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Příprava a charakterizace rekombinantního dermcidinu jako potenciálního vazebného partnera glutamátkarboxypeptidasy II

Bakalářská práce

Vedoucí bakalářské práce:

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Preparation and Characterization of Recombinant Dermcidin as Potential Binding Partner of Glutamate Carboxypeptidase II

Bachelor thesis

Supervisor:

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I affirm this thesis was elaborated individually under the supervision of Doc. RNDr Konvalinka, CSc., that all sources used were cited properly and that any part of the thesis has not been submitted for other degree.

(Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.)

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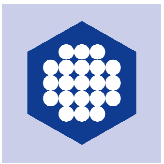

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Brief Contents

English Abstract

A process of forming new blood vessels is necessary for tumour viability and expansion. Without vasculature, tumour stops growing at a size of millimeters. Some tumours, however, undergo an angiogenic switch and start to build up their own vascular architecture. The rate of apoptosis then decreases and the tumour becomes invasive.

There are many factors that control the process of physiological angiogenesis. These might or might not relate to tumour tissue as well.

Glutamate carboxypeptidase II (GCPII; EC 3.4.17.21) is a type II transmembrane glycoprotein with two known enzymatic activities. GCPII expression is upregulated in prostate cancer and also highly expressed in tumour-associated neovasculature even though none of these enzymatic functions was observed on the endothelium. Although numerous researches suggested that GCPII might serve as a receptor, no natural ligand has been identified yet.

Preliminary experiments performed in our laboratory indicated some proteins to be possible natural ligands of GCPII. Therefore, we chose one of them- dermcidin, cloned and expressed this protein in mammalian cells. We investigated its possible interaction with GCPII introducing new detection system utilizing FLAG-tag however, we were not able to approve neither disapprove its interaction *in vitro*.

Key words: angiogenesis; glutamate carboxypeptidase II; GCPII; dermcidin; protein interaction

Český abstrakt

Proces novotvorby cévního zásobení je klíčový pro růst a přežití jakéhokoliv pevného nádoru. Zatímco většina nádorů zůstává nevaskularizována a zpravidla nepřesáhne svou velikostí několik milimetrů, některé novotvary prochází tzv. angiogenním přechodem, v důsledku čehož začne docházet tvorbě krevních tělísek. Počet buněk podstupujících apoptosu se snižuje a nádor se začíná rozrůstat.

Je známo několik dobře prostudovaných faktorů, jež ovlivňují fyziologické děje spojené s novotvorbou cév. Tyto látky se mohou, ale nemusí vyskytovat i v tkáních nádoru.

Glutamátkarboxypeptidáza II (GCPII; EC 3.4.17.21) je transmembránový glykoprotein II. typu, u něhož byly objeveny dvě fyziologicky významné enzymatické aktivity. GCPII je exprimována ve vaskulatuře pevných nádorů, přičemž zde však nebyla pozorována žádná funkce spojená s těmito aktivitami. Přestože se spekuluje o možné receptorové funkci GCPII, přirozený ligand dosud nebyl identifikován.

Předcházející experimenty v naší laboratoři naznačily možnost interakce některých proteinů s GCPII. Jeden z nich- dermcidin byl vybrán jako možný vazebný partner, byl naklonován, exprimován v savčích buňkách a poté byla prověřena jeho možná interakce s GCPII pomocí nového detekčního systému využívajícího kotvu FLAG. Interakci *in vitro* se však nepodařilo potvrdit ani vyvrátit.

Klíčová slova: angiogenese; glutamátkarboxypeptidasa II; GCPII; dermcidin; proteinová interakce

1 Introduction

1.1 Glutamate carboxypeptidase II

Glutamate carboxypeptidase II (GCPII) has been investigated in several tissues in relation to different physiological functions, therefore the nomenclature is not universal and there are several names for the same protein.

GCPII was discovered using a monoclonal antibody 7E11-C5 raised against a human adenocarcinoma cells (LNCaP; lymph node carcinoma of the prostate derived cells) in 1987. The recognized antigen was then called prostate-specific membrane antigen (PSMA) [1].

The same year, an enzyme responsible for cleavage of neurotransmitter N-acetyl-L-aspartyl-L-glutamate (NAAG) was observed independently. Here came its nickname NAALADase (from N-acetylated alpha-linked acidic dipeptidase) still frequently used in the literature [2].

Further researches revealed folate hydrolase activity of GCPII in small intestine where it facilitates absorption of dietary folates. The enzyme was then denominated as folate hydrolase thanks to its ability to cleave γ-linked glutamates from γ-glutamylated folates [3-4].

Even though all these names belong to the very same protein we can come across any of them depending on a topic and specialization of the particular research. For simplification, we decided, according to the recommendation of International Union of Biochemistry and Molecular Biology (IUBMB), to use the name GCPII throughout this thesis.

