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Protein kinase D in astrocytic gliomas – a new approach to control tumor growth

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Die vorliegende Arbeit ist am Institut für Molekularbiologie und Biochemie (Vorstand o. Univ.-Prof. Dr. Wolfgang Graier), Zentrum für Molekulare Medizin der Medizinischen Universität Graz entstanden.

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Introduction

1.1 Tumors of the brain

1.1.1 Generals

Beside stroke, brain tumors represent the second most frequent cause of death among neurological diseases. The incidence rate of brain tumors was 6.7 per 100,000 persons in the US in the year 2000 (Hess, Broglio & Bondy, 2004), 4 per 100,000 in Chile, 10 per 100,000 in Germany and Sweden and 7.3 per 100,000 in Austria in the year 1994 (Kostron, 1999). Gliomas, a group of primary brain tumors (see below), account for approximately 51% of diagnosed central nervous system (CNS) tumors. In the past decades several studies reported an increase in the incidence of brain tumors but besides age, little is known about risk factors and also treatment strategies, especially in gliomas are insufficient (Hess et al., 2004). Thus investigations during the recent thesis were restricted to this group of neoplasms and characteristics of these tumors are discussed in more detail below.

Among the totality of tumors, brain tumors take a special position. They can vary in malignancy but even so-called benign tumors are commonly lethal if not treated adequately. This is primarily due to the osseous skull and therefore every volume-increase has a life-threatening component in the brain. Clinical signs of brain tumors depend on the localization of the tumor-mass and/or repression with of increase intracranial pressure and include seizures, progressive headache, nausea, vomiting, visual abnormalities, motor or sensitive deficits, aphasia, cognitive dysfunction and others (DeAngelis, 2001). The life-threatening component is the tentorial or tonsillar herniation leading to coma and death. Therefore upon clinically suspected brain tumors, Magnetic Resonance Imagine (MRI) or contrast-enhanced Computed Tomography (CT) define the characteristics and localisation of the tumor. However the specific diagnosis must be confirmed by surgical resection or biopsy samples and subsequent histological examination (Behin et al., 2003).

1.1.2 World Health Organization (WHO) classification of brain tumors

Tumors of the brain can be classified in primary and secondary tumors. Secondary tumors are metastases of tumors elsewhere in the body e.g. lung cancer, melanoma or kidney tumors. Primary tumors are those which arise from cells of the brain and they can be classified histologically according to their predominant cell type (**Tab. 1**). The WHO classification also includes the malignancy of the tumors (Grade 1 to Grade 4) which correlates with the average survival times for patients (Kleihues et al., 2002).

1. Tumors of Neuroepithelial Tissue	2. Tumors of Peripheral Nerves	3. Tumors of Meninges
Astrocytic tumors	*Schwannoma*	*Meningioma*
Pilocytic astrocytoma		
Diffuse astrocytoma (fibrillary, protoplasmatic, gemistocytic)		
Anaplastic astrocytoma		
Glioblastoma (giant cell, gliosarcoma)		
Pleomorphic xanthoastrocytoma		
Subependymal giant cell astrocytoma		
Oligodendroglial tumors	*Neurofibroma*	*Mesenchymal tumors*
Oligodendroglioma		
Anaplastic oligodendroglioma		
Mixed gliomas	*Perineurinoma*	*Melanocytic lesions*
Oligoastrocytoma		
Anaplastic oligoastrocytoma		
Ependymal tumors	*Malignant peripheral nerve sheath tumor*	
Ependymoma (cellular, papillary, clear cell)		
Anaplastic ependymoma		
Myxopapillary ependymoma		
Subependymoma		
Choroid plexus tumors	**4. Lymphomas and Haemopoetic Neoplasms**	**5. Germ Cell Tumors**
Glial tumors of uncertain origin		
Neuronal and mixed neuronal-glial tumors	**6. Tumors of the Sellar Region**	**7. Metastatic tumors**
Neuroblastic tumors		
Pineal parenchymal tumors		
Embryonal tumors		

Table 1: Classification of tumors of the central nervous system.
This table has been abridged and modified from the World Health Organization (WHO) classification and gives an overview of all tumors of the brain. The dark shaded area gives an overview of the most common group of primary brain tumors, the astrocytic gliomas.

1.1.3 Astrocytic gliomas

The most common group of primary brain tumors are gliomas which arise from glial tissue. In this group the astrocytic tumors predominate. These are the most common primary brain tumors and are classified according to their malignancy into WHO grade 1 to 4 (Kleihues, Burger & Scheithauer, 1993):

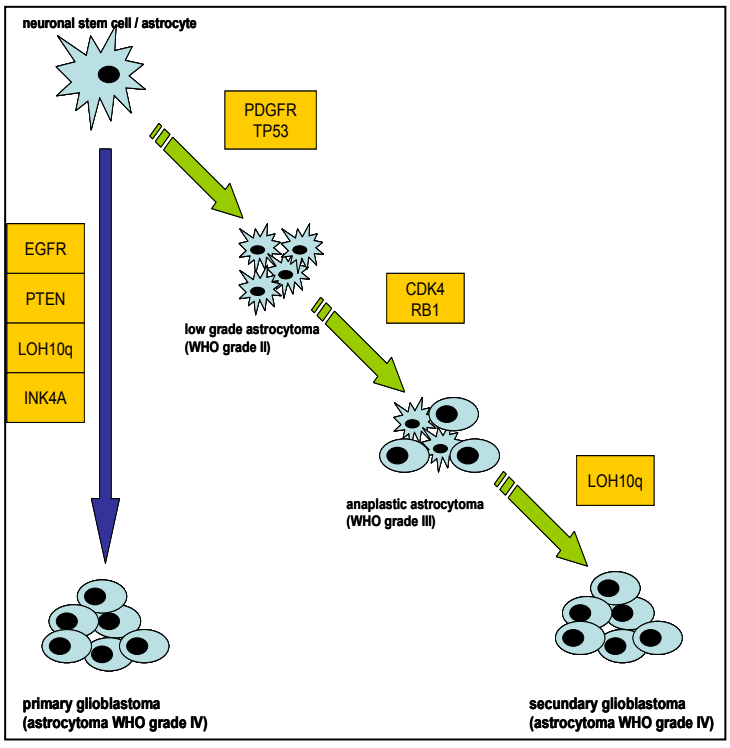
- WHO grade 1 represent pilocytic astrocytomas which are most commonly observed in children. These tumors are not considered to be malignant because they grow slowly and after surgery patients are cured. Therefore gamma knife radiosurgery represents a safe and effective treatment option (Eder et al., 2001).
- WHO grade 2 are low grade astrocytomas which account for approximately 35% of astrocytic gliomas and generally affect people with a mean age of 39 years but are also observed in children. These tumors are potentially malignant due to their infiltrative growth to the adjacent normal brain tissues. The average survival time is 7 years.

- WHO grade 3 are anaplastic astrocytomas. They can arise de novo or progress from a low grade astrocytoma. Histologically they are more cellular, have increased cellular atypia, and show increased cellular proliferation, therefore the average survival time is reduced to approximately 2 to 3 years.
- WHO grade 4 are glioblastomas. These are the most malignant astrocytic tumors and in addition to cellular atypia, increased mitotic index and infiltrative growth they show intratumoral necrosis and vascular endothelial proliferation. Glioblastomas can be arise de novo or progress from an anaplastic astrocytoma. The prognosis is very poor – average survival time between 9 to 12 months - and today there is no adequate therapy available.

Altough little is known about the risk factors to develop astrocytic tumors, it is noteworthy that whites and males have a higher incidence than African-american and females (Hess et al., 2004).

1.1.4 Genetic alterations and signal transduction pathways relevant to astrocytic glioma development

The genetic alterations which are involved in the development and progression of astrocytic gliomas are not yet fully understood, but in the past decades characteristic alterations have been identified which are important for the development of new therapies and drugs (see **Fig. 1**) (Kitange, Templeton & Jenkins, 2003).



◄ Figure 1: Genetic alterations in astrocytic glioma progression.
Two pathways have been proposed in the formation of glioblastoma. In the primary (de novo) pathway (blue arrow) astrocytes or neuronal stem cell directly give rise to glioblastoma, whereas in the secondary pathway (green arrows) a progression from low-grade to high-grade malignancy is shown. CDK4, cyclin-dependent kinase 4; EGFR, epidermal growth factor receptor; INK4A, inhibitor of CDK4; LOH10q, loss of heterozygosity; PDGFR, platelet-derived growth factor receptor; PTEN phosphate and tensin homologue; RB1, retinoblastom gene; TP53, tumor suppressor protein 53. (adapted from Merlo, 2003)

Most glioblastomas arise sporadically (also named the primary or de novo pathway) and are not inherited within families. Recently Singh et al. (Singh et al., 2003) demonstradet that neural stem cells directly give rise to glioblastomas in the primary (de novo) pathway or transform via the secondary pathway (see below) via low grade astrocytomas to glioblastomas. Alterations include mutations in tumor suppressor genes, growth factor (receptor) overexpression or amplification, loss of genes and overactivation of oncogenes and signal transduction pathways. Thus, the development from a low grade lesion into a highly malignant glioblastoma is a multiple step by step pathway (also termed the secondary pathway) and the overall effects of these alterations contribute to the pathophysiology of these tumors (**Fig. 1**) (Merlo, 2003).

Deletion of chromosome band 17p13.1 and mutations of the tumor suppressor gene p53 in a neuronal stem cell or astrocyte is commonly observed as one of the first aberations (Collins, 2002). Patients with Li-Fraumeni syndrome (germline p53 mutations) have an increased risk for gliomas (and other p53 associated tumors) which underline this hypothesis (Guha et al., 1995). The gene product TP53 is a critical regulator of the cellular response to DNA damage which includes inhibition of cell-cycle progression and activation of proapoptotic genes. Thus cells without a functional p53 gene loose the ability to undergo cell cycle arrest and apoptosis (Paunu et al., 2001).

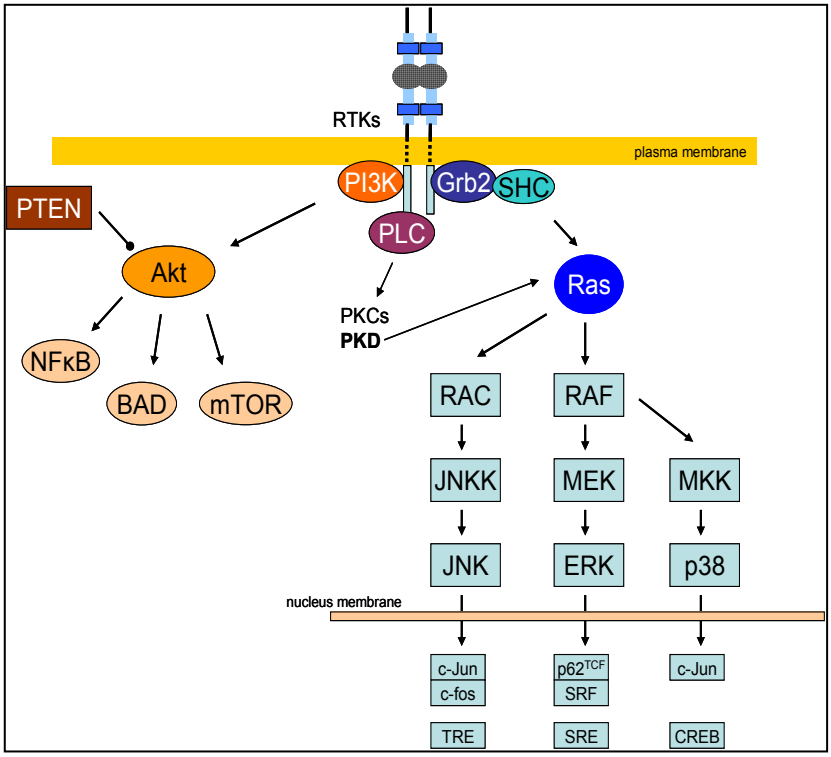
Transition of low-grade to anaplastic astrocytoma is associated with alterations of the retinoblastoma gene (Rb1) and/or amplifications of cyclin dependent kinase 4 (CDK4) and inhibitor of cyclin dependent kinase 4 (INK4A). Increasing activity of CDK4 or loss of Rb1 and INK4A result in unrestricted proliferation of cancer cells (Jen et al., 1994; Ueki et al., 1996).

Another important lesion is overexpression of platelet-derived growth factor (PDGF) and of its receptor (PDGFR) as secondary transformation or overexpression of epidermal growth factor (EGF) and its receptor (EGFR) in the de novo transformation which result in autocrine stimulation (Dai et al., 2001) and activation of proliferation (Claesson-Welsh, 1994) via i) the Ras and ii) the phosphosphatidylinositol-3-OH kinase (PI(3)K) pathway (Holland, 2001) (**Fig. 2**).

i) Ras, a 21 kDa protein family activates the RAC and RAF pathways and leads to induction of extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and the p38 mitogen-activated protein kinase (MAPK) (**Fig. 2**) (Feldkamp, Lau & Guha, 1997).

ii) PI(3)K is directly stimulated in response to activation of receptor tyrosine kinases (RTKs) and activates the AKT pathway which is important for cell-cycle progression, apoptosis and metabolic activity. The AKT pathway is regulated by the protein PTEN (phosphate and tensin homologue deleted from chromosome 10) and its loss in anaplastic astrocytomas and glioblastomas leads to increasing AKT activity that may play a critical role in cellular aggressivness, angiogenesis, migration and invasiveness (Holland et al., 2000).

In addition, upregulation of focal adhaesion kinase (FAK) signaling via integrin- and EGFR pathway promotes cell migration, invasion and angiogenesis (Natarajan, Hecker & Gladson, 2003).



◄ Figure 2: Ras and Akt Signaling pathways in astrocytic gliomas.

BAD, BCL2-antagonist of cell death; CREB, cyclic AMP response element binding protein; ERK, extracellular signal regulated protein kinase; GRB2, growth factor receptor binding protein 2; mTOR mammalian target of rapamycin; JNK(K), Jun kinase (kinase); MEK, MAP kinase-ERK kinase; MKK, mitogen activated protein kinase kinase; NFκB, nuclear factor of kappa gene enhancer in B-cells; PI3K, phosphatidylinositol-3-OH-kinase; PKCs, protein kinases C; PKD, protein kinase D; PLC, phospholipase C; PTEN, phosphate and tensin homologue deleted from chromosome 10; RTKs, receptor tyrosine kinases; SRE, serum response element; TCF, ternary complex factors; TRE, TPA (phorbol ester) responsive element. (adapted from Holland, 2001)

1.2 Protein kinase C plays a pivotal role in tumor cell growth and motility

In all pathways listed above, the large protein kinase C (PKC) familys takes center stage in the regulation of proliferation and invasion of gliomas, but also in many other tumors (Feldkamp et al., 1997). Overexpressed or hyperactivated PKCs are among the most distinguished characteristics of malignant brain tumors (Bredel & Pollack, 1997; Sharif & Sharif, 1999). Thus, PKCs represent potent targets to interfere with glioma cell motility, invasion and proliferation (da Rocha et al., 2002) and may represent new therapeutic avenues (Gleave & Monia, 2005; Zhang et al., 1997).

