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Regulation of the interaction between protein kinase C-related protein kinase 2 (PRK2) and its upstream kinase, 3-phosphoinositide-dependent protein kinase 1 (PDK1)

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Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
Ade motif	adenosine binding motif
ADP	Adenosine diphosphate
AGC	protein kinase A/protein kinase G/protein kinase C
AR	androgen receptor
ATP	Adenosine triphosphate
Akt/PKB	Akt/Protein kinase B
BAD	Bcl-XL/Bcl-2-associated death promoter
$CaCl_2$	Calcium chloride
cAMP	cyclic AMP
CE	Crude extract
CO_2	Carbon dioxide
CPA	Cyproterone acetate
Cre	Cre-recombinase
CT	C-terminal
CT-PRK2	C-terminal fragment of PRK2
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ES cells	Embryonic stem cells
FHL2	Four and a half LIM domains 2
FOXO	forkhead box O
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GLUT	Glucose transporter
GSK-3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HCV	Hepatitis C virus
HEK293 cells	Human embryonic kidney 293 cells

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER-2	Human epidermal growth factor (EGF) receptor 2
HM	Hydrophobic motif
HR	Homology region
HRP	Horseradisch Peroxidase-Labeled
Hsp	Heat shock protein
IEF	Isoelectric Focusing
IgG	Immunoglobulin G
INF-α	Interferon alpha
IRS	Insulin receptor substrate
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
Mck	Muscle creatine kinase
MEF	mouse embryonic fibroblast
MEKK	Mitogen-activated protein kinase/extracellular signal -regulated kinase kinase kinase
mLST8	mammalian lethal with SEC13 protein8
MR	mineralocorticoid receptor
MSK	Mitogen- and stress-activated protein kinase
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
Na_2HPO4	Disodium hydrogen phosphate
NaCl	Sodium chloride
Ni-NTA	Nickel-Nitrilotriacetic acid
NS5B	nonstructural protein 5B
PAGE	PolyAcrylamide Gel Electrophoresis
PAK	Protease Activated Kinase
PC-3 cells	$PTEN^{-/-}$ prostate cancer cells
PDK1	3-phosphoinositide-dependent protein kinase-1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIF	PDK1-interacting fragment
PKA	cyclic AMP dependent protein kinase
PKC	Protein kinase C

PKN	Protein kinase N
PRAS40	Proline-rich Akt substrate of 40 kDa
PRK	Protein kinase C-related kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
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
RBD	Rho binding domain of ROCK-1
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
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
SDS	Sodium dodecyl sulfate
SGK	Serum- and glucocorticoid-inducible protein kinase
SH2	Src homology 2
Sin1	stress-activated-protein-kinase-interacting protein 1
TAU-5	transactivation unit 5
TEMED	N,N,N',N'-Tetramethylethylendiamin
TIF-2	transcriptional intermediary factor 2
TNF	Tumor necrosis factor
TSC	Tuberous Sclerosis Complex protein
Tris-HCl	Tris- hydrochloric acid
UCN-01	7-hydroxystaurosporine
wt	wild type

1. Abstract

The AGC group of protein kinases includes the protein kinase B (PKB, also termed Akt), p70 ribosomal S6 kinase (p70 S6K), p90 ribosomal S6 kinase (RSK), serum and glucocorticoid-induced kinase (SGK) and protein kinase C (PKC) isoforms between others.

Previous publications reported that the AGC family members share a common main mechanism of activation based on three phosphorylation sites, which are located 1- in the activation loop in the kinase domain, 2- in the middle of a tail/linker region C-terminal to the kinase domain (termed the Z/Turn-motif phosphorylation site) and 3- within a hydrophobic motif (HM) at the end of the tail region, respectively.

The mentioned AGC kinases are substrates of the upstream protein kinase activator of the insulin signalling, termed the 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 itself is also a member of the AGC subfamily, but it is atypical since it does not possess a hydrophobic motif. Instead, in most cases, PDK1 interacts with the hydrophobic motif of its substrates through a site termed the PIF-binding pocket within the catalytic domain. This interaction leads to PDK1 activation, which consequently activates the interacting kinase through phosphorylation of its activation loop.

While the role of the hydrophobic motif and activation loop phosphorylation sites in the activation of diverse AGC protein kinases by PDK1 are well characterized, at the start of this thesis, the role of the Z/Turn-motif phosphorylation site was not known. Thus, we decided to evaluate, whether the Z/Turn-motif phosphorylation site was involved in the activity and in the regulation of the interaction between PRK2 and PKCζ with PDK1. Therefore, we mutated the Z/Turn-motif phosphorylation site in PRK2 and PKCζ and we studied the consequences of these mutations on the specific activity and on the interaction with PDK1.

For this purpose, we performed interaction assays and we realized that the Z/Turn-motif phosphorylation site played an essential role in mediating the interaction of PRK2 with PDK1. Actually, a mutation of this site and the resulting lack of phosphorylation at this position increased the binding of PRK2 to PDK1. In contrast, our experiments indicated that the Z/Turn-motif phosphorylation site does not affect the interaction between PKCζ and PDK1. Additionally, we studied the role of the Z/Turn-motif phosphorylation site in regulating the activity of PRK2 and could show that a lack of phosphorylation at this site significantly reduced PRK2 kinase activity *in*

vitro. Further attempts to clarify other aspects of the requirements for the interaction between PRK2 and PDK1 revealed that both a hydrophobic patch and a negatively charged patch, situated in between the hydrophobic motif and the Z/Turn-motif phosphorylation site of PRK2, are essential for the high affinity binding between PRK2 and PDK1. The achieved results are in accordance with a model that defines the role of the Z/Turn-motif phosphorylation site in regulating PRK2 activity in cells: First, a phosphorylation of the Z/Turn-motif phosphorylation site directly activates PRK2 and secondly, a phosphorylation at this position impairs the interaction between PRK2 and PDK1.

1. Zusammenfassung

Die Familie der AGC Proteinkinasen beinhaltet die Proteinkinase B (PKB, die auch als Akt bezeichnet wird), p70 ribosomale S6 Kinase (p70 S6K), p90 ribosomale S6 Kinase (RSK), Serum- und Glukokortikoid-induzierte Kinase (SGK) und Proteinkinase C (PKC) Isoformen.

Frühere Publikationen berichteten, dass sich die Mitglieder der AGC Familie einen gemeinsamen Hauptaktivierungsmechanismus teilen, der auf drei Phosphorylierungsstellen beruht, die sich 1- im Aktivierungsloop in der Kinasedomäne, 2- in der Mitte des zur Kinasedomäne C-terminal liegenden Endes/Binders (als Z/Turn-Motiv-Phosphorylierungsstelle bezeichnet) und 3- innerhalb des hydrophobischen Motivs (HM) am Schluss des Binders befinden.

Die erwähnten AGC Kinasen sind Substrate des vorgeschalteten Proteinkinaseaktivators des Insulinsignalweges, der als 3-Phosphoinositiden-abhängige Proteinkinase-1 (PDK1) bezeichnet wird. PDK1 selbst ist auch ein Mitglied der AGC Familie, aber es ist atypisch, da es kein hydrophobisches Motiv besitzt. Stattdessen interagiert PDK1 in den meisten Fällen mit dem hydrophobischen Motiv seiner Substrate über eine Seite innerhalb der katalytischen Domäne, die als PIF-Bindungstasche bezeichnet wird. Diese Wechselwirkung führt zur Aktivierung von PDK1, welches folglich die interagierende Kinase über die Phosphorylierung ihres Aktivierungsloops aktiviert.

Während die Aufgaben der Phosphorylierungsstellen des hydrophobischen Motivs sowie des Aktivierungsloops in der über PDK1 erfolgenden Aktivierung der verschiedenen AGC Kinasen gut charakterisiert worden sind, war die Aufgabe der C-terminal gelegenen Z/Turn-Motiv-Phosphorylierungsstelle am Anfang dieser Doktorarbeit nicht bekannt. Aus diesem Grund haben wir uns entschieden zu evaluieren, ob die Z/Turn-Motiv-Phosphorylierungsstelle in der Aktivierung und in der Regulierung der Wechselwirkung zwischen PRK2 und PKCζ mit PDK1 beteiligt war. Deswegen, haben wir die Z/Turn-Motiv-Phosphorylierungsstelle in PRK2 und PKCζ mutiert und die Folgen dieser Mutationen auf die spezifische Aktivität sowie auf die Wechselwirkung mit PDK1 untersucht.

Zu diesem Zwecke haben wir Interaktionsexperimente durchgeführt und wir haben festgestellt, dass die Z/Turn-Motiv-Phosphorylierungsstelle eine wesentliche Rolle in der Vermittlung der Interaktion zwischen PRK2 und PDK1 spielt. Tatsächlich, hat eine Mutation dieser Stelle und der daraus resultierende

Phosphorylierungsmangel an dieser Position die Bindung zwischen PRK2 und PDK1 gesteigert. Im Gegensatz dazu, haben unsere Experimente gezeigt, dass die Z/Turn-Motiv-Phosphorylierungsstelle die Wechselwirkung zwischen PKCζ und PDK1 nicht beeinflusst. Außerdem, haben wir die Rolle der Z/Turn-Motiv-Phosphorylierungsstelle in der Regulierung der Aktivität von PRK2 untersucht und wir zeigen, dass ein Phosphorylierungsmangel an dieser Stelle die *in vitro* Kinaseaktivität von PRK2 wesentlich senkt. Weitere Versuche zur Verdeutlichung anderer Aspekte der Bedingungen für die Interaktion zwischen PRK2 und PDK1 zeigten, dass sowohl eine hydrophobische Stelle als auch eine negativ geladene Stelle, die zwischen dem hydrophobischen Motiv und der Z/Turn-Motiv-Phosphorylierungsstelle von PRK2 liegen, unerlässlich sind für die hohe Bindungsaffinität zwischen PRK2 und PDK1. Die erzielten Ergebnisse sind in Übereinstimmung mit einem Modell, das die Rolle der Z/Turn-Motiv-Phosphorylierungsstelle in der Regulierung der Aktivität von PRK2 in Zellen definiert: Erstens, eine Phosphorylierung der Z/Turn-Motiv-Phosphorylierungsstelle aktiviert direkt PRK2 und zweitens, eine Phosphorylierung an dieser Stelle beeinträchtigt die Interaktion zwischen PRK2 und PDK1.

2. INTRODUCTION

2.1. PI3K signalling pathway and PDK1 in health, disease and therapy

The phosphoinositide 3-kinase (PI3K) signalling pathway plays a key role in the regulation of the insulin signalling pathway, cell growth and cell survival. Actually, it regulates diverse biological responses, including glucose metabolism, glycogen, lipid and protein synthesis, gene expression and cell growth/differentiation. For this reason, a misregulation in this pathway is implicated in the development of diabetes, many forms of human cancer and heart failure.

The key effector of the PI3K signalling pathway is the 3-phosphoinositide-dependent protein kinase-1 (PDK1) which is also involved in the phosphorylation of AGC kinase substrates from other signalling pathways.

2.1.1. The insulin signalling pathway

Insulin is a proteohormone with an anabolic effect on skeletal and heart muscle, adipose tissue and liver, which represent key insulin target organs involved in the insulin-mediated regulation of peripheral carbohydrate, lipid and protein metabolism.

An elevated total blood glucose concentration stimulates the secretion of insulin, which binds to its receptor on the plasma membrane. The insulin receptor is a transmembrane heterotetrameric glycoprotein with two extracellular identical α-subunits as insulin-binding sites, and two intracellular identical β-subunits containing a substrate binding site, an ATP- binding site and the tyrosine kinase or the autocatalytic domain, respectively (Löffler Petrides, 7. Auflage). The insulin binding leads to a conformational change of the α-subunits and thus to an abolition of their inhibiting effect on the tyrosine kinase located in the β-subunits. Upon ATP-binding, the tyrosine kinase is able to autophosphorylate certain tyrosine residues of the β-subunits (Löffler Petrides, 7. Auflage), leading to the recruitment and tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2) and their related family of substrate proteins. Then, the phosphorylated IRS proteins act as docking sites for several proteins with Src homology 2 (SH2) domains, such as the p85 regulatory subunit of the class I phosphoinositide 3-kinases (PI3Ks) [140].

Once PI3K is recruited to the membrane, the p110 catalytic subunit of PI3K phosphorylates the glycerophospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at the D-3 position of the inositol ring and converts it to the key

second messenger phosphatidylinositol 3,4,5-triphosphate PtdIns(3,4,5)P_3 (reviewed in [55]). The signal is turned off through the action of the SH2-containing inositol phosphatases (SHIP1 and SHIP2), which convert PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2, or through the action of the lipid phosphatase called Phosphatase and TENsin homologue deleted on chromosome TEN (PTEN), which converts PI(3,4,5)P_3 back to PtdIns(4,5)P_2. (reviewed in [116]).

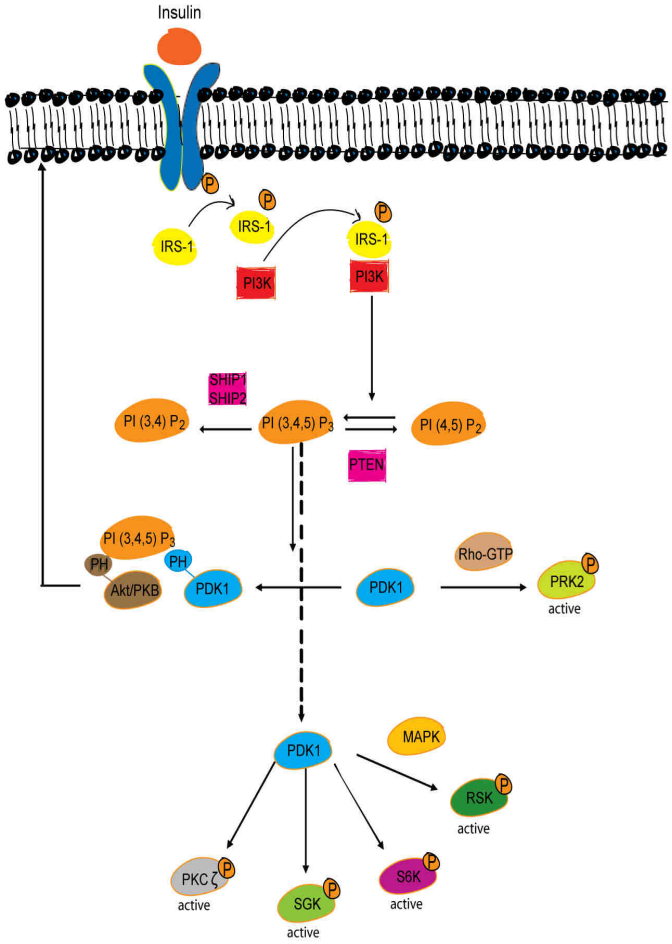

Fig.1. **Insulin/growth factor signalling pathway**. Insulin stimulation enables the tyrosine kinase in the β-subunits of the insulin receptor to autophosphorylate certain tyrosine residues upon ATP-binding, leading to the recruitment and tyrosine phosphorylation of the IRS-proteins and their related family of substrate proteins. Then, the phosphorylated IRS-proteins act as docking sites for the class I phosphoinositide 3-kinases (PI3Ks) which, once recruited to the plasma membrane, lead to the generation of PtdIns(3,4,5)P_3. These second messengers bind to the PH domain of Akt/PKB and PDK1 and promote the co-localization of both AGC kinases to the plasma membrane, where PDK1 is enabled to activate Akt/PKB. In addition, PDK1 activates also other members of the AGC subfamily upon binding to their hydrophobic motifs in a phosphoinositide-independent (conventional PKC isoforms) or phosphoinositide-dependent manner (PKCζ, S6K, SGK). In contrast, the interaction between the related PKC-kinase PRK2 and PDK1 occurs by a Rho-dependent mechanism, while RSK functions in mitogen-activated protein (MAP) kinase pathways.

In particular PtdIns(3,4,5)P_3, but also PtdIns(3,4)P_2, are involved in several cellular processes by interacting with AGC protein kinases containing a certain type of pleckstrin homology domain termed the PH domain, such as the phosphoinositide-

dependent protein kinase 1 (PDK1) and protein kinase B (PKB, also termed Akt) . The generation of the lipid second messengers PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 by PI3K leads to a co-translocation of PDK1 and Akt/PKB to the plasma membrane, enabling PDK1 to phosphorylate and thus activate Akt/PKB (reviewed in [116]).

In addition, PDK1 also activates other AGC kinases through phosphorylation of their activation loops, such as p90 ribosomal S6 kinase (RSK), p70 ribosomal S6 kinase (p70 S6K), serum and glucorticoid-induced kinase (SGK) and protein kinases C isoforms (PKC). In contrast to Akt/PKB, these AGC kinases do not possess a PH domain and their activation by PDK1 occurs in a phosphoinositide-independent manner *in vitro* through the binding of a C-terminal region, termed the " hydrophobic motif " (HM) of the substrates to a docking site in the small lobe of the PDK1 kinase domain, termed the "PIF-binding pocket". Finally, RSK functions in mitogen-activated protein (MAP) kinase pathways [82], whereas the related PKC kinase PRK2 functions in Rho-GTPase signalling [156] (Fig.1).

2.1.1.1. Regulation of the glucose transport

In humans and rodents, skeletal muscle accounts for 80% of insulin-stimulated glucose uptake, representing the most important tissue in the regulation of postprandial glucose homeostasis [10, 52].

The major insulin effects consist in an increased glucose uptake to reduce the total blood glucose concentration and in the stimulation of glucose utilization and storage through the regulation of a variety of specific pathways in the insulin key target organs (Löffler Petrides, 7. Auflage).

The insulin-mediated translocation of the glucose transporter GLUT4 from an intracellular storage site to the plasma membrane and the transverse (T)-tubules in muscle tissue occurs upon activation of PI3K and its downstream targets Akt/PKB, SGK1 and the atypical PKC isoforms (PKCζ and PKCλ) ([42, 48, 49, 99, 100, 106-108, 203, 213], reviewed in [97, 125]).

Interestingly, previous studies showed that acute hyperglycaemia results in increased PDK1 and PKCζ phosphorylation/activity , which occurs parallely in a PI3K-Akt/PKB-independent mechanism [182]. According to recent publications, the maintenance of normoglycaemia in the insulin-resistant state appears to be mediated especially by PKCζ. Actually, studies with rats receiving a continuous glucose infusion revealed that PKCζ, rather than Akt/PKB activity, seems to be regulated by changes in the extracellular glucose concentration, since 5 day of glucose infusion

increased PKCζ activity in the cytosol as well as the translocation of GLUT4 to the plasma membrane of skeletal muscle, thus elevating glucose uptake [209]. Remarkably, on day 15 of continuous glucose infusion, PKCζ has been described to translocate from the cytosol to the plasma membrane with a concomitant reduction of PDK1 activity. This resulted in an increased association between PKCζ and Akt/PKB and in a reduced interaction between PDK1 and Akt/PKB at the plasma membrane. The reduced activity of PKCζ and Akt/PKB and the resulting blunted insulin-mediated GLUT4 translocation eventually led to hyperglycaemia in rats with overt type 2 diabetes, thus underlining the fact that translocation of PKCζ in skeletal muscle appears to play a fundamental role in the formation of type 2 diabetes [209].

While the translocation of the glucose transporter GLUT4 to the membrane in adipose and muscle tissue is mediated by insulin upon activation of PI3K and its downstream targets, liver cells dispose of the insulin-independent glucose transporter GLUT-2, which is regulated by the extracellular glucose concentration (Löffler Petrides, 7. Auflage).

2.1.1.2. Role of the PDK1 substrate Akt/PKB in glycogen synthesis

The activation of Akt/PKB by PDK1 contributes to the regulation of glycogen synthesis. Actually, activated Akt/PKB inhibits glycogen synthase kinase-3 (GSK-3) through phosphorylation, thus preventing GSK-3 from phosphorylating and inhibiting its substrate glycogen synthase especially in muscle and hepatic tissue. As a consequence, glucose storage as glycogen through glycogen synthesis can occur (reviewed in [125]). Besides, the inhibition of GSK3 by Akt/PKB also results in an increased lipid production (reviewed in [125]).

Eventually, Akt/PKB is implicated in the regulation of glucose metabolism through stimulation of the expression of glycolytic enzymes (reviewed in [125]).

2.1.2. Role of the PI3K-PDK1-Akt/PKB-signalling pathway in cell survival

The PI3K-PDK1-Akt/PKB-signalling pathway plays a crucial role in the regulation of cancer cell growth, invasion, survival and tumor progression. Notably, this pathway has been described to be activated in several forms of human cancer [64], in which Akt/PKB directly phosphorylates and inactivates downstream proapoptotic proteins and processes, such as the Bcl-2 family member BAD [50, 210], Forkhead family of transcription factors (FKHRLI or Foxo3a) [19], p70S6K [79], glycogen synthase kinase 3 (GSK3) [46] and caspase-9 [32]. The predominant mechanism through

which Akt/PKB promotes cell growth seems to be through activation of mTOR complex 1 (mTORC1 or the mTOR-raptor complex that comprises mTOR, Raptor and mLST8 and is inhibited by rapamycin [65, 122, 191]), that is involved in the regulation of Akt/PKB mediated cell proliferation and oncogenic transformation (reviewed in [125]). The activation of mTORC1 is mediated by insulin and growth factors in a PI3K-controlled pathway, that comprises Akt/PKB-mediated phosphorylation of PRAS40 (proline-rich Akt substrate of 40 kDa) and tuberous sclerosis complex-2 protein as well as activation of Rheb GTPase [83, 171]. Furthermore, stimulation of mTORC1 occurs through a pathway that implies bidirectional transport of amino acids [148] and the Rag GTPase [101, 170] .

The Akt/PKB family of Ser/Thr protein kinases consists of three members, namely Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ, which possess a high homology (>85%) in their sequence and are largely expressed in several tissues of the human body. Their activation is mediated by a variety of growth factors [43, 50] and overexpression of these Akt/PKB isoforms, which have been described to have isoform-specific functions [193], is tissue-specific [17, 36, 143, 181]. Altogether, Akt/PKB activation is induced by signals that promote cell survival and shelter cells from apoptotic signals.

An inverse correlation between apoptosis and constitutively active Akt/PKB level has been established, indicating that the higher the level of Akt/PKB in the cell, the less the tendency to cell death [98]. Actually, Akt/PKB activation by various oncogenes enables cell proliferation under conditions that normally inhibit cell growth [61, 211].

PTEN is a tumor suppressor gene which is involved in the control of cell proliferation, differentiation and apoptosis. As a PtdIns(3,4,5)P_3-specific phosphatase, it functions to downregulate PI3K activity by removing the 3' phosphate of PtdIns(3,4,5)P_3 and, as a result, generating PtdIns(4,5)P_2. Inactivation of this crucial phosphatase led to elevated levels of phosphoinositides and to an excessive Akt/PKB activity in several types of tumors [98].