1.1.1 Gene

The gene coding for GCPII in human is localized on chromosome 11 and was denominated as *FOLH1*. It consists of 19 exons and 18 introns and ranges about 62 kbp. Even though all the known physiological functions of GCPII are enzymatic, the

extracelular portion shares 26% similarity with transferrin receptor 1 (TfR1) on amino acid level [5].

A region rich in CpG sequences typical for housekeeping and tissue-specific proteins is present in *FOLH1* as well as in almost all genes for neural tissues [6].

A strong prostate-specific enhancer is localized in the third intron and it was shown to be a major regulatory element in androgen dependent repression of transcription of *FOLH1* gene [7].

1.1.2 Protein

GCPII (EC 3.4.17.21) is type II transmembrane glycoprotein (similarly to e.g. transferrin receptor) that exhibits protease activity, which strictly depends on presence of Zn^{2+} ions in its active site. It consists of 750 amino acids and is ranked into M28 peptidase family [8].

1.1.2.1 Structure

GCPII consists of three regions:, N-terminal cytoplasmic tail (amino acid 1-18), transmembrane helix (amino acid 19-43) and C-terminal extracellular domain (amino acid 44-750). [8]

The three extracellular subdomains of GCPII are the protease, apical and helical one that share 29%, 25% and 23% similarity with TfR1, respectively. All of them are involved in substrate recognizing. A 3.5 Å crystal structure of the ectodomain was resolved in 2005 and revealed two-fold symmetric homodimer, which is more compact than TfR1, but the overall similarity is still obvious (Figure 1, page 3) [9-10].

Two zinc ions are present in a large cavity between the three domains forming binuclear active site, coordinated with two histidine, two aspartate, one glutamate residues and bridged with water molecule [9].

Two β-sheets, three α-helices and a helical turn form the apical, protease-associated domain of GCPII. Such structural features were also found in many trafficking receptors and are supposed to be responsible for protein-protein interactions [11].

It was shown that a chlorine and calcium ions are necessary for both enzymatic activities and also for a stability of the whole dimer [12].

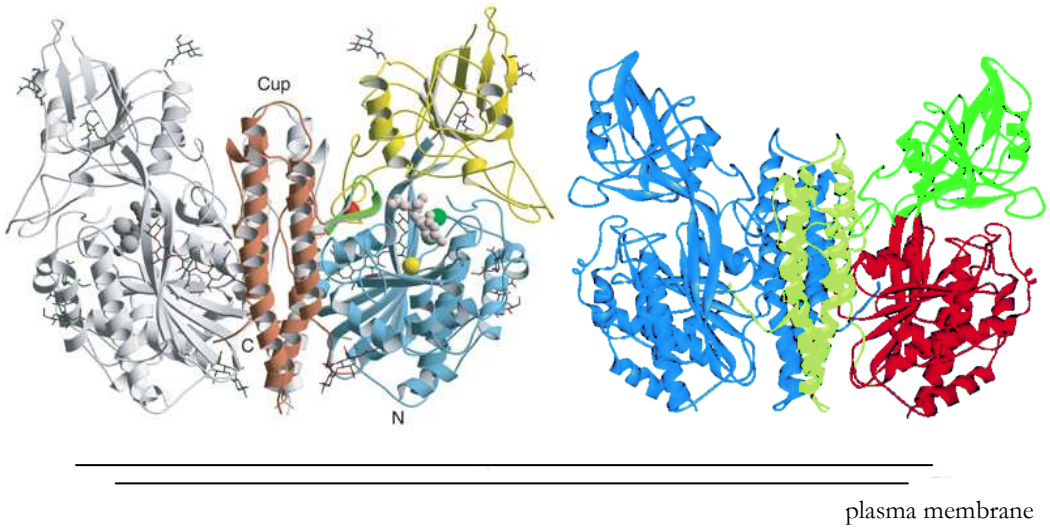

Figure 1: Structure of GCPII Extracellular Portion Homodimer in Comparison with Transferrin Receptor

The structure of GCPII (on the left) shows C-terminal subdomain in orange, protease subdomain in light blue and apical subdomain in yellow. The second monomer is drawn in grey. Green spheres represent zinc ions, the yellow one represent chloride ion and the red one calcium ion. The overall similarity to transferrin receptor (on the right) is obvious. Structures adopted from [10] and [13].

It may be interesting to mention a majority of differences between TfR and GCPII extracellular portion are structural features related to enzymatic characteristics of GCPII [10].

1.1.2.2 Glycosylation

A calculated molecular weight of GCPII polypeptide chain is about 84 kDa but the apparent GCPII molecular weight from different tissue samples differs. An electrophoretic mobility of GCPII from LNCaP cells is about 100kDa [14].

