, ob andere, nicht konservierte, Motive innerhalb des PRK2 C-Terminus ebenfalls an dieser hoch affinen Interaktion beteiligt sein könnten. Diesbezüglich konnten wir zeigen, dass eine neuartig beschriebene hydrophobe Stelle die Interaktion mit PDK1 beeinflusst und dass eine neuartig beschriebene saure Stelle für die Stabilität von PRK2 selbst von Bedeutung ist.

3. Introduction

3.1. The PI3K signalling pathway

The phosphatidylinositol 3-kinase (PI3K) signalling pathway occupies a crucial role in a variety of biological processes. In this manner, it is involved for example in glucose metabolism, glycogen, lipid and protein synthesis, gene expression, cell growth and cell differentiation. As PI3K is assumed to play a main regulatory role in these pathways, the misregulation of this system is implicated in the development of diabetes, many forms of human cancer and heart failure.

One key effector of the PI3K signalling pathway is PDK1. In turn, this kinase is involved in the downstream activation of other AGC-kinase substrates like isoforms of Akt/Protein kinase B (Akt/PKB), SGK, S6K, PKC and PRK.

PI3K is a heterodimeric enzyme, meaning that this protein consists of two polypeptide chains (two subunits), differing in the composition of their amino acid residues and their structure, namely the p85 regulatory subunit and the p110 catalytic subunit. While the p85 regulatory subunit is essential for the interlinkage to special substrates, the p110 catalytic subunit - after recruitment to the membrane phosphorylates phosphatidylinositol (PtdIns), phosphatidylinositol-4-monophosphate (PtdIns(4)P), and phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) in each case at its D3-position, resulting in phosphatidylinositol-3-monophosphate (PtdIns(3)P), phosphatidylinositol-3,4-bisphosphate ($PtdIns(3,4)P_2$), and phosphatidylinositol-3,4,5 trisphosphate ($PtdIns(3,4,5)P_3$). $PtdIns(3,4,5)P_3$ is a major second messenger, that triggers most of known downstream effects of PI3K, for example. downstream of insulin and growth factor (GF) signalling [21, 37].

This activation step is turned off through the action of the lipid phosphatase Phosphatase and TENsin homologue deleted on chromosome TEN (PTEN), that converts $PI(3,4,5)P_3$ back to $PtdIns(4,5)P_2$ via a dephosphorylation at the D3 position [148]. Other phosphatases that are able to dephosphorylate the second messenger $PtdIns(3,4,5)P_3$ are SH2-containing inositol phosphatases (SHIP1 and SHIP2). These 5-phosphatases convert $PtdIns(3,4,5)P_3$ back to $PtdIns(3,4)P_2$ [118].

3.1.1. Insulin and growth factors activate the PI3K signalling pathway

Playing a crucial role in intracellular growth and metabolism, insulin induces signals that are amongst others involved in glucose uptake and disposal, transport of amino acids, transcription of specific genes and DNA synthesis [21, 68, 127] (see Figure 1). These signals are mediated by the binding of insulin to its cell surface receptor that comprises an

intracellular tyrosine kinase domain. Through this binding, the receptor is activated and subsequently autophosphorylated on tyrosine residues and further recruites and rapidly phosphorylates an immediate downstream substrate molecule, insulin receptor substrate 1 (IRS-1) [72, 148]. IRS-1 is a high-molecular-weight cytosolic protein, containing 20 potential tyrosine phosphorylation sites and over 40 potential Ser/Thr phosphorylation sites [21]. Some of the tyrosine phosphorylation sites are located in peptide sequences that are known to associate with proteins containing Src-homology 2 (SH2) domains, like PI3K [135].

Additionally, the IRS-1 is not only a major substrate of insulin receptors but also of insulin-like growth factor -1 (IGF-1) receptors [120, 135]. When phosphorylated and recruited to the membrane, IRS-1 functions as a docking protein, forming a signalling complex with PI3K, Syp (the phosphotyrosine phosphatase SHPTP2) and the Growth factor receptor-bound protein 2 (GRB-2). GRB-2 is a molecule that links IRS-1 to another pathway, the p21ras signalling system [92, 120, 135]. Importantly, the interlinkage of IRS-1 with proteins containing a SH2-domain, like the p85 regulatory subunit of PI3K, leads to their activation.

Further, PI3K is able to interact directly with and is stimulated by several activated growth factor receptors and nonreceptor tyrosine kinases like the platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF1) receptors and the Src-like kinases [27, 71, 131, 141].

3.1.2. Role of the PI3K-PDK1-Akt/PKB pathway in cell survival and cell growth

The best characterized function of PI3K is the activation of Akt/PKB. Akt/PKB is a member of the subfamily of AGC-kinases and a downstream target of the PI3K-PDK1 pathway.

The Akt/PKB family of proteins consist of a central kinase domain with specificity for Ser or Thr residues in substrate proteins, the N-terminus with a pleckstrin homology (PH) domain, that is essential for lipid-protein or protein-protein interactions, and finally the C-terminus, containing an HM that has homology to other protein kinases from the AGC family.

The Akt/PKB family of Ser/Thr protein kinases consists of three members, namely Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, which possess a high homology (>85%) in their sequence and are largely expressed in several human tissues. They all share a similar mechanism of activation: All three isoforms require the phosphorylation of two sites for full activation, one located at the activation loop and one located at the HM [32] (reviewed in [94]). A third phosphorylation site, the Z-/turn motif, is constitutively phosphorylated in Akt/PKB isoforms.

Stimulation of PI3K via tyrosine kinase receptors or other cell surface receptors in response to ligands such as insulin, PDGF, epidermal growth factor (EGF), or fibroblast growth factors (FGFs), results in the development of the second messengers PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. These second messengers bind to the PH-domain of Akt/PKB and recruit Akt/PKB to the plasma membrane. This binding also results in a conformational change of Akt/PKB, which promotes PDK1 to phosphorylate Akt/PKB at Thr308 in the kinase's activation loop. PI3K also enables the phosphorylation at the HM phosphorylation site of the C-terminus at position Ser473 by a kinase complex known as the mammalian target of rapamycin complex 2 (mTORC2) [43, 89, 143].

Activation of Akt/PKB itself is controlled by the tumor suppressor gene PTEN, that negatively regulates the Akt/PKB activation. Interestingly, genetic mutations that inactivate PTEN are known to result in a variety of tumors like endometrium, prostate, lung, and head and neck tumors [94].

Basically, Akt/PKB arises its cell surviving effects by blocking the function of proapoptotic proteins and processes. In this sense, Akt/PKB negatively regulates the function or expression of several Bcl-2 homology domain 3 (BH3)-only proteins. These proteins normally show their proapoptotic effects by binding to and inactivating prosurvival Bcl-2 family members. Akt/PKB does not only inactivate these proteins by phosphorylation, but also inactivates them indirectly via inhibition of their expression. So, Akt/PKB inactivates the BH3-only protein BAD in a direct and an indirect way [33, 138] and inhibits MDM2 (or HDM2 in humans), an E3 ubiquitin ligase that triggers p53 degradation in order to inhibit the proapoptotic function of the transcription factor p53 [98] (reviewed in [94]).

Further, Akt/PKB phosphorylates and inactivates forkhead family of transcription factors (FKHRLI or Foxo3a) [9] and Glycogen Synthase kinase 3 (GSK3) [28], proteins that have a proapoptotic effect, to fulfil its antiapoptotic function. In order to inactivate GSK3, Cross et al. described that Akt/PKB phosphorylates GSK3 isoforms on a highly conserved N-terminal regulatory site (GSK3α-S21, GSK3β-S9), resulting in the inactivation of the kinases [28].

Moreover Akt/PKB acts as an indirect inhibitor of the apoptosis via regulation of a human telomerase reverse transcriptase subunit [70] and regulation of IkB kinase [32].
Another target of Akt/PKB in its antiapototic function is caspase-9, a protein that has to be proteolytically processed and activated by its procaspase to gain its proapoptotic effect. However, activation of Akt/PKB prevents the processing of procaspase-9 via Bcl-2 family members (reviewed in [94]).

These antiapoptotic effects make understandable, why the PI3K-PDK1-Akt/PKB-signalling pathway also plays a crucial role in the regulation of cancer cell growth, invasion, survival and tumor progression and it is not surprising that this pathway has been described to be activated in several forms of human cancers [44]. Further, these effects point out PI3K-PDK1-Akt/PKB as important survival factors that contribute resistance to apoptotic signals (reviewed in [69]).

However, PI3K-PDK1-Akt/PKB pathway not only inhibits apoptosis but also stimulates cellular growth. It is activated by a variety of growth factors as IGF-1, EGF, basic FGF, insulin, interleukin 3, interleukin 6 and macrophagecolony stimulating factor [32].
Interestingly, there are also PI3K-independent mechanisms of Akt/PKB-activation. On this note, under cellular stress conditions, Akt/PKB activation can also be achieved by kinases like the Ca2+/calmodulin-dependent protein kinase (CAM-KK) and the cAMP-dependent protein kinase (PKA) [94], as the activation of Akt/PKB by these two kinases (CAM-KK and PKA) seems not to require phosphorylation of the Ser473 phosphorylation site.

3.1.3. Dysregulation and role of the PI3K-PDK1 signalling pathway in cancer

The PI3K upstream regulators include the Epidermal Growth Factor Receptors (EGFR), 170kDa transmembrane glycoproteins, expressed constitutively throughout the body and found in many epithelial tissues. EGFR are a subfamily of the receptor tyrosine kinases (RTK) that regulate a wide range of cell-functions like cell growth and survival as well as adhesion, migration, differentiation and other cellular processes. Importantly, EGFR (also known as ErbB1) is overexpressed in a number of human cancers including head and neck, bladder, pancreas and breast tumors.

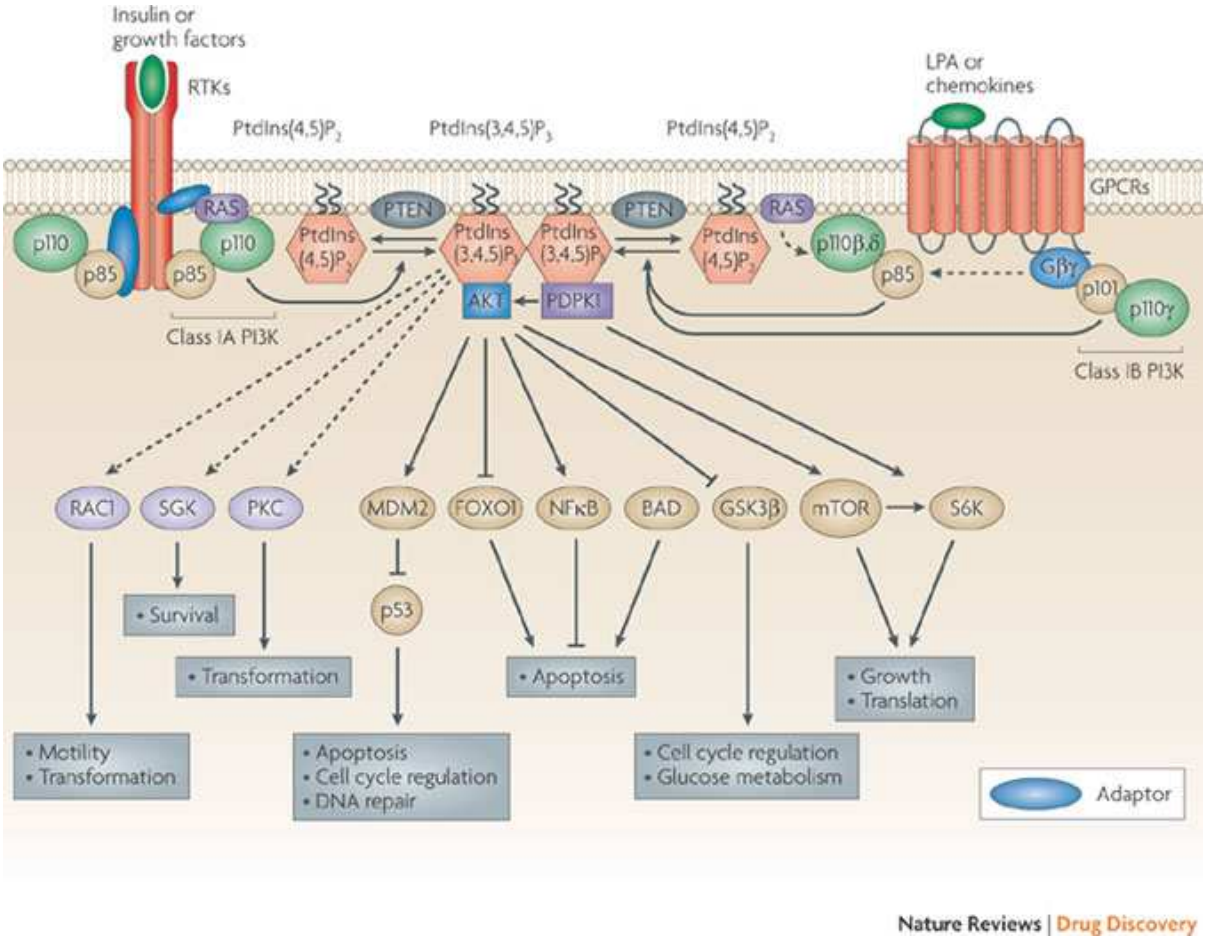

Figure 1: Taken from: Liu et al.: Targeting the phosphoinositide 3-kinase pathway in cancer, *Nature Reviews Drug Discovery 8, 627-644 (August 2009).* The figure provides a schematic overview of the complex Insulin and growth factor down-stream pathway with a focus on PI3K and its effector targets [91]

The fact that the PI3K pathway is a key signal transduction system linking oncogenes and multiple receptor classes to many essential cellular functions and that it is suggested to be one of the most commonly activated signalling pathway in human cancer, highlights the opportunity for cancer therapy by inhibitors that target PI3K isoforms and other major nodes in the PI3K downstream pathway like PDK1, Akt/PKB or mTOR (reviewed in [91]).

Relating to this, it was found that PI3K is mutated in a wide range of human tumors like breast, endometrial, colon and pancreas cancer. Additionally, especially lung cancers show amplifications of a subunit of PI3K. It was possible to demonstrate that two of the most frequent PI3K mutations increase PtdIns(3,4,5)P_3 levels resulting in an activation of Akt/PKB signalling and inducing cellular transformation (reviewed in [91]).

Akt/PKB is the best characterized downstream target of the PI3K signalling pathway, while the relative importance of other downstream protein kinases has not been studied in such detail. Akt/PKB can be found mutated in breast, colon, ovarian and lung cancer, while amplification of Akt/PKB has been reported in various tumour types like gastric, ovarian pancreas, head and neck and breast cancer.

As far as the less characterized PDK1 is concerned, mutations of this enzyme are rarely found in human cancer, while amplification or overexpression of PDK1 was found in up to 20% of breast cancers (reviewed in [91]).

The primary negative regulator of the PI3K-PDK1-Akt/PKB pathway is apparently PTEN [31, 36]. PTEN, an important tumour suppressor, functionally antagonizes PI3K activity through its intrinsic lipid phosphatase activity that reduces the cellular pool of PtdIns(3,4,5)P_3 by converting it back to PtdIns(4,5)P_2. Loss of PTEN results in unrestrained signalling by the PI3K pathway, thereby leading to cancer (reviewed in [91]).

Other regulators of Akt/PKB activity are the Ser/Thr protein phosphatases PP1 and PP2A. These phosphatases are involved in many different cellular processes like glycogen metabolism, cell cycle regulation, protein synthesis and intracellular transport, RNA splicing, and signal transduction (reviewed in [89]). Especially in advanced prostate cancer, elevated levels of cav-1 (caveolin 1) may play an important role in limiting PP2A and PP1 activities, leading to the maintenance of activated Akt/PKB [143]. The PHLPP phosphatase has been more recently described to play a role in Akt/PKB regulation, as it terminates Akt/PKB signaling by directly dephosphorylating and inactivating Akt/PKB [18].

The above mentioned Ser/Thr protein kinase mTOR plays a crucial part in the regulation of cell growth and proliferation, for example by monitoring nutrient availability, cellular energy levels, oxygen levels and mitogenic signals. With respect to its biological functions, it is obvious that dysregulation of the mTOR-pathway is related to cancer. At this point, it is worth mentioning that mTOR exists in two distinct complexes, namely mTORC1 and mTORC2, which is described in more detail in chapter 3.1.4.

In relation to the present thesis, there is one report showing that activity of the PDK1-phosphorylated protein kinase C related protein kinase 1 (PRK1), which is phosphorylated by

PDK1, can be inhibited by PI3K inhibitors. This finding indicates that PRK1 is also activated downstream of PI3K [46]. However, as they used an activation loop phospho-specific antibody to detect a phosphorylated activation loop of endogenous PRK1, it is also possible that, in addition, they detected the level of activation loop phosphorylation of endogenous PRK2, since PRK1 and PRK2 have the same amino acid sequence in this region (peptide antigen for the antibody: RTST(P)FCG, PRK2 (T816): RTS T FCG, PRK1 (T774) RTS T FCG). This shows that PRK1 and perhaps PRK2 activity may be affected by PI3K dysregulation. The PDK1-, PRK- and other PI3K-pathways will be specified in later chapters.

Altogether, this pathway is seen as a very promising therapeutic target for cancer treatment. On the other hand, it is involved in a variety of normal cellular processes, demonstrating that it is a challenge to find a drug strategy that has the most possible success and the fewest side effects at the same time.

3.1.4. Therapeutic potential of targeting the PI3K pathway

Pharmaceutical companies and academic laboratories are currently undergoing a number of different therapeutic strategies to develop drugs to PI3K and other key components in its signalling pathway.

With the knowledge that EGFR/ErbB1 is overexpressed in a number of human cancers, a highly effective therapy was established by treating these growth factor dependent neoplasias with antibodies against the overexpressed growth factor receptor. So, for example in some human epidermal growth factor-2-positive (EGFR-2/HER-2) breast cancer patients, the anti-HER-2 humanized monoclonal antibody trastuzumab (Herceptin) was found to be effective (reviewed in [47]). According to the German Cancer Society (DKG, Deutsche Krebs Gesellschaft), trastuzumab is currently established by default as an adjuvant therapy after operation or polychemotherapy in HER-2-positive breast cancers. In some cases, trastuzumab is also applied in parallel with the chemotherapy.

To target PI3K, numerous PI3K inhibitor chemotypes have been developed. While the inhibitors wortmannin and LY294004 show just little or no selectivity for individual PI3K isoforms and cannot be used therapeutically because of their considerable toxicity, newer inhibitors are in development and in clinical trials (like IC87114).

Inhibitors targeting both mTOR and the p110α subunit in PI3K have been developed. An example for such an inhibitor is BEZ235 (also BKM120, GDC-0941, BGT226 and SF1126). BEZ235 works via binding to the ATP-binding pocket and provides a strong anti-proliferative activity against tumour xenografts that show abnormal PI3K signalling,

including loss of PTEN function or gain of function PI3K mutations. These pharmaceuticals are intended to be used in the therapy of advanced solid tumors (reviewed in [91]).

Another attractive target is Akt/PKB. Various classes of inhibitors have been developed, e.g. lipid-based phosphatidylinositol analogues, ATP competitive inhibitors and allosteric inhibitors. The most clinically advanced substance is the lipid-based phosphatidylinositol analogue perifosine. This inhibitor targets the PH domain of Akt/PKB, which prevents Akt/PKB from binding to PtdIns(3,4,5)P_3 and thereby anticipates the membrane translocation. It stands presently in clinical trials to treat multiple types of cancers (reviewed in [91]).

mTOR is the first kinase of the PI3K pathway that was targeted in the clinic. Known as an antifungal agent, rapamycin was found to have immunosuppressive and antineoplastic properties. Rapamycin associates with its intracellular receptor, FK506-binding protein 12 (FKBP12), which then binds directly to mTORC1 and suppresses mTOR-mediated phosphorylation of its downstream substrates, S6K (at the HM phosphorylation site) and 4EBP1. Later, analogues of rapamycin working with the same mechanism and offering better pharmacological properties, such as temsirolimus (CCI 779) and everolimus (RAD001), were developed as anti-cancer drugs. These inhibitors only inhibit mTOR when this kinase is part of the mTORC1 complex and not when it is part of the mTORC2 complex.

Interestingly, mTOR also presents a potential second target: the mTORC2 complex. mTOR as part of the mTORC2 complex phosphorylates the HM site (Ser473) at the C-terminus of Akt/PKB (reviewed in [91]). Remarkably, it was recently shown that mTORC2 is required for the development of prostate tumors that are induced by PTEN loss [55]. Therefore, a kinase inhibitor of mTOR that can target both mTORC1 and mTORC2, could block the activation of the PI3K pathway more effectively than rapamycin does. Recent studies have described torkinibs and torin1, potent and selective ATP competitive inhibitors of mTOR, to inhibit both mTORC1 and mTORC2 complexes and to impair cell growth and proliferation more effectively than rapamycin. In addition, two ATP competitive mTOR inhibitors, OSI027 and AZD8055, are presently in clinical trials in patients with advanced solid tumours and lymphoma (reviewed in [91]).

Interestingly, it was shown that reduced expression of PDK1 can suppress tumor formation in animal models [8]. This suggests that it might be possible to inhibit tumorigenesis by inhibiting 80% of PDK1 activity (reviewed in [91]).

3.2. PDK1 and its substrates

3.2.1. Regulation of PDK1 and the role of the PIF-pocket

PDK1 is a key protein kinase that regulates the activity of its substrates through phosphorylation. Amongst others, isoforms of Akt/PKB [17, 129], S6K [144], RSK [48] and SGK [82] belong to these substrates. Like its substrates, PDK1 belongs to the group of AGC protein kinases.This name is related to the cAMP dependent protein kinase (PKA), cGMP dependent protein kinase (PKG) and protein kinase C (PKC) [12].

PDK1 possesses an N-terminal kinase catalytic domain and a C-terminal pleckstrin homology (PH) domain [1, 12] and activates its substrates by phosphorylating these kinases at their activation loop [137] (reviewed in [3]). Like all protein kinases, the catalytic core of PKA, the first AGC kinase whose crystal structure has been solved [136], possesses an N-terminal lobe, consisting mainly of β-sheet chains, and a predominantly α-helical C-terminal lobe [60, 136]. The ATP-binding site is located in between the two lobes [67, 78]. At the C-terminal end, PKA contains an extended loop that ends with the sequence Phe-Xaa-Xaa-Phe (where Xaa is any amino acid, FXXF) which is similar to the first part of the HM phosphorylation site of S6K and SGK (Phe-Xaa-Xaa-Phe-Ser/Thr-Tyr, FXXFS/TY) in which the Ser/Thr is the phosphorylated residue [10]. When phosphorylated, this HM folds back onto the catalytic domain and binds to a hydrophobic pocket located between β-4, β-5, α-C and α-B in the small lobe [67, 78]. Phosphorylation of the activation loop bridges the α-C helix in the small lobe with the catalytic and Mg^{2+} positioning loops, and is required (although often not sufficient) for stabilizing active conformations of AGC kinases [13]. Therefore, structurally, the activation of AGC kinases like Akt/PKB, SGK, S6K and others is explained by the coupled action of the phosphorylation at the HM and at the activation loop, that, together, stabilize the α-C helix, which in turn helps to position the ATP binding site residues for catalysis.

In the structure of PKA, the FXXF motif does not contain a phosphorylation site and is buried in a hydrophobic pocket in the small lobe of the PKA catalytic domain [78]. Importantly, mutation of either Phe residues drastically reduces PKA activity towards a peptide substrate [7, 42]. In PKA, the FXXF motif is constitutively bound to its pocket and does not participate in the regulation of the activity.