1.2.1 Structure and activation of PKCs

In the large family of phospholipid-dependet serine/threonine kinases, PKCs belong to the PKA, PKG and PKC group (AGC group). PKCs consists of 13 isoforms and are classified on the basis of sequence similarities and their modes of activation (Liu & Heckman, 1998):

- The conventional PKCs (cPKC) includes PKCα, β1, β2 and γ which are all activated by diacylacylglycerol (DAG) or its surrogate phorbol 12-myristate 13-acetate (PMA), phosphatidylserine (PS) and calcium (Ca^{2+}).
- The novel PKCs (nPKC) require DAG and PS but not Ca^{2+} and include the isoforms δ, ε, η and θ.
- The atypical PKCs (aPKC) respond neither to DAG nor to Ca^{2+} but they still require phospholipids such as PS. The aPKC includes the isoforms λ, ι and ζ (Toker, 1998).

Specially the isoforms PKCα and ε are important for invasion (da Rocha et al., 2002) and the isoforms ζ and η are critical for proliferation in human gliomas (Aeder et al., 2004; Donson et al., 2000).

1.3 A new member of serine/threonine kinases: Protein kinase D (PKD)

1.3.1 Structure and expression levels

Recently, a new family of serine/threonine protein kinases has been identified on chromosome 21, namely protein kinase D1 (PKD1) (formerly known as PKCμ), PKD2 and PKD3 (also named PKCυ) which share a unique molecular architecture that is distinct from other AGC kinases (Rykx et al., 2003). Thus PKDs are now classified in a novel subgroup of protein kinases within the calcium/calmodulin-dependent kinases (CaMK) group, which is distinct from PKCs (Lint et al., 2002). This is due to the fact that the catalytic domain of PKDs shows highest sequence similarities with CaMK. High expression levels of PKD are detectable in heart, lung and brain. Nevertheless, when compared to other genes, messenger RNA (mRNA) levels of PKD in normal tissues appeared to be low. In contrast, strong expression was found in a lung carcinoma cell line (A549), the rhabdomyosarcoma Kym I cell line (Johannes et al., 1994) and in mouse skin carcinoma (Rennecke et al., 1999). Due do these findings, it appears reasonable to assume that PKD1 might have a potential role in carcinogenesis. Like all other

isoforms of PKCs, PKDs contain a regulatory and a catalytic domain (**Fig. 3a**). In contrast to PKC family members, the catalytic domain of PKD is distinct and displays different inhibitor and substrate specificity. The NH_2-terminal of PKD contains a pleckstrin homology (PH) domain and lacks the typical autoinhibitory pseudosubstrate sequence (Gschwendt et al., 1996). The regulatory domain which extends from amino acids 1-588 contains two zinc-fingers (CYS1 and CYS2) and the PH domain exerts an inhibitory effect on the catalytic domain (Vertommen et al., 2000). Interactions of these subdomains with lipids or proteins cause full activation of PKD1 and determines intracellular trafficking localization (Iglesias & Rozengurt, 1998; Van Lint et al., 2002). The kinase domain includes amino acid residues 589-918. Phospholipases C (PLC), D (PLD), and PKCε or η are involved in phosphorylation of the activation loop of PKD1 (Ser738 and Ser742) which is subsequently activated (Rykx et al., 2003). Another important phosphorylation-site is position Ser910 located at the C-terminus of PKD1. Ser910 is not trans-phosphorylated by an upstream kinase but represents an autophosphorylation site indicative of PKD1 activation (Matthews, Rozengurt & Cantrell, 1999b).

1.3.2 Intracellular localization and activiation

In resting cells, a large fraction of PKD1 is located in the cytosol. After activation of the regulatory domain, PKD1 can be translocated to the plasma membrane or the nucleus via zinc-finger 2 domain (CYS2) (Matthews et al., 1999a). Retranslocation from the plasma membrane to the cytosol requires activation of the catalytic domain (see above) whereas export from the nucleus is facilitated by the PH domain (Rey et al., 2001). Alternatively zinc-finger 1 domain (CYS1) can mediate the recruitment of PKD1 to the Golgi apparatus (Baron & Malhotra, 2002).

PKD1 is activated in response to a variety of extracellular stimuli (**Fig. 3b**). These include direct interaction of the regulatory domain with PMA, DAG (Rozengurt, Sinnett-Smith & Zugaza, 1997), and Gβγ subunits in the Golgi apparatus (Baron & Malhotra, 2002). Neuropeptides, PDGF, bryostatin, lysophosphatidic acid (LPA) and oxidative stress activate PKD1 via PLC, PLD or PKC dependent pathways leading to phosphorylation of the activation loop (Kam & Exton, 2004; Van Lint et al., 1998; Zugaza et al., 1997). A special issue is activation by caspases. Caspases lead to a cleavage of PKD1 which is followed by abstraction of the regulatory domain which results in fully blown activation (Vantus et al., 2004).

1.3.3 Potential roles of PKD1 in cancer development

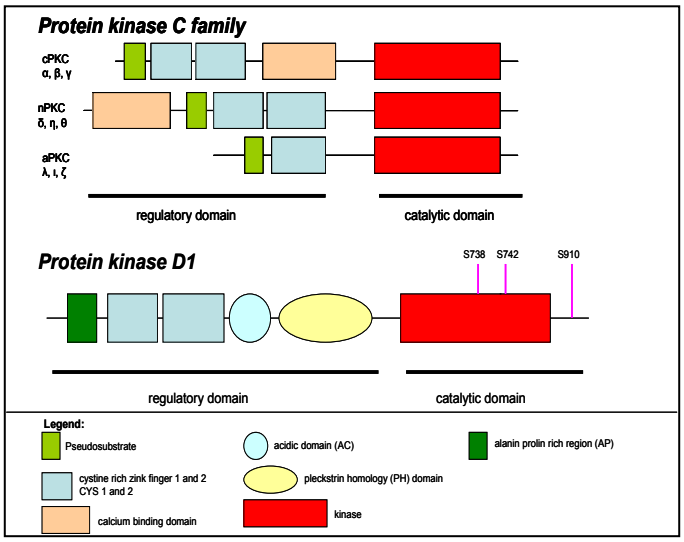
PKD1 is an important modulator or activator of several kinase signal-transduction pathways and plasma membrane proteins (**Fig. 3b**):

- In neuronal and PC12 pheochromocytoma cells PKD1 associates with a transmembrane protein, termed kinase D-interacting protein of 220 kDa (KidIns220), concentrates at the tip of neurites, suggesting a role in extension or growth (Iglesias et al., 2000).
- In breast cancer (the most common tumor in women) PKD1 forms a complex with the actin binding protein cortactin and the focal adhesion protein paxillin in invadopodia (Bowden et al., 1999). Invadopodia are sites of active proteolytic matrix degradation and important for tumor cells who breaking through the basal membrane.
- In prostate cancer (the second most common tumor in men) PKD1 interacts with metallothionein 2A which is associated with cell proliferation and chemoresistance (Rao et al., 2003), and with the transmembrane glycoprotein E-cadherin which is important for cellular aggregation and motility (Jaggi et al., 2005).
- Different observations revealed an important role of PKD1 during apoptosis: PKD1 can be involved in inhibiting apoptosis (Johannes et al., 1998; Trauzold et al., 2003) or sensitize cells to apoptosis (Endo et al., 2000).
- Constitutively active PKD1 activates RIN, a protein that binds to Ras which then activates the MEK-ERK pathway (which is activated in astrocytic gliomas) that induces to DNA-synthesis and proliferation (Rozengurt, Rey & Waldron, 2005).

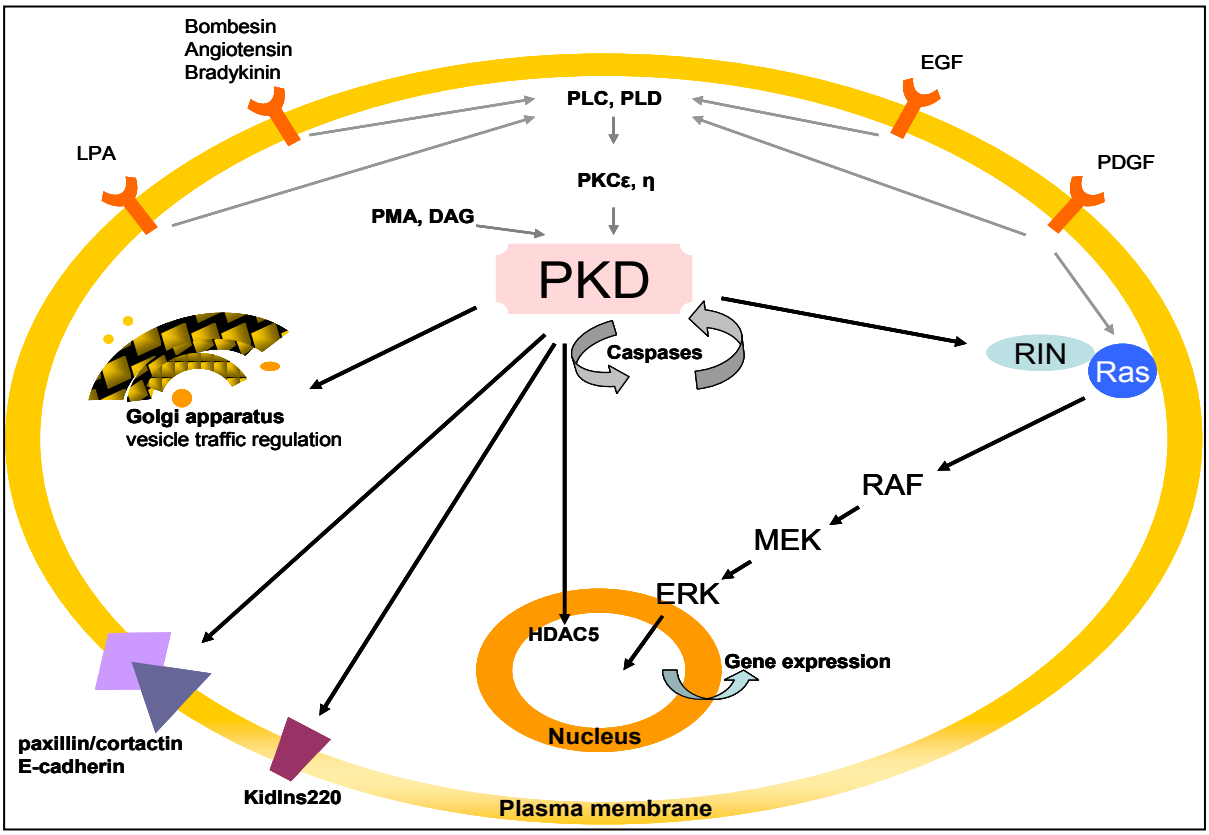
1.3.4 Hypothetical role of PKD1 in glioma growth and invasivness

All these findings revealed an important role for PKD1 in Golgi function, regulating the fission of vesicles from the trans-Golgi network (TGN) (Yeaman et al., 2004), in the regulation of cell proliferation and apoptosis and modification of other kinases and plasma membrane proteins in cancer cells.

The facts that this findings can have important functions in cancer cell growth and motility, that prognosis of patients with glioblastoma are poor and nothing is known about PKD1 in brain tumors, arranged us to investigate the function of PKD1 in astrocytic gliomas.



◄ Figure 3a: Molecular structure of the protein kinase C family and protein kinase D1.
This figure shows the architecture of the subgroups of the PKC family in comparison with the new member of serine/threonine kinases, protein kinase D1. S738/742 (mouse serine 744, 748) are the trans-phosphorylation sites, S910 (mouse serine 916) is the autophosphorylation site. (adapted from Lint, 2002)



▲ Figure 3b: Activation and function of protein kinase D1 (PKD) in cells.
PKD can be activated by various extracellular stimuli via PLC, PLD and PKCs and directly by PMA, and caspases. PKD in turn can activate caspases and RIN, that lead via RAF, MEK, ERK to gene expression and proliferation. At the trans-Golgi PKD regulates the fission of vesicles. The complex of paxillin/cortactin at the plasma membrane of breast cancer cells is regulate by PKD as the trans-membrane protein KidIns220 in the tip of neurites and E-cadherin in prostate cancer. In cardiac tissue PKD1 induces chromatin modification via HDAC5.
DAG, diacylacylglycerol; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; HDAC5, class II histone deacetylase 5; LPA, lysophophadidic acid; MEK, mitogen-activated protein kinase ERK-kinase; KidIns220, kinase D-interacting substrate of 220kDa; PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; PLC, PLD, phospholipase C or D.

1.4 Treatment strategies for malignant astrocytic gliomas

As mentioned above, even when treated, the average survival time of patients with glioblastomas are 9 to 12 months and for anaplastic astrocytomas between 2 to 3 years.

1.4.1 Current treatment

Today standard treatment strategies for malignant astrocytomas include a combination of surgery, chemotherapy, radiation therapy and sometimes hyperthermia (Castro et al., 2003).
Surgery is the major therapeutic managment of malignant and benign brain tumors because after resection tumor patients benefit to alleviate of mass and limitated clinical deficits. However, successful resection of malignant tumors is limited due to diffuse infiltrative growth.
Following surgery to remove as much tumor mass as possible or if the tumor is located in a non-operable region, radiation therapy can be performed. In order to reduce side effects as swelling of the brain and accumulation of apoptotic/dead cells, stereotactic radiosurgery has been developed. This method allows minimized damage of the unaffected brain tissue.
Chemotherapy can be used as primary therapy or as an additional therapy following surgery or radiation. Most of the agents such as nitrosoureas (carmustine, (BCNU®), lomustine (CCNU®)), platinum-based drugs (cisplatin, carboplatin), antimetabolites (methotrexate, fluorouracil) or natural products (vincristine, doxorubicin) have many side effects and most of the tumors develop chemoresistancy. The response of brain tumors to chemotherapy varies depending on tumor type due to the presence of the blood brain barrier (BBB) (Dean et al., 1999) and the activation of multi drug resistance (MDR) genes. Thus patients with anaplastic astrocytoma which have a higher permeability of the BBB respond better to chemotherapy (Doolittle et al., 2002).
Despite the fact that combination of surgery with radiotherapy and new developments such as temozolomide are promising (Stupp et al., 2002), the prognosis of patients with glioblastoma is poor. Only the combination of radiotherapy with concomitant and adjuvant temozolomide resulted in a median survival benefit up to 14 month (Stupp et al., 2005). Therefore, the urgent need to develop novel therapies or to enhance tumor-specific cytotoxic effects is evident (Castro et al., 2003).