Distinct glycosidase-treated cells revealed N-linked glycosylation to amount more than 20 % of the native molecular weight [14].

The deglycosylated GCPII is completely inactive (concerning both its activities) [15], but the glycosylation is supposed to contribute to a proper folding rather than influence the interaction with substrate [16]. Non-glycosylated protein is not recognized by some antibodies against extracellular domain but can be detected by antibodies raised against intracellular domain. This may implicate some oligosaccharides are part of a recognized epitope. Further researches showed that none of the individual N-glycosylations determines proper folding but the protein-carbohydrate contact was clearly defined to be contributing to dimerisation by providing contact at dimerisation domains interfaces [10].

1.1.2.3 Internalization

GCPII undergoes an antibody-induced internalization [17]. Immunofluorescence revealed that the protein accumulates in endosomes regarding to a simultaneous uptake and cell colocalization of GCPII together with labeled transferrin (an endosomal marker). The trafficking is mediated via clathrin-coated pits (identically to TfR). GCPII was also shown to undergo internalization with half-life of about 2 hours without a presence of an antibody [17].

The antibody-induced internalization, on the other hand, is about 3-fold higher and seems to be dose-dependent until reaching its maximum. This kinetics may resemble to some receptor and its natural ligand. A constitutive internalization in LNCaP cells indicates either GCPII recycling from plasma membrane or maybe a presence of the ligand in the medium [17].

Further research revealed MXXXL motif in cytoplasmic tail of the protein to be responsible for the internalization [18]. Also significantly reduced internalization in adaptor protein-2 (AP-2) negative mutants implicates an involvement of AP-2 in these movements [18].

Experiments with prostate cancer cell line PC-3 suggested that GCPII plasmatic tail associate with filamin A *in vivo* and confocal miscroscopy showed that the interaction takes place on plasmatic membrane. The association with filamin A markedly decreases the internalization rate and also proteolytic activity, which could hint at an induced receptor function of GCPII. The association with filamin A connects GCPII to a cytoskeleton and directs the internalized protein to recycling endosomal compartment in contradistinction to

cells that do not produce filamin A and showed GCPII positive signal in cytoplasmic vesicles. [19]

If there is any receptor function of GCPII, its internalization may either control the sensitivity of the cell to a specific external stimulus or/and perform an endocytosis and trafficking of a ligand. These assumptions wait to be experimentally confirmed.

1.1.3 Physiological function

Originally, GCPII was discovered as a prostate membrane protein on LNCaP cells [1]. Later with new antibodies, GCPII expression was mapped in other human tissues [20].

GCPII is abundant in many organs in human and apparently, its physiological function differs regarding to the type of a tissue in which it is expressed. The expression is highly upregulated in malignant hyperplasia of prostate. However, no function has been attributed to GCPII in prostate. To date, GCPII has only two known physiological functions that are both related to its hydrolase activities [2-3].

1.1.3.1 Small intestine

GCPII folate hydrolase activity (or folylpoly-γ-glutamate carboxypeptidase activity) in small intestine facilitates absorption of dietary folates by cleavage of terminal glutamates from folylpoly-γ-glutamate, which can be subsequently transported across the plasma membrane [3-4].

1.1.3.2 Central nervous system

Effects of the neurotransmitter N-acetyl-aspartyl-L-glutamate (NAAG) are dependent on the NAALADase activity of GCPII that is expressed in central nervous system [2, 21].

NAAG is hydrolysed by GCPII while liberating free glutamate that is the most abundant excitatory neurotransmitter in human central nervous system [2]. Changing the equilibrium between glutamate and NAAG by inhibition of GCPII showed neuroprotective effects against neural excitotoxicity caused by increased concentrations of free glutamate in pathological states [22-23].

It was also proved that an inhibition of NAALADase activity decreased accumulation of free extracellular glutamate during middle cerebral artery occlusion in rats (a model for stroke in rat). The inhibition of GCPII has no observable effects on normal animals even though glutamergic signalization is involved in some basic neurological processes such as learning or memory [24].

1.1.3.3 Prostate

Even though GCPII is detected in a wide range of tissues [20], its presence in both normal and hyperplastic prostate is markedly higher [25]. In prostate, it was found a significant correlation between surface expression of GCPII and negative prognosis of the pathological findings [1].

In 1996 U.S. Food and Drug Administration approved Capromab Pendetide (murine antiGCPII antibody 7E11-C5.3 radiolabelled with ^{111}In) with commercial name ProstaScint® as an imaging agent for the identification of prostate metastatic cancer. Since this antibody recognizes the intracellular epitope of GCPII, the imaging is virtually restricted to already death cells [26].

1.2 Angiogenesis and Cancer

In contrast to the opinion of our ancestors, a contemporary paradigm of medicine and molecular biology resembles the very original one – that cancer is relatively generic process with many universal properties but distinct clinical manifestations. From this point of view we hope to be able to provide an insight into these problems and reveal the connections in order to raise further possibilities of development of new treatments.