Remarkably, PDK1 does not possess an HM C-terminal to its catalytic domain like other AGC kinases do. However, PDK1 possesses the equivalent hydrophobic pocket that can be found in the small lobe of its catalytic domain similar to that in PKA. Interstingly, occupancy of the PDK1-interacting fragment pocket (PIF-pocket) activates PDK1. This can

be recognized because peptides that encompass the HM of PRK2 and RSK induce a 4- to 6-fold activation of PDK1 [10, 12, 49]. The PIF-pocket is characterized in more in the chapters 3.3.5.1. and 3.3.5.2. Furthermore, it was shown that mutation in PDK1of residues predicted to form part of this pocket result in reduced or abolished interaction of PDK1 with four of its substrates, namely S6K1, SGK1, PKCζ and PRK2 [6, 11]. Thus, in contrast to PKA, the PIF-pocket of PDK1 plays an important regulatory role.

Interestingly, previous work pointed out that PDK1 could act as a sensor of protein conformation. In this way, the PIF-pocket would allow PDK1 to interact with its substrates when they are in an inactive conformation [13].

As already mentioned, PTEN is frequently mutated in human cancers. This leads to elevated levels of PtdIns(3,4,5)P_3 and to increased Akt/PKB and S6K activity. A study by Bayascas et al. showed that reducing the expression of PDK1 in PTEN+/− mice, markedly protects these animals from developing a wide range of tumors. In this sense, they could show that PDK1 is a key effector in mediating neoplasia as a response to the loss of PTEN [8].

A

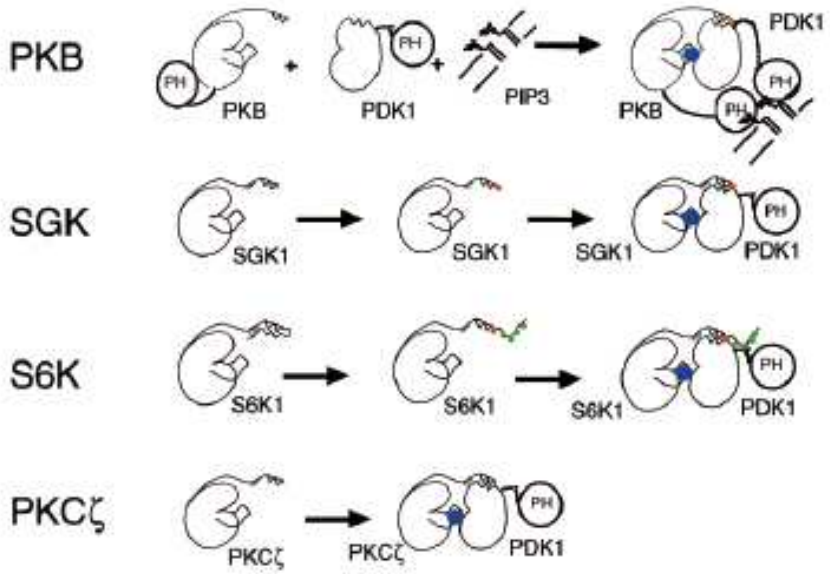

Figure 2A: Taken from: Leslie, Biondi and Alessi: Phosphoinositide-regulated kinases and phosphoinositide phosphatises *Chem Rev. 101(8):2365-80.(August 2001)* [86]: Summary of the model by which PDK1 can recognize, interact, and then phosphorylate different AGC kinase substrates. As described in more detail in chapter 3.2.2., Akt/PKB does not require the PIF-binding pocket on PDK1 to become phosphorylated by PDK1. In contrast, it is the binding of PDK1 and Akt/PKB to the second messenger PtdIns(3,4,5)P3 and PtdIns(3,4)P2 at the membrane of cells that prompts their co-localization, enabling PDK1 to phosphorylate Akt/PKB. The interaction of SGK and S6K with PDK1 is further described in chapter 3.2.3. SGK is a poor substrate for PDK1 until it is phosphorylated at its HM. This converts it into a form that can interact with the PIF binding pocket of PDK1 and hence permits PDK1 to interact with it and to phosphorylate SGK at its activation loop site. S6K is similarly converted into a form that can interact with the PIF binding pocket of PDK1 through a combination of the phosphorylation of the C-terminal Ser-Pro/Thr-Pro in its autoinhibitory domain1 and by phosphorylation of its HM. Thus, for SGK and S6K, it is the phosphorylation of these enzymes at their C-terminal residues that is rate limiting for the phosphorylation of these kinases at their activation loop by PDK1.
As it is specified in chapter 3.2.4.1.2., the atypical isoform PKCζ, in contrast, might be constitutively phosphorylated at its activation loop motif in cells as the HM of PKCζ, which possesses a Glu residue instead of a Ser/Thr residue at the site of phosphorylation, can in principle interact with the PIF binding pocket on PDK1, as soon as it is expressed in a cell, resulting in PKCζ becoming phosphorylated at its activation loop site. Blue circles indicate phosphorylation of activation loop residue, red circles phosphorylation of the HM, and green circles phosphorylation of the Ser-Pro/Thr-Pro residues in the C-terminal autoinhibitory domain of S6K. HMs are marked with two triangles.

B

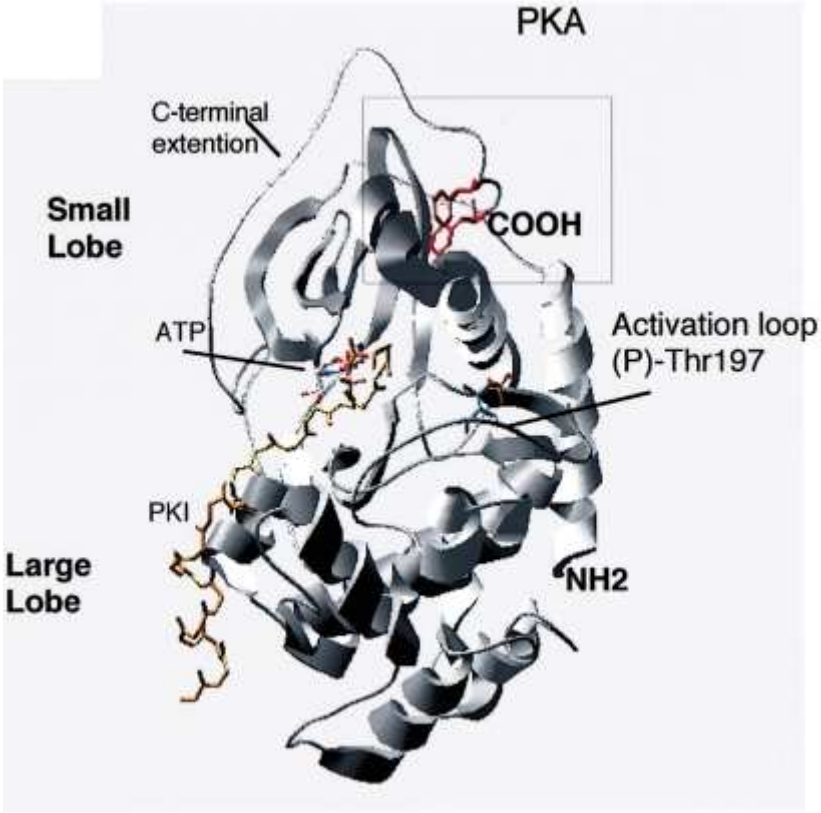

Figure 2B: Taken from Biondi et al: Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA; *EMBO J.;19(5):979-88 (March 2009)* [10];This figure shows the ribbon structure of the PKA–PKI–ATP ternary complex [153] PKI (Protein kinase A inhibitor) is shown in yellow, and the ATP molecule is highlighted. The C-terminal Phe347 and Phe350 are shown in red. The position of phospho-Thr197 (the PDK1 phosphorylation site) in the activation loop is indicated. The C-terminal Phe347-Xaa-Xaa-Phe350 residues of PKA interact with a hydrophobic pocket on the PKA kinase domain, predicted to be conserved in PDK1.

3.2.2. Interaction of Akt/PKB with PDK1

Different substrates of PDK1 are phosphorylated and activated by distinct regulatory mechanisms. For the interaction of Akt/PKB with PDK1, PtdIns(3,4,5)P_3 is required, as PDK1 can only phosphorylate Akt/PKB efficiently in vitro in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 [2, 134]. Both Akt/PKB and PDK1 interact with PtdIns(3,4,5)P3 via their PH-domains. Thus, the PH domains play a crucial role in co-localizing these kinases at the plasma membrane and thereby enabling PDK1 to phosphorylate and activate Akt/PKB [104]. While it is established that Akt/PKB is recruited to the plasma membrane due to PI3K activation [147], it remains open if PDK1 is also translocated to the membrane or if a constant pool of PDK1, that is not further increased by agonists, is constitutively associated with the plasma membrane in unstimulated cells (reviewed in [104]).

The binding of PtdIns(3,4,5)P_3 to the PH-domain does not directly activate Akt/PKB or PDK1 [2, 64], but it seems that binding of PtdIns(3,4,5)P_3 to Akt/PKB induces a large

conformational change (reviewed in [104]). In turn, changing of conformation increases the rate at which Akt/PKB can be phosphorylated by PDK1 [1, 133].

Even though it is not finally resolved, it is possible that the conformational change could result in the exposure of the activation loop residue and/or in the creation of a specific PDK1-binding/docking site.

Interestingly, although PDK1 means „3-phosphoinositide-dependent protein kinase“, all known substrates of PDK1, except Akt/PKB, are phosphorylated efficiently in a phosphoinositide-independent manner in vitro [140] (reviewed in [13]).

3.2.3. Interaction of SGK and S6K with PDK1

The AGC kinases S6K and SGK do not have a PH-domain and are phosphorylated by PDK1 in vitro in a phosphoinositide-independent manner. With substrates other than Akt/PKB, PDK1 specifically interacts with a substrate sequence from the region that is located C-terminally to the catalytic domain within the conserved FXXF HM. As already mentioned, S6K and SGK have a Ser/Thr residue at the HM site. In addition to the phosphorylation of the activation loop phosphorylation site, this HM site also has to be phosphorylated in order to achieve maximal activation [13, 104]. Additionally, there is a temporal difference between Akt/PKB phosphorylation (which is more rapid) and the phosphorylation of SGK and S6K by PDK1. The current model also explains this feature since S6K and SGK require the HM phosphorylation to gain maximal activation [13].

Moreover, further work, where the residue Arg131 of PDK1, that is located in the phosphate-binding pocket of PDK1, was replaced by a Met residue [25, 50], could show that S6K activation is not only related to the phosphorylation of its HM, but also to the additional phosphorylation of the C-terminal residues Ser/Thr-Pro, which is regulated by mTOR [62] and occurs independently of PDK1 [99].

Interestingly, previous work reported that Akt/PKB is indirectly involved in the stimulation of phosphorylation of the HM of S6K by mTORC1 through phosphorylation. This phosphorylation leads to the inactivation of the tuberous sclerosis complex-2 (TSC2).

In contrast, recent studies showed that mTORC2, but not mTORC1, plays a vital role in controlling the HM phosphorylation and activity of SGK [52].

3.2.4. PKC and PRK isoforms

3.2.4.1. The PKC family

PKCs (protein kinase C) compose a family of isoenzymes grouped into three subclasses, based on the composition of domains and membrane targeting modules of the N-terminal regulatory tail that determines the co-factor-dependence of the isoenzyme [111]. Two important components are the C1 domain, acting as the diacylglycerol sensor, and the C2 domain, representing the Ca^{2+} sensor. Each of these comes either in a form that binds ligand or in a form that is lacking determinants that allow ligand binding [23, 59]. On this note, PKCs are divided into conventional, novel and atypical forms. All isoenzymes have an autoinhibitory "pseudosubstrate" sequence N-terminal to a C1 domain.

Conventional PKCs contain functional C1 as well as functional C2 domains, enabling them to respond to diacylglycerol and Ca^{2+} signals. Novel PKCs contain a functional C1 domain, but not a non-ligand-binding C2 domain resulting in the fact that they respond to diacylglycerol, but not to Ca^{2+} signals. In contrast, atypical PKCs contain a non-ligand-binding C1 domain and no C2 domain and consequently, they neither respond to diacylglycerol nor to Ca^{2+} signals (reviewed in [112]). The ten mammalian PKCs contain four conventional isoenzymes (α, βII and the alternatively spliced βI, which differs only in the last 43 residues, and γ) four novel PKCs (δ, ε, η/Λ and θ), and two atypical PKCs (ζ and ι/λ) [100, 113].

The mechanism of regulation of PKCs has been mostly studied in classical PKCs. So it is known, that PKCs are phosphorylated just after synthesis, a process called "maturation". PKC is then kept in an inactive conformation via binding of the pseudosubstrate sequence to the substrate-binding cavity in the sense of a pickup sensor (reviewed in [112]). Thus, in this model, the pseudosubstrate binds intra-molecularly and blocks the active site.

The generation of diacylglycerol and Ca^{2+} and the resulting engagement of the C1 and the C2 domains on the membrane lead to the recruitment of PKC to the membrane. Through this membrane interaction, the energy to release the pseudosubstrate from the substrate-binding cavity is provided, allowing substrate binding and phosphorylation [110, 111].

3.2.4.1.1. Interaction of classical PKCs with PDK1

The PKC family members have three conserved phosphorylation sites in common: The activation loop site, the Z-/turn motif phosphorylation site and the HM phosphorylation site. For the PKC members, phosphorylation is a basic requirement to allow adjacent substrate phosphorylation. Without these preceding phosphorylations, the kinases do not show catalytic activity [112]. The kinase that provides this priming phosphorylation of PKCs at the activation loop is PDK1.

In previous work, PDK1 was shown to be the upstream kinase for conventional [40, 83], novel [20, 83] and atypical [24, 83] PKC family members. In contrast to the PI3K-dependent phosphorylation of SGK, S6K, Akt/PKB and other AGC kinases, the phosphorylation by PDK1, which makes the PKCs catalytically compentent, is a constitutive process for classical PKC family members [112]. These constitutively phosphorylated substrates of PDK1 acquire the active conformation soon after synthesis and their regulation is achieved by other means, such as diacylglycerol and Ca^{2+} binding in classical PKCs (reviewed in [13]). Studies on this topic let suggest that upon synthesis, PKC isoforms interact with PDK1 by means of their dephosphorylated HM and become subsequently phosphorylated at their unmasked activation loop by PDK1. The HM provides a docking site for PDK1 [49] via the PDK1's PIF-binding pocket [12]. A model that has not been proven experimentally suggests that in the active conformation, the C-terminus of substrates is released from the PIF-binding docking site on PDK1. The liberated C-terminus (with the HM) is now accessible for phosphorylation and the phosphorylated HM interacts with the enzyme's own catalytic core (reviewed in [13]).

It is then suggested that phosphorylation of the C-terminus locks this segment on the small lobe of the kinase domain, stabilizing the active conformation and enabling it to bind the N-terminal pseudosubstrate sequence. Further analysis that identified, next to the PIF-pocket, a phosphate binding site that interacts with the phosphate from the phosphorylated HM [50]. This binding pocket has two basic residues that are conserved through AGC kinases that have HM phosphorylation sequences (notably PKC and Akt/PKB, but not PKA) (reviewed in [112]).

As described in more detail under item "The PKC family", in classical PKCs, in order to target PKC to the membrane, generation of $Ca2^{+}$ and diacylglycerol is required. Alexandra Newton suggests that the binding of the C1 and C2 domains on the membrane delivers the energy to release the pseudosubstrate from the substrate-binding cavity, enabling the

downstream signalling. However the molecular details of PKC activation have never been studied to a molecular detail.

Nevertheless, once reaching this active conformation, PKC is thought to be dephosphorylated at its activation loop after agonist stimulation, before degradation (reviewed in [112]). To complete the life circle of PKCs, binding of the molecular chaperone Hsp70 to the dephosphorylated Z-/turn motif stabilizes PKC and enables it to become rephosphorylated and allows it to re-enter the pool of signalling-competent PKCs (reviewed in [112]).

Remarkably, while some PDK1 substrates, as described above in this chapter in more detail, seem to exist in inactive and active conformations crossing their life cycle, other substrates, such as conventional PKC isoforms, appear to be phosphorylated by PDK1 upon synthesis and seem to exist *in vivo* exclusively in stable active conformations [13].

As already mentioned, it was thought that the HM in conventional PKC isoforms would be an autophosphorylation event. However, recent work reported that mTORC2 is required for phosphorylation of the conventional PKCα at its HM (Ser657) [15, 41, 128].

3.2.4.1.2. Atypical PKC isoforms

Atypical PKC isoforms (aPKCs), such as PKCζ, are activated and stabilized by phosphorylation of their activation loop site like other members of the AGC-subfamily of protein kinases. Atypical PKC isoforms (PKCζ and PKCι/λ) possess a Thr residue in a region equivalent to Thr308 (Thr410 in PKCζ [24, 83]) of Akt/PKB in the activation loop. The phosphorylation of this residue is crucial for kinase activation. However, while the other members of the AGC subfamily of kinases possess a phosphorylatable Ser/Thr in their HM, the phosphorylatable Ser/Thr residue is replaced in atypical PKCs by an acidic residue, for example Glu579 in PKCζ. The negatively charged amino acids are able to mimic the phospho-Ser/phospho-Thr residue [121].

As mentioned above, the kinase that phosphorylates the activation loop residue of conventional, novel and atypical PKC isoforms is PDK1 [39, 46]. The kinase domain of PDK1 interacts with a region of atypical PKCs encompassing its HM [5]. Previous work demonstrated that these C-terminal fragments of atypical PKC isoforms (PKCζ and PKCι/λ) interact significantly with the PIF-binding pocket of PDK1 [6]. It was shown that mutation of the conserved aromatic residues in the PKCζ HM inhibits or greatly weakens the interaction of PKCζ with PDK1 and prevent the phosphorylation of PKCζ at its activation loop *in vivo*. This is also the case for PRK2. Interestingly, while mutation of the phosphorylation

mimicking residue of PRK2 (Asp978) to either Ala or Ser importantly diminishes the interaction of PIF and PRK2 with PDK1 [5], a mutation of the equivalent residue in PKCζ (Glu579) did not considerably affect the interaction between PKCζ and PDK1.

Further work showed that the specific activity of the mutant Glu579Ala in PKCζ is only slightly lower compared to PKCζ wild type, suggesting that a negatively charged residue in the HM of the atypical PKC isoform is not required for maximal activity [6].

3.3. Protein kinase C-Related protein Kinases (PRKs)

In addition to the three major classes of PKC isoenzymes, another family of PKC-related protein kinases has been identified, termed the protein kinase C-related kinase (PRK) family.

PRK was first described biochemically, as a protein kinase that showed increased activity upon limited proteolysis and was termed PAK, for Protease Activated Kinase [51]. The activity was also independently termed Protein Kinase N (PKN) [105] and also cloned and found to possess a catalytic domain very similar to PKC isoforms, and named “Protein kinase C-Related protein Kinase” PRK1 and PRK2 [119]. There are at least three different isoforms of PKN (PKNα /PRK1/PAK-1, PKNß/PKN3 and PKNγ/PRK2/PAK-2) in mammals, each of which shows different enzymological properties, tissue distribution, and varied functions [107, 119]. While PRK1 and PRK2 seem to be expressed in all tissues, expression of PKN3 is low in normal adult tissue, but increased in human cancer cells [116] and in early mouse embryonic stages.

Remarkably, the kinase domain fragments of PRK1 and PRK2 represent constitutively active forms, while the corresponding PKN3 fragment exhibited significantly less catalytic activity than the full-length molecule [85]. While PKN3 needs the N-terminal domain for full enzymatic activity, this is not the case for PRK1 and PRK2. Whereas PRK1 and 2 are effectors of small Rho GTPases, as described in more detail below, it has not been clarified yet, if PKN3 is also regulated by these proteins [85].

The kinase that phosphorylates the activation loop of PRKs is PDK1 [39, 46]. As already mentioned previously, the kinase domain of PDK1 interacts with a region of PRK2 encompassing its HM termed the PDK1-interacting fragment (PIF) [5]. Previous work demonstrated that these C-terminal fragments (PIF) of PRK1 and PRK2 interact significantly with the PIF-binding pocket of PDK1 [6].

It is not much known about the regulation of the activation of PRKs. However, work by Flynn et al. provided strong hints, that the activation of PRK1 and PRK2 is phosphoinositide-dependent. They could show that inhibition of PI3K by LY294002 led to a diminished activation loop phosphorylation of PRK1 at its Thr774 residue. As described above under the item “Dysregulation and role of the PI3K signalling pathway in cancer“, it is possible that this is also the fact for PRK2. Interestingly, they could show in addition, that the PRK activation loop phosphorylation induced by PDK1ΔPH (residues 51–404) was also seen to be dependent upon PI3K products, as it was inhibited by LY294002. For this reason they suggested that a separate pathway exists between PI3K and PRK other than through PDK1, perhaps through endogenous GTPases [46].

As they could also show that PDK1 was recruited to the early endosomal compartment in a PRK-dependent manner and that the resulting ternary complex of RhoB-PRK-PDK1 was unaffected by PI3K inhibition, they suggested that the recruitment of the kinases to this membrane compartment is independent of PI3K products, but that the subsequent activation step requires PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 [46].

The role of PRKs as effectors downstream of Rho have been, on the other hand, better characterized.

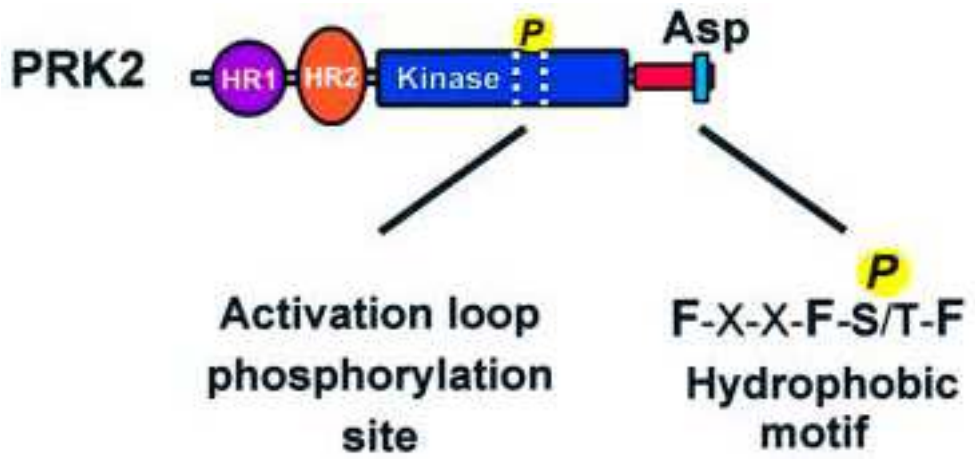

Figure 3: Taken from Frödin et al.: A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation; *The EMBO Journal (2002) 21, 5396 – 5407* [50]; Structural view of PRK2. The graphic illustrates two regulatory features: phosphorylation of the activation loop (stippled area) and phosphorylation of a hydrophobic motif (blue box), located in a tail region (red box) C-terminal to the kinase domain. PRK2 contains a phosphate-mimicking aspartic acid residue.

3.3.1. PRK2 is an effector target of Rho-GTPases

3.3.1.1. Signalling pathways downstream of Rho

PRKs and Rho-associated coiled-coil containing protein kinases (ROCKs) are widely considered to be the protein kinases that mediate phosphorylations downstream of Rho and are both inhibited by the highly specific protein kinase inhibitor Y27632 [34].