1.4.2 Novel and future treatment strategies

Novel therapies focus on new findings about genetic alterations and signal transduction pathways in brain cancer. These include gene therapy, suppression of angiogenesis, activation

of immune response, induction of apoptosis, antibodies and kinase inhibitors (Tremont-Lukats & Gilbert, 2003). Most of the new treatment strategies are currently either in a preclinical development stage or in clinical phase I or II (**Tab. 2**), and are derived from successful applications in other tumors (Rich & Bigner, 2004). It must be kept in mind that no signalling pathway is completly linear or isolated in its activity. Moreover, the pathways interact at multiple levels and the function of any single molecule depends on cell and tissue type. Therefore, different results might be achieved in different tumors and tumor grades. Table 2 give an overview of new drugs and their targets with an up to date summary of observed side effects and outcome success.

A new study revealed that the use of doxorubicin-loaded nanoparticles leads to significantly higher survival time and a reduction of the tumor mass in glioblastoma-bearing rats. In this study doxorubicin was conjugated to polysorbate-coated nanoparticles. This vehicles are able to cross the intact BBB and thus this a new strategy for a non-invasive treatment in glioblastoma (Steiniger et al., 2004). Altough there is no indication of short-term neurotoxicity, further results have to be expected.

Target	Agent	Drug class	Develop-ment stage in glioma	Outcome success: administration / median overall survival time from treatment initiation	Side effects (selection)
EGFR	Gefitinib,Iressa (ZD1839)	TKI	II	50–800mg/d orally / 39 weeks	diarrhoea, skin reaction
	Erlotinib (OSI774)	TKI	II	150mg/d orally / -	diarrhoea, rash
	AEE788	TKI	I	orally / 12 weeks	-
	TP-38	Peptide vaccine	II	- / 23 weeks	mild rash, diarrhoea
PDGFR	Imatinib mesylate (STI571)	TKI	I/II	400mg/d orally / 6 months	haemorrhage, pneumonia
	SU6668	TKI	I	orally / -	haemorrhage
	MLN518/608	TKI	Preclinical	- / -	-
VEGFR	PTK787 / ZK222584	TKI	I	500-2000mg/d orally / -	vein thrombosis, fatigue
	Semaxanib (SU5416)	TKI	I/II	340mg/m2/d iv / -	-
Ras	Tipifarnib (R115777)	FTI	I/II	500mg/2xd orally / 6 months	neutropenia, thrombopenia
	Lonafarnib	FTI	I	150g/2xd orally / -	diarrhoea, neutropenia
mTOR	Rapamycin	Antibiotic	I/II	250mg/d iv / 5 months	neutropenia, rush
	CCI-779	Antibiotic	I/II	700mg/d iv / 5 months	hyperlipidaemia, rush
	RAD001	Antibiotic	Preclinical	orally / -	neutropenia, thrombopenia
	AP23573	Antibiotic	Preclinical	- / -	-
PKC	Tamoxifen	Anti-oestrogen	II	40mg/2xd / 10 weeks	pancytopenia, thrombosis
	LY317615	Small molecule	II		
IL-13	IL-13-PE38QQR	Ligand-toxin-conjugate	I/II	-	-

Table 2: Novel therapies under development for glioma therapy

The agents are listed with their target, drug class stage of development, drug administration and an currently outcome success overview. (Modified from Rich, 2004)

EGFR, epidermal growth factor receptor; FTI, farnesyl transferase inhibitor; IL-13, inerleukin-13; iv, intravenous; mTOR, mammalian target of rapamycin; PDGFR, platelet-derived growth factor receptor; PKC, protein kinase C; TKI, tyrosine kinase inhibitor; VEGFR, vascular endothelial growth factor receptor.

1.5 Central Hypothesis and Aims

Hypothesis

The facts that

i. PKD1 expression is high in the central nervous system,

ii. overexpression of PKCs are among the most distinguished characteristics of malignant brain tumors and definite isoforms are important regulators of proliferation and migration in glioblastomas,

iv. PKD1 might be an important regulator of cell proliferation and invasivness, and

v. nothing is known about PKD1 in astrocytic gliomas

we hypothesised, that PKD1 might contribute to glioblastoma growth and/or invasivness.

Aims

Based on evidence presented above the following Aims were pursred:

i. To compare expression patterns of PKD1 in human astrocytic glioma biopsy material (WHO grade II – IV) and A172 glioblastoma cells.

ii. To isolate human primary tumor cells from astrocytic gliomas (WHO grade III – IV) for further examination.

iii. To examine activation patterns and downstream signaling of PKD1 in A172 cells and primary tumor cells.

iv. To clarify the impact of PKD1 on cell proliferation.

v. To silence PKD1 expression by RNA interference and analyze the consequences on tumor cell growth and invasivness.

Chapter 2

Materials and Methods

2.1 Materials

Cell culture

DMEM "high glucose" with L-Glutamine and Pyruvate	Gibco (Vienna, Austria)
Fetal calf serum	PAA Laboratories (Linz, Austria)
Penicillin / Streptomycin	Biochrom (Germany)
Trypsin	Biochrom (Germany)

Plasticware

petri dishes (10cm diameter)	Greiner (Germany)
25cm^2 cell culture flasks	
75cm^2 cell culture flasks	
pipette tipps, disposable pipettes	
disposable cell scrapers	
6-well cell culture plates	Costar (Vienna, Austria)

Laboratory Equipment

pipettes	Gilson (Middleton , WI)
centrifuges and rotors	Beckmann, Sigma, Heraeus
SDS-gel electrophoresis devices	Bio-Rad (Vienna, Austria)
Densitometer	Herolab (Germany)
PVDF membrane	Pall (Vienna, Austria)

Chemicals

all solvents	Sigma (Vienna, Austria)
all other chemicals	Boehringer Mannheim (Austria)
Western blotting Detection Reagens ECLplus	Amersham (Vienna, Austria)
PKC inhibitor Goedecke 6976	Calbiochem (Vienna, Austria)
PMA	Sigma (Vienna, Austria)
Bodipy FL C5 ceramide	Molecular Probes (Oregon, USA)
Brefeldin A	Cambio (Great Britain)

Antibodies and growth factors

PDGF-BB growth factor	Weizmann Science Park (Israel)
EGF growth factor	Leinco (Missori, USA)
GFAP	Dakocytomation (Vienna, Austria)
Cy-2	Amersham (Austria)
Cy-3	Amersham (Austria)
panPKD	Santa Cruz (CA, USA)
pPKD 744/746	Cell Signaling (Germany)
pPKD 916	Cell Signaling (Germany)
pERK1/2 and total ERK	Cell Signaling (Germany)
goat anti rabbit	Pierce (Rockford, USA)

Electroporation and RNA interference

Nucleofector II	Amaxa Biosystems (Germany)
Mouse Astrocyte Nucleofector Kit	Amaxa Biosystems (Germany)
all siRNA´s (PKD, Lamin A/C, GFP)	Dharmacon (Austria)
GFP-PKD wildtype and kinase dead (in pcDNA3 vector)	gift from Johan Van Lint (Leuven, Belgium)

2.2 Methods

2.2.1 Cell culture

The human glioblastoma cell line A172 is a valid in vitro model for the experiments performed during the present thesis and was already used before by other investigators (Choi et al., 2004; Davis, Dertien & Syapin, 2002; Knupfer et al., 2001; Vassbotn et al., 1994). The cell line was derived from a glioblastoma removed from a 53 year old male and was maintained in Dulbeccos Modified Eagle Medium (DMEM) „high glucose“ supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin in an atmosphere of 5% CO_2 at 37°C. Cells were splitted once a week.

2.2.2 Isolation and culture of primary glioma tumor cells

Tumor tissue specimens were obtained from patients during open surgical resection of astrocytic gliomas in cooperation with Prof. Eder (Department of Neurosurgery, Graz). The WHO grade was diagnosed according to perioperative diagnosis on cryostat sections by a neuropathologist. After removal the biopsies were transferred immediately to liquid nitrogen (for analysis of protein expression patterns or immunohistochemical studies) or prewarmed DMEM „high glucose“ supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (for isolation and culture of primary tumor cells) and transported to the laboratory within 20 minutes.

To investigate protein expression patterns, the biopsy material was homogenized in liquid nitrogen, resuspended in RIPA-buffer (Tris-HCl, 50 mM; pH 7.4; NP-40, 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; PMSF, 1 mM; aprotinin, leupeptin, pepstatin, 1 µg/ml each; Na_3V0_4, 1 mM; NaF, 1 mM), sonicated, and centrifuged to remove debris and insoluble proteins. The supernatant was removed, the protein content analyzed using the Bradford assay and equal amounts of protein was separated on SDS-PAGE gels prior to immunoblotting.

For primary tumor cell isolation and subsequent cell culture, the human biopsy samples (after transport) were washed in DMEM and blood vessels were removed as good as possible. Then the samples were mechanically homogenized by staggered roller blades, the homogenized material was rinsed with PBS again and plated in medium on 25 cm^2 flasks. After two days in culture the non-adherent material was removed and adherent cells were further cultured. When the cells were confluent, they were trypsinized and transferred to new flasks for further amplification.

2.2.3 Immunohistochemical characterization

Tumor tissue specimens were obtained from patients during open surgical resection as described above. Thereafter the tissue samples were embledded in Tissue Tec OCT and serial cryosections (5 µm) in a cryostat (Microm HM 500 OM; Microm, Walldorf, Germany) were made. Cryosections were collected on glass slides and air dried for 2 h at 22°C. Before staining, the samples were thawed, fixed once more in acetone for 5 min (22°C), rehydrated in PBS for 5 min. and blocked with protein block for 15 min. The sections were incubated with monoclonal rat anti-human glial fibrillary acid protein (GFAP) (1:10), followed by incubation with a Cy-2 labeled goat anti-rat antibody (1:100). Afterwards the sections were incubated with rabbit anti-PKD1 antibody (sc-935, D-20, 1:50), followed by incubation with Cy-3 labeled goat anti-rabbit antibody (1:400). Sections were mounted with Moviol and analysed on a confocal laser scanning microscope (Leica TCS NT, Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with an argon-krypton laser (Egger et al., 2001).

2.2.4 Electroporation and transfection of glioblastoma cells

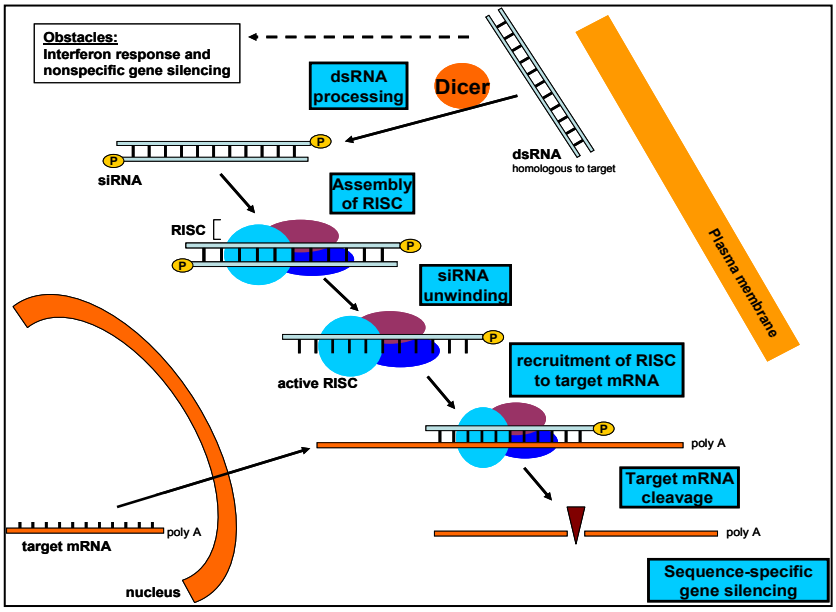
Since our preliminary examination and other investigators (Bell et al., 1998) showed that current available liposomal transfection reagens are toxic for A172 cells or result in poor transfection efficacy, we used the electroporation Nucleofector technology from Amaxa (Gresch et al., 2004). A172 cells or primary glioblastoma cells were plated on 10 cm Petri dishes ($2x10^6$ cells) supplemented with medium (see above). Before transfection, cells were briefly washed with phosphate-buffered saline (PBS), detached with trypsin and centrifugated (1000 U/min for 5 min). Then, the supernatant was removed and the cells were resuspended in 100 µl mouse astrocyte Nucleofector solution (Amaxa) at room temperature. The cell suspension was mixed with 3µg eGFP-PKD1-DNA (for visualising PKD1 in living cells with fluorescence microscopy) or 1.5 µg and 3 µg dsRNA (for RNAi experiments, see below) and transferred to cuvettes followed by electroporation with program T-20 (for A172 and primary cells) according to the manufacturers suggestions. After electroporation cells were transferred into 10 cm Petri dishes with medium and cultured as described above.

2.2.5 RNA interference (RNAi)

2.2.5.1 General

First discovered in plants, where this technique is known as post-transcriptional gene silencing, RNA silencing or RNA interference (RNAi) occurs in a wide variety of eukaryotic organisms (Fire et al., 1998) (Nobel Prize in Physiology or Medicine 2006). RNAi can cause the degradation of virtually any RNA and is thus an important method to examine gene function in somatic cells with enormous potential for therapeutic applications (Stevenson, 2004). For example, RNAi is currently explored as a technique to inhibit the expression of genes involved in oncogenesis or infectious diseases in vitro and in vivo. Among a number of other cancers RNA interference was also successfully applied to glioblastoma cells: Lakka and colleagues (Lakka et al., 2004) have silenced cathepsin B and matrix metalloproteinase-9 (MMP-9) using hairpin RNA (hpRNA) interference. These authors have shown that application of plasmid DNA expressing hpRNA for cathepsin B and MMP-9 completely regressed pre-established glioma tumors for almost four months in a mouse tumor model.