A majority of human solid tumours stop growing and stay at a dormant state (**avascular phase**) in a microscopic size (millimeters). For further expansion a presence of a network of blood vessels is necessary providing the tissue with oxygen and nutrients. Vasculature also later ensures a distribution of growth factors and other regulatory molecules. About 10 % of all tumours undergo an **angiogenic switch**, which is brought by an imbalance of angiogenic regulators and subsequent change of phenotype (**vascular phase**) [27-28].

A tumour before an angiogenic switch is harmless because the rate of proliferation and apoptosis of tumour cells are comparable. After the change of phenotype the proliferation stays similar but the rate of apoptosis becomes much lower. The tumour starts to expand [27-28].

The percentage of **neoplasms** that undergo angiogenic switch and the time in that it happens are surprisingly predictable regarding to internal conditions and type of a tumour; reviewed in [28-30].

1.2.1 Terminology

Angiogenesis is handy but general expression that is not always describing the situation properly. Reader, going through the literature, meets other distinct expressions, some of which are widely spread terms with not well-established meanings. For **neovascularization** (also **vasculogenesis**, **vasculoneogenesis**) and **intussusceptions** we would like to propose following definition noting it might not be the only right one:

Neovascularization, as its prefix indicates, is a process of forming of new blood vessels when there are no pre-existing ones. We can observe post-traumatic neovascularization after e.g. **cardiac ischemia**, neovascularization during embryonic development and, importantly, neovascularization is a key process in tumour growth (reviewed by [29]).

Intussusception (also splitting angiogenesis), on the other side, is an increase of a number of capillaries by splitting and rearrangement of endothelial cells. An increase of the number of new cells in this case does not correspond to an increase of the number of new blood vessels. The local cell proliferation is lower than during neovacularization and this type of formation of blood vessels occurs mostly during embryonic development where energy resources need to be saved [31-32].

In fact the two processes have close relation one to each other and could not be sharply separated *in vivo*. Word "angiogenesis" will be further frequently used as a general term comprehending both previous meanings.

1.2.2 Angiogenesis in Normal State

Angiogenesis is a natural process occurring during embryonic development, menstrual cycle (uterine lining), wound healing and also after an experience of **tissue hypoxia** [29].

In angiogenesis blood vessels proliferate or may undergo a remodeling. A change of a phenotype and an acquisition of new structural and functional properties is a natural way of providing nutrients and oxygen into enlarging tissues. Even though the structure of blood vessels related to a particular organ differs in some features, a hierarchy of venules, capillaries and arterioles is always distinguishable [33-34].

1.2.3 Angiogenesis in Inflamed Tissues

Four main signs of inflammation are ***rubor***, ***tumour***, ***calor*** and ***dolor***. A process of vascular remodeling causes the first three clinical manifestations. Finally such changes ensure plasma leakage and leukocyte efflux [35].

The basement membrane of endothelial cells is shared with mural cells - **pericytes** in physiological architecture of capillaries and post-capillary venules. The organization stays conserved during inflammation in contrast to a tumour growth. However, endothelial cells change their phenotype, which causes remodeling of capillaries into venules within days (Figure 2, page 9). Such vessels are then leaky due to the expression of molecules promoting endothelial gap formation. Endothelial cells also proliferate and release substances inducing leukocyte rolling, attachment and migration [35]. These changes can be (antibacterial drug) [36-38] and mostly cannot be stopped by the inhibition of vascular endothelial growth factor (VEGF) signaling pathway [34]. The regulation can be therefore assumed to be dependent on other factors, expression of which may be driven with the whole complex process of inflammation.

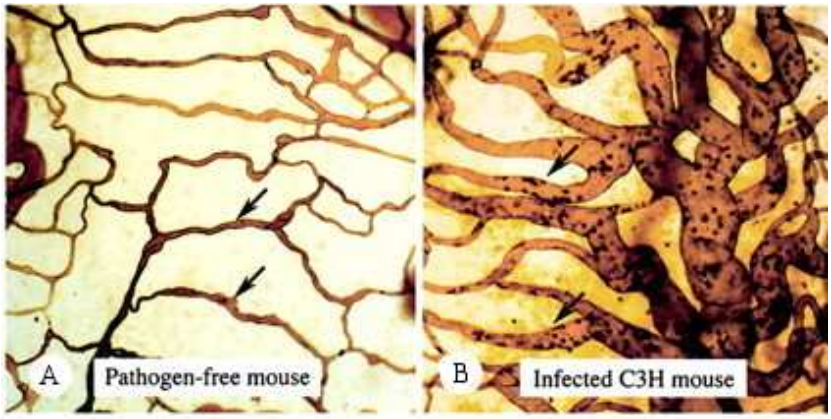

Figure 2: Vascular Remodeling in Inflamed Tissue

Comparison of the microvasculature in the tracheal mucosa of pathogen-free and *M. pulmonis*-infected mice stained with *Lycopersicon esculentum* **lectin**. (*A*) Pathogen-free rat: simple pattern of normal vasculature, with straight capillaries (*arrows*) enlacing **cartilaginous** ring. (*B*) *M. pulmonis*-infected rat at 4 weeks: abundant, curved, capillary-size angiogenic vessels (*arrows*), some of which are located in focal regions of lymphoid tissue. Figure adopted and revised from [35].