2.1.3. PDK1 and animal models

2.1.3.1. Role of PDK1 in insulin signalling in vivo

Several studies showed that insulin exhibits a regulatory function in the hepatic glucose homoeostasis through a pathway involving the insulin receptor, IRS2, PI3K,

PDK1 and PKBβ [135]. In this regard, mice lacking PDK1 exclusively in hepatic cells (termed L-PDK1$^{-/-}$ mice) showed a failed stimulation of the activity of the PDK1 substrate Akt/PKB upon insulin treatment [133, 135, 199]. Besides, the hepatic glycogen content as well as the hepatic protein production were considerably reduced [135]. Accurate analysis revealed that the absence of PDK1 in the liver in the L-PDK1$^{-/-}$ mice did not affect the blood glucose concentrations, the plasma insulin levels, the hepatic cell volume, the total islet volume and islet morphology [135].

The results achieved in this study [135] indicated that the hepatic PI3K/PDK1/AGC kinase pathway did not appear to be rate-limiting for glucose uptake and storage under normal feeding conditions, while it seemed to be crucial for the distribution of the blood glucose upon injection of a large amount of glucose [135].

In contrast to other mouse strains displaying mutations in the insulin-signalling pathway, all of the L-PDK1$^{-/-}$mice developed liver failure and died at a young age [135].

Further studies focused on the effects of deleting the PDK1 gene specifically in pancreatic β cells (βPDK1$^{-/-}$mice) [80]. The results indicated that PDK1 plays a significant role in regulating the distribution of insulin. Remarkably, PDK1 deletion led to reduced insulin levels and increased blood glucose levels of the βPDK1$^{-/-}$mice which resulted in the death of all male βPDK1$^{-/-}$mice because of uncontrolled diabetes [80].

As expected, the phosphorylation of the PDK1 downstream substrates Akt/PKB and p70 S6 kinase in the βPDK1$^{-/-}$mice was significantly inhibited. Since ribosomal protein S6 plays a fundamental role in protein synthesis, its reduced activity in βPDK1$^{-/-}$ resulted in a reduced β-cells size. Notably, mice with an ablated p70 S6 kinase 1 showed a reduced β cell size rather than a lower β cell number [159].

Recently, homozygous PDK1$^{K465E/K465E}$ mice have been generated by mutating Lys465 in the PH domain of PDK1 to a Glu residue [13]. Since Lys465 plays a key role in the interactions with the D3 and D4 phosphates of PtdIns(3,4,5)P3, its mutation abolished the binding of PDK1 to phosphoinositides and its localization at the plasma membrane [134]. Interestingly, the mentioned mutation led to a significant glucose and insulin intolerance despite normal plasma glucose levels in the PDK1$^{K465E/K465E}$mice. The results presented in this study [13] suggest that PDK1$^{K465E/K465E}$ mice compensate for insulin resistance in the first period through

increased insulin production as well as, in the case of young animals, enhanced islet-cell mass [13]. However, when islet cells are exhausted and not capable anymore of producing enough insulin to assure normal glucose homeostasis, levels of blood glucose are elevated. In contrast, in the case of older PDK1$^{K465E/K465E}$ mice, the islet volume was reduced, indicating an initiating failure of the islet cells. Nevertheless, older PDK1 $^{K465E/K465E}$mice still possessed increased blood insulin and normal blood glucose levels [13].

Furthermore, this study [13] underlined the fact that the PI3-kinase/PDK1/PKB/mTORC1/S6K signalling pathway may play a decisive role in the regulation of cell size. Actually, male and female PDK1$^{K465E/K465E}$ mice were ~35% smaller from birth than control PDK1+/+ littermate. In addition, in comparison to the according organ size in the wild-type littermate mice, the brain, the spleen and the testes of PDK1 $^{K465E/\ K465E}$ mice were smaller [13]. Remarkably, the size of the kidneys was not significantly affected in the PDK1 $^{K465E/\ K465E}$mice.

Notably, this study constituted the first genetic evidence in an animal model to highlight the fact that the interaction of PDK1 with phosphoinositides is required for optimal activation of Akt/PKB, since the insulin-stimulated activation of Akt/PKBα and Akt/PKBβ was significantly diminished, but not abolished, in the PDK1$^{K465E/\ K465E}$mice. As a consequence, the reduction of Akt/PKB activation in PDK1$^{K465E/K465E}$ mice led to a decrease in insulin-mediated activation of mTORC1 and thus to a reduced phosphorylation of S6K1 at Thr389 [13]. Remarkably, the phosphorylation of Akt/PKB substrates, such as PRAS40, GSK3 and FOXO1 in insulin-stimulated PDK1 $^{K465E/K465E}$ mice was either not markedly inhibited or only modestly decreased, still allowing downstream signalling [13].

2.1.3.2. Role of PDK1 in tumorigenesis in vivo

Recent work evaluated whether suppression of PDK1 expression impairs tumorigenesis in PTEN$^{+/-}$mice [11].

Since PDK1$^{-/-}$mice died before embryonic day E9.5, hypomorphic mice (PDK1$^{-/fl}$ PTEN$^{+/-}$mice) with markedly reduced PDK1 activity and protein levels in all tissues and littermate control mice (PDK1$^{+/fl}$ PTEN$^{+/-}$mice) were generated [11].

Remarkably, about ¾ of PDK1$^{+/fl}$ PTEN $^{+/-}$mice developed several types of tumors [35, 162, 203], while the proportion of PDK1$^{-/fl}$PTEN$^{+/-}$mice that developed tumors was 2.5- to 7.5-fold lower [11]. Accurate analysis revealed that the tumors of both genotypes were similar and displayed activated forms of Akt/PKB and S6K,

which resulted in an increased cell proliferation within the tumor [11]. Importantly, the reduced PDK1 activity by 80-90% in the employed PDK1 hypomorphic mice could be responsible for a protection or a delay in the onset of tumorigenesis in these animals [11].

Taken together, this study emphasizes the key role of PDK1 in mediating tumor development in tissue lacking PTEN [11]. Thus, PDK1 represents a promising target in the fight against cancers with increased levels of PtdIns(3,4,5)P_3 and/or Akt/PKB and S6K activity [11].

2.1.3.3. Role of PDK1 in heart failure in vivo

Based on the finding that a hyperactivated PDK1/AGC kinase signalling pathway is involved in the occurance of cardiac hypertrophy [185] and on the observation that mice lacking PDK1 died at day E9.5 of embryogenesis because of diverse abnormalities [111], mice lacking PDK1 exclusively in skeletal and heart muscle (mPDK1$^{-/-}$mice) were generated to better clarify the role of PDK1 after birth/development [133].

Unlike other genetic models of the PI3K pathway, all mPDK1$^{-/-}$mice between 5 and 11 weeks of age suddenly died because of the development of cardiomyopathy and of heart failure. This result was presumably due to the loss of PDK1 in mPDK1$^{-/-}$ mice, thus leading to a completely abolished activity of the PDK1 substrates S6K and Akt/PKB [133]. In contrast, PDK1 hypomorphic mice developed markedly hypotrophic hearts, but not heart failure [111].

Further analysis detected an important reduction of the heart muscle mass and of the volume of cardiomyocytes of mPDK1$^{-/-}$hearts in comparison with mPDK1$^{+/+}$mice, while the number of cardiomyocytes in both genotypes was similar, underlining the crucial role of the PI3K/PDK1/AGC kinase pathway in the regulation of cell size in all tissues (reviewed in [110], [15, 41, 45, 176, 177]).

Altogether, it can be concluded that PDK1 regulates heart growth and viability and is involved in cardioprotective signalling [133].

In addition to the knock-out mutations described above, a knock-in mutation was performed by mutating the residue Leu-155 of the PIF-pocket of PDK1 to Glu (this mutation will be described in 2.2.2.2.), thus resulting in a disruption of the PIF-binding pocket of PDK1. In this way, PDK1$^{fl/L155E}$MckCre$^{+/-}$ mice were generated, in which PDK1 [L155E] was expressed rather than PDK1 wild type specifically in skeletal and heart muscle [12]. As expected, insulin stimulation of the skeletal and

heart muscle of mice expressing PDK1 wild type or the mutant PDK1[L155E] induced similar phosphorylation and activation of Akt/PKB and Akt/PKB substrates, while S6K was not phosphorylated in the muscle tissue of mice expressing PDK1[L155E] [12].

As described above, a lack of PDK1 in heart muscle in conditional knock-out mice, in which neither S6K nor Akt/PKB were activated, led to the death of these mice at 5-11 weeks of age because of heart failure, after having developed a dilated cardiomyopathy [133]. In this study the mice expressing PDK1[L155E] survived normally without developing any symptoms of heart failure [117], indicating that the lack of Akt/PKB activity represents the major reason for the appearance of heart failure in mice lacking PDK1 in cardiac muscle [12].

2.1.4. Therapeutic strategies for the inhibition of the PI3K signalling pathway

As already mentioned, the PI3K-PDK1-Akt/PKB-signalling pathway plays a crucial role in the regulation of cancer cell growth, invasion, survival and tumor progression. For this reason, it represents a promising target for the development of anticancer drugs.

Previous work described three potent and selective inhibitors of PDK1 termed BX-795, BX-912 and BX-320 to act as competitive inhibitors of PDK1 activity by binding to the ATP-binding site of PDK1 [64]. Consistent with this, BX-795, BX-912 and BX-320 have been described to block efficiently the phosphorylation of PDK1 substrates, such as Akt/PKB, S6K1 and PKCδ, as well as PDK1 phosphorylation at the activation loop residue Ser421 in PC-3 cells.

Recently, the effects of BX-795 on the cell cycle have been further elucidated in *in vivo* experiments with PDK1 $^{+/+}$ and PDK1 $^{-/-}$ ES cells. The experiments presented revealed that, in contrast to the results mentioned above, not only PDK1, but also additional several protein kinases were strongly inhibited by 1µM BX-795 [188]. In order to study the effects of BX-795 on PDK1 activity in ES cells more specifically, PDK1 with an enlarged ATP binding site has been developed by mutating the large hydrophobic amino acid L159 in PDK1, referred to as the gatekeeper residue, to glycine (L159G). The performed experiments showed that BX-795 inhibits not only PDK1, but also Cdk1, Cdk2, and Aurora A, B and C with similar potencies [188] [6]. Thus, the G2/M arrest observed in these essays and at least part of the antitumor activity described in allograft models, is probably achieved either by Aurora/Cdk inhibition, combined PDK1/Aurora/Cdk inhibition, or by another target not yet studied. Interestingly, this study revealed that specific inhibition of PDK1 does not

affect intrinsic cell viability when cells are grown in high serum, but it importantly sensitizes cells to apoptosis caused by cellular stress. Altogether, it has been demonstrated that the lack of PDK1 in cells inhibits tumor formation and that inhibition of PDK1 activity results in sensitization to cellular stresses and decreased tumor formation [188]

Further studies showed that the BX-compounds inhibited tumor cell growth and promoted apoptosis through caspase-3/7 activation of a considerable number of tumor cell lines. Eventually, the BX compounds significantly inhibited anchorage-dependent growth of tumor cell lines and showed efficacy in an *in vivo* tumor model [64]. Another promising anticancer drug is 7-hydroxystaurosporine (UCN-01) which has been shown to inhibit directly PDK1 *in vitro* and *in vivo* [1, 175].

Trastuzumab (Herceptin) is an anti-HER2/neu receptor monoclonal antibody which binds to the extracellular domain of the human epidermal growth factor (EGF) receptor 2 (HER-2) tyrosine kinase receptor [34], thus, upstream of PI3K and the PDK1 signalling. As a humanized $IgG_{1,}$ it binds to Fcγ receptor III (RIII) and mediates potently antibody-dependent, cell-mediated cytotoxicity (ADCC) [189]. The substantially increased expression of HER2 on cancer cells versus normal epithelial tissues allows selective targeting of malignant cells [113].
Previous publications showed that trastuzumab is capable of enabling PTEN to inhibit Akt/PKB and to induce growth arrest [142]. Interestingly, the treatment of HER-2 overexpressing breast cancer cells with anti-HER2- antibodies inhibited HER2-HER3 association and, since HER3 contains several consensus binding sites for the p85 PI3K subunit, this inhibition prevented the activation of the PI3K-Akt/PKB signalling [84, 114, 201], resulting in decreased enzymatic activities of Akt/PKB and PDK1 [113]. Indeed, Trastuzumab can disrupt the ligand-independent HER2/HER3/PI3K complex in both trastuzumab- sensitive and -insensitive cells [189]. Thus, the enhanced sensitivity to docetaxel and other cytotoxic drugs in breast cancer therapy could be due to the interruption of the PI3K-Akt/PKB pathway.

Previous studies reported that PTEN loss, which is commonly observed in breast tumors, causes trastuzumab resistance by activating the PI3K/AKT pathway [142, 189]. Consistent with this, trastuzumab-resistant cell lines have been shown to usually contain activating ‘‘hot spot’’ PI3K mutations or being PTEN null. Interestingly, recent work has demonstrated that trastuzumab-resistant, HER2-amplified breast cancer cells harboring PI3K mutations are sensitive to GDC-0941, a

highly specific and efficacious PI3K inhibitor in combination with trastuzumab as well as in the treatment of trastuzumab-resistant cells and tumors [189]. Actually, trastuzumab and GDC-0941 inhibit synergistically proliferation and cell viability in trastuzumab-sensitive cells and the combination of GDC 0941 with trastuzumab has been shown to significantly reduce the GDC-0941 concentration that is necessary to achieve the threshold of caspase activation and apoptosis [189].

Furthermore, recent studies reported that trastuzumab induces radiosensitization of HER-2 overexpressing breast cancer cell lines through the PI3K-Akt/PKB signalling pathway [120]. The ability of HER2-antibody to downregulate a considerable number of genes especially involved in cell cycle, cell growth/maintainance and DNA repair/replication has been linked, at least in part, to the inhibition of the PI3K-Akt/PKB signalling [113].

2.2. PDK1

PDK1 plays a central role in the activation of the AGC subfamily of protein kinases. PDK1 is a 63kDa serine/threonine kinase consisting of an N-terminal catalytic domain and a non-catalytic C-terminal PH-domain [3, 183]. The PH-domain binds with high affinity to the second messengers PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, emphasizing the fact that PDK1 can be regulated by PI3K. The gene coding for human PDK1 is located in chromosome 16p 13.3 [3, 183] .

PDK1 itself is a member of the AGC kinase family, but is atypical since it does not possess a region located C-terminal to the catalytic domain termed the hydrophobic motif. Instead, PDK1 possesses an unoccupied PIF-binding pocket on the N-terminal smaller lobe of its kinase domain, in which the residues including Lys-115, Leu-155, Ile-119 and Gln-150 form a hydrophobic pocket that enables PDK1 to interact with the hydrophobic motif of its AGC substrates [22, 24].

Further work described a phosphate-binding site located next to the hydrophobic pocket of PDK1, which recognizes the phosphorylated serine/threonine residue in the hydrophobic motif of PDK1 substrates. Actually, the obtained results suggest that PDK1 uses Arg131 in the C-helix and the N-terminal basic residues Lys76 and Gln150 in order to identify phosphoserine/phosphothreonine or mimicking acidic residues in the hydrophobic motif of its substrates [25, 70].

As typical among AGC subfamily members, PDK1 activation requires phosphorylation of its activation loop residue Ser-241. Remarkably, PDK1 is presumably able to autophosphorylate at this residue *in vivo*, so that this AGC kinase

is constitutively phosphorylated at Ser-241 [198]. Structural analysis of the PDK1 kinase domain showed that the phosphorylated Ser-241 residue coordinates and aligns crucial catalytic motifs, like the αC-helix of the N-terminal lobe [25]. The position and/or formation of the αC-helix play a decisive role in the regulation of most kinases, since the αC-helix has been described to contribute important residues to coordinating ATP as well as hydrophobic and phosphate binding residues to the hydrophobic motif pocket [25]. This is in agreement with recent studies of our laboratory which showed that the interaction of the C-terminal region of PRK2 (PIFtide) or small molecule compounds with the hydrophobic motif/PIF-binding pocket of PDK1 leads to the stabilization of the αC helix and encourages allosteric effects on the ATP binding site, hence activating PDK1 [86].

Previous studies suggested that the interaction of the phosphorylated hydrophobic motif of diverse AGC kinases with the PIF-binding pocket of PDK1 induces a conformational change of PDK1, thus stimulating PDK1 catalytic activity. This model suggests that the hydrophobic motif both recruits and activates PDK1 [21, 22, 71]. In this regard, it has been demonstrated that the interaction of PDK1 with a polypeptide comprising the hydrophobic motif of PRK2 (the PDK1-interacting fragment of PRK2 (PIF)) increased ~4 fold the rate at which PDK1 phosphorylated its substrate T308tide peptide (a peptide substrate that encompasses the activation loop of PKBα) [22].

2.2.1. Interaction of PDK1 with substrates

The activation of many members of the AGC family of protein kinases by PDK1 is stimulated by insulin and growth factors, which promote the interaction of the AGC kinases with PDK1, rather than directly activating PDK1 (reviewed in [134]).

The AGC protein kinases are activated through phosphorylation of a serine/threonine residue in the hydrophobic motif and phosphorylation of a serine/threonine residue in the activation loop within the kinase domain. PDK1 phosphorylates the activation loop of Akt/PKB [3, 184], S6K [4, 53], RSK [94, 163], SGK [104, 157], conventional and novel PKC isoforms [59, 112], atypical PKC isoforms [37, 57, 112] and PRK [67]. The phosphorylatable Ser/Thr residue in the hydrophobic motif (encompassing the sequence Phe-Xaa-Xaa-Phe-*Ser/Thr*-Phe/Tyr) is surrounded by three aromatic residues and its phosphorylation or a phosphate-mimicking acidic residue at its place are required for an efficient interaction of S6K, SGK, RSK, but not Akt/PKB, with PDK1 [7, 24, 71].

As already mentioned, a phosphate-binding site next to the hydrophobic pocket of PDK1 functions to recognize the phosphorylated serine/threonine residue in the hydrophobic motif of PDK1 substrates. Importantly, sequence alignments and structural studies suggest that AGC subfamily members possess equivalent phosphate-binding and hydrophobic pockets in the small lobe of their kinase domains [70, 134]. The sites equivalent to the PIF-binding pocket and equivalent to the phosphate-binding pocket play a crucial role in the regulation of intramolecular as well as of intermolecular activation steps of a number of AGC kinases [70].

Further analysis demonstrated that the intramolecular interaction of the phosphorylated hydrophobic motif with its phosphate-binding pocket protects the phosphate from dephosphorylation by phosphatases and, additionally, it stimulates catalytic activity of the growth factor-activated AGC kinases. In all AGC kinases studied up to now, phosphorylation of the activation loop is required for activity. However, the phosphorylated hydrophobic motif is not capable of activating the kinase on its own. Nevertheless, a synergistic stimulation of catalytic activity results from the phosphorylated form of both the hydrophobic motif and the activation loop, as both phosphates are thought to stabilize the kinase domain in a closed and active conformation via the αC-helix [70, 81]. Thus, crystallography data shows that the regulatory PIF-pocket provides a structural link between the hydrophobic and phosphate-binding pockets and the phosphoserine in the activation loop. In agreement with this, the binding of polypeptides or small compounds to the PIF-binding pocket stabilizes the αC-helix [86].

Based on these findings, the following mechanism of activation of the AGC kinases by PDK1 has been suggested: in the inactive conformation, the unphosphorylated hydrophobic motif of the AGC kinase substrate is exposed. Upon phosphorylation of the hydrophobic motif phosphorylation site, the hydrophobic motif interacts with PDK1, which then phosphorylates the substrate at the activation loop. This results in the stabilization of the αC-helix and the formation of the hydrophobic and phosphate-binding-pockets in the kinase domain of the AGC kinase substrate, which interact with their own phosphorylated hydrophobic motif upon releasing PDK1 (reviewed in [21, 134]). This model would imply that PDK1 only detects inactive conformations of its substrates, since the phosphorylated hydrophobic motif of the active kinase displays a high affinity for its own hydrophobic pocket in order to achieve a stable conformation, not being available for interaction with PDK1 anymore

(reviewed in [21]). This intramolecular interaction, in synergy with the phosphorylation of the activation loop, significantly promotes kinase activity [70].

Recent work suggested that PDK1 on its own may not be capable of properly binding the adenine ring and efficiently transferring the phosphates of ATP to a protein substrate [166]. The hypothesis presented suggests that an active ATP binding pocket can only be achieved upon binding of the Ade motif of a substrate kinase to the N-lobe of PDK1, thus increasing the catalytical efficiency of PDK1. The Ade motif corresponds to C-terminal PDK1-interacting residues in the active site that normally contributes intramolecularly to the ATP-binding pocket in AGC kinases [166]. If this was correct, then the docking of the C-terminal Ade motif from the substrate would contribute to the binding of ATP and to the positioning of the glycine rich loop for catalysis, suggesting that the C-terminus of AGC kinases has both a *cis* and *trans* function [166].

2.2.2. PDK1 substrates

2.2.2.1. Akt/PKB

All Akt/PKB isoforms contain an N-terminal PH domain followed by a kinase catalytic domain and a C-terminal tail containing the hydrophobic motif (reviewed in [116]).

In contrast to other AGC subfamily members, the interaction between Akt/PKB and PDK1 is not regulated by the hydrophobic motif of Akt/PKB and the PIF-binding pocket of PDK1. The interaction between Akt/PKB and PDK1 is rather regulated by the binding of the two AGC kinases to PtdIns(3,4,5)P_3/PtdIns(3,4)P_2. This interaction leads to the co-localization of both AGC kinases to the plasma membrane, where PDK1 is enabled to activate Akt/PKB through phosphorylation of the residue Thr308 [28, 116, 173]. Further experiments indicated that the translocation of Akt/PKB to the plasma membrane could be sufficient for maximal activation of the kinase through phosphorylation of Thr308 and Ser473 (reviewed in [116]). The binding of PtdIns(3,4,5)P_3 to Akt/PKB does not lead to the activation, but rather to a conformational change of Akt/PKB, which increases the rate at which the kinase is phosphorylated by PDK1 (reviewed in [134]).

Recent publications reported that the residue Ser473 of Akt/PKB, which is located in the hydrophobic motif encompassing the sequence Phe-Xaa-Xaa-Phe-*Ser/Thr*-Phe/Tyr [116], is likely to be phosphorylated in a mTOR complex 2 (mTORC2) dependent manner *in vivo*. mTORC2 is composed of mTOR, Rictor, Sin1,

mLST8 as well as Protor and is insensitive to acute rapamycin treatment [35, 38, 69, 77, 91, 92, 172]. Besides, Sin1 has been shown to be required for the assembly of mTORC2 and for the phosphorylation of the hydrophobic motif site of Akt/PKB [69]. The activity of mTORC2 has been reported to be regulated by PI3K, but to be insensitive to amino acids or energy stress [75].