Mechanism of gene silencing (**Fig. 4**): Double-stranded RNAs (dsRNAs) precursors that vary in length and origin are processed to short interfering RNAs (siRNAs) by the endonuclease known as Dicer (Elbashir et al., 2002). The resultant siRNAs are 21 to 24 nucleotides in length, are double-stranded, and have 3' overhangs of 2 nucleotides. These siRNAs are incorporated into the RNA-induced silencing complex (RISC). RISC is directed by the unwound antisense siRNA strand to homologous target RNAs which undergo cleavage. This



◄ Figure 4: Mechanism of gene silencing by RNA interference.
dsRNA is recognized and processed by Dicer, an RNase III enzyme, into siRNA. siRNA are incorporated into RISC which unwinds the duplex siRNA and pairs the target mRNA with the sequence complementary siRNA. An yet unidentified RNase (Slicer) withhin RISC degrade the mRNA. As a result, the protein production is become to silenced.
dsRNA, double-stranded RNA; mRNA, messenger RNA; RISC, RNA-inducedsilencing complex; siRNA, short interfering RNA.
(adapted from Meister & Tuschl, 2004)

mRNA processis is affected by an RNase named Slicer (the identity of Slicer is not yet known) (Meister & Tuschl, 2004).

2.2.5.2 RNA silencing

To design target-specific siRNA duplexes, sequences of the type AA(N19)UU from the open reading frame of the human PKD1 mRNA were chosen, in order to obtain a 21-nucleotide(nt) sense and 21-nt antisense strand with symmetric 2-nt 3' overhangs of identical sequence. A BLAST search against the human genome sequence identified 10 (out of 68) potential siRNA PKD1 sequences that did not show significant homology to other gene products. Three siRNA duplexes targeting PKD1, one duplex targeting GFP and a duplex targeting Lamin A/C were purchased from a commercial supplier (Dharmacon).

Three different target sequences of protein kinase 1 (PKD1):

PKD1-01: 5‘-AAU UGG CGA AGU GAC CAU UAA-3‘
PKD1-02: 5‘-AAU CAC CAU CAG AGU CGU UUA-3‘
PKD1-03: 5‘-AAG AAC CAA CUU GCA CAG AGA-3‘

Target Sequence of green fluoresence protein (GFP):

GFP: 5‘-GGC TAC GTC CAG GAG CGC ACC-3‘

Target Sequence of Lamin A/C:

Lamin A/C: 5‘-CTG GAC TTC CAG AAG AAC A-3‘

Transient transfection of glioblastoma cells were carried out using the Nucleofector technology from Amaxa (see above).

2.2.6 Invasion assay

Spheroid invasion assays were performed with control, mock-transfected, scrambled and PKD1 silenced A172 glioblastoma cells. During this part of the thesis a heterologous confrontation system where fetal chick brain aggregates where confronted with glioblastoma cell spheroids was used. Briefly, before reaggregation of the spheroids glioblastoma cells (control, mock-transfected, scrambled and silenced cells) were stained with DiI and embryonic chick brain spheroids were stained with DiOC. These dyes are suitable to stain living cells, are non-toxic, are stable for up to five days and allow fluorescence imaging of the invasion process. The spheroids were then mechanically confronted (touching each other) forming a cancer-host spheroid pair. These pairs were incubated at standard conditions.

Images of the confronted pairs were obtained every 24 hours as described by Ahammer and collegues (Ahammer, DeVaney & Tritthart, 2001). The remaining volume of brain aggregates or tumor spheroids were analyzed by fractal capacity dimension determination.

2.2.7 Growth assay

Control, mock-transfected, scrambled and PKD1 silenced primary and A172 glioblastoma cells were plated at a density of 25.000 or 50.000 cells on six well trays and cultured under standard conditiones in the absence or presence of 20 ng PDGF-BB and the PKC inhibitor Goedecke (Gö) 6976 (2 and 20 μM). At 24 h intervals the cells were trypsinized and counted manually by a hemacytometer.

2.2.8 Activation pattern assay of PKD1 by relevant stimulants

A172 and primary glioblastoma cells were grown to confluence in 10 cm Petri dishes under standard conditions (see above). To test the activation pattern of PKD1 by relevant stimulants, the cells were incubated in the presence or absence of 100 nM phorbol-12-myristate-13-acetate (PMA), 1 to 20 ng PDGF-BB or 10 to 100 ng EFG and 20 nM to 20 μM of the PKC inhibitor Gö6976. After stimulation the cells were washed two times with PBS and lysed in ice-cold extraction RIPA-buffer (Tris-HCl, 50 mM; pH 7.4; NP-40, 1%; Na-deoxycholate, 0.25%; NaCI, 150 mM; EDTA, 1 mM; PMSF, 1 mM; aprotinin, leupeptin, pepstatin, 1 μg/ml each; Na_3V0_4, 1 mM; NaF, 1 mM), sonicated, and centrifuged for 10 min, at 13.000 U/min. The supernatant was removed, the protein content was analyzed using the Bradford method (Bradford, 1976) and equal amounts of protein were separated on SDS-PAGE gels for immunoblotting.

2.2.9 SDS-PAGE and Immunoblotting

Sodium Dodecyl Sulfate – Polyacrylamid Gel Electrophoresis (SDS-PAGE) was performed on 7% SDS-polyacrylamid-gels with electrophoreses at 130 V for 90 min in a Bio Rad Miniprotean Chamber. For Western blot analysis, protein fractions (20 to 150 μg/sample) were electrophoretically transferred to polyvinylidenfluoride (PVDF) membrane for 60 – 120 min at 150 mA (4 °C). After blocking of non-specific binding sites of PVDF membranes with 5% non-fat milk, immunochemical detection was performed with sequence specific rabbit

primary antibodies. PVDF membranes were incubated overnight at 4°C with primary antibodies against panPKD1 (sc935, D-20), phosphorylated PKD1 (Ser 738/742 and Ser 910) or phosphorylated ERK1/2. Immunoreactive bands were visualized with peroxidase-conjugated anti-rabbit immunoglobulin (dilution 1:5000, incubated for 2 h by 4°C) by the ECL detection method. Films were analyzed by densitometry.

Chapter 3

Results

3.1 Expression of PKD1 in human astrocytic glioma tissue of different severity

As already outlined in the introduction, PKD1 is highly expressed by many tumor cells and in brain tissue. In addition, PKD1 has important functions in cell growth, invasion and Golgi vesicle trafficking. The first aim of the present thesis was to clarify whether PKD1 is expressed by human astrocytic gliomas *in vivo*. Therefore, tumor tissue specimens were obtained during open surgical resection by neurosurgeons. Tumor typing and grading were preoperatively diagnosed on cryostat sections by a neuropathologist. The biopsy material was homogenized in liquid nitrogen and analyzed by western blotting technique using polyclonal PKD1 antibodies and the expression levels of 3 astrocytomas WHO grade II, 6 astrocytomas WHO grade III and 8 glioblastomas (WHO grade IV) were evaluated (**Fig. 5**). It is important to note that PKD1 expression levels clearly correlate with tumor grading. Immunoreactive PKD1 levels in glioblastoma multiforme (GBM) are 3-fold higher then in astrocytoma grade II. The glioblastoma cell line A172 has similar expression levels compared with glioblastomas *in vivo* and was used as valid *in vitro* model for further experiments.

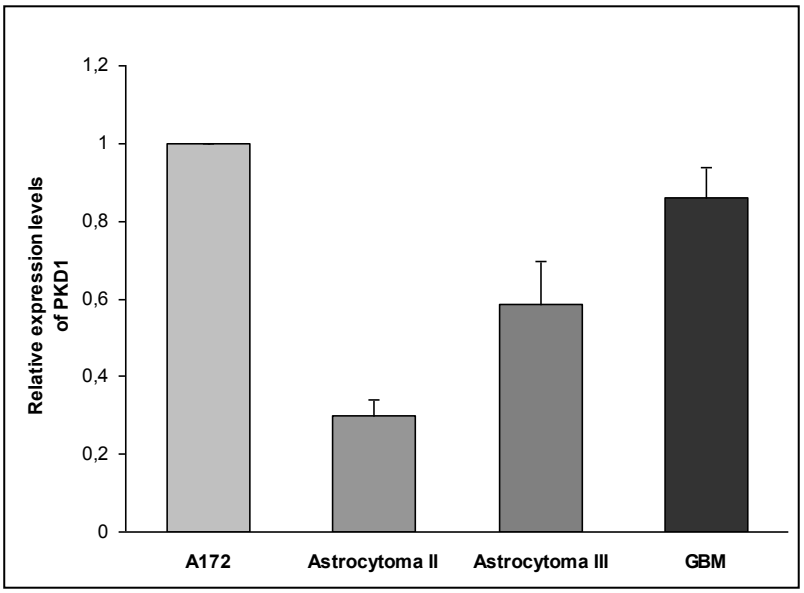


Figure 5: Relative PKD1 expression levels of human astrocytic gliomas.
Astrocytoma biopsy samples of different severity (WHO grade II, III and IV (glioblastoma multiforme; GBM)) were analyzed by immunoblotting. The biopsy material was homogenized in liquid nitrogen, sonicated and centrifuged to remove insoluble material. The supernatant was removed and identical amounts of protein was loaded on 7 % SDS-PAGE gels. Proteins were transferred to PVDF membranes and analyzed by a polyclonal PKD1 antibody using the ECL method. Band intensities were evaluated densitometrically.
Expression levels of total PKD1 in glioblastoma cell line A172 (n=3), astrocytoma grade II (n=3), astrocytoma grade III (n=6) and glioblastoma (GBM) (n=8) are shown. Results shown represent mean ± SD of the band intensities.

To reveal PKD1 expression and cellular localization in GBM, immunohistochemical studies were performed (**Fig. 6**). Following immunohistochemical studies on sections from glioblastomas obtained during open surgical resection, distinct and pronounced colocalization of PKD1 with GFAP-positive cells was observed (**Fig. 6 A-C**). The tissue was removed in the vicinity of the centre of the tumor to limit cross-contamination with non-transformed astrocytes and oligodendrocytes (these would also stain GFAP positive). Because most glioblastoma cells are GFAP-positive these findings indicate that tumor cells express PKD1. Arrows in Fig. 6C depict colocalization of GFAP and PKD1 pointing towards the possibility that PKD1 is predominantly present on the plasma membrane. Further examination with HLA-DR I (a plasma membrane localized protein) antibodies confirmed predominant localization of PKD1 at the plasma membrane of glioblastoma cells (**Fig. 6 D-F**). This fact could indicate the presence of the active (phosphorylated) form of PKD1 at the plasma membrane of glioblastoma cells *in vivo*. To confirm this assumption isolation of primary tumor cells from glioblastoma biopsies were performed (see below).

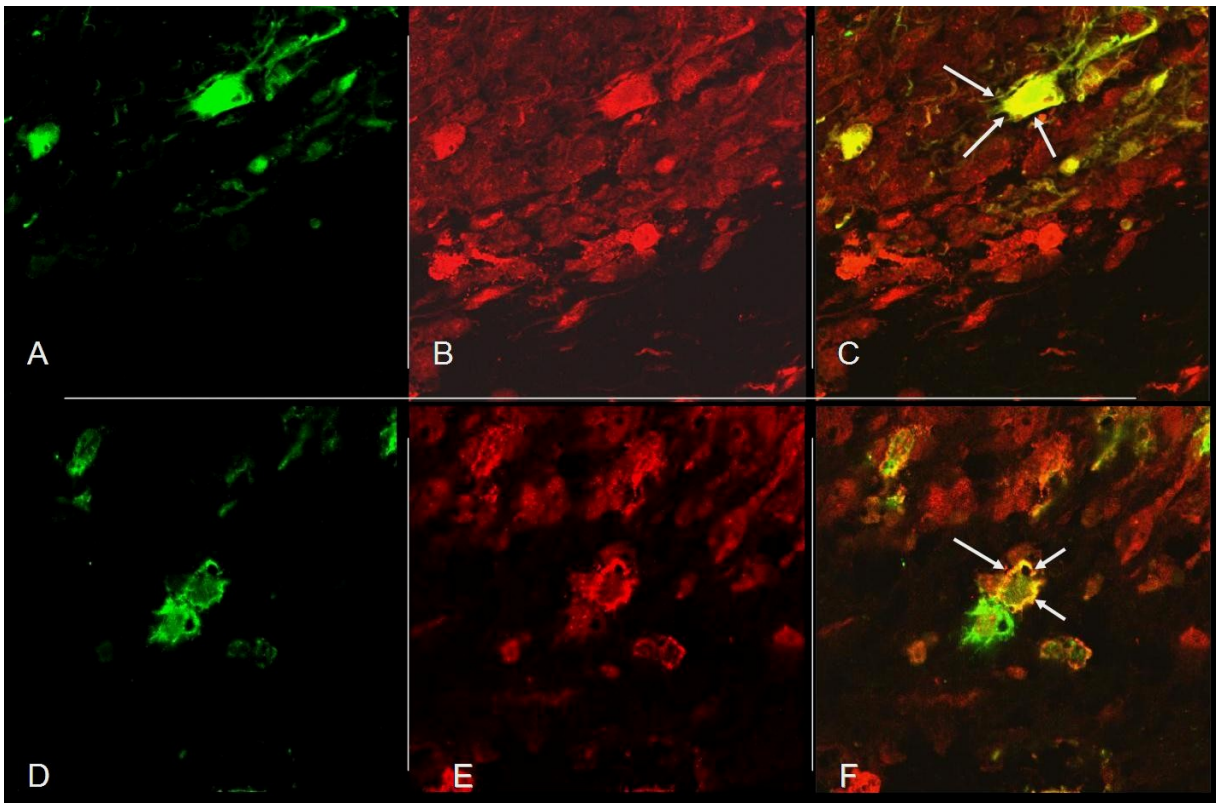


Figure 6: Immunohistochemical staining for GFAP, HLA-DR I and PKD1 in a human glioblastoma section.
Cryosections of a human glioblastoma (WHO grade IV) were thawed, fixed and rehydrated in PBS. After blocking, the sections were incubated with monoclonal GFAP antibody in A or HLA-DR I antibody in D, followed by incubation with a Cy-2-labeled antibody (green; A**,** D). Afterwards the sections were incubated with polyclonal rabbit anti-PKD1 antibody, followed by incubation with Cy-3-labeled antibody (red; B, E).
A: localization of GFAP; **B:** localization of PKD1; **C:** merge of A and B (arrows indicate colocalization of GFAP and PKD1 and indicate predominantly localization of PKD1 on the plasma membrane)
D: localization of HLA-DR I; **E:** localization of PKD1; **F:** merged image of D and E (arrows showing colocalization of HLA-DR I and PKD1, both on the plasma membrane)

3.2 Activation pattern of PKD1 in primary glioblastoma cells

As a first step for these experiments primary glioblastoma tumor cells were isolated from tumor tissue specimens. Prior to biopsy transport from the operating room to the laboratory in prewarmed cell culture medium, a neuropathologist has diagnosed the slides. The tissue was then washed, blood vessels were removed, the remaining tissue was mechanically homogenized and plated on cell culture flasks (**Fig. 7A**). After one day, non-adherent, detachable material was removed and the adherent cells were further cultured for five days (**Fig. 7B**). Four further tumor specimens from other patients were treated in the same way to establish several primary glioblastoma cell cultures for further studies.