1.2.4 Angiogenesis in Tumours

1.2.4.1 Structure

Blood vessels in tumours cannot be easily described from the point of view of mammalian vascular biology. All basic elements of blood flow, such as **endothelial cells**, pericytes and **basement membrane** are malformed (Figure 3, page 10). Endothelial cells occurring in the tissue do not always form vessels and inversely, a flow of erythrocytes do not always pass through any lumen. For instance, even though antibodies against integrin do not usually interact with endothelial cells in healthy tissues, some integrins are accessible in tumours. This may indicate that such cells overexpress proteins on their luminal surface and it is presumed this may be caused by a lost of polarity which contributes to a decreased ability to form a lumen [29, 33-34].

Some vessels are not even a member of a circulation, as was shown by histochemistry [39].

In addition, because of a high number of **extravasated** erythrocytes in certain samples it is hard to differentiate between blood flow and **hemorrhage** [29].

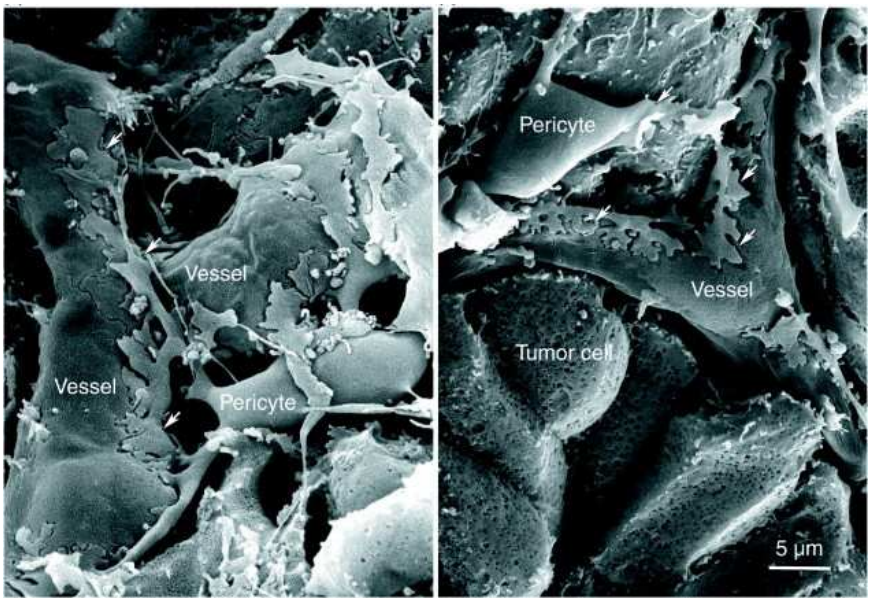

Figure 3: Abnormalities in Tumour Blood Vessels I

Scanning electron micrographs of pericytes on the surface of abnormally shaped pancreatic islet derived tumour vessels in mice (on both sides). Due to their loose association with tumour vessels, pericyte cell bodies are not located on the wall of the vessel, but some pericyte projections (arrows) contact endothelial cells. Figure adopted and revised from [34]

Tumour endothelial cells are beyond control of the cell cycle, proliferate and their association with pericytes becomes occasional or pathologicaly loose. Endothelial cells form long **filopodia** and transcellular gaps reaching almost 1 micrometer (Figure 4, page 11). It is not surprising that these defects are responsible for some cases of blood leakage. A leakiness of these "vessels" in general alters an entry of cells ensuring immunity response and enables drugs to extravasate and reach tumour tissue. On the other hand, the distribution is not uniform and the accessibility of these compounds then may be uneven. In addition, the phenomenon complicates drug delivery via blood stream due to an augmented **interstitial pressure** in some parts [34, 40-41].