Interestingly, previous studies reported that Akt/PKB is still activated in a significant manner in mTORC2-deficient cells [77, 91, 178], since it is still phosphorylated at Thr308 by PDK1 in a mTORC2-independent way. Furthermore, the loss of crucial mTORC2 subunits in Sin1 [91]- or mLST8 [77]-deficient MEFs did not significantly affect the phosphorylation of Akt/PKB substrates such as GSK3 and TSC2. However, a significant reduction of the phosphorylation of FOXO (forkhead box O) 1 (Thr24) and FOXO3a (Thr32) in cells lacking mTORC2 activity [77, 91] has been interpreted as related to a different substrate specificity of Akt/PKB phosphorylated at Thr308 in comparison to Akt/PKB phosphorylated at both Ser473 and Thr308 [91]. An additional explanation consists in the fact that inhibition of FOXO phosphorylation is due to the lack of SGK activity in mTORC2-deficient cells [74], as SGK isoforms have been previously shown to be able to phosphorylate FOXO efficiently [30, 192].

Akt/PKB has been found to activate mTOR complex 1 (mTORC1 or the mTOR-raptor complex) through phosphorylation and thus inactivation of the Tuberous Sclerosis Complex protein 2 (TSC2) within the TSC1-TSC2 complex. TSC2 is a GTPase-activating protein and its inactivation leads to an increase in GTP bound Rheb. Rheb-GTP activates mTORC1, which is able to phosphorylate downstream targets such as the residue Thr389 in the hydrophobic motif of S6K1 (reviewed in [125]).

In response to tyrosine phosphorylation, PDK1 re-localizes, while the activated Akt/PKB moves to the cytoplasm and nucleus, where it phosphorylates several downstream targets. The very rapid activation of Akt/PKB as well as of PI3K in cells could be due to the dependence of their activation on the formation of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2. In contrast, the activation of other AGC kinases like S6K1 and SGK1 occurs more slowly. This temporary difference results from the time required by PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 to activate the S6K1/SGK1 hydrophobic motif kinase(s) which are most probably rate limiting for the activation of S6K1 and SGK1 *in vivo* [24].

2.2.2.2. S6K and SGK

The AGC subfamily members S6K1 and SGK1 are activated *in vivo* through phosphorylation of the residues equivalent to Thr308 (Thr252 in S6K1 and Thr256 in SGK1) and Ser473 (Thr412 in S6K1 and Ser422 in SGK1) of Akt/PKB. Although the phosphorylation of both AGC kinases at their activation loops and hydrophobic motifs depends on PI3K activation *in vivo*, it is still not clear how PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 are involved in the regulation of S6K1 and SGK1 activation. As both AGC kinases, in contrast to Akt/PKB and PDK1, don't possess a PH domain, they do not interact with PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 is not required for their *in vitro* phosphorylation by PDK1.

The phosphorylation of the Ser/Thr residue in the hydrophobic motifs of S6K and SGK significantly enhances the interaction of both kinases with PDK1 [197]. Recent studies demonstrated that the hydrophobic motif of SGK1 is phosphorylated by activated mTORC2 [74]. S6K activation is also not only related to the phosphorylation of its activation loop and hydrophobic motif, but also to the additional phosphorylation of the C-terminal residues Ser/Thr-Pro, which is regulated by mTOR [90] and occurs independently of PDK1 [129].

The interaction of PDK1 with the hydrophobic motif of SGK1 and the resulting phosphorylation of SGK1 at its activation loop residue by PDK1 does not require the binding of phosphoinositide to PDK1 [23]. In agreement with this, the binding of PtdIns(3,4,5)P_3 to PDK1 is not necessary for the activation of SGK1 *in vivo* [13].

Biochemical work first showed that SGK and S6K required binding to the PIF-binding pocket on PDK1 for their phosphorylation and activation in vitro [24]. The essential role of the PDK1 PIF-binding pocket in the activation of S6K and SGK *in vivo* was later elegantly confirmed by performing a knock-in mutation in embryonic stem (ES) cells in order to disrupt the PIF-pocket. The residue Leu155 was mutated to glutamate in both copies of the endogenous PDK1 gene, generating the PDK1 construct PDK1$^{155E/155E}$. The obtained data showed that, even if the PDK1 mutant was fully active in the PDK1$^{155E/155E}$ knock-in cells, it failed to activate S6K1 and SGK1, confirming that the activation of these AGC kinases is dependent on their interaction with an intact PIF-pocket [39].

Similarly, biochemical and crystallography work first characterized the requirement of Arg131, as part of the PIF-pocket associated phosphate binding site of PDK1, on the phosphorylation of S6K and SGK in vitro [25, 70]. Further work

generated PDK1$^{131M/131M}$ knockin ES cells in which the residue Arg131, that is located in the phosphate-binding pocket of PDK1, was replaced by a Met residue [40]. This mutation has been previously reported to inhibit the interaction of PDK1 with a phosphopeptide encompassing the hydrophobic motif of RSK [70]. Again, the results from this study [40] corroborated that the phosphate-binding pocket of PDK1 plays a crucial role in allowing the maximal activation of S6K and SGK by PDK1 [39].

2.2.2.3. The PKC family

The PKC family is divided into three major classes of PKC isoenzymes: the conventional PKCs (α, βII and the alternatively spliced βI, and γ), the novel PKCs (δ, ε, η/L and θ) and the atypical PKCs (ζ, ι/λ) [147]. Similarly to PKA and PKB/Akt, all PKC kinases have a conserved kinase core and a C-terminal extension with two conserved phosphorylation sites: the turn motif and the hydrophobic motif.

The main difference among the PKC isoenzymes consists in the composition of domains and membrane targeting modules in the regulatory N-terminal moiety. All isoenzymes have an autoinhibitory “pseudosubstrate” sequence N-terminal to a C1 domain. The C1 domain is the diacylglycerol sensor and the C2 domain is the Ca^{2+}sensor. While conventional PKCs respond to diacylglycerol and Ca^{2+} signals, novel PKCs bind to diacylglycerol, but not to Ca^{2+} signals. In contrast, atypical PKCs, which comprise PKCζ, respond to neither diacylglycerol nor Ca^{2+} [147].

In conventional PKC isoforms, the binding of the pseudosubstrate sequence to the substrate-binding cavity keeps PKC in an inactive conformation. The generation of diacylglycerol and Ca^{2+} and the resulting engagement of the C1 and the C2 domains on the membrane, leads to the recruitment of PKC to the membrane. This membrane interaction provides the energy required for the release of the pseudosubstrate from the substrate-binding cavity, rendering PKC available for substrate binding and phosphorylation [58, 146].

Previous work showed that the catalytic activities and signal transduction functions of PKCα, PKCε and PRK1 are regulated in a decisive way by their extreme C-terminal tail [121].

2.2.2.3.1. Interaction of the PKC isoforms with PDK1

PDK1 preferentially interacts *in vitro* with the unphosphorylated hydrophobic motif of newly synthesized PKC in a phosphoinositide-independent manner [180]. This model is supported by further experiments with PDK1$^{131M/131M}$-knockin ES cells, in which the

residue Arg131 in the phosphate-binding pocket of PDK1 was mutated to Met, or PDK1$^{L155E/L155E}$ knock-in cells with a disrupted hydrophobic pocket [40]. The achieved results underlined the fact that the hydrophobic pocket rather than the phosphate-binding pocket of PDK1 plays a key role in enabling PDK1 to phosphorylate the interacting conventional or novel PKC kinase [40].

The phosphorylation of PKC at the activation loop has been shown to be necessary for PKC kinase stability and activity (reviewed in [146]). Upon phosphorylation of its substrate at the activation loop [73], PDK1 is released from the C-terminal docking site of PKC. Phosphorylation by PDK1 has been described to be rate-limiting in the maturation of PKC, as it allows the phosphorylation of the PKC isoforms at the Z/turn motif and the hydrophobic motif [14]. Additional experiments revealed that phosphorylation of the Z/turn motif occurs prior to phosphorylation of the hydrophobic motif [60] and eventually, the phosphorylation of these C-terminal sites leads to conformational rearrangements of PKC, which result in a more thermally stable, protease- and phosphatase-resistant conformation [147]. Then, the phosphorylated PKC enzyme is released into the cytosol, where it is kept in a catalytically competent, but inactive conformation by the bound pseudosubstrate sequence. An increase in the level of intracellular diacylglycerol and Ca^{2+} results in the interaction of conventional PKCs with the membrane and the energy achieved from this interaction is used to release the pseudosubstrate from the substrate-binding cavity. This step results in the localization of mature PKC at the membrane and thus allows PKC interaction with its substrates and downstream signalling [146, 149, 190]. It was originally thought that the Z/turn-motif and hydrophobic motif in conventional PKC isoforms were autophosphorylation events [14]. However, recent work reported that mTORC2 is required for phosphorylation of the conventional PKCα at its hydrophobic motif (S657) [26, 60, 172].

Notably, while some PDK1 substrates have the ability to exist in inactive and active conformations, 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 [21].

Atypical PKCs differ from the other classes of PKC isoenzymes as they respond to neither Ca^{2+} nor diacylglycerol, but appear to show an increase in activation loop phosphorylation upon PI3K activation. Actually, these enzymes

display a high basal level of phosphorylation at the activation loop which is increased to 2- or 3-fold after activation of PI3K [147].

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 protein Kinase (PRK) family. The PRKs (also termed PAK for Protease Activated Kinase [4, 47, 72] and PKN for Protein Kinase N [139]) have been described to comprise three members: PRK1, PRK2 and PRK3 [155]. These kinases show a close homology to PKC isotypes in the C-terminal located kinase catalytic domain, but they are characterized by a distinct amino-terminal regulatory domain, since, unlike the PKCs, the PRKs do not possess a C1 domain, but two distinct conserved regulatory domains, termed HR1 and HR2 [138, 155].

In our project we studied the interaction of PDK1 with the atypical PKCζ and the PRK family member PRK2. Therefore, we will further focus on the characteristics and the functions of these two AGC kinases.

2.2.2.3.2. Atypical (PKCζ) and related PKC isoforms (PRK2)

Like other members of the AGC subfamily, the atypical PKC isoforms (PKCζ and PKCι) and the related PKC isoforms (PRK1 and PRK2) possess a Thr residue in a region equivalent to Thr308 of Akt/PKB, whose phosphorylation is crucial for kinase activation (Thr-410 in PKCζ [37, 112], Thr-774 in PRK1 and Thr-816 in PRK2 [56, 67]). These PKC isoforms also possess a C-terminal hydrophobic motif which, as general among the AGC subfamily members, acts as a “docking site” for PDK1.

While the aromatic residues in the hydrophobic motifs of the mentioned PKC isoforms are conserved, the phosphorylatable Ser/Thr residue is replaced by an acidic residue, namely Glu-579 in PKCζ and Asp-978 in PRK2. These negatively charged amino acids are able to mimic the phosphoserine/ phosphothreonine residue [156].

Previous work demonstrated that the C-terminal fragments of atypical PKC isoforms (PKCζ and PKCι) and the PKC-related kinases (PRK1 and PRK2) interact significantly with the intact PIF-binding pocket of PDK1 [7]. A mutation of the conserved aromatic residues of the hydrophobic motif of PRK2 or PKCζ importantly reduced the affinity of these kinases for PDK1 and prevented the phosphorylation of PRK2 and PKCζ at their activation loops *in vivo* [7]. Remarkably, the residue Asp-978 in the hydrophobic motif of PRK2 has been revealed to be necessary for the recognition and phosphorylation of PRK2 by PDK1 *in vivo* [7]. However, a mutation of

the equivalent residue in PKCζ (Glu-579) did not considerably affect the interaction between PKCζ and PDK1 [7]. Consistent with this, the specific activity of Glu579Ala-PKCζ was only slightly lower compared to PKCζ wild type, suggesting that a negatively charged residue in the hydrophobic motif of the atypical PKC isoform is not required for maximal activity [7].

The observation of a clear correlation between the activity of wild type and mutant PRK2 and PKCζ and the phosphorylation status at their activation loop was in agreement with previous findings indicating that the activation of PKCζ [37, 57, 112], PRK1, and PRK2 [56, 66] occurs upon phosphorylation of their activation loop. Indeed, previous work demonstrated that the PRK1 kinase domain is phosphorylated at its activation loop by PDK1 *in vitro* in a time-dependent- and phosphoinositide-independent manner, resulting in an increased kinase activity [67]. In addition, the performed experiments clearly showed that activation of endogenous and exogenous PRK2 *in vivo* requires phosphorylation at the activation loop residue Thr816. Furthermore, the induction of an increased PRK2 phosphorylation level at the activation loop led to increased PRK catalytic activity [67].

The phosphorylation of PRK2 and PKCζ at their activation loop by PDK1 *in vivo* has been shown to be inhibited, when the C-terminal fragment of PRK2 containing the PIF-fragment was overexpressed as a GST-fusion protein (termed GST-CT-PRK2) in cells [9].

Interestingly, phosphorylation of PRK1 and PRK2 was significantly diminished in PDK1$^{-/-}$ES cells. In contrast, 24h incubation with PDK1 inhibitors did not affect the phosphorylation of the mentioned AGC kinases [188], emphasizing a possible structural role of PDK1 in maintaining activation loop phosphorylation and supporting the observation of a direct binding of PDK1 to PRK1 and PRK2 [66]. However, it also raises the possibility that the activation loops of diverse AGC kinases show differences in their accessibility to phosphatases with different activities [188].

2.2.2.3.2.1. The PDK1-interacting fragment of PRK2 (PIF)

The protein kinase C-related kinase-2 (PRK2) interacts with the PIF-binding pocket of PDK1 through a region termed the PDK1-interacting fragment (PIF), which encompasses the hydrophobic motif of PRK2 [8]. The hydrophobic sequence motif of PIF (Phe-Xaa-Xaa-Phe-Asp-Tyr) is similar to that found in other AGC kinases, except for the residue corresponding to Ser-473, which is an aspartic acid (Asp-978) in PRK2.

Previous work reported that the residues Lys115 and Leu155 of the PIF-binding pocket of PDK1 are predicted to participate in a hydrophobic interaction with the residues equivalent to Phe974 and Phe977 of PIF. A mutation of the residues that form the PDK1 PIF-binding pocket either abolished or importantly decreased the affinity of PDK1 for PIF [22].

The hydrophobic motif of PRK2 has been described to function both to recruit and activate PDK1, since its binding to the PIF-binding pocket augmented the catalytic activity of PDK1 several fold [22]. Interestingly, the hydrophobic motif of PRK2 (PIF) has been described to interact with >1000-fold higher relative affinity with PDK1 than the hydrophobic motifs of S6K1, SGK1 and Akt/PKBα [24]. In addition, chimeric proteins where the hydrophobic motifs of Akt/PKB and SGK were replaced by PIF (ΔPH-Akt/PKBα, full-length Akt/PKBα or SGK1) led to the phosphorylation of the mentioned kinases at their activation loops in the absence of stimulus and to constitutive activity [24]. The high affinity of PIF for PDK1, which distinguishes PRK2 from the other AGC kinases S6K1, SGK1 and Akt/PKB in a decisive way, suggests that the hydrophobic motif of PRK2 contains additional residues responsible for the high affinity of PIF for PDK1, which are missing in the hydrophobic motifs of S6K1, SGK1 and PKB [24]. Since the C-terminus of PRK2 has such high affinity for PDK1, it is of interest to know if PRK2 coud potentially block the PIF-binding pocket of PDK1 and thus regulate the availability of the PDK1 PIF-binding pocket.

2.3. PRK2 is an effector target of Rho-GTPase

The Ras-related Rho family of proteins, which includes the Rho, Rac, and CDC42 GTPases, mediates several signalling pathways involved in the rearrangement of the actin cytoskeleton [109, 150, 151, 164, 165], in the changes in nuclear gene expression [20, 44, 85, 132], in mitosis and cytokinesis [154, 202], [78], [93]. Stimulated by guanine nucleotide exchange factors (GEFs), GTPases are active when bound to GTP [27] and inactive when bound to GDP [174].

In the past, several proteins have been found to bind specifically the activated form of Rho-GTPase, but not Rac or CDC42. These effector targets of the activated Rho-GTPase, which possess in part a similar structure to PKC and myotonic dystrophy kinase proteins, are: p120 PKN, p150 ROKα (RhoA-binding kinase) and ROKβ, p160 ROCK (Rho-associated coiled-coil containing protein kinase) and p164 Rho kinase [5, 89, 118, 119, 126, 195].

Remarkably, previous studies defined PRK2 as an “ubiquitous and relatively abundant effector target of the Rho-GTPase” [194], since, in contrast to the other Rho-binding kinases, it has been detected in many tissues and cell lines. Besides, it has been reported that PRK2 is involved in Rho-mediated organization of actin cytoskeleton [194], keratinocyte cell-cell adhesion [31] and suppression of epithelial cell transformation by farnesyltransferase inhibitors [208]. The finding that PRK2 is activated by the GDP- or GTP- bound forms of RhoA or by Rac, but not by CDC42 or Ras [194], distinguishes this AGC kinase from the protein kinases listed above in a decisive way. Indeed, the binding to Rho did not seem to substantially activate the Rho-binding kinases p160 ROCK, p150 ROKα and p164 Rho kinase [89, 119, 126]. In contrast to the findings mentioned above, recent experiments revealed that the GTP-bound but not the GDP-bound form of RhoA is able to activate PRK2 in cells [121].

Additional assays showed that RhoA-GTP interacts with the homology region 1 (HR1) of PRK2 and PRK1/PKN, especially with the N-terminal repeat termed HR1a [212]. The HR1 domain is located N-terminal of PRK2 and the related kinase PKN/PRK1 and consists of three tandem copies of a 60-70 amino acid sequence [212]. The binding of RhoA-GTP to HR1 appeared to disrupt an autoinhibitory intramolecular interaction, consequently increasing PRK phosphorylation and further activation [5, 137, 194, 195]. The Rho kinases (ROKα/ROCK-II/Rho-kinase and ROKβ/ROCK-I/p160 ROCK) that are involved in the mediation of Rho-induced stress fiber formation are characterized by a different Rho-binding structure [144], termed RBD [212]. However, according to recent publications, the binding of RhoA to the HR1 region of PRK1 does not suffice to achieve activation of PRK1 [68]. Actually, further experiments showed that full activation of PRK1 by RhoA and downstream signalling requires the extreme C-terminal tail of five residues of PRK1 [121], while full activation of PRK2 by RhoA in cells necessitates the C-terminal segment of seven amino acid residues of PRK2 [121].

Remarkably, cells expressing the kinase-deficient PRK2 protein exhibited a disruption of fibroblast actin stress fibers and an increased level of subcortical actin, emphasizing the role of PRK2 in the regulation of the cytoskeleton through a Rho-regulated signalling pathway [194].

Recent work demonstrated that PRK2 plays a crucial role in mitosis, since PRK2-depleted cells were delayed in G2/M progression and failed to undergo

abscission, finally resulting in binucleated cells [174]. During mitosis, PRK1 and PRK2 are phosphorylated in dependence of Rho GTPases, Cdc25B and cyclin/Cdk1 activity [174].

PRK2 is known to be a lipid-activated protein kinase that is activated by acidic phospholipids [47, 153]. Cardiolipin represents the most efficient acidic phospholipid activator of PRK2, which has been described to prompt PRK2 autophosphorylation [47]. However, it has been shown that the last seven residues in the C-terminus of PRK2 negatively regulate the activation of PRK2 by cardiolipin [121].

One publication reported that PRK1 and PRK2 interact with PDK1 in a Rho-dependent manner, as the presence of GTPase-deficient RhoA enhanced the complex formation between the two AGC kinases [67]. Interestingly, the activation loop phosphorylation level of endogenous PRK1 and PRK2 in HEK293 cells was significantly elevated by transfecting both PRK kinases with either PDK1 or the GTPase-deficient-RhoA or -RhoB. Besides, the phosphorylation level of PRK was not further increased through the combination of transfected Rho plus PDK1 implying that further requirements are implicated in PRK phosphorylation [67].

Based on these findings, the following model has been proposed: 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. At this stage, PRK is able to autophosphorylate and 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 [68]. 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 [67].

2.4. Regulation of PRK2 by several cellular pathways

The execution of apoptosis is mediated in a decisive manner by caspases which consist in a family of cysteine proteases with aspartate specificity. Previous work

showed that full-length human PRK2 is specifically proteolyzed by caspase-3 at Asp117 (in the N-terminal regulatory domain) and Asp700 (in the C-terminal kinase domain) *in vitro*. Besides, PRK2 is cleaved rapidly during Fas- and staurosporine-induced apoptosis *in vivo* by caspase-3 or a caspase-3-like subfamily member. Both principal cleavage sites of PRK2 and PRK1 *in vivo* are located within the N-terminal regulatory domain [47].

Relying on the finding that several protein kinases, such as the p21-activated kinase PAK2 [167], MEKK-1 [33] and the PKC isoforms PKCδ [62, 76] and PKCθ [50] are cleaved and activated by caspase-3 during apoptosis, it has been speculated that PRK2 cleavage during apoptosis might deregulate its activity, since both PRK isoforms PRK1 and PRK2 have been reported to be activated by limited tryptic proteolysis, possibly by removal of their N-terminal inhibitory domain [47].

PRK2 proteolysis at aspartate 117 and 700 by caspases during apoptosis leads to the generation of a 36-kDa C-terminal fragment (corresponding to the amino acid residues 700-984), termed C1 [47], from which the residues 862-908 are required for the binding of the C1 fragment of PRK2 to Akt/PKB. This fragment comprises the C-terminal region of PRK2 including an incomplete catalytic domain and the PIFtide region (residues 908-984) [8] that has been described above to interact with the PIF-binding pocket of PDK1.

According to this study [105], the N-terminus of PRK2 appeared to interfere with the interaction between the C-terminal fragment of PRK2 and Akt/PKB. Accurate analysis suggested that the C-terminal fragment of PRK2 is generated during apoptosis and specifically associates with Akt/PKB *in vivo* at an early apoptotic stage, while Akt/PKB itself is not cleaved. The association between the PRK2 fragment and Akt/PKB has been found to inhibit Akt/PKB activity by completely abolishing its phosphorylation at Ser473 and, to a smaller extent, at Thr308. In addition, the Akt/PKB downstream signalling was specifically inhibited *in vivo*, while Akt/PKB translocation to the membrane was unaffected. Besides, the Akt/PKB-mediated protection against TNF-induced apoptosis was significantly abolished in the presence of wild type PRK2 or C-terminal PRK2 [105]. However, the conclusions drawn by the authors in this study did not consider that the C-terminus of PRK2 interacts with PDK1. In addition, posterior work concluded that the affinity of PIF to Akt/PKB is ~ 5mM, which is extremely low affinity (crystal structure paper by Bradford). Based on current knowledge, we can 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.

Altogether, several studies demonstrated that PRK2 is regulated by several different regulatory pathways in cells [130, 155] which are thought to rely on a protein-protein-interaction induced release of an intramolecular pseudosubstrate inhibition of PRK2 kinase activity. In this sense, PRK2 has been shown to be activated by RhoA, protease-catalyzed cleavage, cardiolipins and the mitogen-activated protein kinase (MAPK) kinase kinase MEKK2 [47, 186, 207].