The Ras-related Rho subgroup of small GTPases consists of nine Rho-like proteins: RhoA, RhoB, RhoC, Rac1, Rac2, cdc42, g25k, RhoG and TC10. Each of these proteins is at least 50% identical in amino acid sequence to any other in the subgroup and about 30% identical to Ras. Rho-like proteins act as molecular switches, changing from an inactive state when bound to GDP and to an active state when bound to GTP [114], regulated by guanine nucleotide exchange factors (GEFs). These factors control the relation of the GDP-bound to the GTP-bound form [16]. Rho and Rac regulate signalling pathways linking growth factor receptors to the assembly and organization of the actin cytoskeleton [114, 125]. Cdc42 is also involved in the formation of actin-based structures. Rho, Rac and Cdc42 stimulate the assembly of focal adhesion complexes at the plasma membrane and are involved in

controlling cell polarity [125]. Activation of Cdc42 leads to the activation of Rac and Rho in vivo [81, 115, 125]. Additionally, Rho controls the assembly of actin stress fibers and focal adhesion complexes, Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles and Cdc42 stimulates the formation of filopodia [117].

Furthermore, the Ras-related Rho family of proteins mediates signalling pathways involved in changes in nuclear gene expression. So, RhoA, Rac1 and Cdc42 activate transcription by activating transcription factors [26, 58, 103]. Rho, Rac, and Cdc42 control signal transduction pathways that are essential for cell growth [117].

Rho is also involved in mitosis and cytokinesis. In this sense, RhoA plays a critical role in G1-S progression of the cell cycle [56, 63, 150] and Rho, Rac and Cdc42 stimulate cell cycle progression through G1 and subsequent DNA synthesis (reviewed in [117]).

Previous studies showed that the GTP-bound form of RhoA is able to bind and activate PRKs [4, 146]. Other effector targets of activated Rho-GTPases are ROCK-I [14], that is homologous to myotonic dystrophy kinase [4], as well as ROCK-II [87, 88, 96].

3.3.1.2. PRK2 as an effector target of Rho

Recent studies highlighted PRK2 as the major Rho-associated kinase in most tissues. In contrast to the other kinases mentioned above, PRK2 is a potential effector target of both Rho and Rac. The interaction between PRK2 and Rho is nucleotide-independent, while GTP is needed for the interaction with Rac and in both cases, the interaction results in a considerable increase of kinase activity. In contrast, ROCK-I and ROCK-II do not appear to be activated substantially upon binding to Rho [61, 87, 96].

Moreover, as mentioned above, PRK1 and PRK2 activity is found in most tissues and cell lines, while expression of the other Rho effectors seems to be limited. However, PRK2 was described as „the predominant Rho-associated kinase“ and was detected at substantially greater levels than PRK1 [142].

Like PRK1 and PKN3, PRK2 has a Rho binding domain, named HR1 (homology region 1, also called CZ region (charges aa and Leu-zipper-like sequence) or ACC-region (antiparallel coiled-coil region), that is highly charged and sufficient for the interaction with Rho. HR1 consists of three tandem copies of a 60-70 amino acid sequence and is located at the N-terminus of PRK1 and PRK2 (HR1a, b, c). Primarily, RhoA-GTP interacts with the at the very N-terminus HR1 repeat (HR1a) [154]. Comparable data is available for PRK1 [45]. At the present, it is assumed that the binding of RhoA-GTP to HR1 disrupts an autoinhibitory

intra-molecular interaction in PRKs, consequently increasing PRK phosphorylation by PDK1 and resulting in further activation [107, 142, 146].

Although there is only little information of how Rho GTPases interact with PRKs, today it is considered that the binding of RhoA-GTP to the HR1 domain of PRKs is required for optimal activation of PRKs by RhoA [90]. Now it is suggested that PRK2 behaves as most other Rho effectors, meaning that it is activated by the active GTP-bound form of Rho to mediate its effect [90]. This finding fits into a model proposed by Flynn et al. in 1998: the binding of a GTPase to PRK enables the interaction of its C-terminal region with PDK1, which consequently phosphorylates PRK at the activation loop in the presence of PtdIns(3,4,5)P_3. In turn, this fits to the findings of Flynn et al. in 2000, that the phosphorylation of the activation loop of PRK2 seems to depend on PI3K [46]. Additionally, RhoA-GTP is also able to bind to the Rho-binding motif of other Rho kinases (ROCK-I and ROCK-II), termed RBD (Rho binding Kinase of ROCK-I) [154].

3.3.1.3. Function of the Rho-PRK2 pathway

A crucial function of the Rho-PRK2 pathway is the regulation of actin cytoskeletal organisation, corresponding to the observation that cells expressing the kinase-deficient PRK2 protein showed a disruption of fibroblast actin stress fibers and an increased level of sub-cortical actin [142]. Moreover, Rho GTPases regulate PRK2 to control entry into mitosis and exit from cyto-kinesis [130].

Controlled by Rho GTPases, PRK2 is involved in the abscission of the midbody at the end of the cell division cycle and in the activation of mitotic cyclin/Cdk1 complexes at the G2/M transition via activation of a phosphatase, named Cdc25B. In this sense, PRK2-depleted cells have been shown to be delayed in G2/M progression and to fail to undergo abscission, finally resulting in binucleated cells [130].

3.3.1.4. Role of Rho in the PDK1-PRK2 affair

It was shown that the in vivo and in vitro activation of PRK1 and PRK2 involves the activation loop phosphorylation by PDK1. As mentioned above, the interaction of PRK1 and PRK2 with PDK1 is thought to be dependent upon Rho, as Rho influences the activation loop phosphorylation by controlling the ability of PRKs to bind to PDK1 resulting in an increased activation loop phosphorylation of endogenous PRKs in the presence of Rho [46].

Interestingly, previous work showed that transfection of both PRK kinases with either PDK1 or the GTPase-deficient-RhoA or -RhoB significantly elevated the activation loop phosphorylation level of endogenous PRK1 and PRK2 in HEK293 cells. Remarkably, the level of phosphorylation of PRK was not further increased through co-transfection of Rho and PDK1, even if a high stoichiometry of phosphorylation had been excluded, implying that further requirements are needed in PRK phosphorylation [46].

To explain these findings Flynn et al. proposed the following model: the binding of a GTPase to PRK enables the interaction of its C-terminal region with PDK1, which consequently phosphorylates PRK at the activation loop in the presence of PtdIns(3,4,5)P_3 [46]. After activation loop phosphorylation, PRK is able to autophosphorylate and to be further activated. However, the formation of the Rho-PRK-PDK1 complex and the resulting PRK activation loop phosphorylation *in vivo* can only occur after modification of Rho through prenylation, indicating that for the PtdIns(3,4,5)P_3-dependent phosphorylation of PRK a prior assembly on a membrane is necessary. By contrast, *in vitro*, the HR1 domains of PRK1 and PRK2 are capable of binding to bacterially expressed and thus non-prenylated Rho-GTP [45]. Altogether, while the recruitment and the maintenance of the RhoB-PRK-PDK1 complex is independent of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 and is rather regulated by protein-protein interaction, these PI3K products are necessary *in vivo* for the activation of PRK2 by PDK1 through activation loop phosphorylation upon binding of the C-terminal fragment of PRK2 to PDK1 [46].

3.3.2. Biological functions of PRK2

Of major interest, PRK1 has been shown to activate androgen receptors (AR) [101], to phosphorylate histone H3 at Thr11 [102] and to regulate transcription. Interestingly, inhibition of PRK1 blocks AR-induced tumour cell proliferation, making PRK1 a promising therapeutic target. Similarly, PRK2 also induces H3 phosphorylation [102] and PKN3 is required for malignant prostate cell growth [85]. The most notable role described for PRK2, on the other hand, may be to control entry into mitosis and exit from cytokinesis [130].

3.3.2.1. PRK2 is required for hepatitis C virus replication

It was found in 2004 that PRK2 is the specific cellular kinase phosphorylating the hepatitis C Virus (HCV) NS5B protein. NS5B protein is the viral RNA-dependent RNA polymerase required for replication of the HCV RNA genome, suggesting that HCV RNA replication is regulated by NS5B phosphorylation through PRK2 [74]. Subsequent work on this could show

that suppressing PRK2 activation by HA1077 (also known as Fasudil) and Y27632, two kinase inhibitors which exhibit a high selectivity for PRK2 and rho-kinase (ROCKII), block the phosphorylation of the HCV RNA-dependent RNA polymerase and lead to reduced HCV RNA levels. These findings identify PRK2 inhibitors as potential antiviral drugs that act by suppressing HCV replication via inhibition of viral RNA polymerase phosphorylation [75].

3.3.2.2. A wide field of functions with attention to apoptosis

PRK2 is involved in many cellular pathways like cytoskeletal regulation, cell adhesion, apoptosis, regulation of meiotic maturation, regulation of entry into mitosis and exit from cytokinesis and in signalling to the cell nucleus [107].

PRK2 is required for actin reorganisation in a Rho-GTPase dependent manner [142]. In lamellipodia-like structures, regions of large actin turnover, it was shown that PRK2 has a strong co-localization with protein Tyr phosphatase-basophil like (PTP-BL), a large non-transmembrane protein Tyr phosphatase implicated in the modulation of the cytoskeleton, that binds to PRK2 [54]. Further, PRKs are involved in the regulation of organization of intermediate filaments, like the subunits of neurofilament (NF), vimentin, and glial fibrillary acidic protein (GFAP) [97, 106]. Interestingly, PRKs are also found in degenerative neurites within senile plaques implicating that PRKs could be involved in the Alzheimer disease [73].

In keratinocytes, PRK2 is found to be involved in the control of cell-cell adhesion. It links the activation of Rho and the activation of Fyn, a regulator of cell-cell-adhesion [19].

PRK2 is also embraced in the apoptotic model. PRK2 is cleaved in the early stages of apoptosis and it was shown *in vitro* that PRK2 binds Akt/PKB and prevents its phosphorylation at both Ser473 (located at the HM) and Thr308 (located at the activation loop) [80]. It is further suggested that this inhibits the full activation of Akt/PKB and thereby the Akt/PKB downstream signalling and consecutively its anti-apoptotic effects, like the protection against TNF-induced apoptosis [80].

The execution of apoptosis is mediated in a decisive manner by caspases which consist in a family of cysteine proteases with aspartate specificity. Normally, caspases exist in cells as catalytically inactive proenzymes. During the induction of apoptosis, they are proteolytically processed and activated, what is a crucial event in apoptosis. Previous work showed that full-length human PRK2 is rapidly and specifically proteolyzed by caspase-3 at Asp117 (in the N-terminal regulatory domain) and Asp700 (in the C-terminal kinase domain) *in vitro* during the induction of apoptosis. Interestingly, with respect to the development of cancer, mutations of the aspartic residues Asp700 to glutamic acid and Asp117 to alanine prevented cleavage at

these sites. Further, proteolysis at this N-terminal site (Asp117) probably facilitates subsequent cleavage at Asp700. In addition, PRK2 is cleaved rapidly during Fas- and staurosporine-induced apoptosis *in vivo* by caspase-3 or a caspase-3-like subfamily member (reviewed in [29]).

Based on the result that distinct protein kinases, like the PKC isoforms PKCδ and PKCθ, the p21-activated kinase PAK2 and MEKK-1 are cleaved and activated by caspase-3 during apoptosis, it has been suggested that PRK2 cleavage during apoptosis might deregulate its activity, since the two PRK isoforms PRK1 and PRK2 have been reported to be activated by limited tryptic proteolysis, possibly by removal of their N-terminal inhibitory domain (reviewed in [29]). According to the model described above, absence of the N-terminal cleavage site (Asp117) would also hinder the cleavage at Asp700 at the C-terminus in a PRK enzyme lacking the N-terminus.

PRK2 proteolysis at aspartate residues by caspases during apoptosis induces the creation of a 36-kDa C-terminal fragment (corresponding to the amino acid residues 700-984, named C1). One report suggests that the C-terminal region of PRK2 that is cleaved from the inhibitory N-terminal region *in vitro* can bind Akt/PKB [80]. However, the C1 fragment does not contain a complete kinase domain structure required for protein Ser/Thr kinase activity and therefore cleavage at this site does not activate PRK2. Koh et al. suggest that the protein-protein interaction between the C1 fragment region and Akt/PKB is critical and that this binding leads to the inhibition of the growth factor-induced Akt/PKB activity [80]. The authors found that the interaction of the C-terminal region of PRK2 with Akt/PKB inhibits the phosphorylation of Akt/PKB at the residues Ser473 and Thr308, leading to a specific down-modulation of the protein kinase activities. In turn, this inhibition causes the inhibition of the downstream signalling of Akt/PKB. So, the Akt/PKB-mediated phosphorylation of BAD, a pro-apoptotic Bcl-2 family protein, is highly inhibited by the PRK2 C-terminal fragment. This fragment blocks the anti-apoptotic activities of Akt/PKB in vivo, while Akt/PKB translocation to the membrane is unaffected. Further, in additional experiments, Akt/PKB-mediated protection against TNF-induced apoptosis was significantly abolished in the presence of wild type PRK2 or C-terminal PRK2 [80]. On the other hand, this fragment comprises the C-terminal region of PRK2 including the PIFtide region which consists of the residues 908-984 [5] and has been described above to interact with the PIF-binding pocket of PDK1.

However, the conclusions drawn by Koh et al [80] did not consider that the C-terminus of PRK2 interacts with PDK1. In addition, posterior work showed that the affinity of

PIF to Akt/PKB is extremely low. Based on current knowledge, we can now hypothesize that the cleavage of PRK2 during apoptosis would prompt the selective blockage of the PIF-binding pocket of PDK1 by the C-terminal PIFtide region of PRK2. In this way, cleavage of PRK2 along apoptosis would affect PDK1 downstream signalling.

With respect to further biological functions of PRK2, it was shown that this enzyme is also involved in meiosis. During early development, oocytes arrest late in G2 of the first meiotic cell cycle. Hormonal stimulation results in the resumption of meiosis, known as meiotic maturation [95] (reviewed in [107]).

Another cellular pathway where PRK2 is involved is signalling to the nucleus. The kinase translocates from the cytoplasm to germinal vesicles during the meiotic maturation in starfish oocytes [132]. There, PRK2 may regulate the early events during meiotic maturation and the potential roles of PRK2 are the activation of Cdc2/CyclinB, translation initiation and actin cytoskeletal changes. Several reports showed that PRK2 is also embraced in the regulation of transcription [22, 124] (see also 3.3.3.1.). In this context, PRKs could be implicated as a downstream effector of Rho in transcriptional responses in cardiomyocytes, which is associated with cardiac hypertrophy [107]. Interestingly, in addition to PI3K, cAMP may be an upstream regulator of PRK2, as the increase of intracellular elevation of cAMP blocks PRK2 [107].

3.3.3. PRKs in prostate cancer

3.3.3.1. Involvement of PRKs in prostate cancer

Recent work by Metzger et al. showed that PRK1 and PRK2 are involved in the genesis of prostate cancer. To understand how these kinases influence cellular pathways leading to a malignant growth, it is necessary to have a look on the androgen receptors (ARs). Again Rho plays an important role in AR activation and there are two different ways how the Rho signalling pathway induces the activation of the ARs.

On one hand, stimulation of the Rho signalling pathway leads to a translocation of the coactivator FHL2, a protein that contains a highly conserved double zinc finger motif, to the nucleus, resulting in the activation of the AR by FHL2 [108]. Moreover, activation of the Rho signalling pathway induces also a FHL2-independent PRK-mediated transcriptional activation of the AR. This pathway results in a ligand-dependent superactivation of AR-regulated genes. Further, the PRK1 signalling additionally induces the transcriptional activity of mineralocorticoid receptors, progesterone receptors and p160 co-activators [101].

Investigating the role of PRK1 in this context, Metzger et al. showed in 2008 that PRK1 is further involved in posttranslational modifications of histones. It phosphorylates histone H3 at threonine 11 (H3T11) upon ligand-dependent recruitment to AR target genes. The phosphorylation of H3T11 serves as a chromatin mark for transcriptional regulation and enhances demethylation of H3K9 by JMJD2C via removing repressive methyl marks during AR-dependent transcription. Enhancing the JMJD2C-dependent demethylation plays a supporting role in activating the AR-dependent transcription (reviewed in [102]). In short, the phosphorylation of H3T11 leads to an increase of the AR-dependent transcription.

The AR belongs to the steroid hormone receptor family of ligand-activated transcription factors. It has diverse biological functions like cell growth and differentiation, development, homeostasis and various organ functions in the adult [93], for example differentiation, development and maintenance of male reproductive functions and non-reproductive organs [65, 139]. Remarkably, PRK activates the AR both in the presence of adrenal androgens and in the presence of the AR antagonist cyproterone acetate, supporting a novel modell that suggests that the AR activity is controlled by PRK signalling (reviewed in [102]).

In the prostate, the AR is expressed in secretory epithelial cells that respond to androgens. According to the current model of prostate cancer, the AR plays an important role in the development of prostate cancer that mainly originates from epithelial cells.

As described in 1998 by Gregory et al., growth and survival of primary prostate cancer cells is critically dependent on androgens [53]. Nevertheless, despite reduced circulating androgen levels and even in the presence of AR antagonists, most prostate cancers recur and progress to a terminal stage [30].

Evidence that PRK1 is essential for the AR function is supported by the finding that an inhibition or knock-out of PRK1 diminishes the AR-dependent transcription. PRK1 and AR form a complex on chromatin in a ligand-dependent manner resulting in an increased gene expression. Abrogating the PRK1 function overweighs the AR induced phosphorylation of histone 3 at threonine 11 and further inhibits the androgen-induced demethylation of histone H3. According to this, PRK1 is seen as a "gatekeeper of androgen receptor-dependent transcription" [102]. Interestingly, Metzger et al. further describe that in the N-terminus of the AR the transactivation unit 5 (TAU-5) is located, that suffices for activation by PRK1 [101].
Former work already pointed out that Rho family effectors such as PRK1 are overexpressed in human prostate tumors [53, 108]. Metzger et al. showed that in tissue sections obtained from radical prostatectomies there is a significant increase of PRK levels compared to the secretory epithelium of normal prostate tissue and normal basal compartment, where PRK1 is immunhistochemically hardly detected [84, 101]. This increase was shown in all different cancer specimens that were examined. At the same time the expression of the AR in the same cancer specimens is not altered [101].

Having a look on the clinical relevance, the levels of phosphorylated H3T11 and PRK1 correlate with malignancy of prostate cancer and with the Gleason scores [102]. High levels indicate aggressive biology of the tumors. It was shown, that the inhibition of PRK1 drastically reduces androgen induced proliferation of prostate tumor cells. Further, downregulation of PRK1 levels in prostate tumor cells leads to a diminished androgen-induced expression of endogenous androgen receptor target genes like prostate-specific antigen (PSA) or kalikrenin-related peptidase 2 (KLK2).

The remarks above underline the important role of PRK1 in the control of AR-dependent growth of tumour cells and marks PRK1 as a potential target in prostate cancer therapy. Although the studies were centered on PRK1, the authors showed that transfection of cells with PRK2 also had similar effects [101].

3.3.3.2. Involvement of PKN3 in prostate cancer

Not only PRK1 and PRK2 are involved in prostate cancer, but also the third member of the PRK subfamily, PKN3, seems to participate in this cancer. In 2004, Leenders et al. published that PKN3 is required for malignant prostate cell growth. They could show that PKN3 is an effector of chronically active PI3K, that appears to contribute to invasive prostate cancer [85].

PKN3 is regulated by PI3K not only at the level of expression but also at the level of catalytic activity. The loss of PTEN function leads to a chronic activation of PI3K, leading in turn to the chronic activation of PKN3 and is also correlated to an increased metastatic behaviour [145] and further to an increased invasiveness or growth in semi-solid matrices [66, 77, 79].

Like PRK1 and PRK2, PKN3 contains an activation loop phosphorylation site. In the case of PKN3 it is located at position Thr718. In contrast to PRK1 and PRK2, the FL-PKN3 has a considerably higher kinase activity compared to the isolated catalytic domain fragment. In this context, deficiency of the N-terminus causes an inactivity of the kinase. The reason for that seems to be the fact, that the N-terminus overlaps with the kinase domain fragment. This kinase domain fragment is active by itself and phosphorylated at Thr718. It seems that the catalytic domain of PKN3 has a negative regulatory function that is decreased in the presence of the N-terminus in the full-length protein. [85].

Interestingly, relating to the above mentioned involvement of PRK1 and PRK2 in prostate cancer, Leenders et al. suggested that PKN3, but not PRK1 or PRK2, is required for PC-3 ($PTEN^{-/-}$ prostate cancer cells) growth on matrigel (a substrate for cell culture) [109, 123]; cells with increased malignant potential have a growth advantage on matrigel matrix.

As adduced before, PKN3 is expressed in a PI3K-dependent manner and PKN3 expression is upregulated in patient prostate tumor samples. In addition, induced inhibition of PKN3 expression interferes with the formation of lymph node metastases in an orthotopic mouse prostate tumor model. Experiments with mice showed that a decreased level of PKN3 in the primary tumor is related to reduced formation of metastases [85]. PKN3 can be regulated by various signal transduction pathways that mediate cell growth and transformation. In human breast epithelial cells containing the Ras oncogene, PKN3 can contribute to their invasive potential. RasV12, an oncogenic form of Ras, causes an enhanced phosphorylation of MAP kinase, resulting in an increased expression of PKN3, but it has no effect on the PRK1 and PRK2 expression. This shows that PKN3 can also be regulated in a PI3K-independent way by signals that mediate malignant growth in certain cell types (reviewed in [85]).

3.3.4. The structure of PRK2

PRK2 consists of a catalytic domain, formed by a small and a large lobe, flanked by two structurally distinct domains: the N-terminal regulatory domain and the C-terminal tail. PRK2 is a Ser/Thr protein kinase that has a catalytic domain with approximately 50% sequence identity to that of the PKCs and 87% sequence identity to that of PRK1. The ATP-binding site is located between the two lobes of the catalytic domain [13]. As it is known for other kinases, N- and C-terminal regions are important for regulation of the kinase activity.

As already described in more detail under item “PRK2 as an effector target of Rho”, PRK2 has a special regulatory region containing the HR1-region, that is thought to be required for interaction with Rho GTPases [154]. This HR1-region is highly charged and is sufficient for the interaction with Rho. HR1 consist of three homologous stretches of a 60-70 amino acid sequence and is located at the N-terminus of PRK1 and 2 (HR1a, b, c). These stretches are followed by a Leu zipper-like sequence [154] (reviewed in [107]). Between the HR1 region and the catalytic domain, the HR2 region is located, a stretch of about 130 amino acids that has a weak homology to the C2-region of PKC ε and η.

The C-terminal part of this C2-like/HR2 region is thought to have an autoinhibitory effect. It is sensitive to arachidonic acid, leading to an activation of PRK1/2 and PKN3 in vitro, relating to the fact, that in addition to GTPases, PRKs can also be activated by fatty acids in vitro [151]. Admittedly, the kinase activity of PRK2 and PKN3 is significantly less sensitive to arachidonic acid than that of PRK1 [116, 152]. However, the HR1 and the C2-like/HR2-region are conserved among the isoforms PRK1, PRK2 and PKN3 among different organisms (reviewed in [107]).