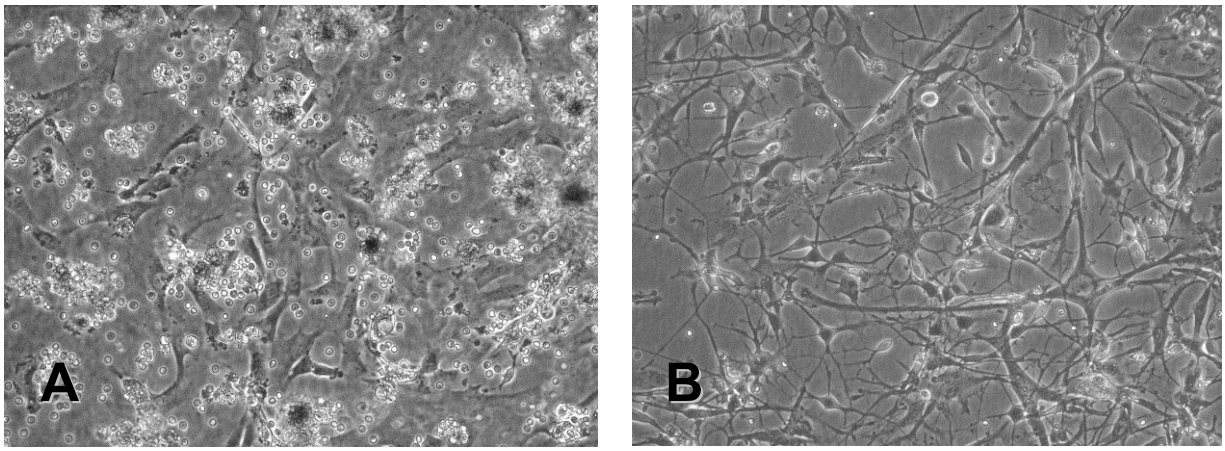


Figure 7: Isolation and culture of primary human glioblastoma cells
A: After washing, removal of blood vessels and homogenization the resulting material was plated in 25cm^2 flasks in DME-Medium supplemented with penicillin and streptomycin. First adherent cells can be seen on the bottom of the flask immediately after plating. The white, punctuate floating material consists of red and white blood cells. The image was taken 1 day after plating. **B:** On the second day the non-adherent material was removed. Adherent tumor cells were further cultured (6 days) and transferred to new flasks until they were confluent. Original magnification: x20.

To evaluate the phosphorylation status and pattern of PKD1 (a major determinant governing intracellular trafficking and translocation of PKD1) in primary glioblastoma cells, cells were plated on Petri dishes and grown to confluency. Confluent cells were stimulated with either phorbolester (phorbol-myristate-acetate, an agonist that activates PKD1 via upstream PKC activation) growth factors (PDGF-BB and EGF, both known to be involved in glioblastoma growth). Untreated cells served as controls to evaluate the phosphorylation status of PKD1 under normal ('resting') conditions. Phosphosite-specific antibodies directed against serine 910 (S^{910}), which is an indicator of PKD1 activation, were used in these experiments. Results

shown in **Fig. 8** demonstrate immunoreactive, phosphorylated (S^{910}) PKD1 in both, non-stimulated and stimulated cells. Although PMA resulted in S^{910} phosphorylation above baseline levels, neither PDGF nor EGF could further stimulate PKD1 phosphorylation in primary glioblastoma cells. These findings indicate that PKD1 is phosphorylated and active in glioblastoma cells, compatible with the plasma membrane location of PKD1 in tumors as observed in immunohistochemical analyses (see above).

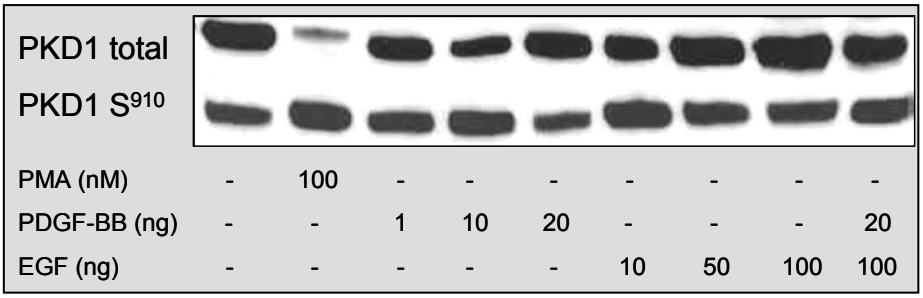


Figure 8: PKD1 activation pattern in primary glioblastoma cells
Primary glioblastoma cells were grown to confluency in 10 cm Petri dishes in DMEM containing FCS, penicillin and streptomycin. Non-stimulated or PMA (100 nM, 20 min.), PDGF-BB (indicated concentrations for 20 min) and/or EGF (10 to 100 ng, 20 min) stimulated cells were washed twice with PBS, lysed and sonicated. PKD1 (total, upper panel, and S^{910} phosphorylated PKD1, lower panel) was analyzed by immunoblotting. One-hundred µg cell protein was applied in each lane.

3.3 Activation of PKD1 in the A172 glioblastoma cell line

To test the activation pattern of PKD1 in the A172 cell line cells were cultured in 10 cm Petri dishes and stimulated with PMA, PDGF, EGF or a combination of both growth factors (**Fig. 9**). Immunoblot analysis (**Fig. 9A**) revealed that PMA induced phosphorylation in the trans- ($S^{738/742}$) and in the autophosphorylation (S^{910}) domain. Also PDGF caused pronounced, concentration-dependent phosphorylation of $S^{738/742}$ and S^{910} while EGF was without effect on the phosphorylation status of the kinase. In line, the addition of both growth factors did not result in synergistic effects. Densitometric evaluation of S^{910} phosphorylation is shown in **Fig. 9B**.

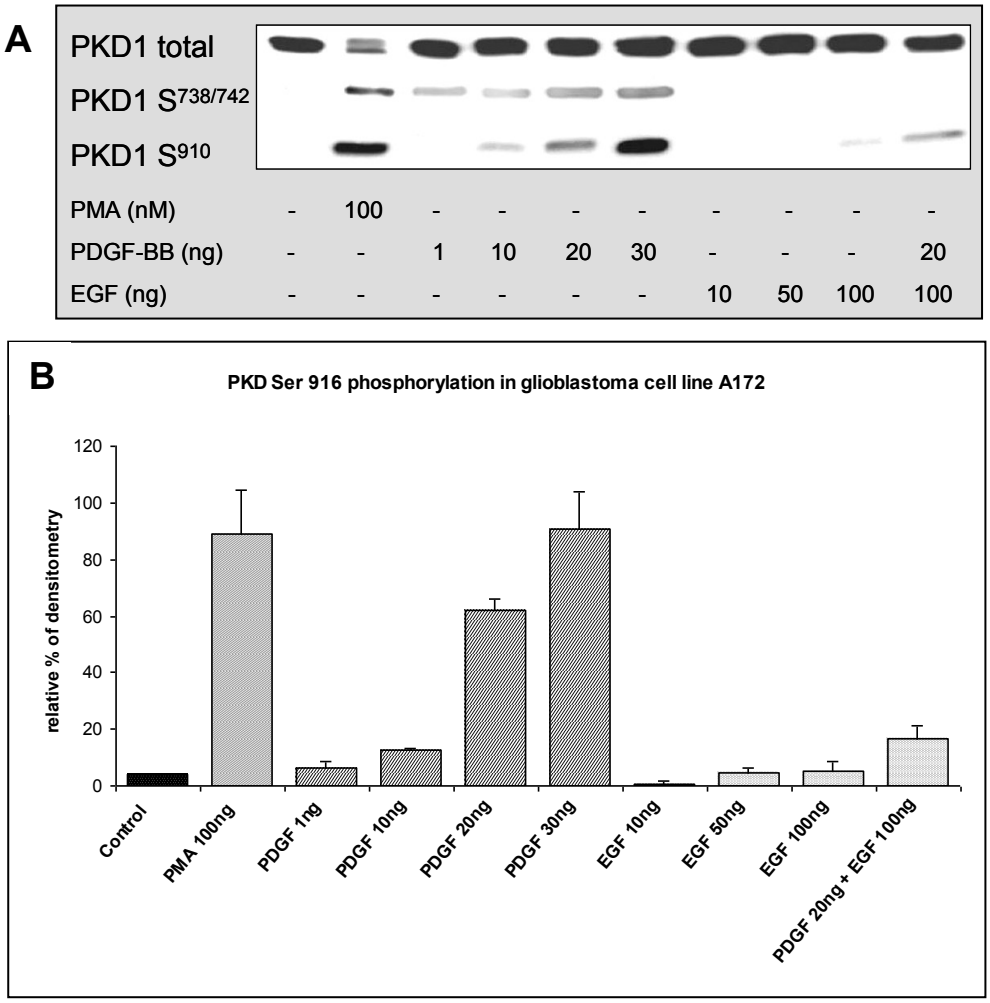


Figure 9: Activation of PKD1 in the glioblastoma cell line A172

Cells were cultured on 10 cm Petri dishes and stimulated in the presence of the indicated amounts of agonists for 15 min. Cells were then washed, scraped in 50 µl RIPA buffer, briefly sonicated and centrifuged to remove insoluble material. **A:** One hundred µg cell protein was applied per lane, separated on 8% gels, transferred to PVDF membranes and incubated with antibodies against total PKD1, and phosphorylated PKD1 ($S^{738/742}$ or S^{910}). Immunoreactive bands were visualized with the ECL method. **B:** Densitometric evaluation of immunoreactive bands, results shown represent mean ±SD from three independent experiments.

3.4 Subcellular trafficking of a PKD1-GFP construct in A172 cells

To determine subcellular localization and trafficking of PKD1 in A172 cells in response to different stimuli, wild-type (wt)PKD1-GFP and a kinase-dead construct were transiently overexpressed in A172 cells and analyzed by confocal microscopy (the constructs are kind gifts

from Dr. Johan van Lint, Belgium). In non-activated cells a diffuse signal for PKD1-GFP was observed throughout the cell, although slight enrichment in perinuclear regions was observed (**Fig. 10**). Addition of PMA (activating PKD1 via activation of upstream PKC's) resulted in rapid translocation of PKD1-GFP (within 10 min) to the plasma membrane. It appears, that PKD1 predominantly localizes to distinct domains at cellular extensions, which could probably resemble invadopodia. This preferential localization at these sites is visible already after 10 min (**Fig. 10 C**) and prominent staining is observed in these cellular structures after 30 min (**Fig. 10H**).

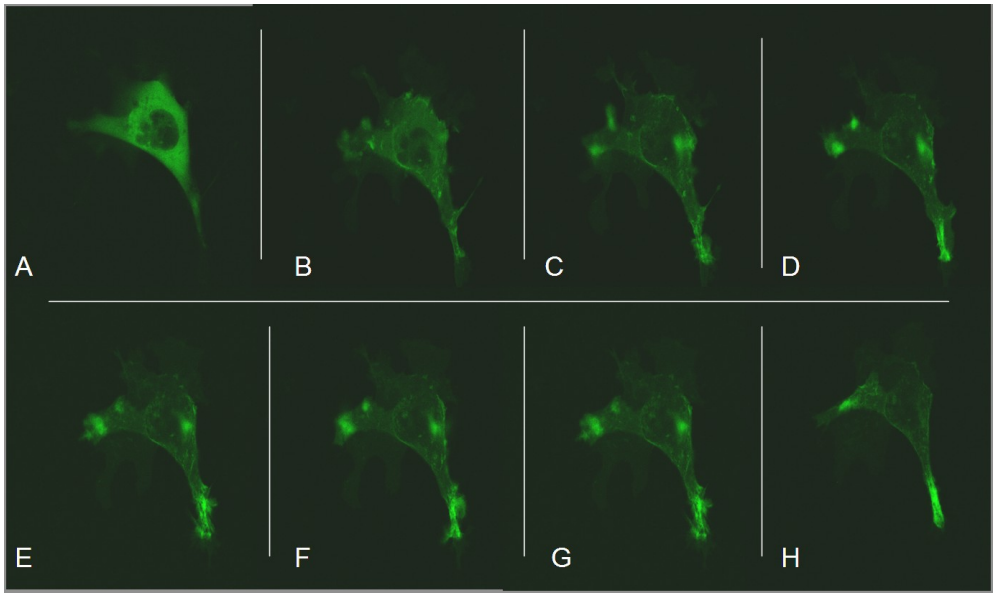


Figure 10: **PMA induces recruitment of PKD1 to the plasma membrane of A172 cells**
A PKD1-GFP construct was transiently overexpressed in A172 cells using the Amaxa nucleofection technology. One hundred µl cell suspension (containing $1.5x10^6$ cells in the solution of nucleofector mouse astrocyte kit) were mixed with 3 µg cDNA and electroporated. Immediately after transfection cells were transferred to prewarmed medium and grown on cover slips. To analyze the effects of PMA stimulation on PKD1-GFP localization, (living) cells on cover slips were transferred to an incubation bath fitted under the optics of the microscope. The images show cells before (A) and after incubation with PMA (100 nM) for up to 30 min (B-H, 5 min intervals).

Having established that the PKD1-GFP construct undergoes intracellular trafficking in response to PMA (as reported for other cell types) we have stimulated A172 cells - that were transiently transfected with the PKD1-GFP construct – in the presence of PDGF (20 ng), which is a more physiological stimulus for glioblastoma cells. One day post-transfection cells were stimulated with PDGF and analyzed by living-cell laser scanning microscopy (**Fig. 11**). Although the cellular response with respect to intracellular trafficking was less pronounced as compared to PMA (Fig. 10), PDGF induced a clear translocation of PKD1-GFP to the plasma membrane (**Fig. 11D and E, arrows**) after an incubation time of 5 min. Thus it is conceivable, that also PDGF, which is a potent tumor mitogen, is capable to induce PKD1 activation and subcellular translocation.