2.5. Role of PRK2 in HCV replication

PRK2 has been reported to bind and phosphorylate HCV NS5B at its N-terminal finger domain (amino acid 1-187) *in vitro* and *in vivo* [102]. In addition, the results presented in this study [102] emphasized the fact that PRK2 kinase activity specifically mediates the phosphorylation of NS5B. HCV NS5B is an RNA-dependent RNA polymerase (RdRp) which plays an important role in the replication of the HCV RNA genome [123, 124, 152]. Actually, a reduced PRK2 level has been demonstrated to correlate with a suppression of NS5B phosphorylation. Consistent with this, PRK2 overexpression markedly increased NS5B phosphorylation and as a result HCV RNA replication, underlining the positive effect of NS5B phosphorylation in HCV RNA replication [102]. The direct physical interaction between PRK2 und NS5B or other mechanisms are thought to be involved in the regulation of apoptosis through prevention of PRK2 cleavage. In this way, HCV-infected cells may have the possibility to sustain NS5B phosphorylation state and escape from the host immune response, which is mediated by liver-infiltrating cytotoxic T lymphocytes [102].

Y27632 and HA1077 inhibitors, which display a high selectivity for PRK2, inhibited PRK2 activity while PRK2 expression levels remained unchanged [103]. These inhibitors suppressed HCV subgenomic RNA replication of an HCV subgenomic replicon RNA [87, 102]. More recently, HA1077 and Y27632 were shown to have the ability to inhibit HCV replication in genotype 2a HCV-infected cells [103].

Experiments performed with Huh7TR-NS cells revealed that PRK2 inhibitors reduced the level of phosphorylated NS5B without affecting the level of total NS5B protein [103]. This result is in line with previously reported assays, in which the PRK2

inhibitor HA1077 specifically inhibited the phosphorylation of full-length, recombinant HCV NS5B by immunoprecipitated PRK2 *in vitro* [102].

Interestingly, the inhibitory effect of HA1077 and Y27632 arises from the competition of both kinase inhibitors with ATP for binding to the target kinase's catalytic domain [29, 191]. However, further experiments in this study [103] showed that the PRK2 inhibitors are not able to directly inhibit NS5B RdRp activity, indicating that the suppression of HCV RNA replication in R-1 cells by PRK2 inhibitors is not due to a direct interference of HA1077 and Y27632 with NS5B RdRp activity [103].

Remarkably, it has been demonstrated that both PRK2 inhibitors potentiate the antiviral activity of IFN-α and the combination of the PRK2 inhibitors with 100 IU/ml IFN-α led to a 99% reduction of viral replication [103]. Interestingly, IFN- α treatment in R-1 cells did not affect neither PRK2 nor PI3K activity [103].

In conclusion, the finding that HA1077 and Y27632 do not have significant cytotoxity [103] and the knowledge that these kinase inhibitors possess antitumor effects through the inhibition of Rho-dependent kinase activity [51, 80, 187, 202, 206], raise the possibility that these compounds could be applied in the therapy of HCV hepatocarcinoma [103].

2.6. Role of PRK in prostate cancer

Interestingly, it has been demonstrated that PKN3, but not PRK1 or PRK2, is necessary for the growth of PC-3 prostate cancer cells on matrigel. Actually, the employment of several types of gene silencing tools, which inhibited the expression of PKN3, disturbed the growth of prostate and breast epithelial cells in 3D assay systems [115].

The experiments performed in this study revealed that an enhancement of the expression level of PKN3 requires a hyperactivated PI3K pathway, since activation of the PI3K signalling appears to be sufficient for elevated PKN3 expression and phosphorylation [115]. Remarkably, PKN3 seems to play a crucial role in the mediation of malignant growth of cells which are characterized by an activation of the PI3K pathway, such as PTEN-defective tumor cells. A decrease of the level of PKN3 in the primary tumors was responsible for the reduction of metastases formation.

Besides, PKN3 is probably involved in the modulation of cell adhesion and/or migration. Indeed, diminished PKN3 levels resulted in cytoskeletal rearrangements and reduced cell motility, leading to a decreased formation of network-like structures on extracellular matrix [115].

Regulation of PKN3 is mediated by several signal transduction pathways which are involved in the mediation of cell growth and transformation. According to this, transformation of cells by oncogenic Ras has been shown to increase the expression level of PKN3 and consequently to contribute to the invasive capability of the cells [115].

Surprisingly, several studies showed that the malignant potential of tumor cells can be better elucidated by analyzing growth in 3D cultures using extracellular matrix components than in 2D culture conditions [169, 196, 200]. Actually, tumor cells with increased malignant growth are advantaged on matrigel matrix consisting of basement membrane components [141, 160]. However, no impairment of matrigel growth has been detected after inhibition of PRK1 and PRK2 [115].

Remarkably, while PRK1 and PRK2 seem to be ubiquitously expressed, PKN3 exhibits a low expression level in normal adult tissue, while its expression level is increased in human cancer cells [115, 153] and in early mouse embryonic stages [115]. Interestingly, in contrast to the results mentioned above, the expression of PKN3 in certain cell types appears to take place independently of PI3K as well [115].

Further work concentrated on the role of the RhoA effector protein kinase C-related kinase PRK1 in the transcriptional activation of the androgen receptor (AR) [131]. In this regard, RhoA has been described to initiate the translocation of the co-activator FHL2 from the cell membrane to the nucleus, thus determining the transcriptional activation of genes that depend on FHL2 and AR and resulting in the activation of the AR by FHL2 [161]. Previous work revealed that the androgen receptor and PRK1 interact both *in vivo* and *in vitro* [131]. Furthermore, it has been shown that ligand-dependent superactivation of AR-regulated genes can be achieved by activating PRK1 signalling through extracellular stimuli or through co-expression of PRK1 mutants with constitutively active RhoA [131].

Remarkably, the transcription activation unit termed TAU-5 [95, 96], which is located between amino acids 360 and 528 and is necessary for full activation of the androgen receptor [18, 96], has been shown to be sufficient for activation by PRK1 [131]. In this way, the TAU-5 has been described as a novel, signal-inducible transactivation domain [131]. Interestingly, PRK1 signalling promotes the transcriptional activity of mineralocorticoid receptor (MR), progesterone receptor (PR) and p160 co-activators, thus emphasizing the crucial role of the PRK1 signalling pathway for the transcriptional regulation of nuclear receptors. Furthermore,

according to the results presented in this study [131], PRK1 as well as Rho family members are highly overexpressed in prostate tumours and an agonist-dependent coactivation of AR-regulated target genes can be achieved by stimulating the Rho signalling pathway [161].

Altogether, the data presented in this study provide evidence for the role of PRKs in RhoA-regulated activation of AR [131]. Furthermore, it has been demonstrated that the AR is stimulated by PRKs in different cell types and that the AR and PRK1 associate both *in vivo* and *in vitro* [131].

Further assays in this study [131] revealed that AR-TAU-5 represents the first transactivation function that is inducible by PRK. Besides, PRK1 signalling has been described to be independent of FHL2 [131]. The data presented further showed that TIF-2, AR and PRK1 form a functional complex which mediates PRK1 signalling [131]. Taken together, this study provides evidence that PRK1 is clearly overexpressed in prostate carcinoma and that PRK1 and AR are co-expressed *in vivo* [131].

Strikingly, PRK1 is capable of activating AR in the presence of agonists as well as in the presence of adrenal androgens or even when the antagonist cyproterone acetate (CPA) is present. The fact that PRK signalling together with ligands controls the transcriptional activity of AR is the reason why prostate cancer growth can occur even in parallel to a therapy based on androgen ablation [131].

2.7. Role of the Z/Turn-motif phosphorylation site in the mechanism of AGC kinase activation

The growth factor-activated members of the AGC subfamily are characterized by a common main mechanism of activation, which consists of three conserved phosphorylation sites. These three sites are situated in the activation loop in the kinase domain, in the middle of a tail/linker region C terminus to the kinase domain, and within a hydrophobic motif (HM) at the end of the tail region, respectively [81].

Intense work concentrated on the functions of the hydrophobic motif and the activation loop phosphorylation sites in the mechanism of activation of the AGC subfamily members [22, 70, 179, 204, 205].

In contrast to the knowledge acquired about the hydrophobic motif and activation loop phosphorylation sites, very little was known about the third phosphorylation site in the middle of a tail/linker region C terminus to the kinase domain, also termed the Z/Turn-motif phosphorylation site. Its mutation appeared to

importantly reduce kinase activity and phosphorylation of the hydrophobic motif of some AGC subfamily members [16, 127, 128, 136, 147, 156, 197]. Recent work performed in collaboration with our research group during the preparation time of this thesis [81] suggested that the Z/Turn-motif phosphate controls the activation of the AGC kinases by promoting zipper-like the interaction of the Z/Turn-motif phosphorylation site and the hydrophobic motif with the kinase domain [81]. This interaction directly stimulates the activity of the AGC kinases by stabilizing the active kinase conformation and in some cases, by protecting the hydrophobic motif phosphorylation site from dephosphorylation by phosphatases.

Altogether, 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. However, while the Z/Turn-motif phosphate alone or together with the activation loop phosphate does not possess an activating effect, it synergistically enhances the stimulation of kinase activity mediated by the hydrophobic motif phosphate in collaboration with the activation loop phosphate [81]. Of note, Lim et al. 2008 state in their paper than an intact hydrophobic motif or turn motif are not required for the interaction between PRK2 and PDK1 in cells [121]; however, analysis of their data indicates that the binding of PRK2 to PDK1 is greatly diminished by these mutations. Furthermore, the results presented in the mentioned work revealed that the phosphoacceptor in the activation loop and the turn motif, but not the phosphomimetic Asp-978 at the hydrophobic motif are crucial for the kinase activity of PRK2 [121].

Interestingly, recent studies reported that mTORC2 plays a crucial role in the phosphorylation of the Z/Turn-motif phosphorylation site of Akt/PKB, PKCα and probably of PKCβ I, β II, γ, and ε [88]. In the case of PKCα, phosphorylation of the Z/Turn-motif phosphorylation site does not require intramolecular autophosphorylation [88]. The data of this research group [88] indicate that mTOR kinase activity is necessary for phosphorylation of the hydrophobic motif and of the Z/Turn-motif phosphorylation site of PKCα as well as of Akt/PKB. Besides, their results raise the possibility that mTORC2 does not directly phosphorylate the hydrophobic motif and Z/Turn-motif phosphorylation site of PKCα and the Z/Turn-motif phosphorylation site of Akt/PKB, but leads to the activation of a kinase(s) which

phosphorylates Akt/PKB and PKC, without excluding the possibility of an autophosphorylation mechanism in the case of the hydrophobic motif of PKCα [88].

In addition, mTORC2 dependent phosphorylation of the Z/Turn-motif phosphorylation site of conventional PKCs and Akt/PKB has been described to be decisive for their folding and stability. In the case of a lack of phosphorylation of the Z/Turn-motif phosphorylation site, Hsp90 is supposed to protect in part Akt/PKB and conventional PKCs for stability [63].

2.7. Aims of the project

The introduction highlights the extensive knowledge on the role of PDK1 and PRK2 in signalling and to some degree on their structure and regulation. However, still, we are far from understanding all the molecular details involved in their regulation. In the present study we aimed at evaluating the molecular mechanisms of recognition between the AGC upstream kinase PDK1 and its substrates, in particular of the PKC-related kinase PRK2 and the atypical PKC isoform PKCζ. In this regard, we were interested in studying the role of the Z/Turn-motif phosphorylation in the regulation of the interaction between the mentioned AGC kinases and its role in the mediation of PRK2 kinase activity.

3. Materials and methods

3.1. Materials

3.1.1. Antibodies

Table 1. Primary and secondary antibodies employed

	Epitope	Crude extract (dilution)	Pull down (dilution)	Company
Primary antibody: c-Myc (9E10): sc-40	Myc-tag	1:800	1:400	Santa Cruz Biotechnology
Secondary antibody: Anti-Mouse IgG (Whole Molecule) Peroxidase Conjugate		1:10000	1:5000	Sigma
Primary antibody: Anti-Flag M5 monoclonal antibody (mouse)	FLAG-tag	1:800	1:400	Sigma
Secondary antibody: Anti-Mouse IgG (Whole Molecule) Peroxidase Conjugate		1:10000	1:5000	Sigma
Primary antibody: GST (B-14): sc-138	GST-tag		1:1000	Santa Cruz Biotechnology
Secondary antibody: Anti-Mouse IgG (Whole Molecule) Peroxidase Conjugate			1:5000	Sigma
Primary antibody: Anti-phospho-PRK (rabbit antiserum)	Anti-phospho-activation Loop		1:1000	upstate
Secondary antibody: Goat anti-Rabbit HRP Conjugate			1:5000	BIO-RAD
Primary antibody: PKC beta 2 (phospho T641) antibody	Anti-Z/Turn-motif phosphorylation site		1:1000	GeneTex, Inc.
Secondary antibody: Goat anti-Rabbit HRP Conjugate			1:5000	BIO-RAD

3.1.2. Buffers and solutions

All chemical products have been purchased in the highest available purity from the companies Sigma, Roth, Fluka and Merck. All buffers and solutions were prepared with high quality water (Aqua ad iniectabilia Braun).

HBS (2X):	275 mM	NaCl
	2,8 mM	Na_2HPO4
	55 mM	HEPES
Lysis buffer:	50 mM	Tris-HCl pH 7.5
	270 mM	Sucrose
	1 mM	Sodium Ortho-Vanadate
		pH 10
	1 mM	EDTA
	1 mM	EGTA
	10 mM	Sodium B glycerophosphate
	50 mM	Sodium fluoride
	5 mM	Sodium pyrophosphate
	1% (by mass)	Triton X-100
	0.1% (by volume)	β-mercaptoethanol
	1 tablet	protease inhibitor mixture
		per 50 ml of lysis buffer
Electrophoresis buffer:	25 mM	Tris
	250 mM	Glycine (electrophoresis
		grade)
		pH 8,3
	0,1%	SDS
Towbin transfer buffer (1X):	24 mM	Tris
	192 mM	Glycine
	20%	Methanol

TBS/Tween:	10 mM	Tris-Cl (pH 8.0)
	150 mM	NaCl
	0,05%	Tween-20
Coomassie Brilliant Blue R250 (staining solution):	0,25 g	Coomassie Brilliant Blue
	90 ml	Methanol:H_2O (1:1)
	10 ml	Glacial acetic acid
Destaining solution:	10 ml	Acetic acid
	30 ml	Methanol
	60 ml	deionized/distilled H_2O
Wash Buffer A:	50 mM	Tris pH 7.5
	0.1 mM	EGTA

SDS-PAGE loading buffer : Roti-load
[Roth; 1X: 50 mM Tris-Cl, (pH 6.8)
100 mM dithiothreitol
2% SDS (electrophoresis grade)
0.1% bromophenol blue
10% glycerol]

3.1.3. Equipment

Hoefer miniVE Vertical Electrophoresis System from Amersham Biosciences
- electrophoresis module with tank
- Blot module
- Hoefer Multiple Gel Caster
- Gel sandwich (Glass plate, notched alumina plate, side spacers)

Cold microcentrifuge / Refrigerated microcentrifuge
- for eppendorf tubes: Mikro 22R, Hettich Zentrifugen
- for falcon tubes: Centrifuge 5804R, Eppendorf

Fluorescent microscope

DU 800 Spectrophotometer (Beckman Coulter)

3.1.4. Other

The oligonucleotides used to perform mutagenesis, the performed transformations, the prepared minipreps and the subsequent sequencing to verify the mutations were performed during the preparation time of our present work [54] for the most part by Lucas Meyer. Besides, Lucas Meyer performed most part of the protein kinase assays presented in our published work [54].

Table 2.

Materias	**Providers**
Complete protease inhibitor cocktail	Roche Applied Science
Glutathione Sepharose	Amersham Biosciences
Ni Sepharose High Performance	Amersham Biosciences
PROTRAN Nitrocellulose Transfer Membrane	Whatman
High Performance chemiluminescence film	Amersham Biosciences
Chemiluminescence detection kit (Roti-Lumin)	Roth
Restore TM Western Blot Stripping Buffer	Pierce
Prestained Protein Molecular Weight Marker (MW: 118 kDa, 90 kDa, 48kDa, 36 kDa, 27 kDa, 20 kDa)	Fermentas
$CaCl_2$, for molecular biology, approx. 99 %	Sigma

3.2. Methods

3.2.1. Cell culture

HEK (human embryonic kidney) 293 cells were kept in cell culture flasks in incubators at 37°C and a CO_2-concentration of 5%. The cell media contained 10% of Gibco Foetal bovine serum and 1% of Antibiotics Antimycotics to Dulbecco's modified Eagle's Medium, DMEM 1° (Invitrogen). 80-90% confluent cells were trypsinised and transferred to cell culture dishes. The splitting of the cell culture dishes began by aspirating the cell media of the cell flask (175 cm^2), adding 3 ml of trypsin and leaving the flask for 5 min at 37°C in the incubator. During this incubation period, trypsin digests extracellular proteins which participate in cell attachment to the dish. After this incubation, the cells detached from the flask and the separation of the cells was improved by repeatedly aspirating and releasing the cells in 10 ml media using an appropriate electronic pipette. After this step, we added the necessary volume of cell media to the suspension, mixed well, and distributed the cell suspension in the corresponding flask or dish, achieving 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).

3.2.2. Transient cell-Transfection using $CaCl_2$ protocol

We used the $CaCl_2$ transfection method because it works very efficiently in HEK293 cells.

The first step consisted in splitting the cells into 10 cm dishes containing 10 ml of media (to 10-20% confluency) 5-6 hours prior to transfection. All steps were performed under sterile conditions, thus all solutions except the plasmids were sterilized by filtration. The solutions required for cell-transfection according to the $CaCl_2$ protocol are: 2M $CaCl_2$ (Calcium chloride dihydrate, for molecular biology, approx. 99% from Sigma; 2.92 g of $CaCl_2$ dissolved in 10 ml of high quality water), HBS (2X) and the chosen DNA plasmids.

Then, the $CaCl_2$ /HBS /DNA precipitate was prepared. To this end, we first added 0.5 ml HBS (2X) to individual falcon tubes. In separate tubes we added 60 µl of 2M $CaCl_2$, 10 µg of each DNA and enough Q water to bring the total volume to 0.5 ml. After that, the $CaCl_2$/ DNA mix was added dropwise to the HBS (2X) with a P1000 or similar pipette, mixing gently during the addition. The mix has been added directly to cells by dropping slowly and evenly into medium trying to cover as much of the plate

as possible. Then, the dishes were placed at 37°C / 5% CO_2. After 16-24 hours, the media of the dishes was changed with warm media and further incubated for 16-24 hours prior to cell lysis.
Besides the plasmids coding for the studied proteins, in parallel, we also transfected duplicate dishes with a plasmid coding for the green fluorescent protein (GFP). These controls were used to verify the transfection efficiency with the fluorescent microscope (16-24 h after cell transfection). The percentage of transfected cells varied in the different experiments up to approx. 90% and corresponded to the transfection efficiency.

3.2.3. Protein-Protein interaction assay (pull-down)

For the protein-protein interaction assay (pull-down) we co-transfected HEK293 cells with a plasmid coding for a GST-fusion protein and a plasmid coding for a tagged protein. The specific binding of the glutathione Sepharose resin to GST allowed us to pull-down the GST-fusion protein. After washing, the specific binding of the tagged co-transfected protein to the GST-fusion protein was evaluated by separating the pulled-down proteins on SDS-PAGE and detecting the interacting protein by immunoblotting with a specific antibody against the tag.

All procedures of the protein-protein interaction assay took place at 4° C. 36 h after co-transfection, the cell media was aspirated and the cells were lysed in 0.8 ml lysis buffer/ 10 cm^2 Petri-dish. The lysates were cleared by centrifugation at 14,000 x *g* for 10 min and 30 µl of the supernatant (crude extract, CE) were diluted with 30 µl of SDS-PAGE loading buffer (2X) and heated 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 volume of lysis buffer and resin) on a platform shaker for 2 h. After incubation with glutathione Sepharose resin, the washing of the beads was performed in batch. To this end, the mix was centrifuged at 14,000 x g for 1 minute and the pulled down resin was mixed with 1 ml of wash buffer and immediately centrifuged at 14,000 x g for 1 minute. Then, the wash buffer was aspirated and the whole procedure was repeated four times, namely two washes with lysis buffer containing 0,5 mM NaCl, followed by two further washes with 50 mM Tris pH 7.5 and 1 mM EDTA. After that, the beads were resuspended in 30 µl of SDS-PAGE loading buffer (2X; Roti-load 1 from Roth) and heated 3 minutes at 95°C. The SDS (Sodium dodecyl sulfate) contained in the SDS-PAGE loading buffer is an anionic detergent with the ability to denature the proteins

and apply a negative charge to each protein in proportion to its mass. In this manner, 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.

3.2.4. SDS-PAGE

SDS-PAGE ("Sodium DodecylSulfate PolyAcrylamide Gel Electrophoresis") is a common technique used to separate proteins according to their size. For this purpose, we first prepared SDS-PAGE gels, each consisting of a notched alumina plate, a rectangular glass plate and two side spacers. To prepare a higher amount of gels at the same time, we used a Hoefer multiple gel caster and separated the individual plate assemblies from each other with a thin weighing paper. Then, we mixed the ingredients of the resolving gel solution (Table 3) and poured the mix in the plate assembly. To guarantee a plane surface of the gel and avoid an inhibition of the polymerization reaction of acrylamide / bisacrylamide through the influence of O_2, we covered the gel surface with isobutanol. After a waiting period of about 20 minutes to allow the gel solution to polymerize, we discharged the isobutanol and prepared the stacking solution according to Table 4. Afterwards, we poured the stacking solution onto the polymerized resolving solution in the plate assembly and in addition, we placed appropriate combs on top of the stacking solution to provide bags for sample loading. 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 further down the gel, while larger proteins come across more resistance and consequently remain closer to the starting point.

Table 3.

Protocol to prepare resolving gel solution (10% Acrylamide; for 10 gels):	
H_2O	16 ml
30% acrylamide, 0,8% bisacrylamide	20 ml
1,88 M Tris/HCl pH 8,8	12 ml
0,5% SDS	12 ml
10% ammonium persulfate	300 µl
TEMED (N,N,N',N'-Tetramethylethylendiamin)	50 µl

Table 4.

Protocol to prepare stacking gel solution (10% Acrylamide; for 10 gels):	
H_2O	8,7 ml
30% acrylamide, 0,8% bisacrylamide	3,3 ml
0,625 M Tris /HCl pH 6,8	4 ml
0,5% SDS	4 ml
10% ammonium persulfate	100 µl
TEMED (N,N,N',N'-Tetramethylethylendiamin)	20 µl

Upon having placed the electrophoresis module with the secured gel in the tank filled with electrophoresis buffer, we loaded the wished amount of each sample in the bags of the stacking gel. Besides, we loaded ~5 µl of the prestained protein molecular weight marker in one position to be able to establish the molecular weight of the loaded proteins in the following steps. Then, we ran the gel for about 40 min by a constant current of 25 mA/gel. The electric current applied across the gel triggered the negatively charged proteins to migrate across the gel towards the cathode. The stacking gel focuses the samples in the gel, while the resolving gel has the main function to separate the proteins based on their size and support the formation of visible bands.