At the C-terminus of PRK2 and all other members in the PKC superfamily, there is a segment of approximately 70 amino acid residues that possesses the lowest sequence similarity among the PKC superfamily members compared to any other domain. Although there is this low similarity at this domain, the region contains a conserved Z-/turn motif and the HM (Phe-Xaa-Xaa-Phe-Ser/Thr(P)-Phe/Tyr, where Xaa is any amino acid) (reviewed in [90]). As already mentioned, instead of a phosphoacceptor Ser/Thr residue, the HM of atypical PKC- and PRK-subgroups have a negatively charged Asp/Glu residue (Asp978 in PRK2 [121]) mimicking a phosphate. Interstingly, in 2008, Lim et al. referred that at the extreme C-terminus of PRKs, there is a segment of 10–15 amino acids beyond the HM, that is least conserved both in terms of amino acid sequence identity and the length of sequence amongst all members of the PKC superfamily. They suggested that the extreme C-terminal tail plays a role in the catalytic competence of PRK2 and in the regulation of PRK2 by Rho,

as they could show that the extreme C-terminal segment is critical for the full activation of PRK2 by RhoA in cells ina GTP-dependent manner. A part of the sequence with high relevance is the HM, that is located C-terminally to the catalytic domain.

3.3.5. Mechanism of activation of PRKs and other AGC kinases

The members of the AGC family of kinases have in common, that they have three phosphorylation sites regulating more or less their activity: The activation loop, the Z/turn-motif phosphorylation site and the HM phosphorylation site. While the activation loop and the HM phosphorylation sites are well investigated, the Z-/turn motif phosphorylation site has just been characterized in recent work by Hauge et al. [57].

Most protein kinases of the AGC family are activated through phosphorylation of their activation loop by their upstream kinase PDK1.

These AGC kinases phosphorylate a considerable amount of cellular proteins and in doing so they regulate cellular division, survival, metabolism, transmembrane ion flux, migrative behaviour and differentiation. The kinases are activated by partly distinct signalling pathways. Akt/PKB, S6K and SGK are for example downstream mediators of PI3K, while RSK and MSK are effectors of ERK and ERK/p38 mitogen-activated protein (MAP) kinases and PRK2 is an effector target and controlled by Rho GTPase (reviewed in [46]). The differential responsiveness to upstream pathways partly depends on the fact that the kinases contain different signalling modules flanking the kinase domain. These are for example a PH domain in Akt/PKB, a Rho-binding domain in PRK, a special inhibitory domain in S6K and a MAP kinase activated kinase domain in RSK and MSK [50].

However, although the AGC kinases have divergent regulation mechanisms, they all require phosphorylation of a Ser or Thr residue in the activation loop within the kinase domain and they all require phosphorylation of the HM [50]. As described before, this HM is characterized by three aromatic amino acids surrounding the Ser/Thr residue that becomes phosphorylated: Phe-X-X-Phe-Ser/Thr-Phe/Tyr. In PRK and atypical PKCs the HM contains a negatively charged amino acid (aspartic acid or glutamic acid) that mimics the phospho-Ser/phospho-Thr [50, 121]. The phosphorylation of the HM promotes the interaction of the HM of substrates with the PIF binding pocket of PDK1 thereby activating PDK1 to phosphorylate the activation loop phosphorylation site. Thus, in distinct AGC-kinases, the phosphorylation of the HM creates a specific docking site that recruits and activates PDK1, which then phosphorylates the activation loop. For an efficient interaction with PDK1, the HM must be phosphorylated or it must contain a phosphate mimicking acidic residue [6, 11,

49, 50]. Interestingly, Akt/PKB and MSK require phosphorylation in their HM, although Akt/PKB does not appear to use the motif for PDK1 docking [13] and MSK is not a target of PDK1 [50, 149].

By homology with other AGC kinases it can be speculated that in the active conformation the HM of PRK2 would fold back onto the catalytic domain and bind to a hydrophobic pocket located between β-4, β-5, α-C and α-B (the PIF-pocket) in the small lobe. Furthermore, as in PKA, it is expected that the activation loop phosphorylation further bridges together the α-C helix in the small lobe with the catalytic and Mg^{2+} positioning loops and is participates on the stabilisation of active conformation of PRK2 [67, 78]. Biochemical experiments suggest that the Z/turn-motif phosphate of PRK2 binds to a phosphate binding site on the small lobe of the kinase domain [57].

3.3.5.1 The hydrophobic motif (HM) and the the PDK1-interacting fragment (PIF)

As a result of yeast two-hybrid screening, Antonio Casamayor found that PDK1 interacts with a certain region of PRK2, the PDK1-interacting fragment (PIF) [10]. This fragment includes the 77 amino acids lying immediately C-terminal to the kinase catalytic domain of PRK2 [119]. In this region there is a moderate sequence homology between members of the AGC-family including the HM (Phe–Xaa–Xaa–Phe–*Ser/Thr*–Phe/Tyr). The C-terminal HM of PRK2 (Phe-Xaa-Xaa-Phe-Asp-Tyr) is similar to that found in Akt/PKB, except that the residue equivalent to Ser473 is an aspartic acid (Asp978). Importantly, mutations of conserved aromatic residues of the HM or mutations of the Asp978 to Ala or Ser greatly diminish the affinity between PIF and PDK1 [5].

Most notably, the authors suggested that the binding of PIF to PDK1 converted this enzyme into a protein kinase that could phosphorylate, not only the activation loop, but also the HM of Akt/PKB and therefore suggested that the Akt/PKB HM- kinase could be PDK1 [5]. However, it was not noted by the authors at the time that PIF also interacted with Akt/PKB. Reinterpretation of the data and further experiments established that PIF did not convert PDK1 into a HM-kinase but that PIF prompted the autophosphorylation of Akt/PKB at the HM [10]. Therefore, most of the experiments from the Balendran et al. paper [5] require re-interpretation and the main conclusion of the paper is not correct.

On the other hand, it was later found that the isolated HM of PRK1, PKCζ and PKCι also interacted with the kinase domain of PDK1 [6]. More interestingly, mutation of HM residues affected the interaction between PRK2 and PDK1. This result provided evidence that

PRK2 binds to PDK1 via these residues. As mentioned above, PDK1 activates PRK2 by phosphorylating the activation loop residue. Mutation of the HM residues affect their phosphorylation by PDK1 [6]. Thus, it was concluded that the HM of PRK2 (PIF) acts as a docking site that enables the recruitment of PDK1 and the posterior phosphorylation of the substrates [6].

Interestingly, using a peptide substrate (T308tide), it was possible to show that PDK1 is activated directly by PIF [10]. It was also shown that PIF does not alter the K_m of PDK1 for ATP [10].

3.3.5.2. The HM-/PIF-bindining pocket of PDK1

By modelling based on the structure of PKA, it was found that PIF interacts with a hydrophobic pocket on the small lobe of the kinase domain of PDK1 [10]. The pocket is different from s the ATP- and substrate-binding sites. The PIF-binding pocket at the kinase domain of PDK1 acts as a "docking site", enabling it to interact with and enhance the phosphorylation of its substrates like PRK2 [11]. Using molecular biolgy and biochemistry tools it was first characterized that the PIF-pocket comprises Lys115, Ile119, Gln150 and Leu155. This assumption is supported by the fact that mutations of these residues lead to a either abolished or significantly diminished affinity of PDK1 for PIF. The residues Lys115 and Leu155 of PDK1 participate in an hydrophobic interaction with the residues Phe974 and Phe977 of PIF [10]. Mutants of PDK1 at Leu155 do not interact with the HM of PRK2 [10] and are also unable to form a complex with the C-terminal fragments of PRK1, PKCζ and PKCι [6]. The crystal structure of PDK1 further allowed the detailed characterization of the PIF-pocket [12].

Interestingly, follow up work using the kinases in cell lines, confirmed that the PIF-binding pocket of PDK1 is essential for activation of S6K and SGK, but not for Akt/PKB [11]. Phosphorylation of the HM of S6K and SGK promotes their interaction with the PIF-binding pocket of PDK1 and their activation loop phosphorylation by PDK1. On the other hand, this pocket is not needed for the phosphorylation of Akt/PKB, showing that the PIF-binding pocket functions as a substrate recognition site that is only required for distinct substrates [11]. Further, the phosphorylation of S6K and SGK at both their activation loop and HM, like that of Akt/PKB, is dependent on PI3K activation. S6K and SGK do not possess a PH domain and do not interact with PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 like Akt/PKB does [11]. This fact underlines the variety of ways of activation within a family of kinases, while it is

pointed out that the interaction of the PIF-binding pocket and the C-terminal part of many AGC-kinases plays a crucial role in the course of activation.

A phosphate-binding site next to the hydrophobic pocket of PDK1 recognizes the phospho-Ser/phospho-Thr in the HM [12, 50]. Frödin et al. could further show that RSK2, S6K, Akt/PKB, MSK1 and SGK contain a similar phosphate-binding pocket in their kinase domain and that they use the phosphate-binding pocket to interact with their own phosphorylated HM, resulting in large stimulation of kinase activity in synergy with activation loop phosphorylation [50]. They also suggested that the phosphate-binding pocket is a key regulatory feature of the >40 human AGC kinases in which it is conserved. Teh study extended the possibility of using the PIF-pocket and its associated phosphate binding site as an potential target for drugs aimed to activate or inhibit AGC kinases, as an alternative to the commonly targeted ATP-binding site [50].

3.3.5.3. Role of the Z-/turn motif phosphorylation site in the mechanism of AGC kinase activation

The Z-/turn motif phosphorylation site is located in the middle of the C-terminal tail region in the AGC-kinases Akt/PKB, S6K, RSK, MSK, PRK and PKC. It got its name because PKA, the first AGC-kinase whose structure was solved, also contains a phosphorylation site in the middle of its tail region, that was called “turn motif” because the phosphate binds nearby residues within the tail and thereby stabilizes a turn in the tail. Anyhow, the PKA “turn motif” phosphate does not interact with the catalytic core.

However, this tail phosphorylation site of the growth-factor activated AGC-kinases mentioned above is not equivalent to the turn motif of PKA, since the “Z” phosphate binds specifically to a phosphate binding site on the catalytic core in other AGC kinases. The phosphorylation sites of the turn motif and of the activation loop work in a cooperative manner, as the turn motif phosphate binds to a phospho-Ser/Thr-binding site above the glycine-rich loop within the kinase domain. This binding promotes an association of the tail with the kinase domain and serves to deliver the HM to its binding site in a zipper-like manner, inducing stabilization of the HM in its kinase-activating binding site. This stablilization directly leads to stimulation of the kinase activity. Because of the zipper-like function of the tail phosphate and the dissimilarity to the turn motif of PKA, Hauge et al. provided the name “Z (zipper) site” for it. In the growth-factor-activated AGC-kinases, the tail phosphate binds a phospho-Ser/Thr-binding site in the kinase domain next the

hydrophobic pocket [57]. Nevertheless, since the site in AGC kinases has been traditionally named “turn motif”, along this thesis we name the site “Z-/turn motif”.

The AGC-kinases are allosterically activated by the Z-/turn motif phosphate via HM-mediated stabilisation of the αC-helix. There are hints that in a subset of the growth-factor activated kinases the tail phosphate also controls the phosphorylation state of the HM. Moreover, the Z-/turn motif phosphate is found to protect S6K and MSK from dephosphorylation. Mutations of this site significantly reduce kinase activity and in some AGC-kinases also the HM-phosphorylation (reviewed in [57] and [121]).

Taken together, the three conserved phosphorylation sites cooperate with each other in the stimulation of AGC kinase activity during stimulus-induced activation by coordinating the shift of the AGC kinase catalytic domain from the inactive, open conformation to the active, closed conformation and vice versa. Though, the Z-/turn motif phosphate alone or in combination with the activation loop phosphate does not have an activating effect. Rather, it synergistically enhances the stimulation of kinase activity mediated by the HM phosphate in collaboration with the activation loop phosphate [57].

3.3.5.3.1. PRK2 and PKCζ are phosphorylated at the activation loop and Z-site in vivo and mutation of the Z-/turn motif phosphorylation site in PRK2 increases the interaction with PDK1

The Z-/turn-motif phosphorylation site is functionally conserved in many AGC kinases. Further, the HM serves for docking to PDK1, although in PRK2, PKCζ, S6K and SGK the alignment of the C-terminal region of different AGC kinases shows a low degree of identity along this segment of the kinases (see Figure 4).

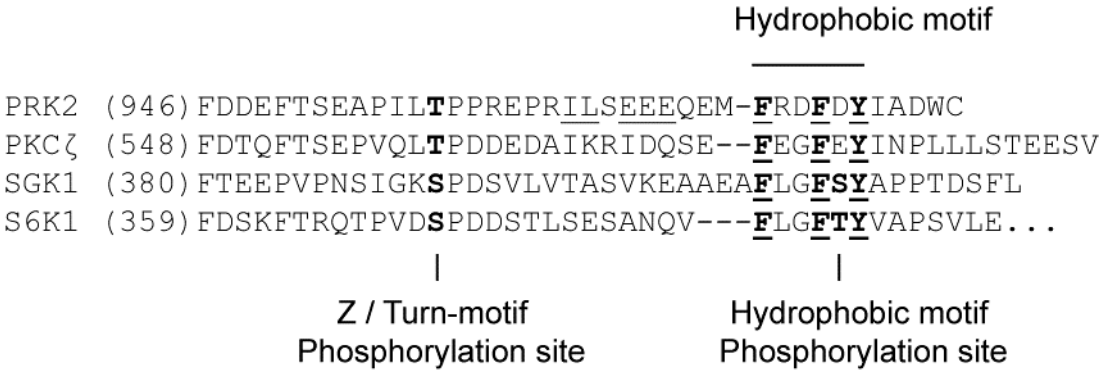

Figure 4.: Alignment of the C-terminal amino acid sequence of PRK2 with the equivalent region of selected AGC subfamily kinases. Taken from Dettori et al. Regulation of the interaction between protein kinase C-related protein kinase 2 (PRK2) and its upstream kinase, 3-phosphoinositide-dependent protein kinase 1 (PDK1). *J Biol Chem;284(44):30318-27; (October 2009)* [35]: The Z/Turn-motif phosphorylation sites are in boldface, the hydrophobic motif phosphorylation site is underlined and shown in boldface. The hydrophobic residues Ile965/Leu966 and the negatively charged residues Glu968-Glu969-Glu970 of the C-terminal fragment of PRK2 are underlined. The residues Thr389 in S6K1 and Ser113 in SGK1 correspond to the hydrophobic motif phosphorylation sites as Thr412 in S6K1 and Ser422 in SGK1, respectively. The numbering differs according to the long and short S6K1 splice variants.

In previously unpublished work, Ricardo M. Biondi and Nik Morrice (with support from Dario Alessi) found that PRK2 and PKCζ were phosphorylated *in vivo* at two sites, the activation loop and the Z-/turn motif phosphorylation site. Moreover, they found that phosphorylation of PRK2 or PKCζ is not dependent on IGF1, as cells stimulated with IGF1 and cells without this stimulation showed an identical phosphorylation pattern. They further found that no other phosphorylation sites could be identified. Because of this fact, we wanted to evaluate, whether the Z-/turn motif phosphorylation could regulate the interaction with PDK1 in these kinases. In addition, Rosalia Dettori et al. found that mutation of the Z-/turn motif phosphorylation site of PRK2 (Thr958Ala) increased the interaction of PRK2 with PDK1. In contrast, parallel experiments with PKCζ mutated at the Z-/turn motif phosphorylation site (Thr560Ala) did not show an effect on the binding of PKCζ to PDK1, suggesting that phosphorylation at this site did not play a role in regulating PKCζ interaction with PDK1.

3.3.5.4. How is the interaction of atypical PKCs and PRKs with PDK1 regulated?

In our laboratory, we were interested to know how atypical PKCs and PRKs regulate the interaction with PDK1. In this line, Rosalia Dettori and myself have studied the role of the N-terminal region and the C-terminal region in the regulation of these kinases.

As part of the studies on the regulation of the interaction between PRK2 and PDK1, Rosalia Dettori transfected HEK 293 cells and performed pull-down assays, *in vitro* protein-protein interaction assays, SDS PAGE gel electrophhoresis and immunoblotting. She additionally performed *in vitro* interaction assays in the presence of low molecular weight compounds and purification of GST-fusion proteins expressed in HEK293 cells. I designed oligonucleotides to perform the mutagenesis, performed DNA transformation into chemically competent *E. coli*, the mini plasmid preps and the subsequent sequencing to verify the mutations. I further performed transfection of HEK 293 cells and purification of GST-fusion proteins and *in vitro* interaction assays. Finally, I performed all PRK2 activity assays. The studies on the regulation of the interaction between PRK2 and PDK1 were published in 2009 [35]. The present thesis also provides first evidence that PRK2 forms oligomers and that this oligomerization is mediated by N-terminal regions. Furthermore, I provide evidence that PRK2 activity is inhibited intermolecularly by N-terminal regions (manuscript in preparation).

4. Materials and Methods

4.1. Materials

4.1.1. Buffers and solutions

We obtained all chemical products from the companies Sigma-Aldrich (St. Louis, United States), Roth (Karlsruhe, Germany), Fluka (St. Louis, United States), Merck (Darmstadt, Germany), BIO-RAD (Hercules, United States), GeneTex, Inc. (San Antonio, United States). Antibodies were obtained from Upstate Biotechnologies (Millipore, Billerica, United States) and Santa Cruz Biotechnology (Santa Cruz, United States).

The buffers and solutions we used were prepared with high quality water from Braun (Melsungen, Germany).

Table 1: Buffers and solutions

Buffer/Solution	**Ingredients**	**Concentration of ingredients/ Amount of ingredients**
Coomassie Brilliant Blue (Staining solution)	Coomassie Brilliant Blue R250 Methanol:H_2O (1:1) Glacial acetic acid	0,25 g 90 ml 10 ml
Destaining solution	Acetic acid Methanol Deionised/distilled H_2O	10 ml 30 ml 60 ml
Electrophoresis buffer	TRIS Glycine (electrophoresis grade) pH 8,3 SDS	25 mM 250 mM 0,1%
HBS (2X)	NaCl Na_2HPO4 HEPES	275 mM 2,8 mM 55 mM
Lysis Buffer	Tris-HCl pH 7.5 Sucrose Sodium Ortho-Vanadate pH 10 EDTA EGTA Sodium B glycerophosphate Sodium fluoride Sodium pyrophosphate Triton X-100 β-mercaptoethanol protease inhibitor mixture	50 mM 270 mM 1 mM 1 mM 1 mM 10 mM 50 mM 5 mM 1% by mass 0,1 % by mass 1 tablet per 50 ml of lysis buffer
SOC Media *	Tris-HCl pH 7.5 Tryptone Yeast Extract	900 ml 20 g 5 g

	5M NaCl 1M KCl 1M $MgCl_2$ 1M $MgSO_4$ 1M glucose Deionised/distilled H_2O Then autoclaved	2 ml 2,5 ml 10 ml 10 ml 20 ml Adjusted to 1 L
TBS/Tween	Tris-HCl (pH 8.0) NaCl Tween-20	10 mM 150 mM 0,05 %
Towbin transfer buffer (1X)	Tris Glycine Methanol	24 mM 192 mM 20%
Wash Buffer A	Tris pH 7.5 EGTA	50 mM 0,1 mM

***** For preparation of this, 0.5 g NaCl, 5 g yeast extract and 20 g tryptone were added to 950 ml of deionized H_2O. Upon shaking, we added 10 ml of a 250 mM solution of KCl and adjusted the pH to 7.0 with NaOH. Then, we filled up the solution to 1 liter with deionized H_2O and sterilized by autoclaving it for 20 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle. Upon autoclaving and cooling to 60 °C or less, we added 20 mM of glucose and 5 ml of a sterile solution of 2 M $MgCl_2$. Then, we aliquoted SOC medium in 1 ml eppendorf tubes and kept them at -20°C until required.

4.1.2. Antibodies

Primary and secondary antibodies employed

All primary antibodies used for immunoblotting detection, together with their corresponding secondary antibody, are detailed below.

Epitope: Anti-phospho-activation-loop:

Primary Antibody: Anti-phospho-PRK (rabbit antiserum)

(dilution): 1:1000
Company: Upstate Biotechnologies (Millipore, Billerica, United States)

Secondary Antibody: Goat anti-Rabbit HRP Conjugate

(dilution): 1:5000
Company: BIO-RAD (Hercules, United States)

Epitope: Anti-Z-/turn-motif phosphorylation site

Primary antibody: PKC beta 2 (phospho T641) antibody

Probed in Pull-down (dilution): 1:1000
Company: GeneTex, Inc. (San Antonio, United States)

Secondary antibody: Goat anti-Rabbit HRP Conjugate

(dilution): 1:5000
Company: BIO-RAD (Hercules, United States)

Epitope: FLAG-tag

Primary antibody: Anti-Flag M5 monoclonal antibody (mouse)

To probe Flag in Crude Extracts (dilution): 1: 800
To probe Flag in Pull-down (dilution): 1:400
Company: Sigma-Aldrich (St. Louis, United States)

Secondary antibody: Anti-Mouse IgG (Whole Molecule) Peroxidase Conjugate

To probe Flag in Crude Extract (dilution): 1:10000
To probe Flag in Pull-down (dilution): 1:5000
Company: BIO-RAD (Hercules, United States)

Epitope: GST-tag

Primary antibody: GST (B-14): sc-138

To probe GST in Pull-down (dilution): 1:1000
Company: Santa Cruz Biotechnology (Santa Cruz, United States)

Secondary antibody: Anti-Mouse IgG (Whole Molecule) Peroxidase Conjugate

(dilution): 1:5000
Company: BIO-RAD (Hercules, United States)

Epitope: Myc-tag

Primary antibody: c-Myc (9E10): sc-40

To probe Myc in Crude Extract (dilution): 1:800
To probe Myc in Pull-down (dilution): 1:400
Company: Santa Cruz Biotechnologies (Santa Cruz, United States)

Secondary antibody: Anti-Mouse IgG (Whole Molecule) Peroxidase Conjugate

To probe Myc in Crude Extract (dilution): 1:10000
To probe Myc in Pull-down (dilution): 1:5000
Company: Sigma-Aldrich (St. Louis, United States)

4.1.3. Equipment

Fluorescent microscope (Carl Zeiss AG, Jena, Germany)

DU 800 Spectrophotometer (Beckman Coulter, Fullerton, United States)

Thermocycler (PE Applied Biosystems, Foster City, United States)

Applied Biosystems 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, United States)

Typhoon 9410 high performance gel and blot imager (GE Healthcare, Chalfont St Giles, Great Britain)

ep*Motion* automated pipetting system (Eppendorf AG, Hamburg, Germany)

Hoefer miniVE Vertical Electrophoresis System (Amersham Biosciences, Freiburg, Germany)

- electrophoresis module with tank
- Blot module
- Hoefer Multiple Gel Caster
- Gel sandwich (Glass plate, notched alumina plate, side spacers)

Refrigerated microcentrifuges

- for 1,5/2 ml tubes: Mikro 22R (Hettich Zentrifugen, Tuttlingen, Germany)
- for 15/50 ml tubes: Centrifuge 5804R (Eppendorf AG, Hamburg, Germany)

4.1.3. Peptides

The polypeptides T308tide, Crosstide and PIFtide were synthesized by Dr Wolfgang Nastainczyk (Medicinal Biochemistry, Homburg) and the purity verified by HPLC. Polypeptides used were at least 75% pure. The identity of polypeptides was confirmed by N-terminal sequencing and mass spectrometry.The polypeptides PIFtide EEE/AAA: REPRILSAAAQEMFRDFDYIADWC and PIFtide IL/AA: REPRAASEEEQEMFRDFDYIADWC (>75% purity) were synthesized by JPT Peptide technologies (Berlin, Germany).