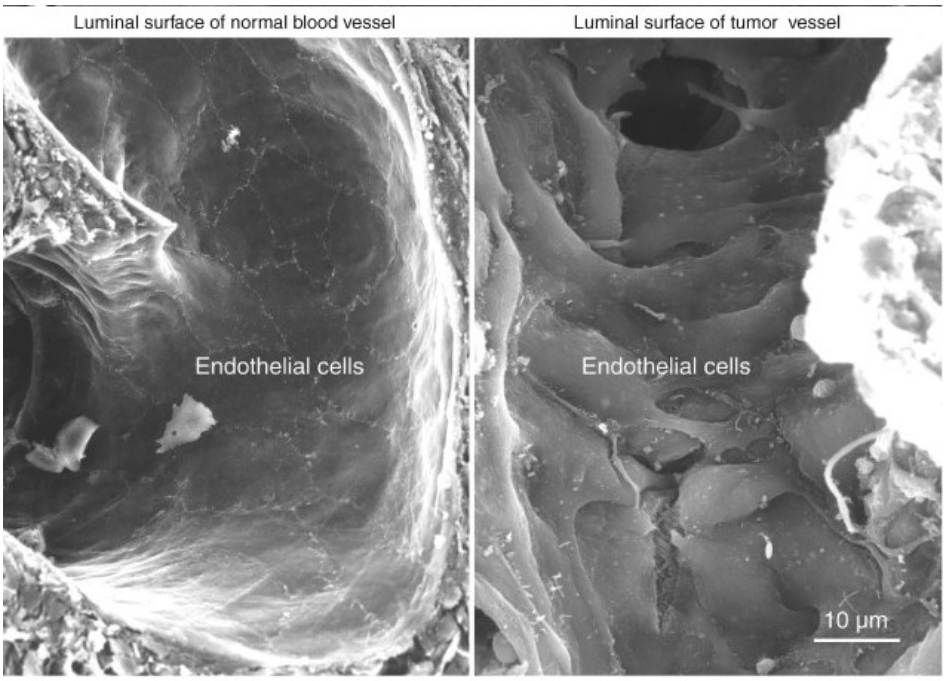

Figure 4: Abnormalities in Tumour Blood Vessels II

Scanning electron micrographs comparing the smooth, tight endothelial cell monolayer covering the luminal surface of normal blood vessel compared to disorganized endothelium with transcellular gaps of vessel in a pancreatic islet tumour. Blood was removed by vascular perfusion of fixative. Figure adopted and revised from [34]

The size of vessels, its organization and spacing differ notably in each type of tumour (e.g. diameters between micrometers and hundreds of micrometers). The only common characteristics that can be observed in almost every tumour is a presence of extravasated erythrocytes and an absence of arteriole-capillary-venule hierarchy [29].

Tumour vasculature is morphologically defective but altered blood flow together with enhanced leakiness are able to provide oxygen and nutrients for the extensively enlarging tissue. Vasculature makes part of every malignant tumour and therefore its presence determines survival of the tissue. For this reason tumour vasculature is regarded to be one of main targets of anticancer research [29].

1.2.4.2 Tumour Markers and Visualization

1.2.4.2.1 Endothelium

A gene expression in tumour endothelial cell is diverse and there is no known antigen that could selectively and universally mark tumour vasculature in humans. For a specific visualization we take advantage of another approach.

Only a functional vasculature can be visualized by lectins unambiguously, because lectins bind to a glycoprotein structures typically expressed on a luminal surface (tumour endothelium does not always form lumen) [29, 39]. However, any endothelial cell can be stained by antibodies against CD31 (also PECAM-1- platelet endothelial cell adhesion molecule) regardless of whether it forms a vessel or not. CD31 immunoreactivity is more complex and allows us also to visualize some sprouts that are not detectable by lectin binding. The differential staining enables to show defective arrangement universally and may facilitate mapping of tumour angiogenesis (Figure 5). In the histochemical point of view, tumour vasculature in general behaves the same as vessels undergoing remodeling and angiogenesis in normal conditions. These observations were in accord with those from scanning electron microscopy [33].

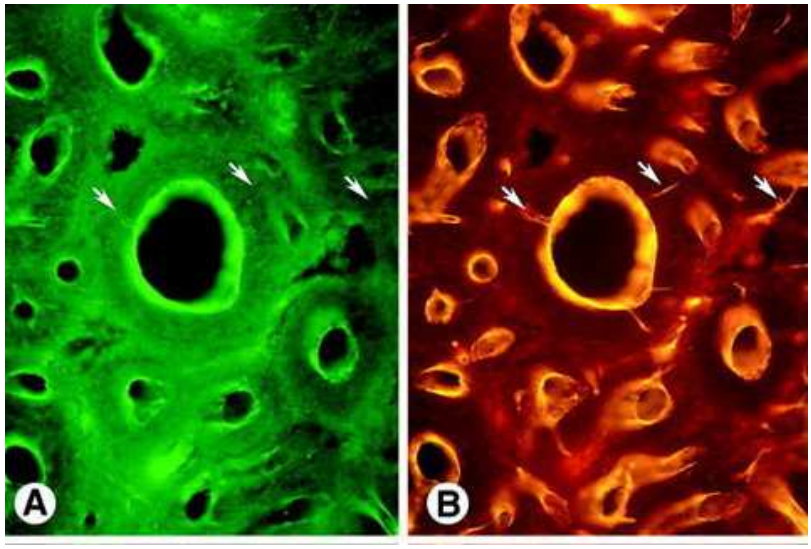

Figure 5: Differential Staining of Blood Vessels Facilitates Mapping of Abnormalities