3.2.5. Immunoblot

After SDS-PAGE, the separated proteins in the gel were transferred to a nitrocellulose membrane for immunoblot. 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. The electrophoresis transfer conditions for blotting proteins in Towbin buffer were 300 mA and 25 V for 1h. Thus, the molecules in the gel migrate to the nitrocellulose membrane by transferring from the black side (cathode) towards the red side (anode) of the module, as typical for negatively charged macromolecules such as proteins run in a SDS gel.

The nitrocellulose membrane was then incubated in a blocking solution (TBS/Tween containing 2.5% of non-fat dry milk) for 1h. After that, the membrane was incubated for 1 h with the monoclonal primary antibody diluted in TBS/Tween containing 2.5% non-fat dry milk. After 4 washes with TBS/Tween during 45 minutes,

the membrane was subjected to a further 1h of incubation with the secondary antibody, which recognized the primary antibody. In the case of using phospho-specific antibodies, we exchanged the 2.5% dry milk-non-fat with 2.5% Albumin, Bovine (Fraction V, Sigma), while the rest of the protocol remained the same. After 4 more washes with TBS/Tween, the antibody detection was performed by using the kit of Roti-Lumin from Roth. The latter is a luminol-based chemiluminescent substrate designed for use with peroxidase-labeled (HRP) reporter molecules, such as the secondary antibodies employed in our experiments. In the presence of hydrogen peroxide, HRP converts luminol to an excited intermediate dianion. This dianion emits light on return to its ground state and in this way it creates the final signal visible on the chemiluminescence films.

3.2.6. Pull-down with chemical compounds

The following chemical compounds have been used to study their effects on protein interaction:

Table 5.

Chemical Compound	Concentration	Company
DMSO (Dimethyl Sulfoxide)	5 µl of 100% conc.	Roth
Orthovanadate (stock)	100 mM	LCLaboratories
LY 294002 (stock)	1mM	Alexis Biochemicals
Okadaic acid (stock)	1mM	LCLaboratories

The chemical compounds LY294002 and Okadaic acid were diluted in DMSO, a frequently used solvent for small organic molecules. Orthovanadate was diluted in high quality water. The HEK293 cell co-transfection was performed according to the usual protocol and 2 hours prior to cell lysis, the cell media of each dish was exchanged for 5 ml of warm media containing 5 µl of the according chemical compound (final concentration of the chemical compound in the dish: 1/1000). In the case of the higher concentrated Orthovanadate, we achieved the final concentration of 1mM by adding 50 µl of the chemical compound to 5 ml of media. Then, upon a waiting period of 2 hours, in which the dishes were incubated at 37°C / 5% CO_2, the cells were subjected to cell lysis, pull down, SDS-PAGE and immunoblot as described above.

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

The purification of GST-fusion proteins expressed in HEK293 cells allowed us to perform quantitative protein kinase assays and *in vitro* pull downs. To purify the GST-fusion proteins, we first transfected HEK293 cells with plasmids coding for the desired proteins fused to GST following the $CaCl_2$ protocol, e.g. 20 dishes (10cm). From this point onwards, all procedures were performed at 4°C with pre-cooled solutions. Then, we lysed the transfected HEK293 cells as described above and centrifuged the cell lysate at 3700 rpm for 15 minutes. We then incubated the supernatant for 2 hours 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). In this way, glutathione Sepharose beads bind to GST and permit us to pull-down the GST-fusion proteins by centrifugation. Upon centrifugation of the resin-extract mix at 3700 rpm for 2 minutes, we discarded the supernatant and subjected the pulled-down GST-fusion proteins to the following washes: 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. Then, we resuspended the washed proteins in 600 µl of Wash Buffer A containing sucrose 0.26M, 0.1% β-Mercaptoethanol and 400 µl of Glutathione (200 mM). The whole mixture was incubated on ice during at least 30 minutes, with vey mild sporadic gentle shaking. The purified protein fraction was filtered through Spin columns with Collection tube (Spin-X, Sigma-Aldrich) and the protein concentration was measured by Bradford (Pierce). Finally, we aliquotted the purified protein, froze it in liquid nitrogen and kept it at –80°C. In order to analyze the purity of the purified proteins, one aliquot was thawed, run on SDS-PAGE and stained with Coomassie Brilliant Blue.

3.2.8. Estimation of protein concentration by Bradford

The Bradford protein assay is a colorimetric assay used to determine the total protein concentration in solutions, relying on the observation that the dye Coomassie Brilliant Blue G-250 binds to protein. By taking into consideration that the absorbance maximum for the anionic blue form of the dye, which binds to protein, shifts from 465 nm to 595 nm upon protein binding, an increase in protein concentration consequently leads to an increase in absorbance at 595 nm. The measurements were performed by using the spectrophotometer with visible light at a wavelength of

595 nm and the “Coomassie Plus protein assay” solution from Pierce. 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. The protein sample was pipetted in a 1 ml plastic cuvette (Sarstedt) containing 800 µl of the Coomassie Plus protein assay solution. In addition, another plastic cuvette containing exclusively the Coomassie Plus protein assay solution was used as the reagent blank. First of all, the blank cuvette was placed in the machine and the absorbance was read and considered 0.00 Abs. Then, the cuvette containing the purified protein was placed in the spectrophotometer and the measurements were performed. The achieved results permitted us to calculate the protein concentration by considering that 1 µg of protein in 800 µl of Coomassie Plus reagent corresponds to 0,055 Abs. The coefficient was obtained using BSA as standard.

3.2.9. Protein kinase assay (using phosphocellulose p81 papers)

The protein kinase assay allows us to test the intrinsic activity of the studied proteins. The assay was performed in a final volume of 20 µl containing 2 µl of 500 mM Tris pH 7.5, 1% β-mercaptoethanol, 2 µl of 100mM Mg, 2µl of 1mM ATP and 50-200 ng of PRK2. 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%, 1/100). At this stage, we spotted 35 µl of the samples onto p81 phosphocellulose papers (Chromatography paper p81, fromWhatman) and washed the papers 4 times during 1h with phosphoric acid (88%, 50 ml in 10 l of deionized water). The last wash was performed with technical ethanol to help in the drying process. Then, the next step consisted in drying the papers and finally count radioactivity with the “phospho imager” (Typhoon, GE Healthcare). 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 that amount of kinase that catalyzed the phosphorylation of 1nmol of substrate in 1 min.

3.2.10. Pull-down using purified proteins

Part of the experiments performed *in vivo* have been repeated *in vitro* in order to exclude the influence of intracellular factors on the investigated interaction patterns. The *in vitro* experiments were performed at 4°C by mixing the according purified GST-PRK2 fusion proteins expressed in HEK293 cells with purified His-tagged PDK1 protein.

The experiment consisted in mixing the established GST-fusion protein together with a mix comprising 12 µl of high quality water and the needed amount of Sucrose 0.26 M solved in Buffer A to achieve the final volume of 18µl. Then, we incubated the whole mixture with 2 µl of His-PDK1 (1mg/ml) for 35 minutes. After this, we further incubated the mixture with 20 µl of Nickel (Ni-NTA) Sepharose resin for 2 hours at 4°C in order to bind the resin to the His-tagged PDK1 protein. After that, we washed the pulled-down proteins in batch following the usual pull-down protocol and subjected the obtained proteins to SDS-PAGE and immunoblot as explained above. However, this method showed a significant unspecific binding of GST to Nickel (Ni-NTA) Sepharose resin in the absence of His-PDK1, not allowing us to come to any reliable conclusions. Thus, the protocol of *in vitro* assays has to be improved further on, possibly by decreasing incubation time of the employed proteins with Nickel (Ni-NTA) Sepharose resin, increasing washing and including excess of an unrelated protein, such as BSA, to avoid unspecific binding.

3.2.11. Plasmid transfection of bacteria and preparation of plasmid DNA maxi prep

The first step consists in preparing the SOC Medium (0.5 g NaCl, 5 g yeast extract and 20 g tryptone 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 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.

The plasmids required for the transfection of HEK293 cells were prepared using maxi prep kit (“JET star” from Genomed) or a mega-prep kit (Qiagen). First, we

transformed DH5α $CaCl_2$ chemically competent bacteria with the plasmid of interest. To this end, 30-50 µl of competent bacteria were aliquoted in an eppendorf tube and mixed with 2 µl of the appropriate DNA. After an incubation time on ice of about 20 minutes, the mixture had to be heat shocked at 42°C for 40 seconds and incubated again on ice for 2 minutes. Then, we added 450 µl of 42°C heated SOC medium to the eppendorf tube and shaked the tube for about 45 minutes at 37°C. Afterwards, we plated the mixture on agar plates containing ampicilline and incubated them overnight at 37°C. The next step consisted in pipetting 4 ml of LB medium (25 g of Luria Broth (Sigma) were added to 1 l of deionized H_2O and sterilized by autoclaving) and 4 µl of Carbenicillin (concentration: 50 mg/ml) to a PP-tube (greiner bio-one). Then, we added one peaked colony of bacteria from the according agar plates to the PP-tube and incubated it for at least 8 hours at 37°C and a speed of 220 rpm. This preparation was used to inoculate ~2 l Erlenmeyer containing 300 ml LB medium.

We established the DNA concentration of the plasmids by setting the spectrophotometer at UV-light and 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. Experiments and results

4.1. The Z/Turn-motif phosphorylation of PRK2 and PKCζ

Previous work showed that several members of the AGC family of protein kinases interact with PDK1 through their hydrophobic motif (e.g. S6K, RSK and SGK). Phosphorylation of S6K, RSK and SGK at the HM phosphorylation site triggers the binding of the HM to the PIF-pocket of PDK1. In this manner, the interaction of S6K, RSK and SGK with PDK1 is regulated by the phosphorylation of the HM (see alignment, Fig.1). However, PKCζ and PRK2 possess a hydrophobic motif but don't possess a hydrophobic motif phosphorylation site at the equivalent position. Therefore, the interaction of PRK2 and PKCζ with PDK1 cannot be regulated in a similar manner. Former unpublished work of Ricardo Biondi and Dario Alessi showed that these two AGC kinases were phosphorylated at their activation loop and at the Z/Turn-motif phosphorylation sites. Thus, these were the only two phosphorylated residues observed after inorganic ^{32}P labelling of HEK293 cells which had been transfected with vectors expressing GST-PRK2 and GST-PKCζ.

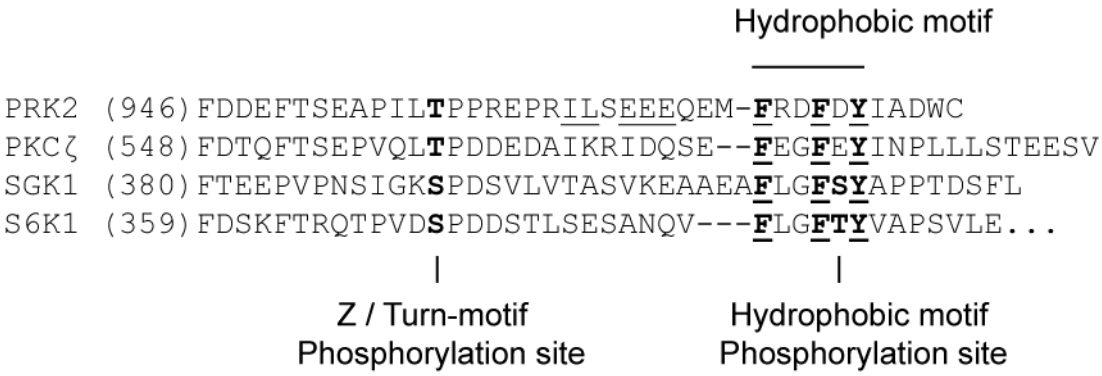

FIG. 1. **Alignment of the C-terminal amino acid sequence of PRK2 with the equivalent region of selected AGC subfamily kinases**. The Z/Turn-motif phosphorylation sites are in boldface, the hydrophobic motif phosphorylation site is underlined and shown in boldface. The hydrophobic residues Ile965/Leu966 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 are the same 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.

4.2. Mutation of the Z/Turn-motif phosphorylation site of PRK2 increases its interaction with PDK1

In order to study the effect of the Z/Turn-motif phosphorylation site on the ability of PRK2 to interact with PDK1, we co-transfected HEK293 cells with plasmids coding for Myc-PDK1 together with constructs coding for GST-PRK2 wt or GST-PRK2 with a

mutated Z/Turn-motif phosphorylation site (GST- PRK2 [Thr958Ala], where the Threonine residue is mutated to an Alanine residue, and GST- PRK2 [Thr958Glu] with a Glutamic acid residue in place of the Threonine residue). As a control, we also co-transfected Myc-PDK1 with a plasmid coding for the C-terminal 67 residues of PRK2 fused to GST (GST-CT-PRK2), which was previously characterized to interact with high affinity with PDK1 [8]. To better evaluate the obtained results, we prepared duplicates for each co-transfection. The cells were lysed 36 h after transfection and the clarified lysate was incubated with glutathione-Sepharose resin. Since glutathione-Sepharose binds to GST, the use of this resin allowed us to pull-down the GST-fusion proteins by centrifugation. Afterwards, we washed the pulled-down proteins 4 times in batch and subjected them to SDS-PAGE and immunoblot. By using anti-Myc as primary antibody, we aimed at detecting the presence of Myc-PDK1 through the specific binding of the antibody to the Myc-tag. However, Myc-PDK1 would only be found in the pull-down if Myc-PDK1 interacted with high affinity with GST-PRK2. In addition, we prepared a Coomassie staining to evaluate the expression level of the GST-fusion proteins. Furthermore, we performed an anti-Myc immunoblot on the clarified lysate to verify the equal expression of Myc-PDK1 in all samples.

By looking at Fig.2A bottom panel, we realized that the expression level of Myc-PDK1 in the crude extract was similar in all cases. Furthermore, we verified the presence of anti-Myc signal on the pull down corresponding to the co-transfection of Myc-PDK1 with GST-PRK2 wt, indicating that Myc-PDK1 bound to GST-PRK2. Interestingly, GST- PRK2 [Thr958Ala], even if lower expressed than GST-PRK2 wt (see Coomassie staining, top panel), seemed to pull-down more Myc-PDK1 protein than GST-PRK2 wt. In accordance with previous work, a high level of interaction was detected between GST-CT-PRK2 and Myc-PDK1.

At this point we wanted to confirm that the interaction between the studied proteins, namely PRK2 and PDK1, was not mediated by unspecific interactions and that the achieved results were indeed due to the specific interaction between the proteins themselves. In order to verify that PRK2 [Thr958Ala] had increased capacity to interact with PDK1, we then co-transfected GST-PDK1 with FLAG-tagged versions of PRK2 wt and Thr958 mutants. In this experiment, we pulled-down GST-PDK1 and verified the interaction by probing the pull-down with anti-FLAG. The results presented in Fig.2B showed that the FLAG-PRK2 mutants were similarly expressed

in the FLAG-immunoblot of crude extract (bottom panel). Importantly, the FLAG-immunoblot of the pull-down confirmed that the interaction between PDK1 and PRK2 [Thr958Ala] was stronger than with PRK2 wt. Again, similarly to Fig.2A PRK2 [Thr958Glu] had somehow intermediate level of interaction. Thus, this result further supported the previous finding that the mutation of the Threonine residue of the Z/Turn-motif phosphorylation site to Alanine increases the binding of PRK2 to PDK1.

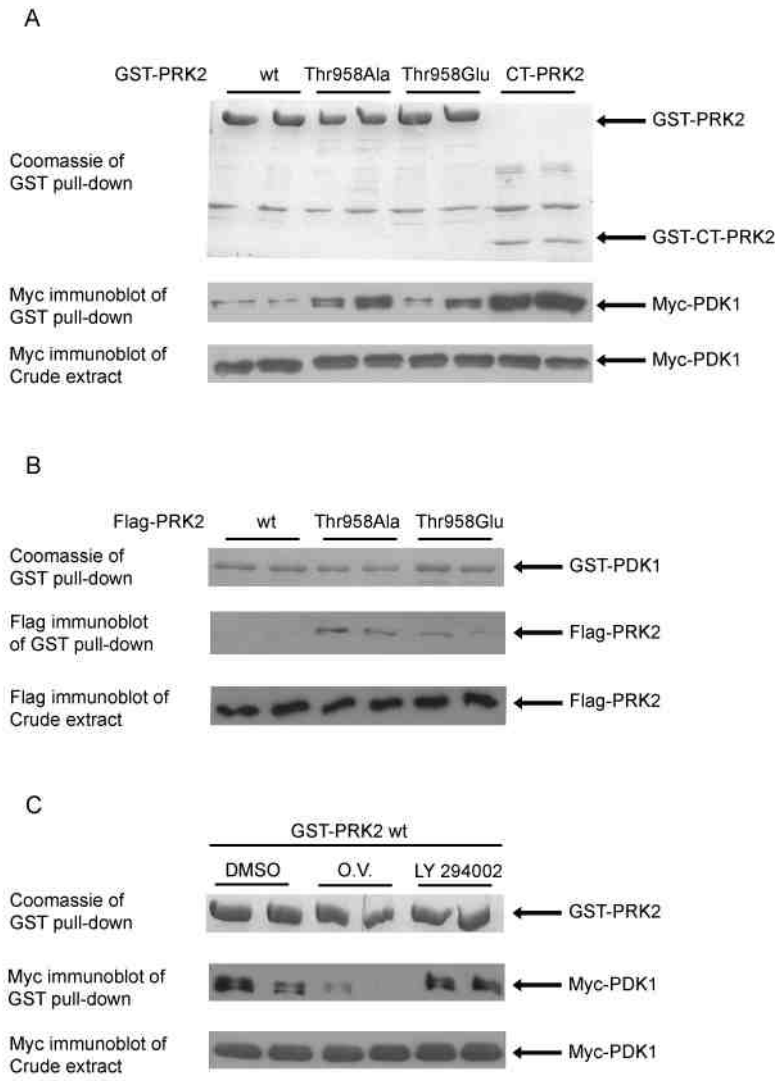

FIG. 2. **The interaction between PRK2 and PDK1 is increased by mutating the Z/Turn-motif phosphorylation site of PRK2.** *A*, HEK293 cells were transiently trasfected with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST-PRK2 wt or the mutants GST-PRK2 [Thr958Ala] and GST-PRK2 [Thr958Glu]. *B*, HEK293 cells were transiently transfected with DNA constructs expressing GST-PDK1 together with constructs expressing either Flag-PRK2 wt or the mutants Flag-PRK2 [Thr958Ala] and Flag-PRK2 [Thr958Glu]. *C*, HEK293 cells were transiently transfected with DNA constructs expressing Myc-PDK1 together with constructs expressing GST-PRK2 wt. *C*, 2 h prior to cell lysis, the transfected cells were incubated with the according chemical compounds, namely DMSO, Orthovanadate (O.A.) and LY294002. 36 h post-transfection the cells were lysed and the cell lysate was incubated with glutathione-Sepharose resin in order to pull-down the GST-constructs. The pulled-down proteins were subjected to SDS-PAGE, stained with Coomassie

Blue, and immunoblotted using an anti-Myc antibody to detect Myc-PDK1 that bound to GST-PRK2 (*A*) or an anti-FLAG antibody to detect FLAG-PRK2 that bound to GST-PDK1 *(B)*. To verify the similar expression of Myc-PDK1(*A,C*) or of the wild type and mutant FLAG-PRK2 (*B*), the total cell lysate was subjected to SDS-PAGE and immunoblotted using anti-Myc antibody (*A,C*) or anti-FLAG antibody (*B*). Duplicates of each condition are shown.

Since the interaction between PRK2 and PDK1 appeared to be dependent on the phosphorylation status of the Z/Turn-motif phosphorylation site of PRK2, we decided to evaluate whether the employment of chemical compounds which are known to affect the phosphorylation of protein kinases *in vivo* would also affect the binding of PRK2 to PDK1. To this end, we employed the protein tyrosine phosphatases inhibitor Orthovanadate and the selective PI3 kinase (PI3K) inhibitor LY294002. Besides, we also used the solvent DMSO as a control.

Similarly to the experiments described above, we co-transfected HEK293 cells with Myc-PDK1 and GST-PRK2 wt, however, 2 h prior to cell lysis, we incubated the transfected cells with warm media containing the according chemical compounds, namely, DMSO as a control, Orthovanadate and LY294002. Upon cell lysis, we pulled-down the GST-fusion proteins with glutathione-Sepharose resin and washed them several times in batch. After that, we subjected the pulled-down proteins to SDS-PAGE and immunoblot with Anti-Myc. By looking at Fig.2C, we realized that the expression level of Myc-PDK1 in the Myc-immunoblot of crude extract as well as the amount of GST-PRK2 wt shown in the Coomassie staining was similar in all cases. Interestingly, Orthovanadate abolished the interaction between GST-PRK2 wt and Myc-PDK1, as shown in the Myc-immunoblot of GST pull-down. Since Orthovanadate inhibits tyrosine phosphatases and thus is expected to cause hyperphosphorylation at the tyrosine residues in the cell, it is likely that an unidentified tyrosine kinase mediates phosphorylation of PRK2, thus impairing its interaction with PDK1. Strikingly, LY294002 did not appear to affect the binding of PRK2 to PDK1 in the Myc immunoblot of GST pull-down, suggesting that the PI 3-kinase pathway does not regulate the interaction between PDK1 and PRK2. However, this result is inconsistent with previous work [67] and will be further treated in the discussion.

In addition, we also took into consideration the role of the hydrophobic pocket of PDK1, which is responsible for the interaction of the kinase with its substrates. Thus, the hydrophobic motif within the PDK1-interacting fragment (PIF) sequence was shown to interact with PDK1 hydrophobic PIF-binding pocket. The hydrophobic pocket of PDK1 is located in the small lobe of its kinase domain and contains several

residues, from which Leu 155 forms part of the hydrophobic binding site and participates in a hydrophobic interaction with the residues equivalent to Phe974 and Phe977 in the hydrophobic motif of PRK2 (present in PIF) [22]. The mutation of the Leu 155 residue (Leu155Glu) of the HM/PIF-binding pocket of PDK1 led to a loss of interaction with PRK2 in all cases, indicating the essential role of this region for the measured interaction (data not shown).

4.3. Mutation of the Z/Turn-motif phosphorylation site in PKCζ does not affect the interaction with PDK1

In parallel experiments, we analysed the effect of the mutation of the Z/Turn-motif phosphorylation site in PKCζ. For this purpose, we co-transfected HEK293 cells with plasmids coding for Myc-PDK1 together with plasmids coding for GST-PKCζ or GST-PKCζ [Thr560Ala]. Thereafter, we followed the same protocol as for the pull-down experiments between PDK1 and PRK2 (Fig.2A-B).