4.1.3.1. T308tide

T308tide is a synthetic dodecapeptide, corresponding to the sequences surrounding the PDK1 phosphorylation site of Akt/PKB. The sequence encompasses the residues 307-320 of PKBα with two arginine residues added to the C-terminus to make the petide bind to p81 paper (aa code KT*FCGTPEYLAPEV-RR) [38]. T308tide is phosphorylated by PDK1 at the residue equivalent to Thr308 of PKBα. In the presence of PIFtide, PDK1 activity towards T308tide is

increased up to 4-fold. This increase in PDK1 activity is observed with either FL-PDK1 or forms lacking the N- or C-terminal non-catalytic regions [10]. This peptide is a poor substrate for PDK1, but a peptide comprising T308tide fused to the PDK1-binding motif of PIF is a vastly superior substrate for PDK1 [10]. Mutations of K115 or L155 lead PDK1 to phosphorylate T308tide 3- to 5-fold more rapidly compared to wt PDK1 (in the absence of PIF), while these mutants are not activated by PIF. T308tide: KTFCGTPEYLAPEVRR

4.1.3.2. PIFtide

Previous work by Biondi et al. demonstrated that a 24 amino acid fragment of PIF (termed PIFtide) that encompasses the HM of PRK2 which binds to a hydrophobic pocket on the small lobe of the kinase domain of PDK1, termed the `PIF-binding pocket'. PIFtide interacted with the PIF-binding pocket of PDK1 with >1000-fold higher affnity than the S6K1, SGK1 and PKB hydrophobic motif peptides [11]. PIFtide: REPRILSEEEQEMFRDFDYIADWC
The polypeptides PIFtide EEE/AAA: REPRILSAAAQEMFRDFDYIADWC and PIFtide IL/AA: REPRAASEEEQEMFRDFDYIADWC (>75% purity) were synthesized by JPT Peptide technologies.

4.1.3.3. Crosstide

Crosstide: GRPRTSSFAEG

4.1.4. Oligonucleotides

Here I list the “forward” and “reverse” (-r) oligonucleotides that I designed to make site-directed mutagenesis of the residues indicated within square brackets. All oligonucleotides were obtained from Operon (Huntsville, United States):

PRK2 mutations:

PRK2[Thr958Ala]:
5´ GAAGCACCTATTCTGGCTCCACCTCGAGAACC 3´
PRK2[Thr958Ala]-r:
5´ GGTTCTCGAGGTGGAGCCAGAATAGGTGCTTC 3´

PRK2[Lys670Glu]:
5´ GAAGAGGACATTTTGGAGAGGTGCTTTTAGCTGAATATAAAAACAC 3´
PRK2[Lys670Glu]-r:
5´ GTGTTTTTATATTCAGCTAAAAGCACCTCTCCAAAATGTCCTCTTC 3´

PRK2[Lys686Met]:
5´ GAGATGTTTGCTATAATGGCCTTAAAGAAAGGAG 3´
PRK2[Lys686Met]-r:
5´ CTCCTTTCTTTAAGGCCATTATAGCAAACATCTC 3´

PRK2[Lys689Ser]:
5´ GTTTGCTATAAAAGCCTTAAGCAAAGGAGATATTGTGGCTCG 3´
PRK2[Lys689Ser]-r:
5´ CGAGCCACAATATCTCCCTTTGCTTAAGGCTTTTATAGCAAAC 3´

PRK2[TST814-816AAA] (Thr-Ser-Thr sequence mutated all to Ala):
5´ GGGATATGGAGATAGAGCAGCCGCATTTTGTGGCACTCCTG 3´
PRK2[TST814-816AAA]-r:
5´ CAGGAGTGCCACAAAATGCGGCTGCTCTATCTCCATATCC 5´

PRK2[Ile-Leu965-966Ala-Ala]:
5´ CACCTCGAGAACCAAGGGCAGCTTCGGAAGAGGAGCAGG 3´
PRK2[Ile-Leu965-966Ala-Ala]-r:
5´ CCTGCTCCTCTTCCGAAGCTGCCCTTGGTTCTCGAGGTG 3´

PRK2[EEE968-970AAA] (Glu-Glu-Glu sequence mutated all to Ala):
5´ CCAAGGATACTTTCGGCAGCGGCGCAGGAAATGTTCAGAG 3´
PRK2[EEE968-970AAA]-r:
5´CTCTGAACATTTCCTGCGCCGCTGCCGAAAGTATCCTTGG 3´

SGK mutation:

For this mutation, I performed the mutagenesis with the HM mutated plasmid pEBG2T-ΔN-SGK[Ser422Asp], that we already had in stock.

SGK[Ser422Asp-Ser401Ala]:
5´ CCAACTCCATTGGCAAGGCCCCTGACAGCGTCCTC 3´
SGK[Ser422Asp-Ser401Ala]-r:
5´GAGGACGCTGTCAGGGGCCTTGCCAATGGAGTTGG 3´

S6K mutation:

For this mutation, I performed the mutagenesis with the HM mutated plasmid pEBG2T-T2-S6K[Thr412Glu], that we already had in stock

S6K[Thr412Glu-Ser394Ala]:
5´CCAAGTTTACACGTCAGGCACCTGTCGACAGCCCAG 3´
S6K[Thr412Glu-Ser394Ala]-r:
5´CTGGGCTGTCGACAGGTGCCTGACGTGTAAACTTGG 3´

4.1.5. Sequencing Primers

In order to design our primers, we chose sequences with a length of about 18-30 nucleotides, depending on the melting temperature and the ability to build hydrogen bonds (number of G-C base pairs respectively A-T base pairs). To calculate the melting temperature (T_m), we used the Wallace rule: T_m= 2x (A+T) + 4x (G+C).

The T_m was intended to be in between 55°C and 65°C for our primers.

GST600F: 5´ TAGCATGGCCTTTGCAGGGCTG 3´
S6K-439F: 5´ GCTCATACAAAAGCAGAACGG 3´
S6K-1151F: 5´ CTGAAGAGGATGTAAGTCAG 3´
SGK-415F: 5´ GAGGAGAAGCATATTATGTCG 3´
SGK-850F: 5´ GGAGCTGTCTTGTATGAGATG 3´
PRK2-380F: 5´ TCCAAATAATGACCCTCGTTGTTC 3´
PRK2-380R: 5´ GAACAACGAGGGTCATTATTTGGAG 3´
PRK2-1080F 5´ GGTTGGAGTCCAAGTGAAACCAG 3´
PRK2-1662F: 5´ CCCTCAACTAGCACCTCCA 3´
PRK2-2299F: 5´ GCTTGTGTAGTTCTTGGGTTG 3´
PRK2-2439F: 5´ GGAGATAGAACAAGCACATTTTGTGGCACTCC 3´
PRK2-2439R: 5´ GGAGTGCCACAAAATGTGCTTGTTCTATCTCC 3´ 3
PRK2-2820R: 5´ TGGTAGGTATAAATGGTGGC 3´
PRK2-2882F: 5´ CTCCACCTCGAGAACCAAG 3´

Sequence of PRK2 with primers and oligonucleotides: the first 3 nucleotides the primer binds to and the mutated amino acids are written in bold type:

```
ggagcgcaaatggcgtccaaccccgaacgggggggagattctgctcacggaactgcagggg
 G  A  Q  M  A  S  N  P  E  R  G  E  I  L  L  T  E  L  Q  G

gattcccgaagtcttccgttttctgagaatgtgagtgctgttcaaaaattagacttttca
 D  S  R  S  L  P  F  S  E  N  V  S  A  V  Q  K  L  D  F  S

gatacaatggtgcagcagaaattggatgatatcaaggatcgaattaagagagaaataagg
 D  T  M  V  Q  Q  K  L  D  D  I  K  D  R  I  K  R  E  I  R

aaagaactgaaaatcaaagaaggagctgaaaatctgaggaaagtcacaacagataaaaaa
 K  E  L  K  I  K  E  G  A  E  N  L  R  K  V  T  T  D  K  K

agtttggcttatgtagacaacattttgaaaaaatcaaataaaaaattagaagaactacat
 S  L  A  Y  V  D  N  I  L  K  K  S  N  K  K  L  E  E  L  H

cacaagctgcaggaattaaatgcacatattgttgtatcagatccagaagatattacagat
 H  K  L  Q  E  L  N  A  H  I  V  V  S  D  P  E  D  I  T  D
                    →PRK2-380F   PRK2-380R←
tgcccaaggactccagatactccaaataatgaccctcgttgttctactagcaacaataga
 C  P  R  T  P  D  T  P  N  N  D  P  R  C  S  T  S  N  N  R

ttgaaggccttacaaaaacaattggatatagaacttaaagtaaaacaaggtgcagagaat
 L  K  A  L  Q  K  Q  L  D  I  E  L  K  V  K  Q  G  A  E  N

atgatacagatgtattcaaatggatcttcaaaggatcggaaactccatggtacagctcag
 M  I  Q  M  Y  S  N  G  S  S  K  D  R  K  L  H  G  T  A  Q

caactgctccaggacagcaagacaaaaatagaagtcatacgaatgcagattcttcaggca
 Q  L  L  Q  D  S  K  T  K  I  E  V  I  R  M  Q  I  L  Q  A

gtccagactaatgaattggcttttgataatgcaaaacctgtgataagtcctcttgaactt
 V  Q  T  N  E  L  A  F  D  N  A  K  P  V  I  S  P  L  E  L

cggatggaagaattaaggcatcattttaggatagagtttgcagtagcagaaggtgcaaag
 R  M  E  E  L  R  H  H  F  R  I  E  F  A  V  A  E  G  A  K

aatgtaatgaaattacttggctcaggaaaagtaacagacagaaaagcactttcagaagct
 N  V  M  K  L  L  G  S  G  K  V  T  D  R  K  A  L  S  E  A

caagcaagatttaatgaatcaagtcagaagttggacctttaaagtattcattagagcaa
 Q  A  R  F  N  E  S  S  Q  K  L  D  L  L  K  Y  S  L  E  Q

agattaaacgaagtccccaagaatcatcccaaaagcaggattattattgaagaactttca
 R  L  N  E  V  P  K  N  H  P  K  S  R  I  I  I  E  E  L  S

cttgttgctgcatcaccaacactaagtccacgtcaaagtatgatatctacgcaaaatcaa
 L  V  A  A  S  P  T  L  S  P  R  Q  S  M  I  S  T  Q  N  Q
```

tatagtacactatccaaaccagcagcactaacaggtactttggaagttcgtcttatgggc
 Y S T L S K P A A L T G T L E V R L M G

tgccaagatatcctagagaatgtccctggacggtcaaaagcaacatcagttgcactgcct
 C Q D I L E N V P G R S K A T S V A L P

→PRK2-1080F

ggttggagtccaagtgaaaccagatcatctttcatgagcagaacgagtaaaagtaaaagc
 G W S P S E T R S S F M S R T S K S K S

ggaagtagtcgaaatcttctaaaaaccgatgacttgtccaatgatgtctgtgctgttttg
 G S S R N L L K T D D L S N D V C A V L

aagctcgataatactgtggttggccaaactagctggaaacccatttccaatcagtcatgg
 K L D N T V V G Q T S W K P I S N Q S W

gaccagaagtttacactggaactggacaggtcacgtgaactggaaatttcagtttattgg
 D Q K F T L E L D R S R E L E I S V Y W

cgtgattggcggtctctgtgtgctgtaaaatttctgaggttagaagatttttagacaac
 R D W R S L C A V K F L R L E D F L D N

caacggcatggcatgtgtctctatttggaaccacagggtactttatttgcagaggttacc
 Q R H G M C L Y L E P Q G T L F A E V T

ttttttaatccagttattgaaagaagaccaaaacttcaaagacaaaagaaaatttttca
 F F N P V I E R R P K L Q R Q K K I F S

aagcaacaaggcaaaacatttctcagagctcctcaaatgaatattaatattgccacttgg
 K Q Q G K T F L R A P Q M N I N I A T W

ggaaggctagtaagaagagctattcctacagtaaatcattctggcaccttcagccctcaa
 G R L V R R A I P T V N H S G T F S P Q

→PRK2-1662F

gctcctgtgcctactacagtgccagtggttgatgtacgcat**ccc**tcaactagcacctcca
 A P V P T T V P V V D V R I P Q L A P P

gctagtgattctacagtaaccaaattggactttgatcttgagcctgaacctcctccagcc
 A S D S T V T K L D F D L E P E P P P A

ccaccacgagcttcttctcttggagaaatagatgaatcttctgaattaagagttttggat
 P P R A S S L G E I D E S S E L R V L D

ataccaggacaggattcagagactgtttttgatattcagaatgacagaaatagtatactt
 I P G Q D S E T V F D I Q N D R N S I L

ccaaaatctcaatctgaatacaagcctgatactcctcagtcaggcctagaatatagtggt
 P K S Q S E Y K P D T P Q S G L E Y S G

attcaagaacttgaggacagaagatctcagcaaaggtttcagtttaatctacaagatttc
 I Q E L E D R R S Q Q R F Q F N L Q D F

aggtgttgtgctgtcttgggaagaggacattttggaaaggtgcttttagctgaatataaa
R C C A V L G R G H F G **K** V L L A E Y K
670*E*
aacacaaatgagatgtttgctataaaagccttaaagaaaggagatattgtggctcgagat
N T N E M F A I **K** A L **K** K G D I V A R D
686*M* **689*S***
gaagtagacagcctgatgtgtgaaaaaagaatttttgaaactgtgaatagtgtaaggcat
E V D S L M C E K R I F E T V N S V R H

ccctttttggtgaacctttttgcatgtttccaaaccaaagagcatgtttgctttgtaatg
P F L V N L F A C F Q T K E H V C F V M

gaatatgctgccggtggggacctaatgatgcacattcatactgatgtcttttctgaacca
E Y A A G G D L M M H I H T D V F S E P
→PRK2-2299F
agagctgtattttatgct**gct**tgtgtagttcttgggttgcagtatttacatgaacacaaa
R A V F Y A A C V V L G L Q Y L H E H K

attgtttatagagatttgaaattggataacttattgctagatacagagggctttgtgaaa
I V Y R D L K L D N L L L D T E G F V K
→PRK2-2439F
attgctgattttggtctttgcaaagaaggaatgggatat**gga**gatagaacaagcacattt
I A D F G L C K E G M G Y G D R **T S T** F
PRK2-2439R← **814*AAA*816**
tgtggcac**tcc**tgaatttcttgccccagaagtattaacagaaacttcttatacaagggct
C G T P E F L A P E V L T E T S Y T R A

gtagattggtggggccttggcgtgcttatatatgaaatgcttgttggtgagtctcccttt
V D W W G L G V L I Y E M L V G E S P F

cctggtgatgatgaagaggaagtttttgacagtattgtaaatgatgaagtaaggtatcca
P G D D E E E V F D S I V N D E V R Y P

aggttcttatctacagaagccatttctataatgagaaggctgttaagaagaaatcctgaa
R F L S T E A I S I M R R L L R R N P E

cggcgccttggggctagcgagaaagatgcagaggatgtaaaaaagcacccatttttccgg
R R L G A S E K D A E D V K K H P F F R
PRK2-2820R←
ctaattgattggagcgctctgatggacaaaaaagtaaagccaccatttatacctaccata
L I D W S A L M D K K V K P P F I P T I

agaggacgagaagatgttagtaattttgatgatgaatttacctcagaagcacctattctg
R G R E D V S N F D D E F T S E A P I L
→PRK2-2882F
a**ctc**cacctcgagaaccaaggatactttcggaagaggagcaggaaatgttcagagatttt
T P P R E P R **I L** S **E E E** Q E M F R D F
965*AA*966 968*AAA*970
gactacattgctgattggtgttaa
D Y I A D W C -

Figure 5: Nucleotide and amino acid sequence of PRK2 with starting points of the primers we used and the mutations we created

4.1.6. Other

Complete protease inhibitor cocktail tablets were from Roche (Basel, Switzerland).

Protein concentration was estimated using Coomassie Brilliant Blue R250 from Perbio (Waltham, United States).

Glutathione sepharose 4B was from Amersham Pharmacia Biotech (Amersham Biosciences, Freiburg, Germany).

Chemiluminescent substrate used for western-blot (Roti-lumin) was from Roth (Karlsruhe, Germany).

Western-blot stripping buffer (Restore) was from Pierce (Waltham, United States).

Dulbecco's modified Eagle's medium (DMEM) was from Sigma-Aldrich (St. Louis, United States).

Fetal bovine serum was from Gibco (Carlsbad, United States).

Okadaic acid was from Calbiochem, Orthovanadate from Sigma-Aldrich (St. Louis, United States), LY294002 and Y27632 from Sigma-Aldrich (St. Louis, United States).

Purified human Rho (Rho-GST) was from Chemicon International (Temecula, United States).

Pfu turbo was from Stratagene (San Diego, United States).

DpnI was from New England BioLabs (Ipswich, United States).

Luria Broth medium was from Sigma-Aldrich (St. Louis, United States).

Prestained Protein Molecular Weight Marker (MW: 118 kDa, 90 kDa, 48kDa, 36 kDa, 27 kDa, 20 kDa) was from Fermentas (Burlington, Canada).

$CaCl_2$, for molecular biology, approx. 99 %, was from Sigma-Aldrich (St. Louis, United States).

QIAprep Spin Miniprep Kit was from QIAgen (Venlo, Netherlands).

14 ml-PP-tubes were from Greiner bio-one (Kremsmuenster, Austria).

DyeEx product purification kit was from QIAgen (Venlo, Netherlands).

Mega plasmid purification kit was from QIAgen (Venlo, Netherlands).

Antibiotics/Antimycotics (AA) solution (1000x concentrated) was from Sigma-Aldrich (St. Louis, United States).

Trypsin was from Sigma-Aldrich (St. Louis, United States).

Spin columns with Collection tube were from Sigma-Aldrich (St. Louis, United States).

Chromatography paper p81 was from Whatman (Maidstone, Kent, United Kingdom).

4.2. Methods

4.2.1. Mutagenesis for plasmids with approximately 1-2 Kb insert

For mutagenesis of small plasmids like pCMV5, we used a standard protocol. First, we prepared a mix following the protocol (Table 2):

Table 2

10x concentrated Pfu buffer	2,5 µl
Template (plasmid) DNA	1 µl
Oligonucleotide 1 (10uM)	1,25 µl
Oligonucleotide 2 (10uM)	1,25 µl
10uM dNTPs	0,5 µl
H_20	18 µl

Then the PCR was started. After reaching 94°C, we added 0,5 µl Pfu Turbo as a Hot-Start. The PCR was processed according to the following protocol (Table 3):

Table 3

Temperature (°C)	Time (min)	Cycles
94	2	1
94 55 68	0,5 1 12-14	16-18
4	infinite/hold	

For large plasmids, such as pEBG2T vector, we used the following protocol (Table 4):

Table 4

10x concentrated Pfu buffer	2,5 µl
Template (plasmid) DNA	1 µl
Oligonucleotide 1 (10uM)	1,25 µl
Oligonucleotide 2 (10uM)	1,25 µl
10uM dNTPs	0,5 µl
H_20	17,5 µl

Then the PCR was started. After reaching 94°C, we added 1 µl Pfu Turbo as a Hot-Start for the larger plamids and used the following PCR protocol (Table 5):

Table 5

Temperature (°C)	Time (min)	Cycles
94	2	1
94 55 68	0,5 1 22-25	18
4	infinite/hold	

4.2.2. Transformation and Miniprep

After PCR for mutagenesis, we added 0,5 µl of DpnI to the PCR product and incubated it at 37°C for at least 1h. 40 µl of chemically competent *E. coli* DH5α bacteria were added in an 1,5 ml-tube and placed it on ice. We then added 1 µl of the PCR product/DpnI mix from mutagenesis and incubated it on ice for approximately 30 minutes. After that, I applied a heat shock at 42°C for 40 seconds. Then the mix was put on ice again for 2 minutes before adding 450 µl of 42°C warm SOC-media. Now, this mix was shaken for 1 hour at 37°C. After that, we plated the mixture on agar plates containing carbenicillin and incubated these plates at 37°C over night. Then, we picked one colony of bacteria from the according agar plate and added it to the PP-tube containing 4 ml of LB medium (25 g of Luria Broth in 1 l of deionized H_2O, then autoclaved) and 4 µl of carbenicillin (concentration: 50 mg/ml) and incubated it for at least 8 h at 37°C at a speed of 220 rpm. The following day we performed a mini plasmid prep following the QIAprep Spin Miniprep Kit protocol.

4.2.3. Sequencing

For sequencing the Miniprep DNA, we established the following mix listed at table 6:

Table 6

Miniprep DNA	1,25 µl
Sequencing primer	1 µl
H_2O	5,75 µl
Big Dye seq solution	2 µl

Then the purified mix was amplified using the following PCR protocol:

Table 7

Temperature (°C)	Time (min)	Cycles
96	1	1
96 50 60	0,5 0,05 4	35
4	infinite/hold	

After sequencing PCR, the PCR product was purified by using DyeEx product purification kit and the DNA sequences were analysed using an automatic DNA sequencing (Applied Biosystems 3100 Genetic Analyzer).

After verifying the sequences, the plasmids required for the transfection of HEK293 cells were prepared using a mega plasmid purification kit following the QIAgen protocol.. Afterwards, we measured the DNA concentration of the plasmids by setting the spectrophotometer at UV-light at a wavelength of 260 nm. Then, we pipetted 3 µl of DNA and 1 ml of high quality water in a quartz cuvette and measured the absorbance. The DNA concentration of the plasmid was calculated by considering that 1 Abs corresponds to 50 µg/ ml of DNA.

4.2.4. Cell culture

HEK293 cells were maintained in culture in a humidified 5% CO_2 atmosphere at 37°C in complete medium consisting of DMEM (Invitrogen) supplemented with 10% fetal bovine serum (GIBCO) and 1% of Antibiotics Antimycotics (AA).

When the cells were 80-90% confluent, we trypsinised and transferred them to cell culture dishes or to fresh cell culture flasks. The splitting of the cell culture dishes began by aspirating the cell media from the cell flask (175 cm^2), adding 3 ml of trypsin and leaving the flask for 5 min at 37°C in the incubator in order to let the cells detach from the flask.

Then, we repeatedly aspirated and released the cells in 10 ml media in order to disrupt clumps of cells and re-plated single cells. After that, we added the necessary volume of cell media to the suspension, mixed well and distributed the cell suspension in the corresponding flask or dish, resulting in a final dilution of the cells in each flask or dish of 1/10 (25 ml for 175 cm^2 flasks or 10 ml for 10 cm diameter 20 cm^2 Petri-dishes).

4.2.5. Transient transfection of HEK 293 cells using $CaCl_2$ protocol

For the transfection of 293 cells, we used 10 cm dishes (medium: 10ml).

We splitted cells 5 to 6 h before transfection to give them 10-20% confluence at the moment of transfection.

In a next step, in order to prepare the $CaCl_2$ /HBS/DNA precipitate, we added 0.5 ml HBS (2X) to individual 15 ml tubes. In separate tubes we added 60 µl of 2M $CaCl_2$, 10 µg of DNA and enough high quality water to bring the total volume to 0.5 ml. Then, we added the $CaCl_2$/DNA mix to the HBS (2X) dropwise with a pipette while carefully mixing. After that, we slowly added the mix drop by drop on the cells. The dishes were then placed at 37°C in the 5% CO_2 incubator. 16-24 h later, we changed the media of the dishes with fresh preewarmed media and incubated further for another 16-24 h. Then, the cells were placed on ice and lysed. To this end, we first aspirated the cell media from the dishes. Then, we immediately added 800 µl of cold lysis buffer on each dish. After that, cells were scraped with a cell scraper and the mix was aspirated and transferred to a 1,5 ml tube.