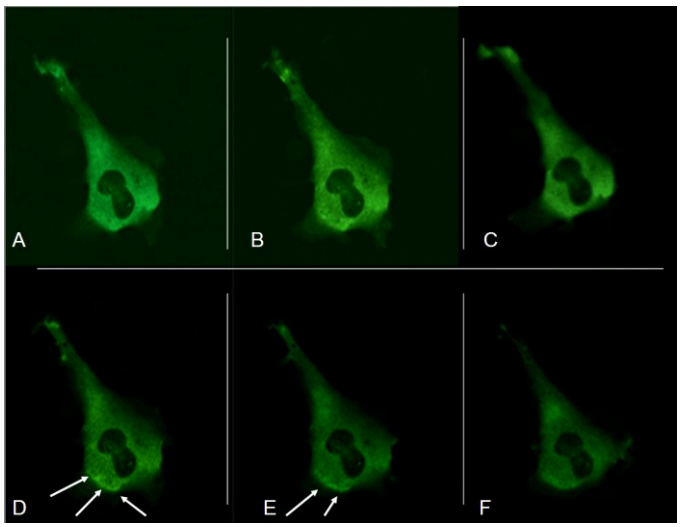


Figure 11: PDGF-induced translocation of PKD1-GFP

A172 cells were transfected with a PKD1-GFP construct exactly as described in the legend to figure 10 subsequent to PDGF-BB addition (20 ng). Cells were visualized after 2 (B), 5 (C), 10 (D), 15 (E) and 20 (F) min. Arrows indicates sites of PKD1 translocation.

The integrity of the kinase domain of PKD1 is a prerequisite for normal intracellular trafficking in response to activation – this also implies that a physiological response is only obtained when PKD1 is directed to the correct subcellular destination after activation. Overexpression of a kinase-dead construct of PKD1 resulted in extensive tubulation of the Golgi (Liljedahl et al., 2001). To be able to visualize the Golgi in A172 cells a specific dye (Bodipy FL) was used. Bodipy FL C5 ceramide is routed across cellular membrane and catabolized like naturally occurring lipids. A172 cells containing an intact Golgi and Brefeldin A-treated A172 cells with disassembled Golgi were incubated in the presence of Bodipy FL C5 and visualized by laser scanning microscopy (**Fig. 12**). In untreated cells (**Fig. 12A**) the Golgi stacks were clearly visible, while in Brefeldin A-treated cells the Golgi stacks were absent due to organelle disassembly (**Fig. 12B**).

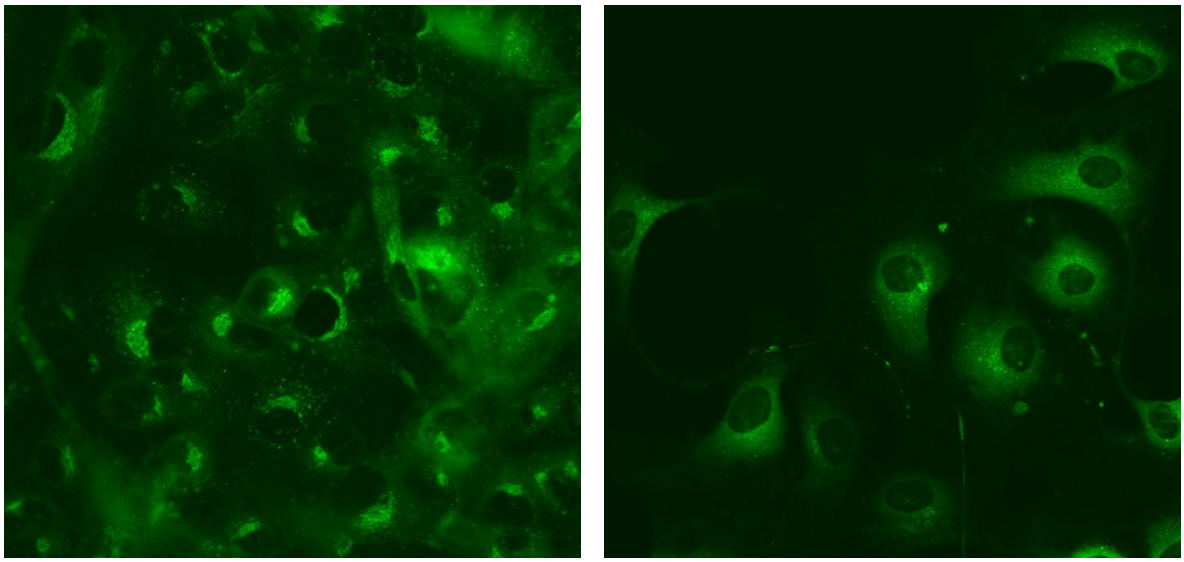


Figure 12: Visualization of the Golgi Apparatus in A172 cells
A: To detect the Golgi Apparatus A172 cells were incubated with Bodipy FL C5 ceramide and visualized by laser scanning microscopy. **B:** In the presence of Brefeldin A (10µg/ml, 10 min) the Golgi stacks were absent due to organelle disassembly.

The next series of experiments was designed to investigate whether overexpression of a kinase-dead PKD1 construct would result in either disassembly or tubulation of the Golgi in a similar manner as reported for HeLa cells (Liljedahl et al., 2001). To test this possibility a PKD1-GFP construct, which was modified by site-directed mutagenesis by substituting a lysine by an asparagine (K618N; this construct was a kind gift from Dr. Johan van Lint, Belgium) in the ATP-binding site was transiently overexpressed in A172 cells. One-day post transfection cells were stimulated with PMA and translocation of the GFP construct was followed by live cell laser scanning microscopy (**Fig. 13**). In unstimulated cells the distribution of the GFP-construct appeared to be roughly similar to results shown in Figs. 10 and 11. However, in response to activation this construct translocated rapidly to (peri)nuclear destinations and much less of the fluorescent product was localized to the plasma membrane after stimulation with PMA. It is currently unclear whether or not overexpression of the $PKD1_{K618N}$-GFP construct results in tubulation of the Golgi in A172 cells as described for HeLa cells (Liljedahl et al., 2001). However, what is clear from these experiments is the fact that mutations in the ATP-binding site that alter the catalytic properties of the kinase results in severely altered intracellular trafficking properties as compared to the wild-type construct in glioblastoma cells.

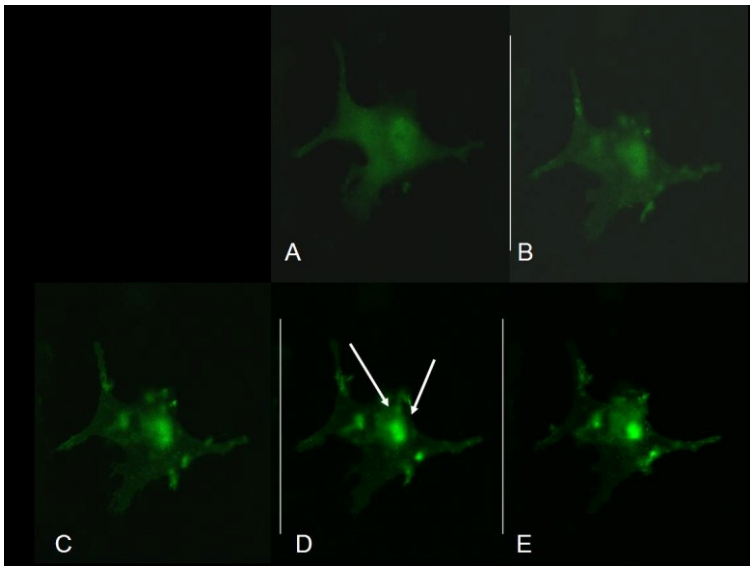


Figure 13: Overexpression of a kinase dead PKD1-GFP construct in A172 cells
A172 cells were transfected with a PKD1K618N-GFP construct exactly as described in the legend to figure 10. Cells were analyzed after 2 (B), 5 (C), 10 (D), and 15 (E) minutes. Arrows indicates sites of preferential PKD1-K618N translocation.

3.5 Effects of the pharmacological PKC-inhibitors Gö6976 on A172 proliferation

Different isoforms of PKC are regulators of proliferation and differentiation via activation of downstream signaling modules in a variety of different cell lines. The critical role of PKC isoenzymes and downstream effectors make them important and suitable targets for anticancer therapy and consequently a variety of potent and selective PKC inhibitors that discriminate between different PKC-isoforms have been developed. Among these inhibitors are the indolocarbazole Gö6976 that discriminates between Ca-dependent and Ca-independent isoforms of PKC (Martiny-Baron et al., 1993) which is also a potent PKD1 inhibitor (Gschwendt et al., 1996). The bisindolylmaleimide Gö6983 is also used as a PKC inhibitor, is, however, a poor inhibitor of PKD1 (Gschwendt et al., 1996). In the experiments described below the efficacy of Gö6976 as an inhibitor of PKD1 activation (S916 phosphorylation) on A172 cell proliferation rates was assessed. In the first set of experiments A172 cells were cultured in 10 cm Petri dishes, incubated in the presence of increasing concentrations of Gö6976 (20 nM to 20 μM) and then stimulated with PMA or PDGF. S916 phosphorylation was

analyzed by immunoblotting as described above, films were then densitometrically evaluated and the band intensity after PMA stimulation in the absence of inhibitors was set 100 % (**Fig. 14**). In PMA stimulated cells Gö6976 inhibited PKD1 S916 phosphorylation by 18, 26, 58 and

75 % (20 nM, 40 nM, 10 µM and 20 µM, respectively). As already mentioned above, S916 phosphorylation in response to PDGF stimulation is less pronounced as compared to PMA (approx. 30 % lower), however, the inhibition rates of S916 phosphorylation by Gö6976 in PDGF stimulated cells was in a similar range as observed for PMA stimulation (reduction by 10, 25, 90 an 92 % in the presence of 20 nM, 40 nM, 10 µM and 20 µM Gö6976, respectively). These results clearly indicate that Gö6976 inhibits activity of PKD1 in a dose dependent manner, independent of the stimulus used.

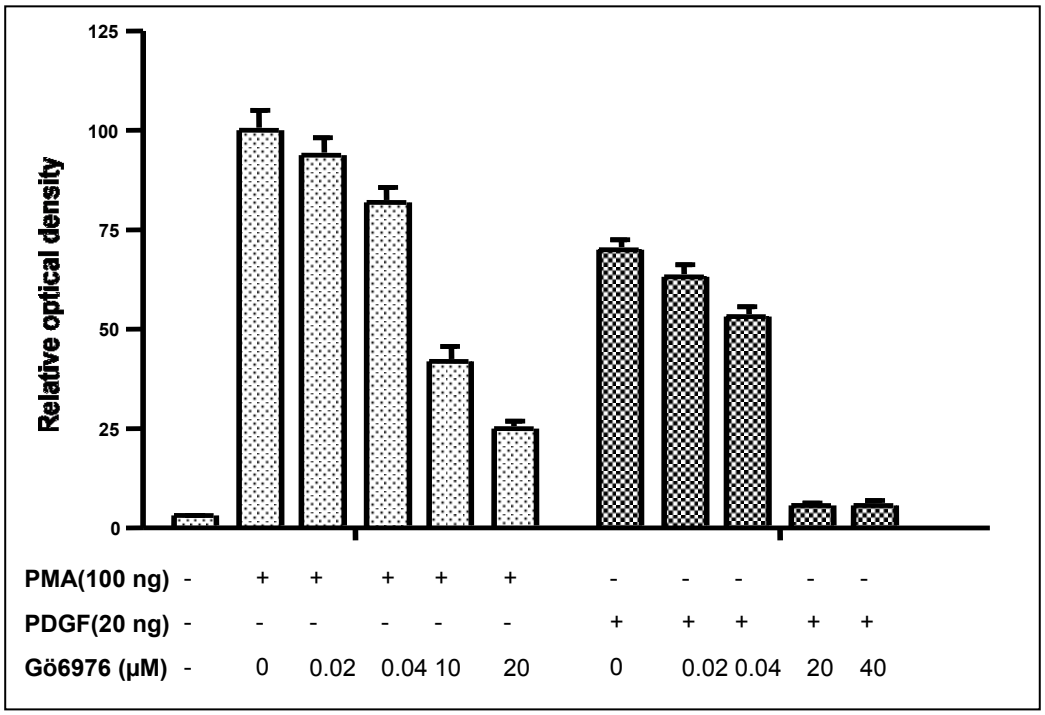


Figure 14: Effects of Gö6976 on PKD1 S^{916} phosphorylation
A172 cells were plated on 10 cm Petri dishes, confluent monolayers were preincubated with the indicated concentrations of Gö6976 (20 min) and then in the presence of PMA (100 ng) or PDGF (20 ng) for 15 min. Cells were placed on ice, scraped in RIPA buffer and S916 phosphorylation status was analyzed by immunoblotting. Data shown represent mean ± SD.

To get an impression about the effects of Gö6976 on the cellular morphology A172 cells were incubated in the presence of 20 µM Gö6976 and photos were taken prior and after (2 d) the incubation (**Fig. 15**). From these slides it appeared that incubation of A172 cells in the presence of Gö6976 resulted in a reduction of cell numbers, hypertrophic cells and the appearance of numerous vacuoles that probably resemble lipid laden cells. Another possibility – that was not further investigated – is the fact that vacuole formation is due to autophagic cell death.

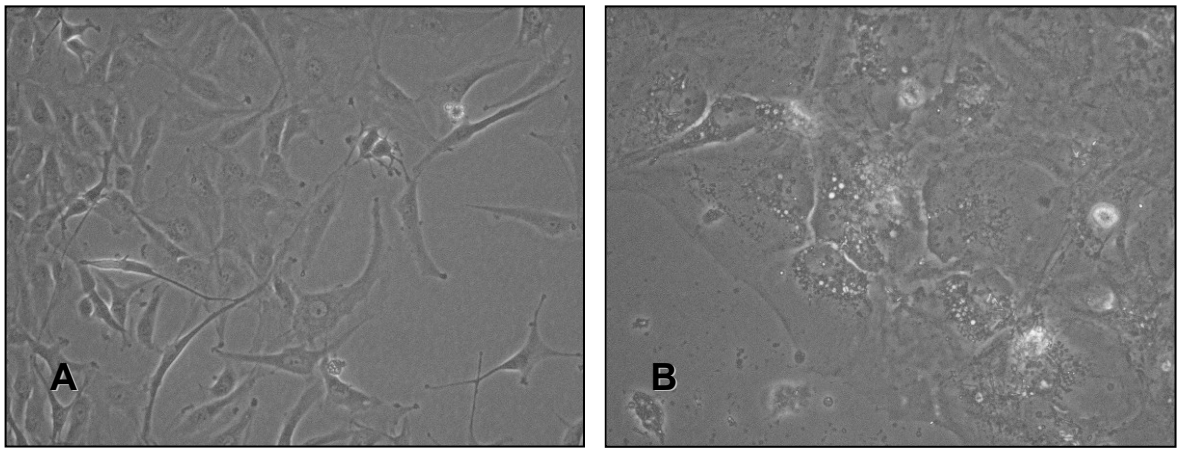


Figure 15: Effect of Gö6976 on A172 cell morphology

Cells were plated in 6-well trays at a density of 60000 cells. After 2 days in culture (A) Gö6976 in DMSO was added (final DMSO concentration 0.15 % v/v). The slide shown in B was taken after 48 h Gö6976 treatment.