However, the results did not reveal any relevant difference in the Myc-immunoblot of the pull-down. In this way, we realized that, in contrast to the results achieved with PRK2, the mutation of the Z/Turn-motif phosphorylation site Thr560 to Ala in PKCζ (Thr560Ala) did not significantly influence the interaction with PDK1 (Fig.3A). As for the experiments with PRK2 described above, we repeated the essay by co-transfecting GST-PDK1 together with FLAG-PKCζ or FLAG-PKCζ [Thr560Ala] and by performing an immunoblot with anti-FLAG. In this manner, we were able to confirm the already described result, since we did not see any particular difference in the interaction level between FLAG- PKCζ or FLAG-PKCζ [Thr560Ala] and GST-PDK1 (Fig.3B). We reasoned that the lack of difference could be due to a surprisingly low level of Thr560 phosphorylation in both constructs of PKCζ. This was unlikely since Ricardo M. Biondi´s and M. Frödin´s previous work suggested that PKCζ was highly phosphorylated at the Z/Turn-motif phosphorylation site and the protein was homogeneous by IEF.

However, in order to further evaluate if a low stoichiometry of phosphorylation of the Z/Turn-motif phosphorylation site could explain this result, we co-transfected the plasmids coding for GST-PKCζ or GST-PKCζ [Thr560Ala] with a plasmid coding for Myc-PDK1 and, in parallel, we repeated the same experiment with the addition of okadaic acid 2 h prior to cell lysis.

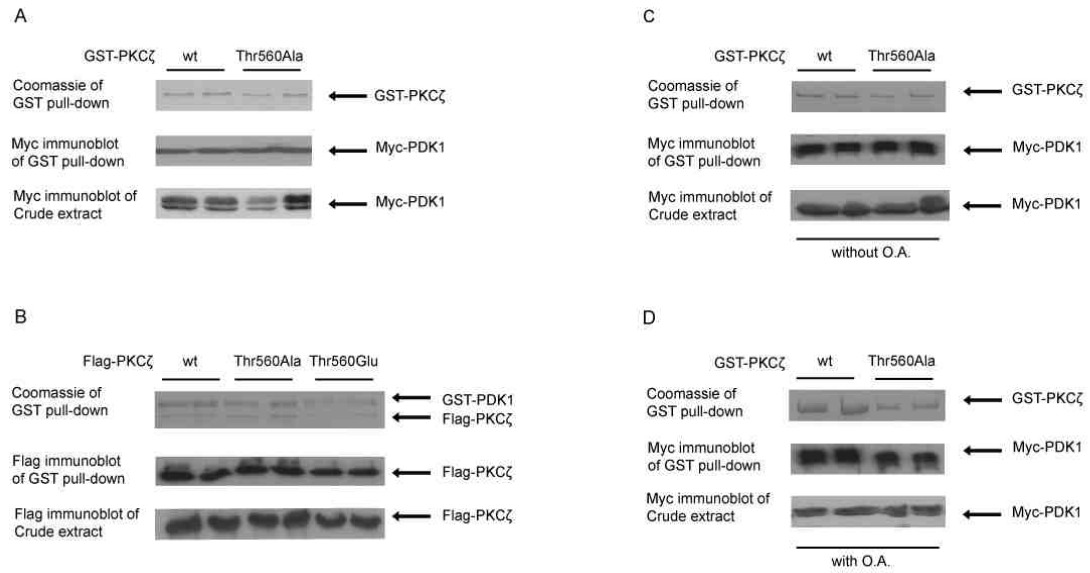

FIG. 3. **Mutation of the Z/Turn-motif phosphorylation site does not significantly influence the interaction between PKCζ and PDK1.** *A*, *C, D* HEK293 cells were transiently trasfected with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST-PKCζ wt or the mutants GST-PKCζ [Thr560Ala] and GST-PKCζ [Thr560Glu]. *B*, HEK293 cells were transiently transfected with DNA constructs expressing GST-PDK1 together with constructs expressing either FLAG-PKCζ wt or the mutants FLAG-PKCζ [Thr560Ala] and FLAG-PKCζ [Thr560Glu]. *D*, 2 h prior to cell lysis, the transfected cells were incubated with Okadaic acid (O.A.). 36 h post-transfection the cells were lysed and the cell lysate was incubated with glutathione-Sepharose resin in order to pull-down the GST-constructs. The pulled-down proteins were subjected to SDS-PAGE, stained with Coomassie Blue, and immunoblotted using an anti-Myc antibody to detect Myc-PDK1 that bound to GST-PKCζ (*A, C, D*) or an anti-FLAG antibody to detect FLAG-PKCζ that bound to GST-PDK1 *(B)*. To establish if the expression level of Myc-PDK1 (*A, C, D*) or of the wild type and mutant FLAG-PKCζ (*B*) was similar in all cases, the total cell lysate was subjected to SDS-PAGE and immunoblotted using anti-Myc antibody (*A, C, D*) or anti-FLAG antibody (*B*). Duplicates of each condition are shown.

Okadaic acid is a potent inhibitor of protein serine/threonine phosphatase. Therefore we used this compound to increase protein phosphorylation levels at the Z/Turn motif phosphorylation site. Unfortunately, this treatment led to a detachment of the HEK293 cells employed for transfection. However, we could conclude that the use of the protein phosphatase did not affect the interaction pattern between PKCζ and PDK1 (Fig 3C-D), further confirming that the phosphorylation of the Z/Turn-motif phosphorylation site does not affect the interaction between PKCζ and PDK1.

Eventually, the results of the interaction between PDK1 and PRK2 or PKCζ suggested that the lack of phosphorylation at the Z/Turn-motif phosphorylation site in PRK2 -due to the mutation of the Threonine residue at this position to Alanine- increased the binding of PRK2 to PDK1; thus, the phosphorylation of the Z/Turn-motif phosphorylation site in PRK2 may inhibit its binding to PDK1 and may play a role for the regulation of PRK2 activity *in vivo*.

In contrast to that, the binding of PKCζ to PDK1 was unaffected by mutation of the Z/Turn-phosphorylation site. For this reason, we conclude that the phosphorylation of

PKCζ at the Z/Turn-motif phosphorylation site cannot regulate the interaction of PKCζ with PDK1.

4.4. Mutation of the Z/Turn-motif phosphorylation site in SGK and S6K does not affect the interaction with PDK1

Previous studies revealed that the interaction of two further members of the AGC family, namely S6K and SGK, with PDK1 is promoted by the phosphorylation of their hydrophobic motifs. In these two cases, the hydrophobic motif phosphorylation prompts the interaction with PDK1 and hence their phosphorylation at the activation loop by PDK1. The presence of the hydrophobic motif phosphorylation site in S6K and SGK distinguishes these two proteins from PRK2 and PKCζ in a decisive way, since PRK2 and PKCζ have a negatively charged residue at its place. Based on the information about PRK2 and PKCζ described above, we decided to investigate whether the Z/Turn-motif phosphorylation site could also play a role in the interaction between PDK1 and its substrates S6K and SGK. In order to simplify the system and thus compare S6K and SGK with the two AGC kinases PRK2 and PKCζ, we generated S6K and SGK constructs with a mutation at the hydrophobic motif phosphorylation site. To this end, we mutated S6K Thr412 to Glutamic acid [S6K-T2(Thr412Glu)] and SGKSer422 to Aspartic acid [SGK(Ser422Asp)]. In addition, the S6K-T2 construct employed in these experiments lacked the last 104 amino acids, while the ΔN SGK construct lacked the N-terminal 60 amino acids. Previous work showed that [S6K-T2(Thr412Glu)] and [SGK(Ser422Asp)] interact significantly with PDK1 and have a comparable intrinsic activity as the respective wild type constructs. By using these two mutants, we avoided a possible dephosphorylation at the hydrophobic motif phosphorylation site and so we were able to evaluate in a convincing manner the effect of mutation of the Z/Turn motif phosphorylation site in S6K and SGK on the interaction with PDK1. Therefore, as performed with PRK2 and PKCζ, we co-transfected HEK293 cells with several S6K and SGK mutants and studied their interaction pattern with Myc-PDK1.

The co-transfection assays were performed with a plasmid coding for Myc-PDK1 together with the plasmids coding for the GST-S6K mutants S6K-T2, S6K-T2[Thr412Glu], S6K-T2[Thr412Glu/Ser394Ala], S6K-T2[Thr412Glu/Thr399Ala] or the GST-SGK mutants ΔN SGK, SGK[Ser422Asp], ΔN SGK [Ser422Asp/Ser401Ala] or the GST-Vector as a control. In the following steps we proceeded as already described above and finally we performed an immunoblot with Anti-Myc. The results

did not reveal any obvious difference between the wild type constructs of S6K and SGK and all the S6K and SGK mutants in their interaction with PDK1 (data not shown), suggesting that most probably the Z/Turn-motif phosphorylation site does not regulate the interaction of these two AGC kinases with PDK1.

4.5. Effect of the Z/Turn-motif phosphorylation site on the activity of PRK2

Based on the fact that the Z/Turn-motif phosphorylation site plays a key role in the interaction between PRK2 and PDK1, we decided to further characterize the Thr958 mutants. Therefore, we first co-transfected HEK293 cells with the appropriate plasmids, incubated the cell lysate with glutathione Sepharose resin and purified GST-PRK2 wt, GST-PRK2 [Thr958Ala] and GST-PRK2 [Thr958Glu].

The purified proteins were subjected to a protein kinase activity assay using a polypeptide (Crosstide) as a substrate. Under our assay conditions, the specific activity of GST-PRK2 wt was approximately 4.8 U/mg. On the other hand, both GST-PRK2 mutants had significantly lower activity (Fig. 4A), suggesting that the Z/Turn-motif phosphorylation site may be required for the activity of PRK2. In order to establish if the Z/Turn-motif phosphorylation was directly involved in this effect, we decided to probe the phosphorylation status of GST-PRK2 wt and mutants. To this end, we subjected the purified protein kinases to immunoblot using as primary antibodies anti-phospho-PRK (Upstate Biotechnologies) to detect the phosphorylation degree at the activation loop phosphorylation site, and PKC beta 2 (phospho T641) (GeneTex, Inc) antibody in order to detect the phosphorylation status at the Z/Turn-motif phosphorylation site. To control the amount of protein loaded in each lane, we stripped the two membranes and incubated them again with Anti-GST as primary antibody. In this way, we verified the loading of the GST-PRK2 wt and mutants used in the experiment (Fig.4). The results shown in Fig. 4B indicated that the GST-PRK2 wt protein was indeed phosphorylated at the Z/Turn-motif phosphorylation site and that this site was not phosphorylated in GST-PRK2 [Thr958Ala] and GST- PRK2 [Thr958Glu]. The analysis of the activation loop phosphorylation site however, indicated that GST-PRK2 wt and GST-PRK2 [Thr958Ala] were phosphorylated at the activation loop at approximately similar levels, but, surprisingly, GST- PRK2 [Thr958Glu] was significantly less phosphorylated at this site (Fig. 4C).

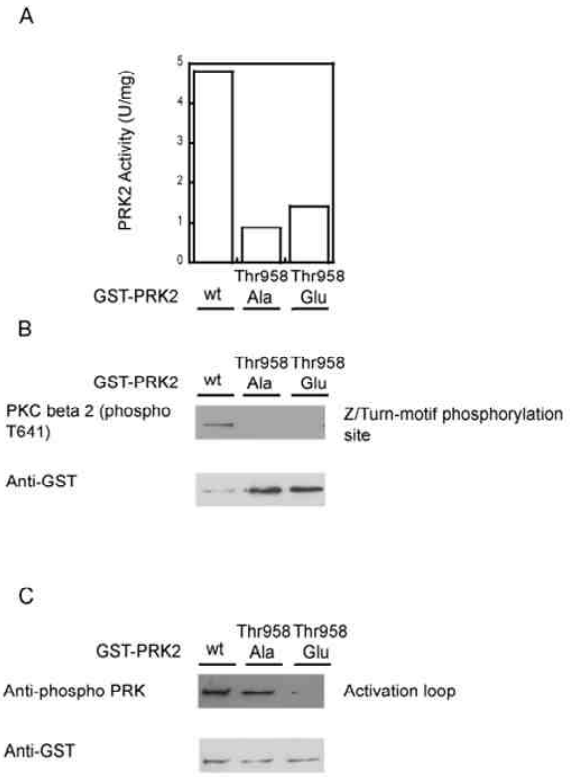

FIG. 4. **The lack of phosphorylation at the Z/Turn-motif phosphorylation site leads to a significant loss of activity of PRK2.** HEK293 cells were transiently transfected with DNA constructs expressing GST-PRK2 wt, GST-PRK2[Thr958Ala] and GST-PRK2[Thr958Glu]. 36 h post-transfection the cells were lysed and the cell lysate was incubated with glutathione-Sepharose resin in order to pull-down the GST-fusion proteins. The pulled-down GST-constructs were purified and either assayed for activity (*A*) or subjected to SDS-PAGE and immunoblotted using a PKC beta 2 (phospho T641) antibody to establish the phosphorylation status at the Z/Turn-motif phosphorylation site (*B*) or an anti-phospho PRK antibody to detect the phosphorylation status at the activation loop (*C*). To verify the equal amount of protein loaded in each lane, the two membranes were stripped and immunoblotted again with an anti-GST antibody (*B*, *C*).

The main difference between GST-PRK2 wt and GST-PRK2 [Thr958Ala] was the lack of Z/Turn-motif phosphorylation of the mutant. The result indicated that the mutation of the Z/Turn-motif phosphorylation site and consequently the lack of phosphorylation at this site in GST-PRK2 [Thr958Ala] led to a significant loss of activity of this mutant in relation to GST-PRK2 wt. The result further suggests that the Z/Turn-motif phosphorylation has a direct role in PRK2 activity.

In spite of low degree of phosphorylation at the activation loop, the activity of GST- PRK2 [Thr958Glu] was higher than in the case of GST-PRK2 [Thr958Ala]. Therefore we concluded that the negatively charged Glutamic acid present in GST-PRK2 [Thr958Glu] in place of the Threonine residue could mimic to a certain degree the phosphorylation at the Z/Turn-motif phosphorylation site, increasing the *in vitro* activity of the mutated kinase.

4.6. Effect of the Z/Turn-motif phosphorylation on the interaction of the isolated C-terminal segment of PRK2 with PDK1

Based on the interaction assays described above, we concluded that 2 distinct general models could explain the increased binding of PRK2 [Thr958Ala] to PDK1. In a first model (Fig.5A-B), we could assume an increased affinity of the C-terminal PRK2 residue to PDK1 in the absence of phosphorylation (Fig. 5A); upon phosphorylation, the affinity of the C-terminal PRK2 segment for PDK1 would be lost (Fig. 5B). In a second model (Fig.5C-D), the phosphorylation of the Z/Turn-motif phosphorylation site promotes an intra-molecular interaction with the PRK2 catalytic domain (Fig. 5C); the mutation would inhibit the intramolecular binding of the C-terminal fragment of PRK2 to PRK2 catalytic domain and thus promote indirectly the interaction of the C-terminal fragment of PRK2 with the PDK1 PIF pocket (Fig.5D). In order to evaluate these two possible explanations, we decided to test the ability of the wild type, [Thr958Ala] and [Thr958Glu] C-terminal fragments of PRK2 to interact with PDK1. To do this, we generated plasmids coding the C-terminal 67 amino acids of PRK2 fused to GST and the corresponding [Thr958Ala] and [Thr958Glu] mutants. We then co-transfected HEK293 cells with plasmids coding for Myc-PDK1 together with plasmids coding for the C-terminal residues of PRK2 as fusion proteins with GST, namely GST-CT-PRK2 wt, GST-CT-PRK2 [Thr958Ala] and GST-CT-PRK2[Thr958Glu].

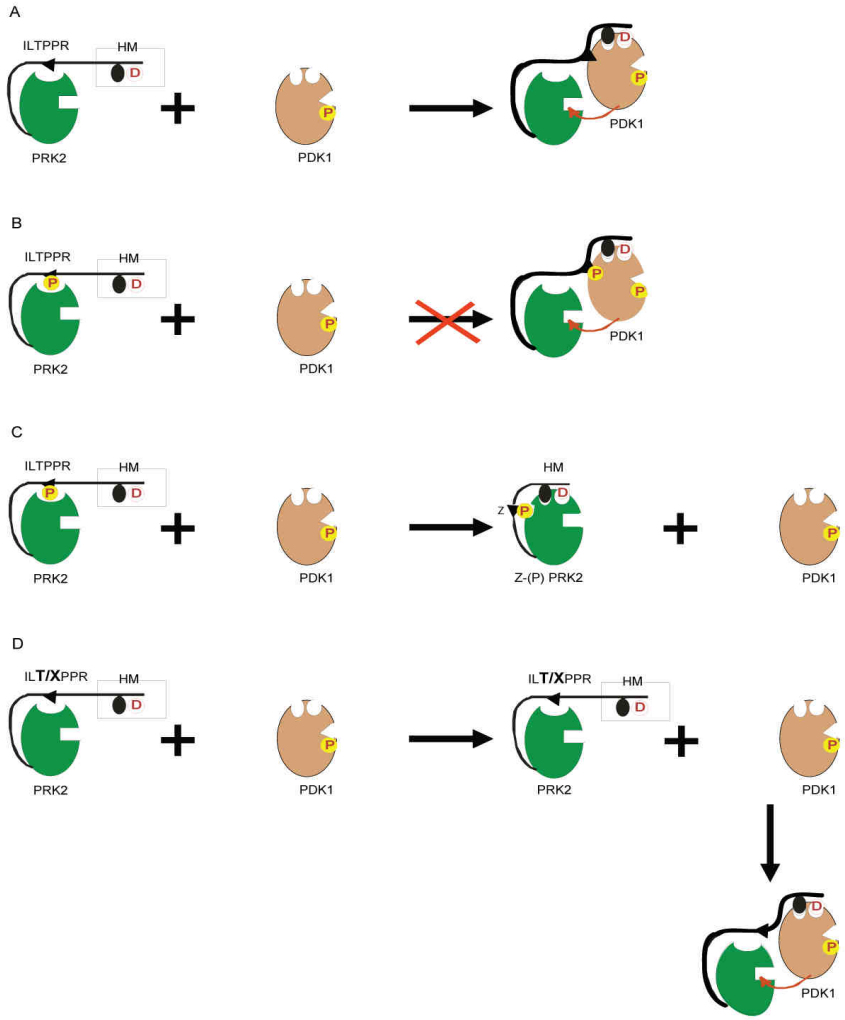

FIG. 5. **Possible explanations for the role of the Z/Turn-motif phosphorylation site in the regulation of interaction between PRK2 and PDK1**. (*A*) An absence of phosphorylation at the Z/Turn-motif phosphorylation site of PRK2 increases the binding affinity of the hydrophobic motif of PRK2 to the PIF binding pocket of PDK1. (*B*) Phosphorylation of the Z/Turn-motif phosphorylation site of PRK2 impairs the binding of the hydrophobic motif of PRK2 to the PIF binding pocket of PDK1. Phosphorylation of the Z/Turn-motif phosphorylation site of PRK2 promotes the binding of the hydrophobic motif of PRK2 to its own catalytic domain (*C*), while mutation of the Z/Turn-motif phosphorylation site of PRK2 leads to a lack of phosphorylation at this position, inhibiting the binding of the hydrophobic motif of PRK2 to its own catalytic domain and thus making it available for interaction with PDK1 (*D*).

After cell lysis, we pulled-down the GST-CT-PRK2 constructs with glutathione-Sepharose resin and washed several times in batch. Interestingly, the Myc immunoblot of the pull down showed that GST-CT-PRK2 wt, GST-CT-PRK2 [Thr958Ala] and GST-CT-PRK2 [Thr958Glu] interacted similarly with Myc-PDK1 (middle panel). In addition, we performed a Coomassie staining in order to verify the

expression level of the GST-CT-PRK2 constructs (top panel). We also performed an anti-Myc immunoblot of the crude extract to check the amount of Myc-PDK1 (bottom panel). In all cases, the expression level was comparable for all employed mutants (Fig.6A).

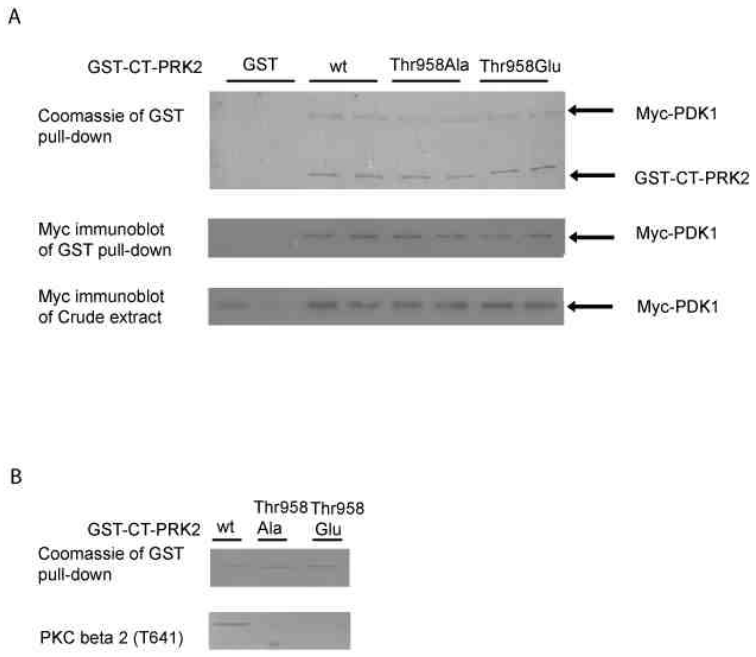

FIG. 6. **The Z/Turn-motif phosphorylation site does not affect the binding affinity of the C-terminal fragment of PRK2 to the PIF binding pocket of PDK1.** *A*, HEK293 cells were transiently trasfected with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST, GST-CT-PRK2 wt or the mutants GST-CT-PRK2 [Thr958Ala] and GST-CT-PRK2[Thr958Glu]. *B*, HEK293 cells were transiently transfected with DNA constructs expressing GST-CT-PRK2 wt or the mutants GST-CT-PRK2 [Thr958Ala] and GST-CT-PRK2 [Thr958Glu]. *A, B,* 36 h post-transfection the cells were lysed and the cell lysate was incubated with glutathione-Sepharose resin in order to pull-down the GST-constructs. The pulled-down proteins were washed several times in batch (*A*) or purified (*B*) and subjected to SDS-PAGE *(A, B)*. The electrophoresed proteins were stained with Coomassie Blue *(A, B)* and immunoblotted using an anti-Myc antibody to detect Myc-PDK1 that bound to GST-CT-PRK2 (*A*) or immunoblotted using PKC beta 2 (T641) to detect the phosphorylation status at the Z/Turn-motif phosphorylation site of GST-CT-PRK2 (*B*). *A*, To verify if the expression level of Myc-PDK1 was similar in all cases, the total cell lysate was subjected to SDS-PAGE and immunoblotted using anti-Myc antibody. *A*, Duplicates of each condition are shown.