In order to verify the transfection efficiency, we additionally transfected two dishes with a plasmid coding for the green fluorescent protein (GFP). After 16 h we could already observe the level of flourescence of the transfected cells using a fluorescent microscope. Maximal fluorescence was observed after 36 h. In our experiments, the level of transfection varied between 50 and 90%.

4.2.6. Protein-Protein interaction assay (GST pull-down)

We co-transfected HEK293 cells with a plasmid coding for a GST-fusion protein and a plasmid coding for a protein fused with another tag**.** As the glutathione Sepharose resin shows a specific binding to GST, we were able to purify the GST-fusion protein. After washing, we evaluated the specific binding to the co-expressed protein by separating the GST-pull-down samples on SDS-PAGE and detection of the interacting protein by immunoblotting with a specific antibody against the tag (epitope e.g. Flag).

The following procedures were performed at 4° C. 36 h after co-transfection, we lysed the cells in 0.8 ml of 4°C lysis buffer from each 10 cm^2 dish. Then, the lysates were centrifuged at 14,000 x *g* for 10 min in order to clear it. After that, 30 µl of the supernatant (crude extract, CE) were diluted with 30 µl of SDS-PAGE loading buffer (2X; Roti-load 1 from Roth (1X: 50 mM Tris-Cl, (pH 6.8), 100 mM dithiothreitol, 2% SDS (electrophoresis grade), 0.1% bromophenol blue, 10% glycerol)). In a next step, we heated this dilution at 95°C for 3 minutes.

The remaining crude extract (approximately 0.8 ml) was incubated with 30 µl of pre-washed glutathione Sepharose resin (pipetted from resin solution containing equal volumes of lysis buffer and resin) on a platform shaker for 2 h. After this incubation the washing of the beads was performed in batch. Therefore, the mix was centrifuged at 14,000 x g for 1 minute and the supernatant was removed. The resin was mixed with 1 ml of wash buffer and immediately centrifuged again at 14,000 x g for 1 minute. After that, the wash buffer was aspirated. We repeated this procedure 4 times: two washes with lysis buffer containing 0,5 mM NaCl and then two washes with 50 mM Tris pH 7.5 and 1 mM EDTA.
In a following step, we resuspended the beads in 30 µl of SDS-PAGE loading buffer (2X; Roti-load 1 from Roth) and heated it for 3 minutes at 95°C.

4.2.7. SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique that is widely used in molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). So, SDS gel electrophoresis of samples having an identical charge per unit mass due to binding of SDS results in fractionation by size. The SDS (Sodium dodecyl sulfate) contained in the SDS-PAGE loading buffer is a dissociating agent used to denature native proteins to individual polypeptides. That means that when a protein mixture is heated to 95°C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g/g of polypeptide. In this process, the intrinsic charges of polypeptides becomes negligible when compared to the negative charges contributed by SDS. Thus, after treatment, polypeptides get a rod like structure possessing a uniform charge density, that is the same net negative charge per unit length. Mobilities of these proteins will be a linear function of the logarithms of their molecular weights. To this end, the negatively charged proteins would be ready for SDS/polyacrylamide gel electrophoresis and the following steps consisting in either staining with Coomassie Blue or immunoblot.

In a first step we prepared the SDS-PAGE gels. These gels generally consist of acrylamide, bisacrylamide, SDS and a Tris-Cl buffer with an adjusted pH. Importantly, the total percent of acrylamide in the resolving gel determines the pore size, which is responsible for the separation of the proteins. Actually, smaller proteins migrate more easily through the pores of the gel, while larger proteins come across with more resistance and consequently remain closer to the starting point. The separating or resolving gel (for our gels consisting of 1,6ml of H_2O, 2,0ml of 30% acrylamide/0,8% bisacrylamide, 0,4ml of 0,625 M Tris /HCl pH

6,8, 0,4ml of 0,5% SDS, 10ul of 10% ammonium persulfate and 2ul of TEMED (N,N,N',N'-Tetramethylethylendiamin) for 10% acrylamide in total) is usually more basic and has a higher polyacrylamide content than the stacking gel (for our gels consisting of 0,87 ml of H_2O, 0,33 ml of 30% acrylamide/ 0,8% bisacrylamide, 1,2ml of 1,88 M Tris/HCl pH 8,8, 1,2ml of 0,5% SDS, 30 µl of 10% ammonium persulfate and 5 l of TEMED for 10% acrylamide in total). The gels were polymerized in a gel caster. First, the separating gel was poured and allowed to polymerize. Then, a thin layer of isopropanol was added. After a 20 minute waiting period to allow the gel solution to polymerize, we discharged the isopropanol, the stacking gel was poured and a comb was placed to create the wells. After the stacking gel was polymerized, the comb was removed and the gel was ready for electrophoresis.

For electrophoresis, we put the electrophoresis module with the secured gel in a tank filled with electrophoresis buffer. Then, we loaded the designated amount of each sample in the pocket of the stacking gel. Additionally, we loaded a prestained protein molecular weight marker in one pocket to allow an estimation of the molecular weight of our loaded proteins in the subsequent workflow. We ran our gels for about 40 minutes with at a current of 25 mA/gel. This current created an electric field across the gel, initiating the negatively charged proteins to migrate across the gel towards the anode. After a certain time (40 minutes in our experiments), the proteins had differentially migrated based on their size: smaller proteins traveled further down the gel, while larger ones remained closer to the starting point.

4.2.8. Immunoblot

In order to make the proteins accessible to antibody detection, we transferred the separated proteins after SDS-PAGE electrophoresis from the gel onto a membrane made of nitrocellulose. To this end, we first prepared the Towbin transfer buffer (1X) and assembled a transfer stack on the black half of the Hoefer Blot module following a strict order: sponge, filter paper, gel, nitrocellulose membrane, filter paper and sponges. After that, we closed the module, filled it with Towbin transfer buffer (1X) and positioned it in the tank, which had to be filled with distilled water. Then, the proteins were transferred via electrophoresis transfer (conditions for blotting proteins in Towbin buffer were 300 mA and 25 V for 1h). Through this procedure, the molecules in the gel migrated to the nitrocellulose membrane by moving from the cathode towards the anode of the module. In a next step, we incubated the membrane in a blocking solution (TBS/Tween and 2.5% of non-fat dry milk) for 1h and subsequently incubated it for another 1 h with the monoclonal primary antibody (diluted in TBS/Tween containing 2.5% non-fat dry milk). During the following 45 minutes, the membrane was

washed 4 times with TBS/Tween. Then, the membrane was incubated for 1 h with the secondary antibody. This secondary antibody recognized and bound to the primary antibody.

When we used phospho-specific antibodies, we exchanged the 2.5% dry milk-non-fat for 2.5% Albumin, Bovine (Fraction V, Sigma), while the rest of the protocol was kept.
Then, we performed another 4 washes with TBS/Tween. After that, we performed the antibody detection with a kit from Roti-Lumin from Roth.

Roti-lumin is a luminol-based chemiluminescent substrate. This chemiluminescent detection method depends on incubation of the immunoblot with a substrate that will luminance when exposed to the peroxidase-labeled (HRP, horseradish peroxidase) reporter molecules on the secondary antibody. In the presence of hydrogen peroxide, HRP converts luminol to an excited intermediate anion. Converting back to its initial state, the anion emits light. This light is then detected on chemiluminescence films. The maximal intensity is reached after 5 minutes and is kept for 1-2 h on a high level.

4.2.10. Purification of GST-fusion proteins expressed in HEK293 cells

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture and was essential for us to perform quantitative protein kinase assays and *in vitro* interaction assays with our proteins of interest.

In order to purify GST-fusion proteins, we first transfected HEK293 cells with plasmids coding for the designated proteins fused to GST following the $CaCl_2$ protocol. After that, we lysed the transfected HEK293 cells as it is described above under the item “Cell culture”. Then, we centrifuged the cell lysate at 4°C and 3700 rpm for 15 minutes. We discarded the pellet and incubated the supernatant at 4°C for 2 h on a platform shaker with the required amount of glutathione Sepharose resin (usually 1.5-2 ml of resin for the equivalent of 20 dishes (10 cm)). This allowed the glutathione Sepharose beads to bind to GST and enabled us to purify the GST-fusion proteins via centrifugation. We centrifuged the resin-extract mix at 4°C and 3700 rpm for 2 minutes and discarded the supernatant. After that, we washed the GST-fusion proteins bound to the resin at 4°C according to the following protocol: 4 washes with Lysis buffer containing in addition 500 mM NaCl, 8 washes with Wash Buffer A containing 0.1% β–Mercaptoethanol and 2 washes with Wash Buffer A containing Sucrose 0.26M and 0.1% β-Mercaptoethanol. In a next step, we poured the washed proteins in 600 µl of Wash Buffer A containing sucrose 0.26 M, 0.1% β-Mercaptoethanol and 400 µl of Glutathione (200 mM) and incubated the solution on ice for at least 30 minutes. After that, the purified protein fraction was filtered through Spin columns with Collection tube (Sigma-

Aldrich) and the protein concentration was measured by Bradford. After that, the purified proteins were aliquotted, frozen in liquid nitrogen and stored at –80°C. Additionally, we run a SDS-PAGE of one sample and stained it with Coomassie Brilliant Blue in order to estimate the purity of the proteins.

4.2.11. Estimation of protein concentration using the Bradford assay

The Bradford assay, a colorimetric protein assay, is based on an absorbance shift in the dye Coomassie when the initial red form of the Coomassie reagent changes and stabilizes into Coomassie blue by binding to protein. During the formation of this complex, two types of bond interaction take place: the red form of Coomassie dye first donates its free electron to the ionizable groups of the protein, which causes a disruption of the protein's native state, consequently exposing its hydrophobic pockets. These pockets in the protein's tertiary structure bind non-covalently to the non-polar region of the dye via van der Waals forces, positioning the positive amine groups in proximity to the negative charge of the dye. The bond is further strengthened by the ionic interaction between the dye and the protein. Binding of the protein stabilizes the blue form of Coomassie dye which has an absorption spectrum maximum historically held to be at 595 nm while the cationic (unbound) forms are green or red. The increase of absorbance at 595 nm is proportional to the amount of bound dye and thus proportional to the amount (concentration) of protein present in the sample. The measurements we performed were done by using the reagent from "Coomassie Plus protein assay"(Pierce, ORT) and the spectrophotometer with visible light of awavelength of 595 nm. The protein amount necessary to permit a reliable measurement of the protein concentration by Bradford was displayed by an alteration of the dark colour of the Coomassie Plus protein assay solution into blue upon protein addition.

We added the protein sample in a 1 ml plastic cuvette (Sarstedt) containing 800 µl of the Coomassie Plus protein assay solution. In addition, another plastic cuvette containing only the Coomassie Plus protein assay solution was used as the reagent blank. To calibrate the machine, the blank cuvette was placed in the machine and the absorbance was read and considered as 0.00 Abs. After that, we included known amounts of a standard protein (BSA) and the Absorbance ploted. The calibration curve revealed a factor of 0.055 Abs/ µg BSA.

To quantitate the concentration of protein in our samples, I pipetted various volumes of our sample into the 800 µl Bradford assay solution and read the absorbance on the spectrophotometer. With the detected absorbance, we were able to to calculate the protein

concentration, as 1 µg of protein in 800 µl of Coomassie Plus reagent corresponded to 0,055 Abs.

4.2.12. Protein kinase assay (using phosphocellulose p81 papers)

In order to test the intrinsic activity of the studied proteins we performed protein kinase assays. The PRK2 activity assays were performed in a 20 µl mix containing 50 mM Tris-HCl pH 7.5, 0.05 mg/ml BSA, 0.1% β-mercaptoethanol, 10 mM $MgCl_2$, 100 µM ATP + [γ^{32}P]ATP (5-50 cpm/pmol), 0.003% Brij, 1-200 ng PRK2 and Crosstide (100 µM) as peptide substrate. PDK1 activity was assayed in the same buffer containing 150 ng PDK1 and T308tide (100 µM) as the substrate. The linearity of the activity tests was verified routinely by performing the assay on serial dilutions of the enzyme. We started the reaction by adding the peptide substrate to a concentration of 100 µM in order to achieve the widest possible linearity in the assay. The peptide was diluted in dilution buffer (50 mM Tris pH 7.5, 0.1% β-mercaptoethanol, 1 mg/ml BSA). We terminated the reaction by adding 20 µl of phosphoric acid (88%, 1/50). At this stage, we spotted 35 µl of the samples onto p81 phosphocellulose papers (Chromatography paper p81, Whatman; peptides bind to this paper according to their positive charge) and washed the papers 4 times during 1 h with phosphoric acid (88%, 1:200 in deionized water). The last wash was performed with technical ethanol to support the drying process. Then, the next steps consisted in drying the p81 papers and exposing them to a screen in a cassette over night and finally in measuring the radioactivity with the "phospho imager" by reading the screen. Proper controls were included in the exposition to the phospho imager in order to estimate the specific activity of the kinase. One unit of activity was the amount of kinase that catalyzed the phosphorylation of 1nmol of substrate in 1 minute.

The activity measurements were performed in duplicates and with less than 10% difference between duplicate pairs. All experiments were repeated at least twice, although most of the experiments were repeated multiple times with similar results.

5. Results

5.1. Influence of phosphorylation of the Z-/turn motif site of S6K and SGK on their ability to interact with PDK1

Work by Biondi et al. showed that the phosphorylation of the HM of RSK, S6K and SGK regulates the interaction with their upstream kinase PDK1. Most recently, our group found that the phosphorylation of the Z/turn-motif site could regulate the interaction of PRK2 with PDK1. Therefore, we wanted to evaluate if the interaction of S6K and SGK with PDK1 can also be regulated their interaction with PDK1 via Z-/turn-motif phosphorylation.

In order to find out if the Z-/turn motif phosphorylation of these kinases could also regulate the interaction with PDK1, we generated mutants which had the HM-phosphorylation site mutated to Glu or Asp, respectively to mimic the phosphorylated HM. Mimicking the phosphorylated HM, these mutated kinases would not be affected by dephosphorylation of the HM, which can happen when the Z-/turn motif phosphorylation site is mutated. Against this background, I further mutated the Z-/turn-motif phosphorylation site to Ala (SGK [Ser401Ala] and S6K[Ser394Ala]).

In further experiments performed by Rosalia Dettori , the interactions of GST-T2-S6K[Thr412Glu] and GST-ΔN-SGK[Ser422Asp] with Myc-PDK1 were compared to the interaction of GST-T2-S6K[Thr412Glu] further mutated at the Ser394 and GST-ΔN-SGK[Ser422Asp] mutated at the Ser401 site. The interaction was evaluated by co-transfection and GST pull-down experiments. Thus, HEK293 cells were transiently transfected with DNA constructs expressing Myc-PDK1 together with constructs expressing GST-T2-S6K[Thr412Glu], GST-ΔN-SGK[Ser422Asp] and the Z-/turn-motif double mutants GST-T2-S6K[Thr412Glu-Ser394Ala] and GST-ΔN-SGK[Ser422Asp-Ser401Ala]. After 36 h, the cells were lysed, the GST-fusion protein purified by affinity chromatography on glutathione-sepharose beads, the product separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue or immunoblotted using an anti-Myc antibody to detect co-purified Myc-PDK1.

GST-T2-S6K[Thr412Glu-Ser394Ala] and GST-ΔN-SGK[Ser422Asp-Ser401Ala] interacted equally well with PDK1 compared to the wildtype constructs (data not shown). This result suggested that in the case of S6K and SGK the Z-/turn motif phosphorylation site did not regulate the interaction with PDK1 under these conditions, as it was described for PRK2.

5.2. Influence of mutation of the Z-/turn motif phosphorylation site within the isolated C-terminal segment of PRK2 on the interaction with PDK1

As mentioned above, our group previously found out that mutation of the Z-/turn motif phosphorylation site of PRK2 led to an increased interaction of full length PRK2[Thr958Ala] with PDK1. Thus, the results clearly showed that GST-PRK2[Thr958Ala] bound higher levels of PDK1 compared to the GST-PRK2 wt. However, the mechanism to explain this effect was not clear.

We considered two options as explanations for the increased binding of PRK2[Thr958Ala] to PDK1. On the one hand an increased binding affinity of the C-terminal residues of PRK2 to PDK1 or, on the other hand, a decreased affinity of this C-terminal fragment of PRK2 to its own catalytic domain. In order to investigate these two possibilities, we decided to mutate the Z-/turn-motif site on the isolated C-terminal region of PRK2 and to test if the mutant C-terminal (C-T) region had increased affinity for PDK1. To this end, I prepared the appropriate mutanted GST-fusion plasmid construct (pEBG2T-CT-PRK2[Thr958Ala]).

The pEBG2T-CT-PRK2 and pEBG2T-CT-PRK2[Thr958Ala] plasmids were then co-transfected with a plasmid coding for Myc-PDK1. The pull-down interaction assay that was performed by other members of the research group verified that both C-terminal mutations of PRK2 at position Thr958 were equally effective in binding Myc-PDK1 (Figure 6A). Further investigation pointed out that the mutant GST-CT-PRK2 having Thr958 mutated to Glu was also not affected in its interaction with PDK1. As a control, using a phospho-specific antibody, we verified that GST-CT-PRK2, but not GST-CT-PRK2[Thr958Ala] or GST-CT-PRK2[Thr958Glu] were phosphorylated at the Z-/turn motif phosphorylation site (Figure 6B). These results suggested to us that the wildtype and both of the C-terminal mutants feature similar affinities towards PDK1.

I further examined the ability of GST-CT-PRK2 sequences to bind to endogenous PDK1. To this end, I transfected HEK293 cells (20 dishes) with either pEBG2T-CT-PRK2, pEBG2T-CT-PRK2[Thr958Ala] or pEBG2T-CT-PRK2[Thr958Glu] and purified the expressed GST-fusion proteins. I measured the concentration of the purified protein and confirmed the purity by SDS-PAGE followed by Coomassie staining. Then, I tested the presence of PDK1 in the different fractions using a PDK1 activity test. In all GST-CT-PRK2 protein fractions PDK1 activity was detectable. Moreover, the protein kinase activity tests pointed out that the ability to co-purify with endogenous PDK1 was not modified in CT-PRK2 derived polypeptides mutated at the Z-/turn motif phosphorylation site (Figure 6C).

Altogether we concluded that the Z-/turn motif phosphorylation did not alter the ability of PRK2 derived polypeptides to interact with PDK1.

In summary, the increased interaction of PDK1 with GST-PRK2[Thr958Ala] was not mimicked by the isolated C-terminal residues of PRK2. This corresponds to the finding from previous plasmon resonance (Bia-Core) studies, which showed that another Z-/turn motif phosphorylated polypeptide had identical binding kinetics to PDK1 as the equivalent non-phosphorylated counterpart [57]. The results thus disproved the hypothesis that the increased binding of PRK2 [Thr958Ala] to PDK1 could be due to an increase in affinity of teh C-terminal region to PDK1.

A

GST-CT-PRK2	GST	wt	Thr958Ala	Thr958Glu	
Coomassie of GST pull-down	-	+	+	+	Myc-PDK1
	-	+	+	+	GST-CT-PRK2
Myc immunoblot of GST pull-down	-	+	+	+	Myc-PDK1
Myc immunoblot of crude extract	+	+	+	+	Myc-PDK1

B

GST-CT-PRK2	wt	Thr958Ala	Thr958Glu
Coomassie of GST pull-down	+	+	+
Phospho-Z/turn-motif immunoblot	+	-	-

C

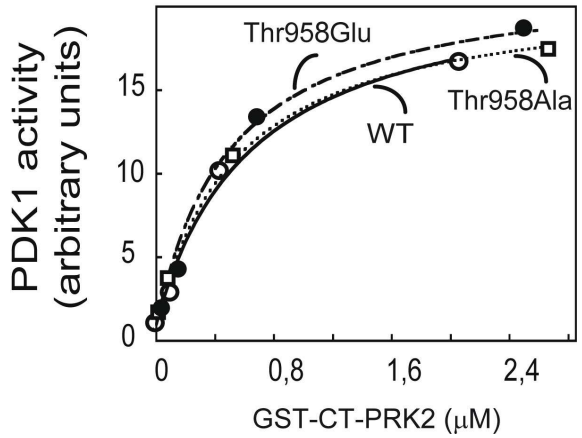

Figure 6: The Z-/turn motif phosphorylation site does not affect the interaction between the C-terminal fragment of PRK2 and PDK1 as the mutation of the Z-/turn motif phosphorylation site within the isolated C-terminal segment of PRK2 does not affect the interaction with PDK1. *A*, Table of a co-transfection of HEK293 cells with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST, GST-CT-PRK2[Thr958Ala] and GST-CT-PRK2[Thr958Glu]. After 36 h the cells were lysed, the GST-fusion protein purified by affinity chromatography on glutathione-sepharose beads, the product electrophoresed on a separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue or immunoblotted using an anti-Myc antibody to detect co-purified Myc-PDK1.
B, Table of an immunoblot with GST-CT-PRK2 proteins with an antibody which recognizes the Z-/turn motif phosphorylation site only when it is phosphorylated. *C*, HEK293 cells were transfected with DNA constructs expressing GST-CT-PRK2 wt or the mutants GST-CT-PRK2[Thr958Ala] and GST-CT-PRK2[Thr958Glu] and the GST-fusion proteins purified by affinity chromatography. The ability of the GST-CT-PRK2 proteins to interact with endogenous PDK1 was analysed indirectly by measuring the PDK1 activity which was co-purified with the GST-fusion protein.

5.3. Influence of the PRK2 Z-/turn motif phosphate binding site on the interaction of PRK2 with PDK1.

As shown above, the non-phosphorylatable C-terminal segment of PRK2 did not show increased affinity for PDK1. Thus, we took into consideration that the increased affinity of PRK2[Thr958Ala] could be due to the dissociation of the phosphorylated C-terminal segment from the catalytic domain of PRK2. Since our group previously characterized the existence of a Z-/turn-motif phosphate binding site on the small lobe of PRK2 [57], we decided to mutate the site and evaluate the effect on the interaction with PDK1. I therefore generated full length PRK2 constructs which had mutated Lys670 to Ser, Lys689 to Glu, and in addition, generated the double mutant construct PRK2[K670S,K689E]. These two Lys-residues were previously described on the ΔN-PRK2 construct by Hauge et al. in 2007 as key residues forming part of the Z-/turn motif phosphorylation site [57].

The Lys689 was predicted to be exposed to the solvent in the absence of Z-/turn motif phosphorylation. Furthermore, its mutation to Glu was planned as a way to completely destruct the Z-/turn motif phosphate binding site by exchanging a positive charge (Lys) for a negative charge (Glu).

To analyze the ability of PRK2 wildtype and the mutants to interact with PDK1, we co-transfected HEK293 cells with plasmids coding for the expression of GST-PRK2 together with a plasmid coding for Myc-PDK1. After cell lysis, GST-PRK2 fusion proteins were pulled-down using glutathione-sepharose resin and analysed for the presence of Myc-PDK1 in the pull-down by western-blot using anti-Myc antibodies. As previously shown using this methodology, we observed that PDK1 interacted with GST-PRK2 and the mutated PRK2[Thr958Ala] showed increased interaction. Most interestingly, in agreement with our assumption, the exchange of charge within the phosphate binding site in PRK2[Lys689Glu], induced a significantly higher level of binding to PDK1 than the wild type PRK2 conterpart (Figure 7A). Interestingly, additional mutation of Lys670 to Ser on PRK2 did not further enhance such increase in binding on PRK2.