To be able to quantitate the impact of Gö6976 on A172 proliferation rates, growth curves were established. Cells were plated at a density of 20000 cells per well and after 48 h agonists or antagonists were added (PDGF 20 ng/well, DMSO 0.15 % v/v), Gö6976 10 and 20 µM, respectively; **Fig. 16**). During these experiments it became evident that DMSO at the concentration used has no detrimental effects on proliferation rates since control cultures and DMSO-supplemented cultures were almost identical and are superimposed in the graph. The addition of PDGF to A172 cultures resulted in a significantly enhanced proliferation rates (at day 6 an increase by 23 % was observed; 262333 vs. 214500 cells, presence and absence of PDGF, respectively). In contrast, the addition of Gö6976 to the culture medium resulted in almost complete growth arrest at both concentrations used.

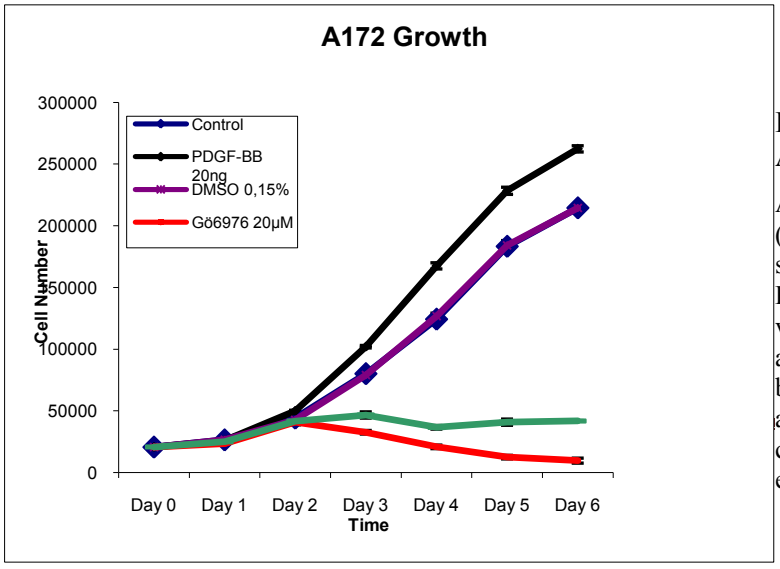


Figure 16: Effect of Gö6976 on A172 cell growth

A172 cells were plated in 6 well trays (20000 cells) and cultured under standard conditions for 2 d. At day 2 PDGF (20 ng/well), DMSO (0.15 % v/v) and Gö6976 (10 and 20 µM) were added. Cells were counted manually by a hemacytometer. Results shown are mean ± SD, controls and vehicle controls (DMSO) are superimposed, error bars are smaller than symbol size.

3.6 Effects of PKD1 silencing by RNA-interference

Activation of PKD1 is known to couple downstream to one or more members of the mitogen-activated protein kinase (MAPK) family, which are potent effectors of cell growth and proliferation. To reveal effects of PKD1 on the proliferative properties of A172 glioblastoma cells, PKD1 was silenced by RNAi using three different 21-mer siRNAs directed against different regions of the PKD1 mRNA. In a first set of experiments the three different constructs were transiently transfected in A172 cells using the Nucleofector technology. Forty-eight hours post transfection the levels of total PKD1; phosphorylated ERK1/2 and total ERK were analyzed by immunoblotting in PDGF stimulated cells (**Fig. 17**). The results of these experiments clearly indicated that mock transfection of A172 cells was without effects on total PKD1, phosphoERK1/2 and total ERK1/2 levels. With regards to the 21-mer siRNAs targeting PKD1 constructs siPKD1 I and II were approx. equally effective in silencing PKD1 expression (a reduction in immunoreactive PKD1 between 50 and 90 % was consistently observed in these experiments). The construct siPKD1 III was less effective in silencing PKD1 expression at the lower concentration (1.5 µg) and showed comparable efficacy to constructs I and II when transfections were performed with three µg siRNA. With regards to pERK1/2 detection it is noteworthy that the active form of ERK1/2 was undetectable in cells where the highest rates of PKD1 silencing occurred (siPKD1 II and III at 3 µg). The levels of detectable total ERK1/2 were unaffected by either treatment.

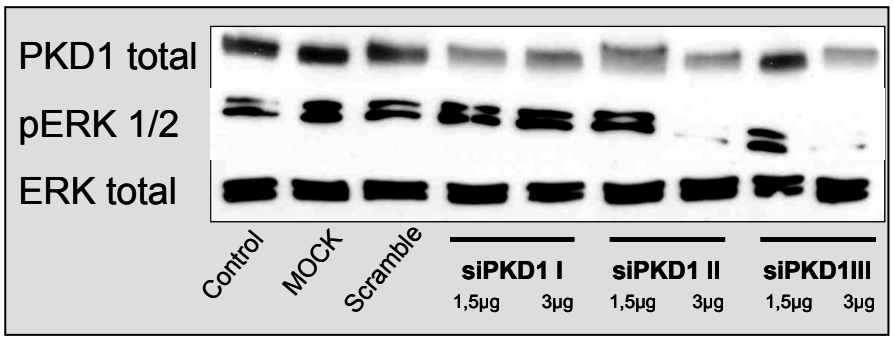


Figure 17: siRNA silencing of PKD1 in PDGF-BB (20ng) stimulated A172 cells
Cells were nucleofected with 21mer oligonucleotides (directed against nt 938-958 of human PKD1 mRNA; 1.5 or 3 µg siRNA) and the expression of total PKD1, pERK1/2 and total ERK1/2 was analyzed 48 h post-transfection by Western blotting.

To get an impression about the duration of PKD1 silencing the kinase was silenced with construct siPKD1 III (3 µg) and PKD1, pERK1/2 and total ERK1/2 expression was analyzed in

a time dependent manner (**Fig. 18**). Results revealed that mock treatment and scrambled siRNA had no effect on the proteins analyzed, the highest rate of silencing was observed at day 3 for PKD1 and at day 2 for pERK1/2. Total ERK1/2 levels were unaffected, indicating that the decrease in pERK1/2 is caused by defective phosphorylation due to a disrupted upstream signaling cascade and not due defective protein synthesis.

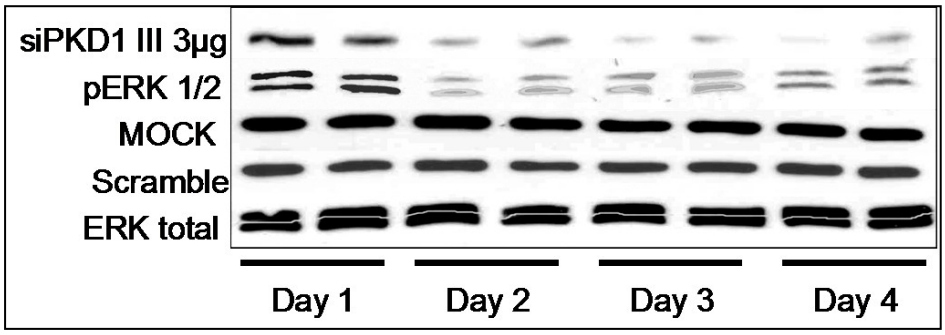


Fig. 18: Duration of PKD1 silencing in A172 cells
Cells were nucleofected with 21mer oligonucleotides (3 µg siPKD1 III) and the expression of total PKD1, pERK1/2 and total ERK1/2 was analyzed in mock, scrambled and siRNA-transfected cells at the indicated time points by immunoblotting.

The strong reduction in phosphorylated ERK1/2 levels in PKD1 silenced cells suggested that PKD1 silencing could be coupled to reduced proliferation rates of A172 cells. To clarify the functional outcome of PKD1 silencing in A172 cells, control, mock, scrambled and PKD1-siRNA transfected cells were used to study cell proliferation in response to PDGF. Neither mock transfection nor scrambled 21mer oligonucleotides affected growth rates while PKD1 silencing resulted in a 3.3-fold reduction of cellular growth rates (**Fig. 19**). These data clearly demonstrate that knockdown of PKD1 results in efficiently reduced tumor cell proliferation.

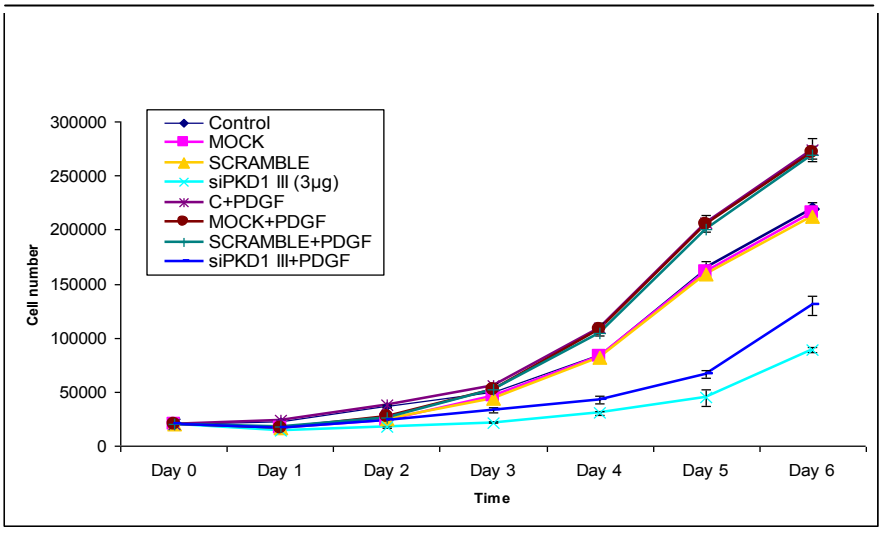


Figure 19: **Effects of PKD1 knockdown by RNAi on A172 cell proliferation**

Wild-type (controls) and transfected (mock, scrambled or PKD1 siRNA) A172 cells (20000 cells were seeded at the beginning of the experiments) were cultured in the absence or presence of PDGF (20 ng/ml). Cells were counted every day and the results represent mean±SD from triplicate dishes.

3.7 Assessment of A172 cell invasiveness in spheroid invasion assays

Invasion of cancer cells into the surrounding tissue is one of the major mechanisms leading to the formation of metastases. To get an indication about the effects of PKD1 silencing on the invasiveness of A172 cells into spheroids of freshly prepared embryonic chicken brain cells *in vitro* spheroid invasion assays were performed. In these experiments A172 cells ('cancer') and ECB ('host') were labeled with fluorescent dyes, and, after reaggregation to a size of approx. 50 µm, the spheroids were mechanically confronted with the ECB spheroids forming cancer-host pairs. These pairs were incubated in a microcultivation chamber and confocal images were recorded using a laser-scanning microscope (**Fig. 20**).

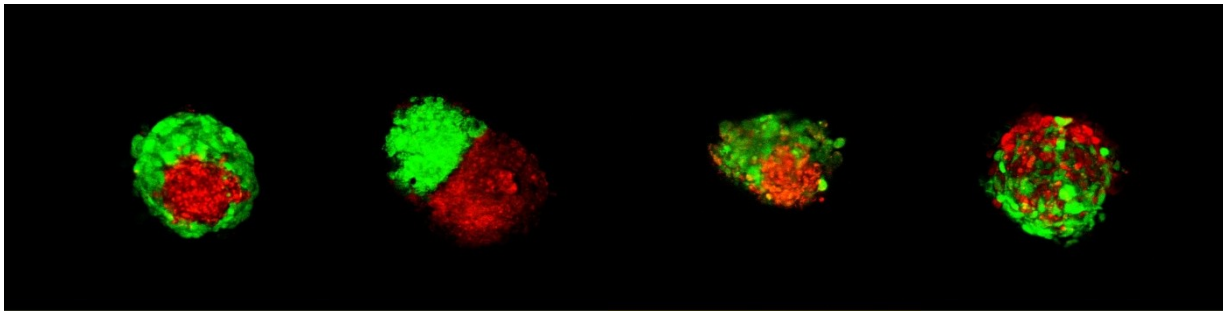


Figure 20: Spheroid invasion assays
Spheroids of mock (A), scrambled (B), PKD1-siRNA (C) transfected A172 cells were mechanically confronted with either embryonic chicken brain cell spheroids (A-C) or with A172 cell spheroids (D). After 48 h the spheroids were analyzed by confocal laser scanning microscopy.

Fig. 20 A demonstrates that mock-transfected A172 spheroids attached to embryonic chicken brain cell spheroids and progressively invaded them. Surprisingly transfection with scrambled siRNA (**Fig. 20 B**) completely inhibited A172 invasion into the host spheroids for yet unknown reasons. In contrast, PKD1 silenced A172 cells readily invaded the host tissue; however the spheroids were consistently smaller in size than compared to mock and scrambled transfected spheroids. Another interesting observation during these studies was the fact that A172 cells have an extremely high invasive potential when the host spheroid is also formed by A172 cells (**Fig. 20 D**).

During the spheroid invasion assays we have observed that PKD1 silenced cells form spheroids that are mechanically more labile than the spheroids formed by control, mock, or scrambled transfected cells. Thus it appeared possible that PKD1 silencing somehow affected adhesion properties of A172 cells. To test this we have analyzed the expression pattern of two proteins involved in cellular adhesion, namely E-cadherin and β-catenin. E-cadherin-β-catenin complexes are linked to the actin cytoskeleton via catenin maintaining structural integrity of tissues. While no changes in E-cadherin expression was observed between wildtype and silenced cells (data not shown), PKD1 silencing resulted in a pronounced reduction of cellular β-catenin levels at day 2 and 3 after RNAi (**Fig. 21**). Thus, disintegration of cellular adhesion complexes due to PKD1 silencing could at least partially contribute to the high invasive properties of silenced cells as observed during spheroid invasion assays.