Thus, the results indicated that GST-CT-PRK2 wt and mutants interacted equally well with PDK1. Such result is not compatible with the first model (Fig. 5A-B) since under such model, the GST-CT-PRK2 [Thr958Ala] mutant would have been expected to interact with higher affinity with PDK1. Therefore, the lack of phosphorylation at the Z/Turn-motif phosphorylation site of PRK2 may impair the binding of the C-terminal fragment of PRK2 to its own catalytic domain and indirectly promote its interaction with the PIF binding pocket of PDK1. However, in order to exclude the possibility that GST-CT-PRK2 was not phosphorylated at the Z/Turn-

motif phosphorylation site, we performed an immunoblot with purified constructs of GST-CT-PRK2 wt, GST-CT-PRK2 [Thr958Ala] and GST-CT-PRK2 [Thr958Glu]. By using anti-phospho-PRK (Upstate Biotechnologies) in order to check the phosphorylation status of the GST-CT-PRK2 constructs at the Z/Turn-motif phosphorylation site, we verified that GST-CT-PRK2 wt, but not GST-CT-PRK2 [Thr958Ala] or GST-CT-PRK2 [Thr958Glu], was phosphorylated at the Z/Turn-motif phosphorylation site (Fig.6B). Altogether, our results support the PRK2-PDK1 interaction model described in Fig. 5C-D.

4.7. PRK2 C-terminal negatively charged patch Glu968-Glu970 and hydrophobic patch Ile965/Leu966 are required for the high affinity interaction of PRK2 with PDK1

As already mentioned, the PDK1-interacting fragment (PIF) of PRK2 represents a "docking site" consisting in a hydrophobic sequence motif defined as Phe-Xaa-Xaa-Phe-Asp-Tyr, which allows PRK2 to interact with PDK1 by binding to the PDK1 PIF-binding pocket. In previous studies it has been proved that the C-terminal hydrophobic motif of PRK2 requires the hydrophobic residues Phe974, Phe977, Tyr979 and the negatively charged residue Asp978*,* for high affinity binding to PDK1. Indeed, mutation of any of the mentioned residues to Alanine abolished the interaction with PDK1 in pull down experiments [8].

We were interested to test if any other residues in the C-terminal fragment of PRK2 would contribute to the interaction with PDK1. Since an isolated polypeptide comprising the last 24 aminoacids of PRK2 possesses high affinity for PDK1 and shorter versions of the polypeptide have decreased affinity, additional high affinity determinants may be located within this region somewhere upstream of the hydrophobic motif. To study this hypothesis, we mutated the C-terminal Glutamic residues 968-969-970 to Alanine as well as the hydrophobic residues Ile965 and Leu966 of GST-CT-PRK2 to Alanine and tested the ability of GST-CT-PRK2 wt and the corresponding mutants to interact with Myc-PDK1 (the aminoacid residues mutated are those underlined in the sequence REPRILSEEEQEM**F**RD**FDY**IADWC).

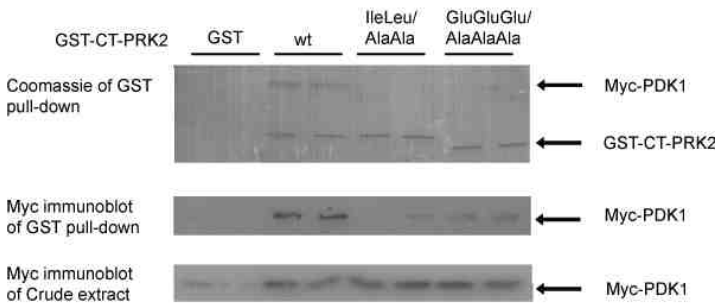

FIG. 7. **Not only the hydrophobic motif, but also further residues within the C-terminal fragment of PRK2 mediate the interaction between GST-CT-PRK2 and PDK1.** HEK293 cells were transiently trasfected with DNA constructs expressing Myc-PDK1 together with constructs expressing either GST, GST-CT-PRK2 wt or the mutants GST-CT-PRK2 [Ile965Ala; Leu966Ala] and GST-CT-PRK2 [Glu968Ala; Glu969Ala; Glu970Ala]. 36 h post-transfection the cells were lysed and the cell lysate was incubated with glutathione-Sepharose resin in order to pull-down the GST-constructs. The pulled-down proteins were subjected to SDS-PAGE, stained with Coomassie Blue, and immunoblotted using an anti-Myc antibody to detect Myc-PDK1 that bound to GST-CT-PRK2. To verify the equal expression of Myc-PDK1, the total cell lysate was subjected to SDS-PAGE and immunoblotted using anti-Myc antibody. Duplicates of each condition are shown.

In order to analyse the effect of the mutations on the interaction with PDK1, we co-transfected HEK293 cells with plasmids coding for Myc-PDK1 and for GST-CT-PRK2 wt and those containing the mentioned mutations. After incubating the cell lysate with glutathione Sepharose resin and washing several times in batch, we performed an immunoblot with Anti-Myc to detect the presence of Myc-PDK1 bound to the pulled down GST-CT-PRK2 wt and mutants. The presence of Myc-PDK1 was greatly reduced in the pull-down from GST-CT-PRK2 [Ile965Ala; Leu966Ala] and GST-CT-PRK2 [Glu968Ala; Glu969Ala; Glu970Ala] (Fig. 7) indicating that there was a very significant loss of interaction with PDK1 when the hydrophobic patch [Ile95; Leu96] or acidic patch [Glu968; Glu969; Glu970] were mutated. These results suggested that the interaction between GST-CT-PRK2 and PDK1 is not only mediated by the hydrophobic motif, but also by other residues within the C-terminal fragment of PRK2.

5. DISCUSSION

Many AGC subfamily members have been described to share a common core mechanism of activation based on three conserved phosphorylation sites, namely the activation loop, the Z/Turn-motif phosphorylation site and the hydrophobic motif (HM) phosphorylation site [21, 81, 158]. Previous studies demonstrated that the phosphorylation of the AGC subfamily members S6K, RSK and SGK at their hydrophobic motif phosphorylation sites stimulates the binding of their hydrophobic motif to the PIF-binding pocket of PDK1, enabling PDK1 to phosphorylate the interacting kinase at the activation loop [24, 71]. In contrast, the conventional PKC isoforms (PKCα, β and γ) appear to interact with PDK1 through their unphosphorylated hydrophobic motif to become phosphorylated at their activation loop (reviewed in [147]). Notably, considerable information had been acquired about the activation loop and hydrophobic motif phosphorylation sites. However, at the start of this thesis, there were still many unanswered questions about the role of the Z/Turn-motif phosphorylation site in the regulation of AGC kinase activities and on the interaction between PDK1 and its AGC kinase substrates.

Given the central role of the PDK1 signalling pathway in the regulation of the insulin signalling, cell growth and cell survival and consequently in the development of diabetes, human cancer and heart failure, we decided to further characterize the interaction between PDK1 and its AGC substrates, especially PRK2 and PKCζ. The knowledge that the PKC-related PRK2 and the atypical PKCζ possess a hydrophobic motif, but instead of a hydrophobic motif phosphorylation site they contain an acidic residue at its place (aspartic acid in PRK2 and glutamic acid in PKCζ, respectively), raised the possibility that the third phosphorylation site, namely the Z/Turn-motif phosphorylation site, could be involved in the interaction of these two PKC kinases with PDK1.

Thus, we decided to investigate the possible role of the Z/Turn-motif phosphorylation site in the regulation of the interaction of PRK2 and PKCζ with their upstream protein kinase activator PDK1 by performing interaction assays, in which we studied the consequences of mutating the Z/Turn-motif phosphorylation site in PRK2 and PKCζ on their interaction with PDK1 and further characterized determinants for the interaction between PDK1 and PRK2. Our experiments revealed

that a mutation of the Z/Turn-motif Threonine phosphorylation site to Alanine increased the binding of the hydrophobic motif of PRK2 to the PIF binding pocket of PDK1. In contrast, the Z/Turn-motif phosphorylation did not affect the interaction between PKCζ and PDK1.

Based on these results, we decided to further investigate the role of the Z/Turn-motif phosphorylation site in regulating the activity of PRK2 and showed that a lack of phosphorylation at this position drastically reduced PRK2 activity *in vitro*. We also characterized other aspects of the requirements for the interaction between PRK2 and PDK1. Our findings suggest that both a hydrophobic patch and a negatively charged patch located in between the hydrophobic motif and the Z/Turn-motif phosphorylation site directly support the binding of the C-terminal fragment of PRK2 (GST-CT-PRK2) to PDK1 and are responsible for the high affinity interaction. Finally, we characterized aspects of the molecular mechanism by which PRK2 mutation at the Z/Turn-motif phosphorylation site increases the interaction with PDK1. The results are compatible with a model in which PRK2 activity may be regulated in cells by the Z/Turn-motif phosphorylation in two ways: 1- Z/Turn-motif phosphorylation directly activates PRK2 and 2- phosphorylation would decrease the interaction of PRK2 with PDK1.

5.1. Role of the Z/Turn-motif phosphorylation site in the regulation of the interaction of PRK2 with PDK1

In order to study the role of the Z/Turn-motif phosphorylation site in the regulation of the interaction between PRK2 and PDK1, we performed interaction assays with Myc-PDK1 together with GST-PRK2 wt, GST-PRK2 [Thr958Ala] and GST-PRK2 [Thr958Glu]. The achieved results (Fig. 1A) revealed that a mutation of the Threonine residue of the Z/Turn-motif phosphorylation site of GST-PRK2 to Alanine (GST-PRK2 [Thr958Ala]) significantly increased the binding of the hydrophobic motif of PRK2 to PDK1. An increased interaction with PDK1 also occured for PRK2 [Thr958Glu], however, to a lower extent than for GST-PRK2 [Thr958Ala]. These important findings were confirmed by further experiments, in which we analysed the interaction between GST-PDK1 and the wild-type and the [Thr958Ala] and [Thr958Glu] mutants of FLAG-PRK2. Again, we observed a higher binding of FLAG-PRK2 [Thr958Ala] to GST-PDK1 compared to FLAG-PRK2 wt, as well as an intermediate level of interaction between FLAG-PRK2 [Thr958Glu] and GST-PDK1 (Fig.1B).

Thus, the results obtained in these experiments suggested that the Z/Turn-motif phosphorylation site can play an important role in the regulation of the interaction between PRK2 and PDK1. Actually, a lack of phosphorylation at this site upon mutation significantly increased the binding of the hydrophobic motif of PRK2 to PDK1 in pull-down experiments. In additional experiments, we generated C-terminal fragments of PRK2 as GST-fusion proteins with a mutated Z/Turn-motif phosphorylation site and analyzed their interaction with PDK1. In these assays we realized that GST-CT-PRK2 wt and GST-CT-PRK2 [Thr958Ala] are both able to interact equally well with PDK1, suggesting that the C-terminal fragment of PRK2 is sufficient to permit the binding to PDK1. Posterior work in our group showed that peptides derived from the C-terminal fragment of PRK2 (PIF) and PIF Thr/Ala interacted equally well with PDK1 and stimulated PDK1 activity with similar AC 50 (the concentration necessary to achieve 50% of the maximal activation), which corresponds to the concentration that is required to obtain half maximal activity. Altogether, the results did not support a model in which the lack of phosphorylation at the HM triggered the binding to PDK1. Rather, our results are compatible with a model in which the phosphorylation of the Z/Turn-motif phosphorylation triggers an increased binding to the Z/ Turn-motif phosphate binding site within the catalytic domain of PRK2. In this manner, the binding to PRK2 catalytic domain would prevent its binding to the PIF pocket on PDK1.

To further characterize the role of the Z/Turn-motif phosphorylation site in the regulation of the interaction between PRK2 and PDK1, we performed interaction assays with Myc-PDK1 and GST-PRK2 wt and evaluated whether the employment of chemical compounds, such as the protein tyrosine phosphatases inhibitor Orthovanadate and the selective PI3 kinase (PI3K) inhibitor LY294002, would affect the binding of PRK2 to PDK1.

Through the inhibition of tyrosine phosphatases, Orthovanadate is expected to augment the phosphorylation status of the tyrosine residues within all proteins in the cell. Remarkably, Orthovanadate seemed to abolish the interaction between GST-PRK2 wt and Myc-PDK1 (Fig.2C), suggesting that the higher level of phosphorylation at tyrosine residues could impair the interaction of the AGC kinase with PDK1.

Thus, it is possible that an unknown kinase, distinct from the serine/threonine kinase PDK1, phosphorylates PRK2 and influences its binding properties for its upstream kinase activator PDK1. One possibility is that the Z/Turn-motif

phosphorylation site-kinase increases its activity upon increased tyrosine residues phosphorylation. This increase in activity would translate to increased Z/Turn-motif phosphorylation and decreased binding to PDK1.

Since PRK2 activity is linked to Rho activation, and Rho GTPases are downstream of multiple Receptor Tyrosine Kinases (RTK), it is tempted to speculate that the Orthovanadate effect may be related to the activation of RTK. Kinases that efficiently phosphorylate phosphorylation sites followed by a Pro residue are typically from the MAPK family or CDK family of protein kinases. Given the RTK link to PRK2 activation, it can be envisaged that MAP Kinases, which are typically activated by RTK, could be involved in the phosphorylation of the Z/Turn-motif phosphorylation site on PRK2, which is likewise followed by a proline residue (Fig.1). Specifically blocking the activity of the Z/Turn-motif phosphorylation site kinase is expected to increase the interaction of PRK2 with PDK1. Based on this indirect method, we can discard the MAP kinase family members MEK1 and its substrates ERK1/2, since the MEK1 kinase inhibitor PD 98059 [168] did not affect the interaction of PRK2 with PDK1 (data not shown).

In our cellular system, LY294002 did not appear to affect the binding of GST-PRK2 wt to PDK1 (Fig.2C). This result suggests that the Z/Turn-motif phosphorylation site-kinase is not a kinase which requires PI3K activation nor is regulated transiently by PI3K signalling. In this way, we can discard any relation between the PI3K downstream kinases (such as PKB/Akt, S6K, SGK, RSK, and their downstream kinase targets, such as GSK3) on the Z/Turn-motif phosphorylation site-kinase activation and the interaction between PDK1 and PRK2. This conclusion is further supported by the finding that the unspecific kinase inhibitor staurosporine (which inhibits several of the above stated kinases) did not affect the interaction between PDK1 and PRK2.

In the past, Flynn and co-workers also studied the effects of inhibiting PI3K activity by using LY294002 on endogenous PRK activation loop phosphorylation [67]. This work reported that while the basal phosphorylation level at the activation loop residue Thr-774 of PRK1 was slightly lower upon inhibitor treatment, the increased signals expected after transfection of PRK1 with either GTPase-deficient and thus overactive Rho or PDK1 was decreased to nearly basal levels by the inhibition of PI3K activity. These results have been interpreted as related to the requirement of a PtdIns(3,4,5)P_3-dependent PDK1 localization/allosteric event for PRK

phosphorylation. However, as the activation loop phosphorylation of PRK by PDK1ΔPH (residues 51-404) was also inhibited by LY294002 and therefore obviously dependent on PI3K activity, it has been assumed that PI3K influences PRK in a pathway that does not involve PDK1, but probably endogenous GTPase.

In apparent contrast, in our experiments LY294002 did not affect the binding of PRK2 to PDK1. This is presumably due to the fact that, while Flynn and co-workers employed endogenous PRK proteins, we used overexpressed PRK2 in our interaction assays. It is possible that the overexpression of PRK2 made the kinase more resistant to outside influences, such as PI3K inhibition. Altogether, the conditions in our assays did not reflect the normal intracellular conditions or the conditions in the experiments performed with endogenous PRK constructs by Flynn and co-workers, thus influencing to some extent our findings. The Orthovanadate experiments, on the other hand, provided evidence that protein phosphorylation can influence the interaction between PRK2 and PDK1 in a cellular environment.

5.2. Effect of the Z/Turn-motif phosphorylation site on the activity of PRK2

In addition to the interaction assays performed to define the role of the Z/Turn-motif phosphorylation site in the regulation of the interaction between PRK2 and PDK1, we decided to evaluate the consequences of mutating the Z/Turn-motif phosphorylation site on PRK2 activity *in vitro*, by subjecting the purified GST-fusion proteins GST-PRK2 wt, GST-PRK2 [Thr958Ala] and GST-PRK2 [Thr958Glu] to a protein kinase assay.

As presented in Fig. 4A, the mutants GST-PRK2 [Thr958Ala] and GST-PRK2 [Thr958Glu] had considerably lower activity than GST-PRK2 wt. The immunoblot assays showed that, as expected, PRK2 wt was phosphorylated at the Z/Turn-motif phosphorylation site, while the mutation of this site in both PRK2 mutants led to a lack of phosphorylation at this position (Fig.4B). Thus, the lack of phosphorylation at the Z/Turn-motif phosphorylation site of GST-PRK2 [Thr958Ala] and GST-PRK2 [Thr958Glu] was associated with an important loss of activity of both GST-PRK2 mutants.

Our findings with the full-length PRK2 constructs agree with the recently presented results by Hauge and co-workers [81], which showed that a mutation of the Z/Turn-motif phosphorylation site or of its phosphate-binding site in the kinase domain of PKBα, S6K1, MSK1, RSK1 as well as of the truncated PRK2 lacking the N-terminal region ($PRK2_{\Delta 1\text{-}500}$) likewise significantly reduced kinase activity in all

cases. These results emphasized the crucial role of the Z/Turn-motif phosphorylation site and of its binding site in mediating kinase activity of the AGC subfamily members [81].

Furthermore, Hauge and co-workers [81] proposed a model, in which the bound Z/Turn-motif phosphorylation site supports the formation of a closed and active kinase conformation. Even if the phosphorylated Z/Turn-motif phosphorylation site has no effect on its own on kinase activity, it directly stimulates kinase activity by synergistically enhancing kinase activation by the hydrophobic motif phosphate in cooperation with the activation loop phosphate. In the case of some AGC kinases, such as S6K1, MSK1 and RSK2, a mutation of the Z/Turn-motif phosphorylation site importantly diminished phosphorylation of the hydrophobic motif, suggesting that the function of this phosphorylation site to promote the binding of the hydrophobic motif to its own hydrophobic pocket, not only stabilizes the phosphorylated hydrophobic motif in the pocket, but also reduces the exposure of its phosphate to phosphatases [81].

Surprisingly, single to triple mutation of the basic residues in the Z/Turn-motif phosphate binding site of PKBα has been described to importantly increase basal and insulin-stimulated kinase activity, which appeared to result from the augmented phosphorylation of the hydrophobic motif and activation loop [81]. For this reason, in an even more general scenario, the Z/Turn-motif phosphorylation site can help regulate the level of phosphorylation and dephosphorylation of the hydrophobic motif and activation loop phosphorylation sites.

Previous publications reported that PDK1 activates both PRK1 and PRK2 by phosphorylating the conserved threonine residue in their activation loops (Thr-774 for PRK1 and Thr-816 for PRK2) *in vitro* and *in vivo*. In addition, an increased phosphorylation status at the activation loop led to a significant activity increase of both PRK kinases [67]. Consistent with these findings, the activity level of GST-PRK2 wt - as the GST-PRK2 construct with the highest phosphorylation degree at the activation loop (Fig.4 C) - was ~4 times higher than the activity level measured for GST-PRK2 [Thr958Ala] or GST-PRK2 [Thr958Glu] (Fig.4A). In parallel experiments performed in our laboratory, the activity of PKCζ constructs with a mutated Z/Turn-motif phosphorylation site was completely abolished. Thus, it appears that the requirement of the Z/Turn-motif phosphorylation site in the full length PRK2 protein is

not as important as the phosphorylation of the Z/Turn-motif phosphorylation site in other AGC kinases, such as PKCζ.

Surprisingly, even if the phosphorylation status at the activation loop was considerably lower compared to GST-PRK2 wt or GST-PRK2 [Thr958Ala] (Fig. 4C), the kinase activity of GST-PRK2 [Thr958Glu] was higher than in the case of GST-PRK2 [Thr958Ala] (Fig.4A). This suggests that the negatively charged Glutamic acid inserted in place of the Threonine residue in GST-PRK2 [Thr958Glu] could be able to mimic the phosphorylated Z/Turn-motif phosphorylation site, thus promoting kinase activation to some extent. However, this assumption is not concordant with recent work, in which mutation of the Z/Turn-motif phosphorylation site to a phosphate-mimicking Glu could replace phosphorylation in PKBα and RSK2, but not in the truncated PRK2 lacking the N-terminal region [81]. It is possible that Glu can substitute the phosphorylation status in the full length PRK2, but not in the isolated catalytic domain of PRK2. It is also notorious that the [Thr958Glu] mutant in the context of the catalytic domain of PRK2 [81] had lower level of activation loop phosphorylation.

Therefore, the effects of mutations in the catalytic domain of PRK2 and in the full length of PRK2 are not always identical. Both forms of PRK2 are equally phosphorylated at the activation loop when the Threonine residue of the Z/Turn-motif phosphorylation site is mutated to Alanine and both forms have decreased specific activity. However, more detailed inspection of the data suggests that the activity of the catalytic domain appears to be more affected by this mutation then the activity of the full length PRK2. A further difference is observed when the Threonine residue of the Z/Turn-motif phosphorylation site is mutated to Glu. In this regard, the full length PRK2 protein has substantially more activity when the Threonine residue of the Z/Turn-motif phosphorylation site is mutated to Glu with respect to the non-mutated form of the kinase. Altogether, we are tempted to speculate that the N-terminus of the kinase does indeed have an effect on the kinase activity. To do this, it should be expected that at least parts of the N-terminus are folding back onto the catalytic domain. However, further experiments to clarify this aspect are required. In order to confirm the above stated observation, an obvious test would be to purify the PRK2 mutant described by Hauge et al. and perform the assay with both forms of the PRK2, in parallel.

Eventually, we can claim that the Z/Turn-motif phosphorylation site is directly involved in the mediation of PRK2 activity.

5.3. Mutation of the Z/Turn-motif phosphorylation site does not increase the binding of the C-terminal fragment of PRK2 to PDK1

According to the results achieved in the interaction assays described above, we developed 2 distinct general models which could elucidate the increased binding of GST-PRK2 [Thr958Ala] to PDK1. The first model suggests a higher affinity of the C-terminal fragment of PRK2 (CT-PRK2) to PDK1 in the absence of phosphorylation at the Z/Turn-motif phosphorylation site (Fig. 5A). The increased binding would be lost upon phosphorylation of PRK2 at this site (Fig. 5B).

To evaluate this possible model, we generated C-terminal fragments of PRK2 as GST-fusion proteins with the according mutations at the Z/Turn-motif phosphorylation site (GST-CT-PRK2 wt, GST-CT-PRK2 [Thr958Ala] and GST-CT-PRK2 [Thr958Glu]) and analysed their capability to interact with PDK1. All the employed GST-CT-PRK2 constructs interacted equally well with PDK1 (Fig.6A). In addition, to assure that GST-CT-PRK2 wt was indeed phosphorylated at the Z/Turn-motif phosphorylation site, we subjected the purified GST-CT-PRK2 constructs to an immunoblot with anti-phospho PRK and showed that GST-CT-PRK2 wt, but not the mutants GST-CT-PRK2 [Thr958Ala] or GST-CT-PRK2 [Thr958Glu], was phosphorylated at this phosphorylation site (Fig.6B). Thus, since the C-terminus phosphorylated at the Z/Turn-motif phosphorylation site interacted equally well as the Ala or Glu mutants of the phosphorylation site, we confidently discard the first model, which suggested that a lack of phosphorylation at the Z/Turn-motif phosphorylation site of GST-CT-PRK2 [Thr958Ala] and GST-CT-PRK2 [Thr958Glu] would increase the affinity of their C-terminal fragments to PDK1.