Taken together, this experiment showed that the two mutations, on the one hand the mutation of the Z-/turn motif phosphorylation site to Ala and on the other hand the destruction of the Z-/turn motif phosphate binding site by mutating it to Glu, led to a similar increase in the binding of PRK2 to PDK1. These observations are compatible with a model in which the phosphorylation of the Z-/turn motif site can decrease the binding of PRK2 to PDK1 by increasing the binding of the C-terminus of PRK2 to its own Z-/turn motif binding site.

5.4. Influence of PRK2 kinase dead on the interaction with PDK1

It is tempting to speculate that PDK1 substrates which tend to interact with PDK1 are the ones which are not phosphorylated at the PDK1 phosphorylation site or which are in an inactive conformation. Nevertheless, this had never been tested for any substrate of PDK1. To test this hypothesis I generated two mutants of PRK2 which were virtually inactive. On the one hand the PRK2 K686M mutant, having a Met instead of the Lys in position 686 at the catalytic site and on the other hand the TST/AAA mutant, which has all three activation loop phosphorylatable residues (Thr814, Ser815 and Thr816) mutated to Ala.

Remarkably, these two kinase dead (K-dead) mutants were expressed at lower levels in cells (Figure 7B). However, we found that the two K-dead PRK2 proteins were able to bind higher levels of Myc-PDK1 compared to the wildtype PRK2 protein. (Figure 7B). It was notable that the PRK2 TST/AAA protein, although hardly expressed, still pulled-down as much Myc-PDK1 as PRK2 wt. These observations indicated that the inactive mutants of PRK2 had an increased affinity towards PDK1.

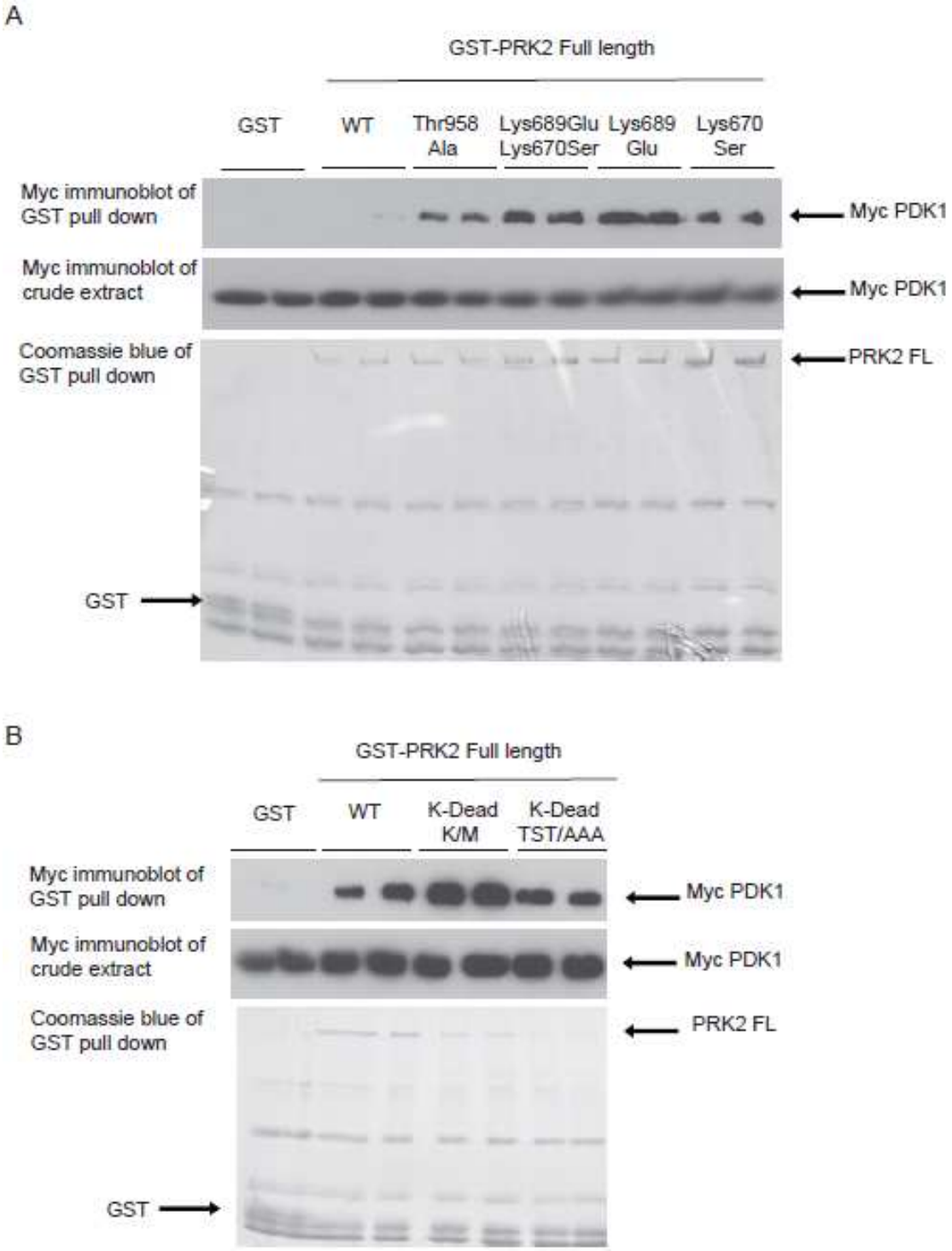

Figure 7: Mutation of the Z-/turn motif phosphate binding site and mutations that abolish activity of PRK2 enhance the interaction with PDK1. HEK293 cells were transfected with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST, or the GST-PRK2 proteins mutated at the Z-/turn motif phosphorylation site (GST-PRK2[Thr958Ala]) or mutated within the predicted Z-/turn motif phosphate binding site (GST-PRK2[Lys689Glu], GST-PRK2[Lys670Ser] and the double mutant GST-PRK2[Lys689Glu; Lys670Ser] (*A*) or mutated at the active site [Lys686Met] or at the activation loop [TST-AAA] *(B).*

Although the two kinase dead (K-dead) mutants were expressed to lower levels in cells, theywere able to bind higher levels of Myc-PDK1

For analysing interaction, HEK293 cells were transfected with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST, GST-PRK2-FL wt or particular mutant. After 36 h the cells were lysed, the GST-fusion protein purified by affinity chromatography on glutathione-sepharose beads, the product electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue or immunoblotted using an anti-Myc antibody to detect co-purified Myc-PDK1. The Duplicates of each condition are shown.

5.5. Influence of the PRK2 C-terminal negatively charged patch Glu968-Glu970 and hydrophobic patch Ile965 /Leu966 on the interaction of PRK2 with PDK1.

As Balendran et. al could show via the mutation of conserved residues within the C-terminus of PRK2 and other AGC kinases, the interaction with PDK1 requires the hydrophobic residues Phe974, Phe977, Tyr979 and the negatively charged residue Asp978 [5]. These residues are equivalent to the HM and the HM phosphorylation site in other AGC kinases. However, the C-terminus of PRK2 interacts with PDK1 with higher affinity than the C-terminus of other AGC kinases [11]. Therefore, we now paid attention on other non-conserved motifs within the C-terminus of PRK2 and wanted to examine, if such motifs could also play a role in the high affinity interaction with PDK1. We pursued this goal in the absence of any crystal structure information describing the molecular interaction between PDK1 and its docking polypeptides.

We transfected HEK 293 cells with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST, GST-CT-PRK2 wt or the mutants GST-CT-PRK2 [EEE/AAA] and GST-CT-PRK2[IL/AA] mutated at an acidic or hydrophobic patch, respectively. After cell lysis, GST-PRK2 fusion proteins were bound to glutathione-sepharose resin and samples were analysed for the presence of Myc-PDK1 by western-blot using anti-Myc antibodies. Actually, we could show by these protein-protein interaction assays that mutation of the three Glu residues 968-969-670 to Ala significantly diminished the interaction of GST-CT-PRK2 with PDK1 and similarly, when GST-CT-PRK2 was mutated at both hydrophobic residues, Ile965Ala and Leu966Ala, the resulting fusion proteins also lost their ability to interact with PDK1 (Figure 8A). The results suggested that the high affinity interaction of GST-CT-PRK2 with PDK1 was not only due to the HM-mediated interactions, but also importantly influenced by other regions within the C-terminus of PRK2.

To further characterize the role of these motifs on the interaction with PDK1, we synthesized PRK2-C-terminus (PIFtide)-derived polypetides having Glu-Glu-Glu[968-970] and Ile-Leu[965-966] mutated to Ala. I then tested the effect of various polypetide concentrations on the activity of PDK1 and evaluated the data using Kaleidagraph software. Interestingly, the EEE/AAA mutant had greatly decreased ability to activate PDK1 (23-fold higher AC50), while the polypetide having the IL mutated to AA had a 3-fold higher AC50 as the corresponding PIFtide control (Figure 8B). We assumed that the greatly affected ability of EEE/AAA to activate PDK1 was caused either due to a loss of binding or to the loss of the ability to activate PDK1. To further analyse this, we confirmed these results by evaluating the binding of the polypeptides to PDK1 using surface-plasmon resonance technology (not shown).

As well as verifying the effect of the C-terminal mutations EEE/AAA and IL/AA of PRK2 on the interaction with PDK1, it was of interest for us to know if these mutants had also effect on the intrinsic activity of PRK2. To this end, I further mutated the equivalent residues in the full length PRK2 protein.

Interestingly, PRK2 IL/AA was normally expressed, had decreased interaction with PDK1 and was active, suggesting that the IL hydrophobic patch affects the interaction with PDK1 but does not greatly affect the PRK2 enzyme. On the contrary, the kinase mutated at the three Glu residues to Ala was hardly expressed. This let us suggest that the C-terminal acidic patch is important for the stability of PRK2 itself.

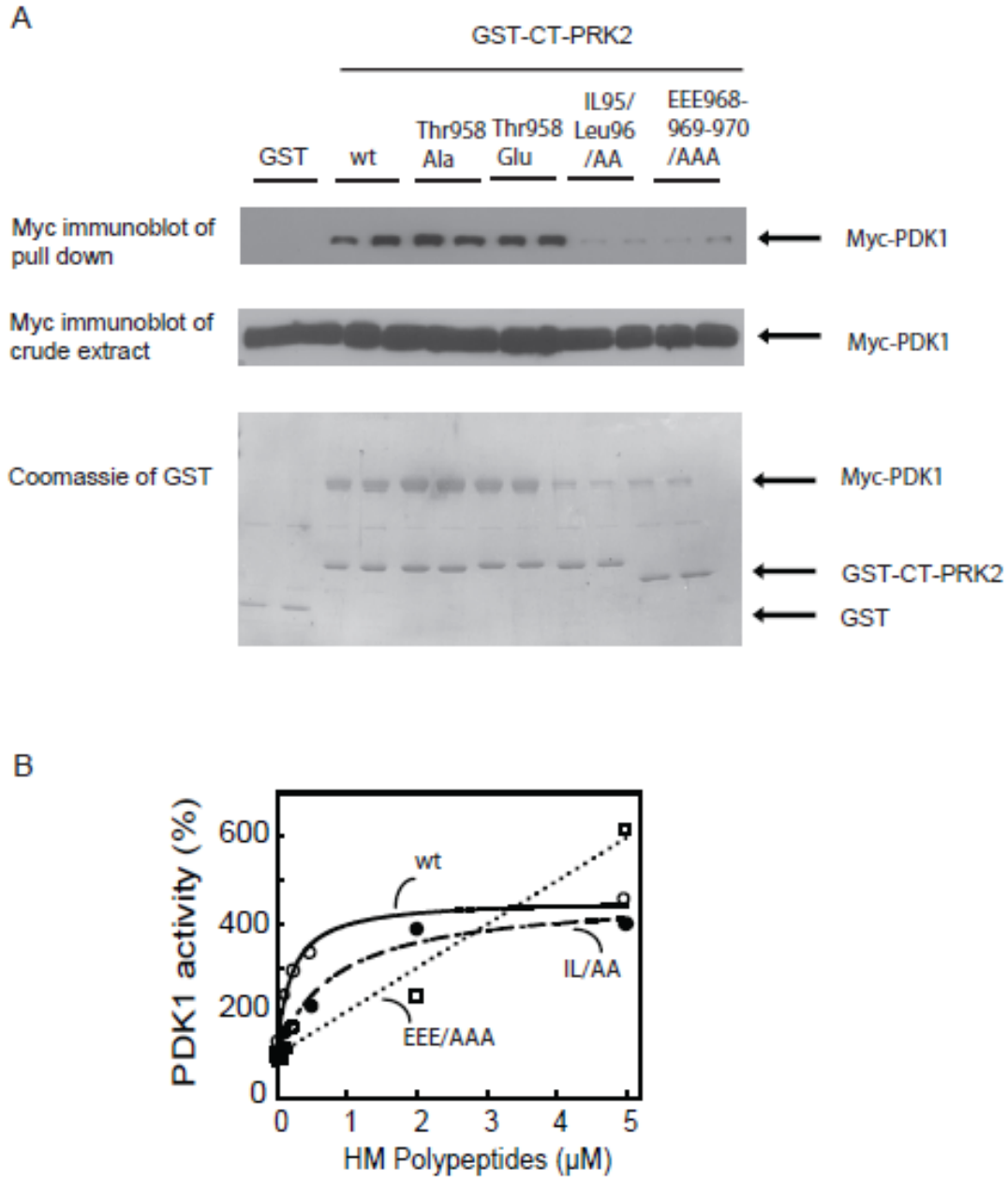

Figure 8: PRK2 C-terminal negatively charged patch Glu968-Glu970 and hydrophobic patch Ile965 /Leu966 are required for the high affinity interaction of PRK2 with PDK1*A:* In order to analyse if other C-terminal residues of PRK2 were important for the interaction with PDK1, GST-CT-PRK2 wt and the mutants GST-CT-PRK2[Ile965Ala; Leu966Ala] and GST-CT-PRK2[Glu968Ala; Glu969Ala; GLu970Ala] were co-expressed with Myc-PDK1 in HEK293 cells using the same protocol as it is described at figure 7.
B: The interaction between the C-terminus of PRK2 and PDK1 was analysed indirectly by measuring the ability of PRK2 C-terminal polypeptides to activate PDK1. The polypeptides (C-terminus PRK2 wt and the PRK2 C terminus-derived polypeptides (PIFtide, REPR**IL**S**EEE**QEMFRDFDYIADWC), PIFtide IL/AA and PIFtide EEE/AAA were analysed for their ability to activate PDK1 *in vitro*. The activity of 0.2 µM PDK1 was measured in triplicates using the peptide T308tide as a substrate. The EEE/AAA mutant had greatly decreased ability to activate PDK1 (23 fold higher AC50), while the polypetide having the IL mutated to AA had a 3 fold higher AC50 as the corresponding PIFtide control.

5.6. Influence of the Z-/turn motif phosphorylation site on the intrinsic activity of full length PRK2 and the isolated catalytic domain of PRK2.

The above mentioned study characterizing the role of the Z-/turn motif phosphorylation on PRK2 by Hauge et al. in 2007 was performed on a PRK2 mutant protein lacking 500 N-terminal residues (ΔN-PRK2) [57]. For the purpose of evaluating if the N-terminal regulatory region was involved in the phosphorylation of the Z-/turn motif site, I also mutated the Z-/turn-motif phosphorylation site of the full length (FL) PRK2. I purified the different GST-fusion proteins and compared the effect of the mutation to the impact on the catalytic domain of PRK2 (ΔN-PRK2) on the specific activity.

By using purified GST-fusion proteins in *in vitro* activity assays, we confirmed that mutation of the Z-/turn motif phosphorylation site in PRK2 substantially diminished the activity of ΔN-PRK2. Similar effects on the activity could be observed on the corresponding full length-mutant protein. Thus, the proteins mutated at the Z-/turn-motif site [Thr958Ala] showed greatly decreased activity. These results indicated that the N-terminal region did not affect the Z-/turn motif phosphorylation site-mediated modulation of PRK2 activity. Altogether, we now confirmed that the Z-/turn-motif phosphorylation is very important for the activity of PRK2. In this sense, its dynamic phosphorylation may be used by nature to regulate the activity of PRK2 in cells.

I further expressed, purified and tested the activity of PRK2 full length proteins comprising the K-dead K/M and TST/AAA mutatants as well as IL/AA and EEE/AAA C-terminal mutants. As already mentioned both kinase dead mutants were expressed at lower levels compared to the wildtype and were essentially inactive. In addition, in most purifications, we could not detect PRK2 EEE/AAA mutant expression suggesting that the protein was unstable. As described for the ΔN-PRK2 construct, the Thr958Ala mutation within the context of the FL-protein also drastically affected the activity without significant changes at the level of phosphorylation at the activation loop.

Interestingly, we also repeatedly observed that the ΔN-PRK2 construct had higher activity than the FL-PRK2 when measured at equal protein concentrations (e.g. 40ng) (Figure 9). A higher specific activity had been previously observed upon limited proteolysis of PRK2 and had been proposed to be due to the cleavage of a pseudosubstrate within the N-terminal extension of the kinase, as it had been described for classical PKCs [76, 107, 122].

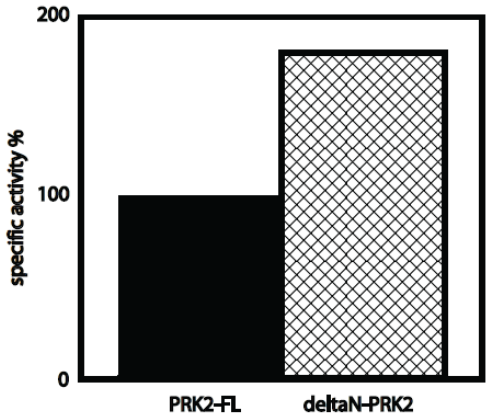

Figure 9: Comparison of specific activity of the PRK2-FL with the ΔN-PRK2 construct: This assay was performed in a 20 µl mix containing 50 mM Tris-HCl pH 7.5, 0.05 mg/ml BSA, 0.1% β-mercaptoethanol, 10 mM $MgCl_2$, 100 µM [γ^{32}P]ATP (5-50 cpm/pmol), 0.003% Brij, 40 ng of the particular PRK2 construct and Crosstide (100 µM). We observed that the ΔN-PRK2 construct had higher activity than the FL-PRK2, when measured at equal protein concentrations (40ng in this case). The specific activity of PRK2-FL is shown to be 100%. Compared to this, the ΔN-PRK2 construct shows a more than 1.8 fold higher (180,4%) specific activity at the given protein concentration.

5.7. Regulation of PRK2 by its N-terminal region.

While performing the above mentioned PRK2 activity measurements, we observed that the specific activity of PRK2 FL could not be accurately measured because it was dependent on the protein kinase concentration in the assay. We realised that lower concentrations gave raise to higher specific activities. Interstingly, the same could be observed with the FL-PRK2-Thr958Ala, but not in any of the ΔN-constructs, where the same specific activity was obtained when 2,5 – 50 ng PRK2 were used. Table 8 shows a comparison between the acivity assay-measured counts of PRK2-FL and the ΔN-PRK2 construct. Remarkably, while the FL protein had greatly decreased activity under some conditions, this was no longer observed when low concentrations of FL-PRK2 were employed in the assays (1.8 nM). In this case, the FL-PRK2 specific activity was 5,9 ± 0,5 pmol product/min/pmol, indistinguishable from the specific activity of ΔN-PRK2 (5,5 ± 0,3 pmol product/min/pmol).

The biochemical characterization of PRK2 indicated that the lower specific activity of FL-PRK2 was clearly concentration-dependent. This was not compatible with an intramolecular inhibition as expected if the N-terminal extension to the catalytic domain in PRK2 would act intramolecularly as a pseudosubstrate as described for PKCs. Rather, the results were compatible with an intermolecular inhibition of activity, mediated by the N-terminal extension to the catalytic core of PRK2.

For deeper investigation, we wanted to examine if PRK2 molecules could form oligomers. To this end, we co-transfected plasmids coding for GST-FL-PRK2 and HA-FL-PRK2 in HEK293 cells, followed by pull-down and anti-HA immunoblotting on the pull-down. GST-FL-PRK2 significantly interacted with HA-FL-PRK2 (Figure 10A). Interestingly, GST-ΔN-PRK2 interaction with HA-FL-PRK2 was drastically diminished in comparison to the interaction between full-length proteins, suggesting that the N-terminal region of PRK2 participated in an intermolecular interaction with another PRK2 molecule.

In order to provide further evidence that the PRK2-PRK2 intermolecular interaction was responsible for the inhibition of full length PRK2, I examined the effect of the K-dead mutant FL-PRK2 TST/AAA on the activity of FL-PRK2 or ΔN-PRK2 by performing a protein kinase assay. Whereas the addition of K-dead FL-PRK2 did not have a detrimental effect on ΔN-PRK2 (Figure 10B, closed circles), it inhibited FL-PRK2 activity in a concentration-dependent manner (open circles). Together, we concluded that the N-terminal region of PRK2 was responsible for the inhibition of its activity by an intermolecular interaction.

As previous work pointed out, Rho-GTP binds to a Rho-binding domain on the N-terminal region of PRK2 and participates in PRK2 activation in cells [107]. So, we wanted to know if Rho-GTP could directly activate PRK2 *in vitro*. To analyse this possibility we tested the PRK2 activity in a protein kinase assay using a concentration where PRK2 is known to be in a partially autoinhibited form and added purified Rho-GTPase. The activity of GST-PRK2 and the GST-ΔN-PRK2 assayed at various concentrations was not affected by the addition of purified Rho, Rho-GDP or Rho-GTP (Figure 11). Thus, our results suggested that Rho did not affect the specific activity of the isolated PRK2 protein *in vitro*.