Blood vessels in tumours are marked by green lectin staining (on the left) or CD31 immunoreactivity (on the right). Like the lectin, CD31 immunoreactivity defines the luminal surface of tumour vessels, but, unlike the lectin, it is also present on sprouts (arrows) radiating from the vessel lining into the tumour. Figure adopted and revised from [39].

In 2000, 46 proteins were discovered comparing gene expression of endothelial cells derived from blood vessels in normal and malignant colorectal tissues. Their expression was 10-fold higher in tumour-associated vasculature compared to normal-derived endothelium, therefore they were called tumour endothelial markers (TEMs) [42]. Four out of nine sequenced and characterized TEMs had residues localized on cell surface and so seemed to be able to be used as antigens *in vivo*. An expression of these proteins was localized and compared to their mouse orthologs. Moreover, these antigens were shown to be also present in developing rodent embryo and therefore the expression was not limited only to tumour angiogenesis [43].

TEMs were present on normal adult tissues as well and their abundance was different over various organ samples. The specificity was not satisfactory. The observations showed three out of four TEMs to be present in both human and mouse tumours [43]. Such findings may indicate rather provocative conception of certain universal properties of a malignant tissue growth in different organisms.

1.2.4.2.2 Pericytes

Pericytes are relatively undifferentiated connective tissue cells that occur about capillaries or other small blood vessels outside the basement membrane.

Nor pericytes express any specific protein which could be used for their identification. Platelet-derived growth factor receptor-β (PDGFR- β), α-smooth muscle actin (α-SMA), desmin and chondroitin sulfate proteoglycan NG2 (NG2, CSPG4, HMW-MAA) are possible antigens that can be used for the specific staining of pericytes because they seem to usually express one or more of them [44-45].

Especially an expression of α-SMA in pericytes is not a phenomenon usual in physiological conditions. For example, pericytes of normal pancreatic islet capillaries express desmin, but no presence of α-SMA can be detected. On the other hand, pericytes in tumour developed from these islets express both these proteins. Even though this case cannot be taken as a rule, pericytes in many tumours show an unusual expression of α-SMA [44].

1.2.4.2.3 Basement membrane

Basement membrane (also lamina) is a thin layer of connective tissue that underlies epithelium.

Simultaneous marking of type IV collagen and CD31 showed that despite numerous abnormalities, basement membrane signal match to pericytes and endothelial cells surface of more than 98 %. These findings together with the fact that type IV collagen is the most specific protein expressed in tumour vascular basement membrane makes type IV collagen a promising target for visualization of tumour vasculature. Unfortunately, these results are not always in agreement with electron-microscope observations [33].

1.2.4.3 Treatment of Pathological Angiogenesis

1.2.4.3.1 Antiangiogenic Therapy

Targeting the cells of vasculature solves some pharmacokinetic problems related to usual chemotherapy (but also brings others, see chapter Structure). It is much more effective to deliver drug to endothelial cells only because the **bioavailability** in the vessel approaches its theoretical maximum and we are not obliged to control the exchange between blood and tissue [29].

There are many tumour cells completely dependent on one endothelial cell. Evidently killing one of them is more effectual than gradual eradicating of tumour mass during chemotherapy [29].

Since cells of blood vessel are genetically stable, antiangiogenic therapy does not cause any selection and thus no development of individual resistance [46].

Finally, this approach has great potential in developing more universal treatment of cancer in general. However, it should be mentioned that tumour vasculature introduces immune cells and distributes chemotherapeutics. Consequently, it need to be considered whether antiangiogenic therapy could alter any resistance of tumour tissue to a natural immune response or drug therapy and therefore prolong its viability; reviewed in [28-29]).

Majority of preclinical models and clinical studies demonstrated that suppression of angiogenesis represents a beneficial part of anticancer treatment. Apparently, this strategy has many advantages. Antiangiogenic therapy or its combination with chemotherapy is now

besides radiation therapy, surgery and chemotherapy one of four common cancer treatments [43].

A balance between positive and negative angiogenic regulators and inhibition of cleavage of extracellular matrix are three main targets of antiangiogenic research. Some drugs are aimed at endothelial cells, others block one or more steps in the angiogenesis signaling pathway or inhibit proteases (for review see [29]).