In a second model, we propose that the phosphorylation of the Z/Turn-motif phosphorylation site promotes an intramolecular binding of the phosphorylated C-terminal fragment of PRK2 to PRK2 polypeptide (Fig. 5C), while a mutation of the Z/Turn-motif phosphorylation site would inhibit this intramolecular interaction and, in this manner, indirectly promote the binding of the C-terminal fragment of PRK2 to the PIF-binding pocket of PDK1 (Fig.5D). Our results are compatible with this second model (Fig. 5C-D). Since the increased binding of the PRK2 [Thr958Ala] mutant to PDK1 was also found using the catalytic domain of PRK2 in follow up work, we conclude that the putative phosphorylation dependent intramolecular interaction does

not require the N-terminal 500 aminoacids of PRK2 and is most likely located within the catalytic domain of PRK2. In parallel work to this research, in collaboration with Prof. Frödin (Copenhagen) our laboratory provided evidence about the existence of a Z/Turn-motif-phosphate binding site on the small lobe of the catalytic domain of multiple AGC kinases, including PRK2. Thus, the mutation of the PRK2 basic residues Arg (665), Lys (670), Lys (689) and His (732) forming part of the putative phosphate binding site resulted in a reduced kinase activity to comparably low levels as obtained by mutating the Z/Turn-motif phosphorylation site of PRK2 [81]. Besides, the phosphorylation level of the Z/Turn-motif phosphorylation site of PRK2 upon mutating the basic residues in the catalytic domain of the kinase was profoundly diminished, suggesting that the binding of the phosphorylated Z/Turn-motif phosphorylation site to the mentioned basic residues protects it from dephosphorylation [81].

If the Z/turn-motif phosphate binding site was indeed participating in the mechanism by which interaction to PDK1 is increased, then it should be possible test this by mutation of residues within the phosphate binding site. Indeed follow-up experiments confirmed that mutation of the Z/turn-motif phosphate binding site also increased the interaction of PRK2 with with PDK1 [54].

5.4. Role of the Z/Turn-motif phosphorylation site in the regulation of the interaction of PDK1 with other AGC substrates

Within AGC kinases, PKCζ is similar to PRK2 in that it possesses a Z/Turn-motif phosphorylation site and does not have a hydrophobic motif phosphorylation site. Surprisingly, in sharp contrast to the findings with PRK2, in parallel experiments we could show that a mutation of the Threonine residue of the Z/Turn-motif phosphorylation site of PKCζ to Alanine (GST-PKCζ [Thr560Ala]) did not affect its interaction with PDK1 (Fig.3A). These results were confirmed by additional interaction assays, in which we analysed the interaction between GST-PDK1 and FLAG-PKCζ constructs (FLAG-PKCζ wt and FLAG-PKCζ [Thr560Ala]). As already observed in the case of the GST-PKCζ proteins, both FLAG-PKCζ constructs interacted equally well with PDK1 (Fig.3B).

In order to exclude the possibility that a low stoichiometry of phosphorylation of the Z/Turn-motif phosphorylation site could be responsible for the obtained results, we performed again the co-transfection of GST-PKCζ wt and GST-PKCζ [Thr560Ala] with PDK1 and in parallel, we repeated this assay with the addition of the protein

serine/threonine phosphatase inhibitor Okadaic acid 2 h prior to cell lysis. Again, no difference could be detected in the interaction between PDK1 and the GST-PKCζ constructs GST-PKCζ wt and GST-PKCζ [Thr560Ala] (Fig.3 C-D), suggesting that the phosphorylation of the Z/Turn-motif phosphorylation site does not affect the interaction of PKCζ with PDK1. Thus, we can conclude that, in contrast to PRK2, the Z/Turn-motif phosphorylation site is not involved in the regulation of the interaction between PKCζ and PDK1.

Interestingly, PKCζ was also shown to possess a Z/Turn-motif-phosphate binding site, similar to PRK2. Thus, it is not easy to explain why the Z/Turn-motif-phosphate could regulate the interaction of PRK2 with PDK1 while not affecting, indirectly, the PKCζ interaction. One possible explanation is the level of affinity between the Z/Turn-motif phosphate and the phosphate binding site. The higher affinity of the Z/Turn-motif phosphate to the corresponding phosphate binding site, the more it is likely to regulate the interaction with PDK1. Alternatively, it is possible that other more complex mechanisms take place in PKCζ. Indeed, PKCζ mechanism of regulation is highly complex, possessing multiple N-terminal regulatory modules: a PB1 domain (involved somehow in activation of PKCζ by interaction with partner proteins), a putative “pseudo-substrate” region (thought to mediate intramolecular inhibition as a pseudo-substrate) and a C1 domain (involved in the regulation of the activation of PKCζ by phospholipids). Thus, it can be reasonably contemplated that the C-terminus of PKCζ, comprising the Z/Turn-motif phosphate, could have a more complex relationship to other portions of the PKCζ protein. In order to test this possibility, future work could evaluate the effect of mutating the Z/Turn-motif phosphate residue in a construct comprising the catalytic domain of PKCζ.

Remarkably, the interaction of PKCζ with PDK1 is likely to be regulated in a decisive way by its hydrophobic motif, since the mutation of the residue Leu155 of the PIF-binding pocket of PDK1 to Glutamic acid (Leu155Glu) significantly reduced the interaction of the GST-PKCζ constructs GST-PKCζ wt and GST-PKCζ [Thr560Ala] with PDK1 (data not shown).

In overexpression systems, SGK and S6K interaction with PDK1 were also not regulated by Z/Turn-motif phosphorylation. Again, it is not clear why this same mechanism does not happen in other AGC kinases different from PRK2 which also have a Z/Turn-motif phosphorylation site and a putative Z/Turn-motif phosphate

binding site, such as SGK and S6K. It is possible that S6K and SGK have evolved to only rely on the HM phosphorylation to regulate the interaction with PDK1.

In the case of S6K, previous work suggested a possible model, in which phosphorylation of its C-terminal autoinhibitory domain induces a conformational change, enabling the hydrophobic motif of S6K to interact with the PIF-binding pocket of PDK1 and thus allowing S6K phosphorylation at the activation loop by PDK1 [24, 40]. In addition, the activation of S6K and SGK by PDK1 is regulated in a decisive manner by the phosphorylation of the hydrophobic motifs of both AGC kinases, as phosphorylation of S6K and SGK at this position enhances their interaction with PDK1 (reviewed in [134]).

In accordance with the finding that the Z/Turn-motif phosphorylation site plays a key role in the regulation of kinase activity among the AGC subfamily members by an intramolecular activation mechanism, a mutation of the Z/Turn-motif phosphorylation site of S6K to Alanine or a mutation of the basic residues situated in its phosphate-binding pocket in the kinase domain markedly reduced S6K activation [81].

Finally, we should contemplate that the studies here presented were either performed *in vitro* or in cellular overexpression systems, where, if low abundant third components were required, these would not be represented. Thus, it is conceivable that the PKCζ Z/Turn-motif phosphate may participate in the regulation of the interaction with PDK1 in *in vivo* physiological situations which were not tested in the present work.

5.5. Role of the N-terminal extension of PRK2 on the interaction with PDK1

To clarify the role of the N-terminal extension of PRK2 on the interaction of the AGC kinase with PDK1, we generated GST-PRK2 constructs with a disrupted auto-inhibitory N-terminal domain (ΔNT-PRK2), namely GST-ΔNT-PRK2 wt and GST-ΔNT-PRK2 [Thr958Ala], and analysed the consequences of these mutations on the interaction with PDK1, *in vivo*. In parallel, we repeated the same experiment with PDK1 and, instead of the GST-ΔNT-PRK2 constructs, we employed the GST-PRK2 full-length constructs GST-PRK2 wt and GST-PRK2 [Thr958Ala] as a control. In this way, we hoped to be able to compare the results obtained with the GST-PRK2 full-length constructs with the data achieved with the GST- ΔNT-PRK2 mutants lacking the N-terminal regulatory domain.

Interestingly, previous publications suggested that the N-terminus is likely to have an inhibiting effect on PRK2 activity, since ΔNT-PRK2 has been shown to possess a ~10-fold higher specific activity than full-length PRK2 [7].

Despite a lower expression level of both GST-ΔNT-PRK2 wt and GST-ΔNT-PRK2 [Thr958Ala] compared to GST-PRK2 wt and GST-PRK2 [Thr958Ala] (data not shown), the results achieved in the pull-down suggested that the GST-ΔNT-PRK2 constructs still interacted with PDK1 (data not shown). For this reason, we can assume that, under these assay conditions, the N-terminus is not required for the intermolecular interaction between PRK2 and PDK1. Besides, according to follow up work, we can state that the GST-ΔNT-PRK2 [Thr958Ala] mutant also interacted better with PDK1 than GST-ΔNT-PRK2 wt.

However, Flynn and co-workers [67] previously suggested an interaction model between PRK2 and PDK1, in which the prior binding of a GTPase (such as RhoA-GTP) to the N-terminal of PRK2 located HR1 domain enables PRK2 to interact with PDK1, which consequently phosphorylates PRK2 at the activation loop in a phosphoinositide-dependent manner. Based on this work, it would be interesting to repeat the experiments performed with the GST-ΔNT-PRK2 and GST-PRK2 full-length constructs together with PDK1 as described above in the presence of a GTPase, such as RhoA-GTP. However, the formation of the Rho-PRK2-PDK1 complex and the resulting activation of PRK2 by PDK1 *in vivo* has been reported to take place only upon prenylation of Rho, indicating that the PtdIns(3,4,5)P_3-dependent phosphorylation of PRK2 by PDK1 requires a prior assembly on a membrane [67]. To avoid this, the planned experiment constellation could also be realized *in vitro*, since under these conditions the HR1 domain of PRK2 has been reported to be able to bind to bacterially expressed and consequently non-prenylated Rho-GTP [68].

Concerning this matter, we attempted to repeat the experiments fully *in vitro* to exclude the influence of other factors which may influence the interaction. For this purpose, we incubated the purified GST-fusion proteins GST-PRK2 wt, GST-ΔNT-PRK2 wt, GST-PRK2 [Thr958Ala] and GST-ΔNT-PRK2 [Thr958Ala] with His-tagged PDK1 and pulled down the amount of His-PDK1 bound to the GST-fusion proteins with Nickel (Ni-NTA) Sepharose. However, we were not able to distinguish between specific and unspecific binding between ΔNT-PRK2 and PDK1 and further efforts are required in order to augment the specificity of this *in vitro* method.

5.6. PRK2 C-terminal acidic and hydrophobic patch as well as the PIF-binding pocket of PDK1 are required for high affinity interaction between PRK2 and PDK1

Previous publications reported that the PDK1-interacting fragment of PRK2 (PIF) interacts with PDK1 by binding to the PDK1 PIF-binding pocket. Besides, the C-terminal located hydrophobic residues Phe974, Phe977, Tyr979 and the acidic residue Asp978 have been shown to be necessary for the high affinity binding of the hydrophobic motif of PRK2 to PDK1 [8].

In our interaction assays, we investigated whether further residues located upstream of the hydrophobic motif in the C-terminal fragment of PRK2 interacted with PDK1. For this purpose, we analysed the consequences of mutating the C-terminal Glutamic residues 968-969-970 to Alanine (GST-CT-PRK2 [Glu968Ala; Glu969Ala; Glu970Ala]) or of mutating the hydrophobic residues Ile965 and Leu966 to Alanine (GST-CT-PRK2 [Ile965Ala; Leu966Ala]) on the interaction with PDK1, in comparison with GST-CT-PRK2 wt. Interestingly, while GST-CT-PRK2 wt interacted with PDK1, we observed a significant loss of interaction between the mentioned GST-CT-PRK2 mutants and PDK1 (Fig.7), indicating that the C-terminal hydrophobic residues Ile965, Leu966 and the negatively charged residues Glu968, Glu969, Glu970 of PRK2 are also essential for the high affinity interaction between PRK2 and PDK1.

Based on these findings, it would be interesting to perform additional experiments in order to detect the exact residues of PDK1 that are involved in the interaction with the mentioned C-terminal residues of PRK2. Applying on the fact that the binding of the hydrophobic motif of PRK2 to the PIF-pocket of PDK1 is the precondition for the interaction between the two AGC kinases and by considering that a mutation of the C-terminal PRK2 residues Ile965, Leu966 and Glu968, Glu969, Glu970 significantly decreased the interaction between PRK2 and PDK1, we can speculate that these PRK2 residues interact with PDK1 residues located close to the PIF-binding pocket. However, it is not clear why mutation of the hydrophobic patch of PRK2 significantly reduced the interaction between the mentioned GST-CT-PRK2 mutants and PDK1 without affecting the interaction of full length PRK2 with PDK1. One possible explanation is that the hydrophobic patch plays a comparable role in the intermolecular binding of the C-terminal of PRK2 to PDK1 as well as in the intramolecular binding to the catalytic core of PRK2 [54]. Besides, recent studies suggested an additional motif located in the C-terminal region of PRK2, termed the

adenosine binding (Ade) motif, which may be involved in a decisive manner in the interaction of the C-terminus of PRK2 with PDK1 [166].

Remarkably, the hydrophobic patch of PRK2 described above is not conserved among the AGC subfamily members that interact with PDK1. As a consequence, it is thought to be responsible for the higher binding affinity of the C-terminal region of PRK2 (PIFtide) to PDK1 in comparison to the C-terminal regions of other AGC kinases [23]. In accordance with this, Leu966 has been recently detected as a PIFtide determinant for interaction with PDK1 [166].

As a summary, the results presented thus far indicate that the CT extension to the catalytic core harbours the main determinants for the interaction of PRK2 with PDK1 [54].

5.7. Regulation of the interaction between PRK2 and PDK1

As already mentioned, Flynn and co-workers reported that PRK2 interacts with PDK1 in a Rho-dependent manner [67], whereas the binding of a GTPase to PRK2 promotes the interaction of its hydrophobic motif with PDK1. As a consequence, the authors suggested that PDK1 phosphorylates the activation loop of PRK2 in the presence of PtdIns(3,4,5)P_3, enabling PRK2 to autophosphorylate and be further activated [67].

Since a mutation of the Z/Turn-motif phosphorylation site of PRK2 to Alanine in our assays increases the interaction between PRK2 and PDK1 *in vivo*, it is possible that this mutation adopts to some extent the promoting effect of Rho-GTPase on the interaction of PRK2 with PDK1, rendering the influence of Rho in the presence of a PRK2 construct with a lack of phosphorylation at the Z/Turn-motif phosphorylation site superfluous.

Since PRK2 does not contain a hydrophobic motif phosphorylation site, but a phosphate-mimicking aspartic acid at the equivalent position, the Z/Turn-motif phosphorylation site seems to be the only C-terminal phosphorylation site which has to be phosphorylated in order to achieve maximal kinase activation, upon phosphorylation at the activation loop by PDK1. Thus, the activation mechanism of PRK2 appears to be less complex in comparison with that of most PKC isoforms, which, upon phosphorylation by PDK1 at their activation loops, are thought to undergo an intramolecular autophosphorylation mechanism at both the hydrophobic motif and Z/Turn-motif phosphorylation sites to achieve maximal activation.

Whether PRK2, in analogy to the PKC isoforms, phosphorylates really itself at the Z/Turn-motif phosphorylation site or if the kinase is phosphorylated at this site by another kinase is not clear, yet. Our experiments were not designed to evaluate this possibility. However, hints to this answer could be obtained by treatment of cells with Yoshitomi compound Y-27632 which is known to inhibit ROCK and PRK2 [2]. If the Z/Turn-motif-phosphorylation is not inhibited, it may be tempted to speculate that the phosphorylation may not be an auto-phosphorylation.

5.8. Role of the phosphorylation sites of PKCζ in kinase stability and activity

As already mentioned, the glutamic acid in place of the hydrophobic motif phosphorylation site in PKCζ is not required for the interaction of PKCζ with PDK1, while a mutation of the aromatic residues of the hydrophobic motif significantly reduced the binding of the AGC kinase to its upstream activator PDK1 [7]. By considering that, as an atypical PKC isoform, PKCζ responds to neither diacylglycerol nor to Ca^{2+} and its activation differs from the intramolecular autophosphorylation mechanism described for conventional PKCs [14], another activation mechanism must exist for atypical PKC isoforms which has not been clarified, yet.

As general among PKC isoforms, PKCζ binds to PDK1 with its C-terminal docking site. PDK1 preferentially interacts with the unphosphorylated hydrophobic motif of PKCs [73] and indeed the lack of a phosphorylation site in the hydrophobic motif of PKCζ does not affect this interaction with PDK1 [7]. Activation loop phosphorylation of atypical PKC isoforms has been reported to be increased upon PI 3-kinase activation, a fact that distinguishes the atypical PKCs from the conventional and novel PKCs (reviewed in [147]). The authors suggest that PtdIns(3,4,5)P_3 assumes the role of tethering PKCζ in the open conformation to membranes, which is accomplished by Ca^{2+} and diacylglycerol in the case of conventional and novel PKCs (reviewed in [147]). Thus, it has been suggested that the recruitment of PKCζ to the membrane would allow its phosphorylation at the activation loop and thus activation by PDK1. However, in overexpression experiments performed by Ricardo M. Biondi and Dario Alessi, they never observed a PI 3-kinase dependence on the activation of overexpressed PKCζ, while this effect was clearly observed in using Akt/PKB. Thus, in our laboratory we are tempted to believe that the mechanism may be more complicated than the postulated model.

Recently, the conventional PKCα has been found to be phosphorylated at the hydrophobic motif by mTORC2 [77]. This finding raises the possibility that additional

pathways, apart from the intramolecular autophosphorylation mechanism presumed until now, could be involved in the activation and stabilization of the conventional and novel, but also of the atypical PKC isoforms. Therefore, the C-terminal Z/Turn-motif phosphorylation site - currently assumed to be an autophosphorylation in all PKC isoforms - could likewise be phosphorylated by a still unknown kinase.

Previous work reported that phosphorylation of the hydrophobic motif phosphorylation site in PKC is not required for kinase function, but rather for kinase stability (reviewed in [145, 147]). This seems also to apply for the equivalent glutamic acid in the hydrophobic motif of PKCζ, since its mutation did not significantly influence PKCζ activity, but it was shown to reduce kinase stability in a particular heat-inactivation test [7].

At this stage, it would be interesting to evaluate the role of the Z/Turn-motif phosphorylation site in mediating PKCζ activity. This phosphorylation site has been described to be necessary for kinase activity of several PKC isoforms, since its mutation led to a relevant loss of kinase stability and activity (reviewed in [147]). Remarkably, previous work reported that negative charge at the turn motif is required and sufficient for the function of mature protein kinase C, since upon enzyme maturation, the Z/Turn-motif phosphate is all that is necessary for the catalytic function of the PKC kinase (reviewed in [145]). According to these findings, the Z/Turn-motif phosphorylation site in PKCζ is presumably necessary for kinase stability and activity. However, the work on PKCζ suggests that this may not be the case for atypical PKC isoforms.

Altogether, the findings thus far suggest that PKCζ activation is dependent especially on activation loop phosphorylation by PDK1 and phosphorylation at the Z/Turn-motif phosphorylation site, while an intact acidic residue in the hydrophobic motif of the kinase appears to be necessary for kinase stability *in vivo*.

Recent studies about the role of the Z/Turn-motif phosphorylation site of the atypical PKCι have revealed that the phosphorylation at this site is less crucial for PKCι compared to its relevance for other analysed AGC kinases [81]. Actually, the results presented showed that mutation of the phosphorylated Z/Turn-motif phosphorylation site resulted in a ~25% reduction of kinase activity. In comparison with the data concerning the loss of kinase activity of other AGC kinases upon performing the same mutations (60%-98% for S6K1 or 40-60% for RSK1 [81]), the results indicate that the reduction in kinase activity observed in this atypical PKC is

not representative of atypical PKCs since our laboratory has shown that the activity of PKCζ constructs with a mutated Z/Turn-motif phosphorylation site was completely abolished. Thus, our analysis of another atypical PKC isoform showed that the Z/Turn-motif phosphorylation site was essential for its activity. We cannot explain such a difference, since both atypical PKC isoforms are rather conserved.

5.9. Conclusion

PDK1 plays a key role in the insulin signalling, cell growth and cell survival by regulating several biological responses, including glucose metabolism, glycogen, lipid and protein synthesis, gene expression and cell growth/differentiation.

Along apoptosis, PRK2 is cleaved and the cleaved CT terminus, can potentially bind the PIF pocket of PDK1, influencing insulin and growth factor signalling. On the other hand, PKCζ interaction with PDK1 may determine glucose uptake in response to insulin signals.

This project helped in the characterization of the PDK1 signalling pathway by studying the interaction between the central AGC protein kinase activator and several AGC kinase substrates, especially PRK2 and PKCζ. In particular, we showed that the Z/Turn-motif phosphorylation site plays a crucial role in the regulation of the interaction between PRK2 and PDK1 and emphasized the direct involvement of the Z/Turn-motif phosphorylation site in the regulation of PRK2 kinase activity. Our results suggest that phosphorylation of the Z/Turn-motif phosphorylation site promotes an intra-molecular interaction with the PRK2 catalytic domain, while a mutation of the Z/Turn-motif phosphorylation site would inhibit the intramolecular binding of the C-terminal fragment of PRK2 to PRK2 catalytic domain, thus promoting indirectly the interaction of the C-terminal fragment of PRK2 with the PDK1 PIF binding pocket.

In summary, in our recent work [54] we propose a general model in which the C-terminal region of inactive PRK2 interacts with the Ade and PIF pocket bindings sites on PDK1. The approximation of PRK2 to PDK1 enhances the intrinsic catalytic activity of PDK1 to phosphorylate the activation loop of PRK2. The following phosphorylation of the Z/Turn-motif phosphorylation site of PRK2 promotes the intramolecular interaction of the Z/Turn-motif phosphate with its binding site, leading to the intramolecular binding of the Ade and hydrophobic motifs to the respective binding sites on the catalytic core of PRK2. The described intramolecular interactions trigger the dissociation of PRK2 from PDK1 and the activation loop and Z/Turn-motif

phosphorylation sites, together with the phosphate mimicking aspartic acid in the hydrophobic motif, contribute locally and allosterically to the stabilization of the hydrophobic motif/PIF-binding pocket and of the active site of PRK2 in the active conformation. The closed-active conformation of PRK2 displays a low affinity for PDK1 and leads to the stabilization of the active conformation of PRK2 by protecting the phosphorylated Z/Turn-motif phosphorylation site from dephosphorylation by phosphatases.

Altogether, our work made a contribution to the elucidation of the mechanism of activation of members of the AGC subfamily of protein kinases. Understanding of the molecular mechanism of regulation of protein kinases is a possible step for the development of new strategies against human disease, such as diabetes, human cancer, heart failure and hepatitis C.

6. REFERENCES

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7. Publications /Acknowledgements

7.1. Publications

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