Table 8: Activity assay-measured counts of PRK2-FL and ΔN-PRK2 construct

Enzyme	Added amount (ng)	Counts (average)	Enzyme	Added amount (ng)	Counts (average)
PRK2-FL-wt	40	4.957.292	PRK2-ΔN-wt	40	8.875.324
	8	2.357.580		8	2.537.639
	2	811.882		2	667.511

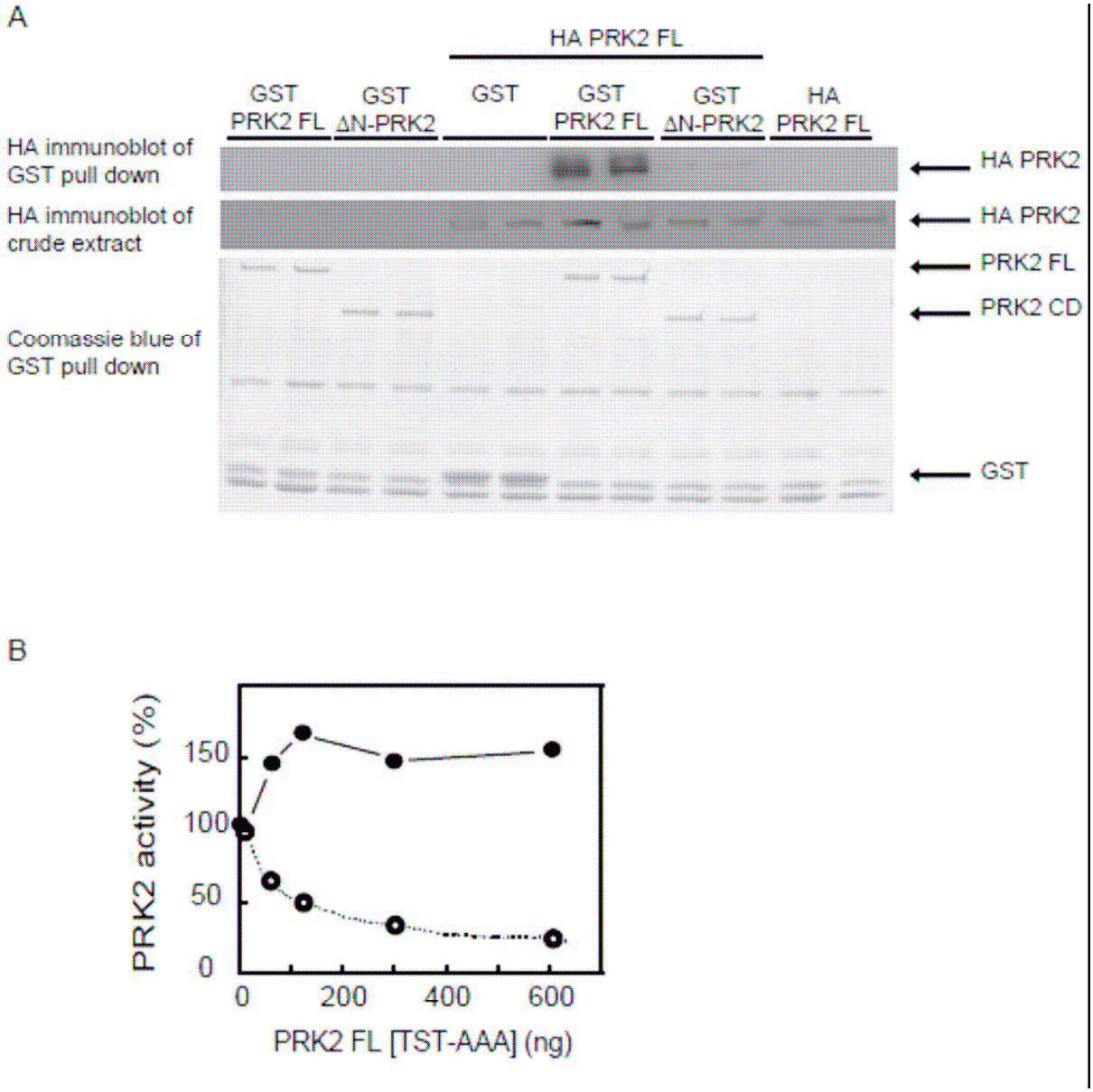

Figure 10: The PRK2 N-terminal region is required for PRK2 oligomerization and inhibits its activity *in trans*. *A*: GST-PRK2 full length (FL), but not GST-ΔN-PRK2 interacted with HA-FL-PRK2. HEK293 cells were transfected with DNA constructs expressing GST, GST-FL-PRK2, GST-ΔN-PRK2 together with a construct expressing HA-PRK2. GST-fusion proteins were isolated and the interaction between GST-fusion proteins and HA-FL-PRK2 was analysed as described in the legend to Figure 6. *B*, Activity of FL-PRK2 (open circles) and ΔN-PRK2 were measured *in vitro* in the absence (100%) or in the presence of the indicated amounts of PRK2 [TST-AAA] as described in the legend of figure 9. The K-dead PRK2 [TST-AAA] mutant inhibits the activity of FL-PRK2 but not ΔN-PRK2.

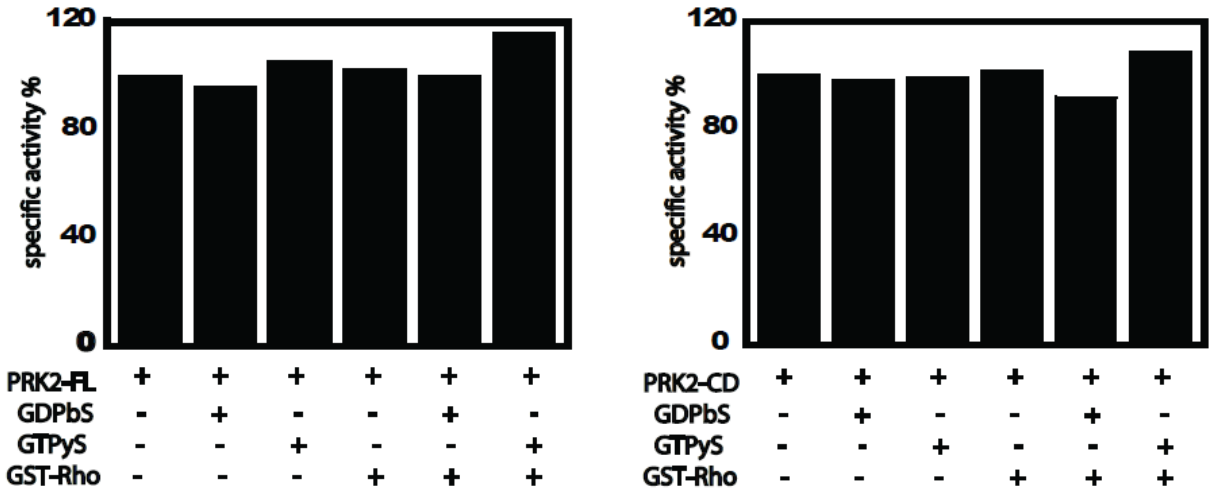

Figure 11: Effect of Rho, Rho-GDP or Rho-GTP on GST-PRK2 and ΔN-PRK2- construct. This figure shows the Effect of Rho, Rho-GDP or Rho-GTP on the specific activity of GST-PRK2 (left) and the ΔN-PRK2-construct (right). We could not observe a mentionable activity increasing effect using Rho, Rho-GDP or Rho-GTP in the kinase assay. The specific activity of GST-FL-PRK2 and -ΔN-PRK2 without adding one of the above mentioned GTP-ases are taken as 100%. This PRK2 activity assays were performed in a 20 µl mix containing 50 mM Tris-HCl pH 7.5, 0.05 mg/ml BSA, 0.1% β-mercaptoethanol, 10 mM $MgCl_2$, 100 µM [γ^{32}P]ATP (5-50 cpm/pmol), 0.003% Brij, 40 ng of the particular PRK2 construct, Crosstide (100 µM) and Rho (10uM), Rho-GDP (10uM) or Rho-GTP (10uM).

6. Discussion

The study of the molecular mechanism of regulation of PRK2 impinges in different aspects of cellular regulation and disease: 1- the mechanism of regulation of PRK2 interaction with PDK1 relates to the broad topic of “protein kinase docking interactions”, which are known to occur in many pathways (specially in the MAP kinase pathway) but the mechanisms of regulation of those interactions are vastly unknown. 2- Protein phosphorylation is widely used by nature for cellular regulation, but the mechanisms are most often not known. This thesis studies the role of protein phosphorylation, in particular, at the Z-/turn-motif site, a widely conserved phosphorylation site in AGC kinases, concluding that it participates both in the regulation of the activity and in the regulation of the docking to its upstream activation loop kinase PDK1. 3- Protein kinases appear to achieve specific regulation by means of N-terminal or C-terminal extensions to the catalytic core. In contrast to the present knowledge on related AGC kinases, this thesis reveals that the N-terminal region inhibits the docking interaction with PDK1 and that it also promotes the oligomerization that inhibits PRK2 activity. 4- The C-terminal region is known to regulate the activity of AGC kinases. In this study we further learned aspects of the C-terminal region of PRK2 that turn out to be important for the high affinity binding to PDK1. 5- The present work finally deals with the different mechanisms of regulation that evolve from a common theme. Thus, our comparison of the mechanism of regulation of PRK2 and other AGC kinases reveal that the regulation of docking interaction by Z-/turn-motif phosphorylation happens on PRK2 but not on other related AGC kinases. Also, our studies on the N-terminal and C-terminal extensions to the catalytic domain unveil a very particular mechanism of regulation of PRK2. Altogether, the work provides information on the mechanism of regulation of PRK2 that may help develop novel therapies for deseases where PRK2 is involved.

Docking interactions have to be regulated and are the substance of the signalling specificity of PDK1 and a group of other kinases. As it is very little known about the regulatory mechanisms that are involved, we wanted to investigate the aspects of PRK regulation in detail. In this sense we examined the interaction between PRK2 and PDK1, but also spotted a light on the PRK2-PRK2 interaction. Actually, we found a new way of regulation of the interaction between these two kinases and discovered that the mechanism that decreases the interaction with the upstream kinase is directly linked to the mechanism of activation of the substrate kinase.

In this work, we studied the N-terminal and C-terminal extensions of PRK2 and learned that not only the C-terminal Z-/turn motif phosphorylation site, but also the N-

terminal segment acts as an inhibitor for the interaction of PRK2 with PDK1. Furthermore, with the work presented in this thesis, we provide evidence that PRK2 forms oligomers with other PRK2-molecules via its N-terminal extension and that this inhibits the activity of PRK2 intermolecularly.

It is already known that AGC-kinases need certain phosphorylation sites for the interaction with their upstream kinase PDK1 and for their activation. Next to the already well-known actvation loop and the HM phosphorylation site belongs the recently characterized Z-/turn-motif phosphorylation site. A common mechanism of activation including these three phosphorylation sites is assumed for up to 26 AGC kinases and was further investigated for Akt/PKB, S6K, RSK, MSK, PRK and PKC, as recently described by Hauge et al. [57]: The phosphorylation sites work in a cooperative manner, as the Z-/turn motif phosphate binds a phospho-Ser/phospho-Thr-binding site above the glycine-rich loop within the kinase domain. This binding promotes an association of the tail with the kinase domain and serves to deliver the HM to its binding site in a zipper-like manner, inducing the stabilization of the HM in its kinase-activating binding site. This stablilization directly leads to the stimulation of the kinase activity.

Most AGC-kinases, like PRKs, PKCs, S6K and SGK, consist of a catalytic domain, formed by a small and a large loop, and N- and C-terminal extensions to the catalytic core, that are important for stabilizing the active conformation of the catalytic domain. Like PKC members, PRKs possess a large N-terminal extension, which contains a putative pseudosubstrate region and a C2-like domain. Further, the N-terminus possesses three HR1 Rho-effector domains. On the C-terminal extension to the catalytic core, PRKs have a docking sequence that allows binding to its upstream kinase PDK1. A recent peptide array study suggested that another motif, termed Ade (adenosine binding) motif, may be important for interaction of C-terminal regions of distinct AGC-kinases with PDK1. It was shown to be conserved in PKA, Akt/PKB and PRK2 and it was predicted to be a PDK1-interacting site for most AGC kinases [126]. Although not formally proved, it is therefore possible that the Ade motif also participates in the interaction of PRK2 with PDK1. However, this study [126] was published after the work at this thesis has been finished and our publication was in revision. The three phosphorylation sites (HM, Z-/turn motif and activation loop) are located at certain regions of the kinases. The activation loop is settled in the kinase domain, while the HM and the Z-/turn motif are located at the C-terminal tail of the enzyme. As already established, in most AGC-kinases the HM is characterized by three aromatic amino acids surrounding the Ser/Thr residue that becomes phosphorylated: Phe-X-X-Phe-Ser/Thr-Phe/Tyr. However, in

PRKs and atypical PKCs the HM contains a negatively charged amino acid (aspartic acid or glutamic acid) that mimicks the phospho-Ser/ phospho-Thr [50, 121].

We know that phosphorylation of the HM of RSK, S6K and SGK regulates the interaction with their upstream kinase PDK1. We also know that PRK2 and PKCζ cannot be regulated through HM phosphorylation as they have an acidic residue instead of a HM phosphorylation site and that PRK2 and PKCζ are phosphorylated at the activation loop and Z-/turn motif site *in vivo*. We further know that the mutation of the Z-/turn motif phosphorylation site in PRK2 increases the interaction with PDK1.

So, according to this, we wanted to verify if the interaction of S6K and SGK with PDK1 could also beregulated by Z-/turn motif phosphorylation. Our experiments could not show that the Z-/turn motif phosphorylation site regulates the interaction with PDK1 in the case of S6K and SGK. In addition, Rosalia Dettori's data show that this is not the case for PKCζ. However, this topic is an interesting subject for further investigation, as the Z-/turn motif phosphorylation in PKCζ, S6K and SGK could actually play a role in the interaction with PDK1, while the methods we used just cannot detect it. In conclusion it can be said that in PKCζ, SGK and S6K, but presumably also in other AGC-kinases like Akt/PKB, phosphorylation of the Z-/turn motif phosphorylation site seems to be important for activaty but does not appear to regulate the interaction with PDK1. In contrast, the phosphorylation of the Z-/turn motif phosphorylation site in PRK2 can regulate the interaction with PDK1. These results made us suggest that the Z-/turn motif phosphorylation plays a special role in the case of PRK2 as a characteristic modulator of PRK2 regulation, differing from other AGC-kinases.

Since the mutation of the Z-/turn motif phosphorylation site of PRK2 threonine 958 to alanine leads to an increased interaction of PRK2 with PDK1, we wanted to further investigate the molecular mechanism. In order to do that we figured out two different explanations that could explain such behaviour. On the one hand an increased binding affinity of the C-terminal residues of PRK2 to PDK1 or on the other hand a decreased affinity of this C-terminal fragment to its own PRK2 catalytic domain. Both explanations indeed fit in a model, assuming that the binding of the C-terminus of PRK2 to PDK1 is in direct competition to the binding of the C-terminus of PRK2 intramolecularly to its own catalytic domain. When phosphorylated at the Z-/turn motif site, the C-terminus of PRK2 would bind to its own Z-/turn motif binding site, while the non-phosphorylated Z-/turn motif had higher affinity to the PIF-binding pocket of PDK1.

We know from PRK2-PDK1 interaction experiments in 293 cells that the increased affinity may not du to an increased binding avidity of the C-terminus of PRK2 towards PDK1,

as mutating the Z-/turn motif phosphorylation site within the isolated GST-CT-PRK2, did not affect the interaction with PDK1. Further, this mutant also had equivalent avidity for endogenous PDK1. However, we could provide evidence that there is a competition between the binding of the C-terminus of PRK2 to PDK1 and to the Z-/turn motif phosphate binding site, as we could show an increased binding of PRK2 to PDK1 when we mutated the putative Z-/turn motif phosphate binding site on PRK2.

Another topic we were interested in, was the further characterization of the C-terminus of PRK2. In order to investigate this part of the kinase in more detail, we examined the negatively charged patch Glu968-Glu970 and the hydrophobic patch Ile965/Leu966 located in this region. We found out that these patches, which are not conserved within the AGC-kinases, are required for the high affinity interaction between PRK2 and PDK1. However, because of the fact that PDK1 is still able to phosphorylate the activation loop of PRK2 IL/AA, it is possible that there is an additional interaction site.

Taken together, it can be said that the C-terminus of PRK2 plays a crucial role in the interaction and in the regulation of the interaction with PDK1, since this region contains key interacting determinants, HM, the Glu968-Glu970 negatively charged patch, the Ile965/Leu966 hydrophobic patch and the regulated Z-/turn motif phosphorylation site. As far as PKCζ is concerned, there must be another mechanism of regulation compared to PRK2 and other AGC-kinases. We could show that although it has no HM phosphorylation site like PRK2its interaction with PDK1 is not regulated by Z-/turn motif phosphorylation.

Work mainly done by Newton's and Parker's laboratories in the nineties indicated that PKCs have a pseudosubstrate sequence at their N-terminal end acting as an inhibitory module providing a sterical blocking of the active site. Binding of the co-factor diacylglycerol was presented to relieve an autoinhibition through conformational changes that unmask the active site. This intra-molecular inhibition via a pseudosubstrate sequence was also assumed for PRK2. In contrast to this assumption, we provide evidence that there is an inter-molecular inhibition of PRK2 activity via the N-terminal extension.

Our PRK2 activity measurements showed that the specific activity of the full length PRK2 could not be accurately measured because it was dependent on the protein kinase concentration in the assay, with lower concentrations giving raise to higher specific activities. In contrast, this was not the case for the PRK2 construct lacking the N-terminal extension. This one showed constant specific activity when tested at different concentrations, as expected. Interestingly, the raise to higher specific activity at lower concentration was also observed for the full length PRK2[Thr958Ala] mutant. These observations are not compatible

with an intra-molecular inhibition as expected if the N-terminal extension to the catalytic domain in PRK2 would act as a pseudosubstrate intramolecularly. Thus, we assumed an inhibition *in trans* with another molecule of PRK2 as the used GST-fusion proteins we employed were essentially pure. For further investigation we tested if PRK2 molecules could form oligomers. In this sense, we co-transfected plasmids encoding GST-FL-PRK2 and HA-FL-PRK2 and in parallel plasmids coding for GST-ΔN-PRK2 with HA-FL-PRK2. We could show that the full length PRK2 construct significantly interacted with HA-FL-PRK2, while the ΔN-construct showed drastical diminished interaction with HA-FL-PRK2. Thus, we could provide evidence that the N-terminal region of PRK2 participated in an inter-molecular interaction with another PRK2 molecule. In further support, we tested the effect of the FL-PRK2 TST/AAA inactive mutant on the activity of FL-PRK2 or ΔN-PRK2, showing that FL-PRK2, but not ΔN-PRK2 activity was inhibited through the addition. In summary, our results support indications that PRK2 molecules are able to form oligomers via their N-terminal extensions and that this binding causes an inter-molecular inhibition.

It was previously described by Vincent and Settleman that PRK2 is an effector target of Rho-GTPases. It was shown that PRK2 was substantially activated by Rho *in vitro* in a nucleotide-independent manner, while the interaction with Rac was completely GTP dependent. They further showed that for activation of PRK2, Rho had to bind to an intact Rho effector domain [142]. Recently, Lim et al. found that the extreme C-terminal segment is critical for the full activation of PRK2 by RhoA in cells in a GTP-dependent manner. They could show in experimental procedures that the addition of GST-GTPγS-RhoA to the immuno-purified wild-type PRK2 resulted in an approximately 1.5-fold activation of PRK2 [90]. However, the Rho binding domains themselves are located at the N-terminal extensions in PRK2. Lim et al. also described that the binding of RhoA to PRK2 could relieve an auto-inhibitory interaction between the N- and the C-terminus of the kinase. They further showed that PRK2 was activated by GST-RhoA *in vitro*. In contrast to this, in the course of our *in vitro* studies using purified PRK2 constructs, Rho, Rho-GTP and Rho-GDP did not exert an effect on full length PRK2 activity. This finding supports the hypothesis that additional proteins may be required to mediate the Rho-dependent effects.

Summing up all findings, we provide evidence for our hypothesis that PRK2 may remains inhibited because of an intermolecular interaction mediated by the N-terminal extension. This oligomerization blocks not only the intrinsic activity but also the interaction with PDK1. Based on the work by others, the model suggests that upon stimulation of Rho, Rho-GTP may bind to the Rho-effector domains within the N-terminal region of PRK2,

releasing the inter-molecular inhibition and enabling the interaction with PDK1. In order to explain the different requirement for Rho-GTP found by others and shown in the present work, we suggest that the interaction between PRK2 and Rho is supported by third proteins. However, this issue remains unproven and would require further investigation in the future.

The model suggested in the past by Biondi et al. considered that the inactive substrate could have higer affinity for PDK1. Anyhow, this had not been previously tested experimentally. In support to our concept that after phosphorylation at the Z-/turn motif site, the C-terminus of PRK2 would bind intramolecularly to its own Z-/turn motif binding site, while the non-phosphorylated Z-/turn motif has higher affinity to the binding site of PDK1, we found that two mutants of PRK2 which were virtually inactive bound PDK1 with a higher affinity than wild type PRK2 did, although they were expressed to a lower extent. In this sense, the complete model would suggest that once phosphorylated by PDK1, the Z/turn motif site of PRK2 becomes phosphorylated and PRK2 achieves the active form, which hides the HM from PDK1 and thus decreases the avidity to interact with PDK1. As an additional regulation, due to the close relationship between the activation loop and the Z-/turn motif phosphorylation sites, it is possible that, in the absence of activation loop phosphorylation, the Z-/turn motif site may becomes dephosphorylated, and with the appropriate stimulation, could trigger the binding to PDK1, consecutively leading to the phosphorylation at the activation loop. Our model further suggests that after activation loop phosphorylation, the Z-/turn motif site would also become phosphorylated by a yet unidentified protein kinase; after Z-/turn motif phosphorylation, the Z-/turn motif phosphate would interact with the Z-/turn motif phosphate binding site and trigger the binding of the C-terminus of PRK2 to its own catalytic domain, stabilizing the active conformation of PRK2. At the same time, since most determinants for PDK1 binding are located within this C-terminus, its intra-molecular binding to the PRK2 catalytic domain would release the upstream kinase PDK1 by decreasing the avidity of the PRK2 C-terminus for PDK1.

This thesis illustrates the complex mechanism of regulation of AGC kinases with a focus on the molecular mechanisms that are involved in the control of PRK2 activation and interaction. As AGC kinases are found to play a crucial role in cancer development and as they are already a target in cancer treatment, finding out more about the regulation of their molecular mechanisms will hopefully specify the therapy of such diseases and increase their effectiveness.

While the drugs used today mostly target whole signalling pathways, characterization of special unique mechanisms of kinases will open the chance to gain a maximal success with

minimal side effects. An example for these special mechanisms is the role of the Z-/turn motif site of PRK2. We could show that this motif has a special function as a regulator of PRK2 activity, while we could not detect this effect in closely related kinases, pointing out the Z-/turn motif phosphorylation site as a possible pharmaceutical target in PRK2-related cancer subtypes.

Another potential target could be the Rho effector domain. According to the hypothesis that blocking this region would hinder Rho to release the intermolecular inhibition with a resulting decreased PRK2 activity in cells, compounds blocking the Rho effector domain may block PRK2 downstream signalling.

With respect to PRKs, especially prostate cancer came to the fore, as recent work by Metzger et al. showed that PRK1 and PRK2 are involved in the genesis of this type of cancer via activation of the androgen receptor. They could show that stimulation of the PRK signalling cascade results in a ligand-dependent superactivation of the AR that according to the current model of prostate cancer plays an important role in the development of this neoplasia. Normally, the AR is expressed in secretory epithelial cells and responds to androgens. Interestingly, although growth and survival of primary prostate cancer cells is critically dependent on androgens, reduced circulating androgen levels and even in the presence of AR antagonists, most prostate cancers recur and progress to a terminal stage [30].

PRKs as targets could open a new strategy in prostate cancer therapy, especially for prostate cancer specimen that are resistant against anti-androgens. A selective inhibition of the PRKs overactivating the AR could be a therapeutic route to fight the progress of such a disease.

The understanding of the mechanism of regulation of PRK2 suggests ways in which cancers could activate this signalling pathway. Since we showed both the Z-/turn motif and the N-terminus act as inhibitors for PRK2 activity, it can be imagined that mutations in this region could be responsible for the high PRK2 activity in different cancer specimens. It is also possible to imagine that PRK2 could be overactive in cancer cells due to loss of its whole N-terminus, for example because of a dysfunction of cleavage proteins or because of mutations. It would be interesting to evaluate if mutations on PRKs happen in cancers where PRK signalling is important. Such data may help us to further understand the molecular aspects of PRK regulation. Most importantly, getting deeper into the mechanisms of regulation opens a new field of an appropriate drug therapy adapted to the particular reason of enzyme dysfunction for personalized treatment.

7. References

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8. Publications/Acknowledgements

8.1. Publications

Dettori R, Sonzogni S, Meyer L, Lopez-Garcia LA, Morrice NA, Zeuzem S, Engel M, Piiper A, Neimanis S, Frodin M, Biondi RM (2009) Regulation of the interaction between protein kinase C-related protein kinase 2 (PRK2) and its upstream kinase, 3-phosphoinositide dependent protein kinase 1 (PDK1). J Biol Chem 284:30318-30327

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