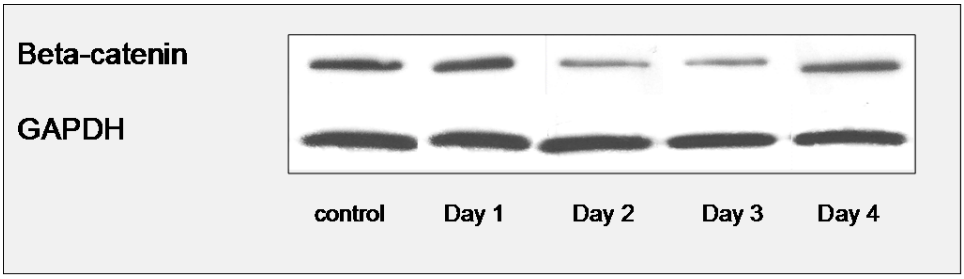


Fig. 21: Silencing of PKD1 in A172 cells reduces cellular β-catenin levels

PKD1 was silenced with the siPKD1 III construct (3 µg) and β-catenin and GAPDH expression was analyzed by immunoblotting.

3.8 Summary of results

In summary the results indicate that

i) PKD1 expression levels correlate with astrocytoma grading.

ii) PKD1 is phoshorylated and active in primary glioblatoma cells and can be activated by PMA and PDGF in the A172 glioblastoma cell line.

iii) Activated PKD1 undergoes intracellular trafficking to the plasma membrane, activation of a construct lacking the kinase-domain result in extensive vacuole formation.

iv) Pharmacological inhibition or silencing PKD1 by RNA-interference significantly reduced proliferation rates of glioblastoma cells and result in mechanically more labile spheroids probably due to reduced cellular β-catenin levels.

Chapter 4

Discussion

4.1 Activation of PKD via the protein kinase C family

It is not surprising that diacylglycerol (DAG) activation of protein kinase C (PKC) has an important role in cancer as PKCs are targets of phorbol esters, which are potent tumor-promoting compounds. However, deciphering the mechanisms by which PKCs control the numerous phenotypes that are displayed by cancer cells remains a challenge. DAG and its prime target PKC regulate many important cellular responses. As such, targeting of the PKC pathway(s) for therapeutic purposes is challenging. The protein kinase D (PKD) family - a novel DAG-activated serine/threonine kinase family (consisting of PKD1, -2, and -3) - now classified within the Ca^{2+}/Calmodulin-dependent kinase (CaMK) superfamily (Rozengurt et al., 2005) - has received considerable interest within the recent years. PKD is involved in a multitude of important cell functions induding cell growth, gene expression, survival, motility, protein trafficking, and immune cell biology. PKD is implicated in pathological processes like cardiac hypertrophy, tumor cell proliferation and metastasis (Rykx et al., 2003).

The findings obtained during the present thesis provide several lines of evidence that PKD1 might be a master regulator of glioblastoma cell growth: First, PKD1 protein mass correlates with astrocytoma grading, second, PKD1 is expressed by glioblastoma cells, third, PKD1 is phosphorylated in response to PDGF (PDGF overexpression has been described in up to two thirds of glioblastoma cases, (Guha et al., 1995)), fourth, the PKC-inhibitor Gö6976 inhibits PKD1 phosphorylation and glioblastoma cell growth, and fifth, silencing of PKD1 expression by RNA interference significantly inhibits glioblastoma cell growth. These findings demonstrate that PKD1 could provide a novel molecular target to interfere with glioblastoma growth.

PKD activation is a two step process, occurring via i) binding for DAG to the Cl domain of PKD and ii) PKC-dependent phosphorylation of PKD. PKCε interacts with the PH domain of PKD and trans-phosphorylates the activation loop at S744 and S748, inducing PKD activation. Although there appears to be a preference for the novel PKCs in PKD activation (Zugaza et al., ; Zugaza et al.), also classical PKCα can activate PKD (Wong & Jin, 2005). Activation of PKD2 and PKD3 appears to follow the same activation patterns (Rey et al., 2006; Sturany et al., 2002). The factors contributing to signal specificity along the PKC-PKD axis is not entirely dear, however, substrate specificity of PKD, the cell type, and the subcellular localization of PKD are major determining factors (Cabrera-Poch et al., 2004; Marklund, Lightfoot & Cantrell, 2003).

4.2 PKD localization determines cellular function

At basal state PKD is primarily localized in the cytosol. After activation, PKD gains intracellular mobility and distributes between different compartments, including the plasma membrane, the Golgi, mitochondria and the nucleus. The ability of PKD to distribute to different subcellular compartments has important functional implications since PKD localized in different compartments carries out different functions (see below). In response to activation of G-protein-coupled receptors or tumor-promoting phorbol esters, PKD translocates to the plasma membrane. This translocation step of PKD is rapid and reversible; because it was demonstrated that PKD rapidly dissociates from the plasma membrane and is distributed back to the cytosol, where, in contrast to other kinases, PKD can stay active over hours (Matthews et al., 2000). This prolonged activation pattern of PKD enables the kinase to propagate extracellu1ar signals in a unique fashion. Results obtained during the present study have revealed localization of PKDl at the plasma membrane (and to a lesser extent in the cytosol) of GFAP-positive cells in glioblastoma sections. This could indicate that, due to autocrine or paracrine activation, PKD is activated in glioblastoma cells and contributes to tumorigenesis (see below). In line, treatment of GFP-PKDl transduced A172 cells with phorbolester induced rapid and sustained translocation of PKDl to the plasma membrane (sites probably resembling invadopodia). Activation of the PDGF receptor pathway in GFP-PKDl transfected A172 cells revealed a comparable pattern of intracellular PKD1 translocation.

Golgi localized PKD was first reported by Prestle and colleagues (Prestle et al., 1996), where the C1 domain mediates binding of the kinase to the trans-Golgi network (TGN). Localization in the TGN depends on local DAG concentrations that mediate binding via the C1a domain (Baron & Malhotra, 2002). Golgi localization of PKD is essential for vesicular protein transport and protein trafficking from the TGN to the plasma membrane (Liljedahl et al., 2001). During the present study overexpression of a kinase-dead PKDl construct on A172 glioblastoma cells resulted in mostly perinuclear localization of the GFP construct. Whether or not this affects Golgi function or results in the tubulation of the TGN as reported for HeLa cells (Liljedahl et al., 2001) is currently unclear.

Finally, PKD1-3 is able to shuttle between the cytosol and the nucleus. The relative distribution between the two compartments depends on the nuclear import and export signals for each isoform The structural determinants governing nuclear import/export localize to the C1a, C1b, and the PH domain of the kinases (Auer et al., 2005; Rey et al., 2001).

The localization of PKD in the nucleus could have important implications on tumor biology by regulating the activity and subcellular localization of histone deacetylases (see below).

4.3 Potential roles of PKD in tumor biology

One of the reasons to initiate the present study was the fact that a major proportion of PKD1 (besides lung) was identified in brain (Johannes et al., 1994), although the physiological function of PKD1 in this organ is still unclear.

In the brain, inappropriately active growth factor signaling is the driving force for proliferation in malignant gliomas. Growth factor pathways involved in glioma growth include EGF, VEGF, PDGF, fibroblast growth factor, and insulin-like growth factor 1. Amplification or activating mutations of genes coding for growth factors and their corresponding receptors, e.g. *FGF* and/or *FGF-receptor, PDGF* and/or *PDGF-receptor,* and *IGF* or *IGF-receptor,* are events that occur in gliomas with high frequency (Dai et al., 2001). As a result of excessive growth factor production autocrine stimulation and increased downstream pathway activity of the corresponding receptors is observed. Of interest, the present study clearly revealed pronounced activation of PKD1 in response to PDGF-BB treatment. PDGF treatment resulted in phosphorylation of PKD1 (trans- and autophosphorylation sites), translocation of the kinase to the plasma membrane, and a concomitant increase of A172 glioblastoma cell proliferation rates. Stimulation of the EGF-receptor pathway was less effective in stimulating PKD1 phosphorylation. Currently the underlying signaling pathways that induce increased cell proliferation are not entirely clear; however, several scenarios are conceivable and are described below:

4.3.1 PKD1 as a regulator of the PDGF-PKD1-Ras-ERK pathway

Activation of RAS (a protein family playing a pivotal role in cellular proliferation, differentiation and apoptosis) occurs directly in response to activation of receptor tyrosine kinases and leads to induction of mitogen-activated protein kinases (MAPK), namely extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase and the p38 MAPK (Feldkamp et al., 1997) resulting in altered proliferative potential of the cells. PKD1 has been implicated in a variety of cellular functions (Van Lint et al., 2002) including DNA synthesis and cell proliferation (Rennecke et al., 1999), NF-KB-mediated gene expression (Storz, Doppler & Toker, 2005), and modulation of the MAPK pathway (Brandlin et al., 2002a; Brandlin et al., 2002b). Another feature of PKD1, which is disparate from the other PKCs, is sustained activity over several hours in response to activation (Oancea et al., 2003) a fact of particular interest for cell proliferation of tumor cells. Other studies revealed that PDGF-receptor increased cell proliferation and cell migration upon stimulation by PDGF in a glioblastoma cellline (Laurent et al., 2003). Along this line it is noteworthy that PDGF is also a

potent activator of PKDl (Bagowski et al., 1999), data in line with findings obtained during the present investigation.

One of the pathways activated in astrocytic gliomas is the MAPK pathway, which by itself is activated through small G-protein RAS family members. Unlike other tumors, activation of RAS in astrocytic gliomas is not due to mutated RAS but has been linked to growth factor signaling. The critical step in determining the cellular response to RAS activation is the physical interaction with RAS interaction partners that function to accept the message and dispatch it appropriately. Importantly, RINl, an inhibitor of RAS is phosphorylated by PKDl (Wang et al., 2002). PKDl mediated phosphorylation of RINl serves as an attenuation mechanism that uncouples RINl from activated RAS and this was suggested as a potential mechanism how PKDl could affect RAS and downstream ERK1/2 activation (Wang et al., 2002), events resulting in enhanced cellular proliferation rates.

Many of the above mentioned findings are entirely compatible with findings obtained during the present study. Silencing of PKDl expression resulted in pronounced reduction of glioblastoma proliferation rates, independent of whether the cells were stimulated with PDGF or not. However, it is noteworthy that PDGF-mediated signaling bypasses PKDl since in comparison to untreated cells, PDGF-stimulated, PKDl silenced cells showed slightly higher proliferation rates. The reasons for this observation are presently not clear, could, however, be related to signaling nodes that are activated in response to PDGF and operate independently of PKD1 activation. Due to the complex signaling networks evoked by PDGF-receptor signaling this would not be surprising. The reduced activation of the MAPK-pathway in PKD1 silenced cells is probably the most plausible explanation for the reduced proliferation rates observed during the present study utilizing A172 glioblastoma cells. Notwithstanding, these findings have to be verified in primary glioblastoma cell cultures and in an in vivo glioblastoma animal model, which will be performed in future studies. An obvious contradiction comes from invasion assays that were performed with embryonic chicken brain cell spheroids: Although these studies made clear that A172 glioblastoma cells have an extremely high invasive potential, PKD l-silenced cells still readily invaded the host tissue (it is noteworthy that the spheroids with PKDl silenced cells were smaller in size and mechanically less stable than the wild-type spheroids). The mechanical destabilization of spheroids established with PKDl-silenced A172 cells could be due to much lower expression of β-catenin, a member of the adherens junction complex and responsible for cell-cell interaction (see below).

4.3.2 PKD1 as a regulator of invasion and inter-cellular adhesion properties

Treatment of small cell lung cancer cells with tumor promoting phorbolesters leads to rapid activation of PKD1 via upstream activation of conventional PKCs. PKD1 was also identified in invadopodia of human breast cancer cells where it forms a complex with cortactin and paxillin (Bowden et al., 1999). Cis-polyunsaturated fatty acids were shown to stimulate integrin-mediated adhesion of human breast carcinoma cells to collagen by activating PKD1 in response to PKCε stimulation (Palmantier et al., 2001). The association of PKD1 with metallothionein 2A (Rao et al., 2003), small proteins known to cause resistance to chemotherapy in several cancer forms) suggests an involvement of PKD1 in tumorigenesis like e.g. human prostate cancer (Jaggi et al., 2005). In pancreatic tumor cells PKD1 is overexpressed (Trauzold et al., 2003) and was shown to phosphorylate E-cadherin contributing to intercellular adhesion events (Trauzold et al., 2003). At adherens junction, cadherins have a number of intracellular binding partners that are involved in the regulation of intercellular adhesion (by stabilizing adherens junctions between adjacent cells) and are also potent regulators of intracellular signaling pathways (the catenins are potent regulators of the wnt--pathway that controls gene expression). Results obtained during the present study provide evidence that PKD1-silencing affects expression patterns of β-catenin: The present thesis provides evidence that PKD1 is also involved in β-catenin synthesis or degradation. This is conceivable since the localization of adherens junction proteins is governed by phosphorylation events: While serine/theorin-phosphorylation favors localization at the plasma membrane (where the adherens junctions are assembled and functional), thyrosine-phosphorylation of these adhesion proteins favors either degradation or translocation to the nucleus. Whether these events are involved in reduced β-catenin mass in PKD1-silenced A172 glioblastoma cells is currently not clear. It is noteworthy that reduced expression of β-catenin and a resulting reduction in intercellular tumor cell adhesion could contribute to disintegration of tumors and favor metastasis and glioblastoma cell spread within the centraI nervous system. Whether or not the focal adhesion pathway (FAK promotes malignant astrocytoma/glioblastoma cell proliferation (Wang et al., 2005)), promotes cell migration, and was suggested to promote tumor angiogenesis (Natarajan et al., 2003) is affected in PKD1-silenced cells remains to be determined.

4.3.3 PKD1 as a regulator of histone acetylation

A hallmark of physiological PKD function was the demonstration of cardiac hypertrophy through nuclear export of class II histone deacetylase (HDAC) 5 that occurs in response to agonist-induced activation of PKD (Vega et al., 2004). Interestingly all four class IIa HDAC's contain the consensus PKD substrate phosphorylation site. HDAC's control chromatin accessibility that in turn is a key mechanism for transcriptional regulation of gene expression. Dysregulated function of HDAC's is a factor contributing to heart failure, skeletal muscle defects, and cancer. Studies along this line are currently ongoing in PKDl silenced A172 glioblastoma cells.

In summary the results obtained during the present thesis indicate that intervention (either pharmacological or via RNA interference) might be a promising, useful, and novel pharmacological approach to interfere with the progression of glioblastoma growth and thus improve the prognosis for affected patients. Future in vivo studies are currently on their way and will clarify these important neurological issues.

Chapter 5

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