1.2.4.3.2 Drug Kinetics

Majority of chemotherapeutics hit cells in mitosis, which means that the more often a cell divides the more probably it will be affected. A proliferation of tumour-associated endothelial cells can be only poorly impaired with usual episodic dosing (chemotherapy cycle) because their cell cycle has much lower rapidity in comparison to tumour cells [46]. In addition, typical rest periods (after each chemotherapy cycle) allow cells to recover after the treatment. An administration of **low doses regiment** or **metronomic therapy** rather than **maximal tolerated dosing** is then preferable because of the efficacy and side effects above all [47].

Another phenomenon should be taken into account in relation with vasculogenesis in tumours. Similarly to many types of chemotherapy, antiangiogenic treatment also seems to exhibit **biphasic response** (especially drugs related to VEGF receptor - VEGFR) [48]. Numerous inhibitors of angiogenesis have U-shaped **dose response curve**, which means that its low doses may promote tumour growth (negative hormetic effect).

These two facts play crucial role in determining therapeutic dose but first and foremost make us realize that any antiangiogenic research should be considered from more than just a **dynamic** point of view.

1.2.4.3.3 Vascular endothelial growth factor

Most important proangiogenic regulators are basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), transforming growth factor-β (TGF- β), placental growth factor (PGF) and vascular endothelial growth factor (VEGF) (reviewed in [29]).

Every rapid tissue enlargement such as tumour growth leads to an oxygen deprivation - tissue hypoxia that alters expression of many genes. One of them – VEGF is a mitogen that by stimulation of permeability of blood vessels facilitates consequently distribution of other growth factors. These regulatory molecules have, among others, undisputable effect to a recontribution to an up-regulation of VEGF expression. [29].

VEGF expression is upregulated during tissue hypoxia and is involved in normal and also pathologic angiogenesis. It triggers a wide range of intracellular signals. Several receptors and their splice variants are known. Vascular endothelial cell-associated VEGF receptor is a homo or hetero-dimer with tyrosine-kinase activity. Its activation is linked to diverse signaling pathways (including phospholipase C and **RAS/RAF/MEK/ERK** pathway), which positively regulate cell survival, proliferation, and motility. VEGF regulates angiogenesis on distinct levels (e.g. migration and mitosis of endothelial cells) and changes overall state of the blood vessel (chemotaxis of white blood cells, vasodilatans) [29, 49].

VEGF and VEGF-related signaling pathway is still the most popular target of antiangiogenic research. Three out of seven antiangiogenic drugs approved by US FDA are indirect inhibitors of angiogenesis targeting VEGF receptor (VEGFR). These agents prevent VEGF to bind to its receptor (bevacizumab), inhibit Raf and VEGFR kinase (Sorafenib) or inhibit multiple tyrosine kinases related to VEGFR (sunitinib) [50].

1.2.4.3.4 VEGF Inhibitors in Tumour Tissue

Two types of inhibitors were tested to determine an effect of VEGF on tumour vasculature. First was Axitinib a small molecule inhibiting VEGF receptors and related tyrosine kinase receptors and the second one called VEGF-trap, a construct of the extracellular domain of VEGFR-1 and VEGFR-2 that specifically entraps ligands [51-53].

Treated Lewis lung carcinoma and pancreatic islet-derived tumour (RIP-Tag2 tumours) showed a suppression of vascular sprouting, endothelial fenestrations disappeared, some vessels lost the **patency** (an opened or unobstructed state of a passage) and the whole tumour vasculature decreased. These effects were evident after 1 day of treatment. A vascular regression in treated RIP-Tag2 tumours after 7 days was 70% and a continuing inhibition decreased their volume. [51-52].

A 50% reversion of vasculature in Lewis lung carcinoma was reached within 4 days after withdrawal. Vessels become functional almost at the same time. It should be said the original density of vascularization is not exceeded even after longer periods of regrowth [51-52]. This fact may hint a presence of some internal rules or predicted architecture of a tumour. A presence of empty sleeves of basement membrane after anti-VEGF therapy indicates that, as in normal tissue, residues of basement membrane serves as a scaffold for revascularization. These relics of basement membrane in tumours were referred to be an important target of anticancer research [52].

The treatment via VEGF signaling pathway inhibition was reproducible on the very same sample [51].

1.2.4.3.5 VEGF Inhibition in Physiological Conditions

In spite of the essential role of VEGF is played during embryonic development; the inhibition of its signaling pathway even in an adult organism triggers an avalanche of reactions; reviewed in [29]. All of them should be taken into account as side effects of the inhibition treatment.

The changes are dose-dependent and apparently differ in each organ. Major manifestations include reduction in **endothelial fenestrations**, regression of capillaries and reduction in endothelial cell expression of VEGFR-2 and VEGFR-3. It may be interesting to mention that in the inhibition experiments little or no regression of capillaries was observed in brain, retina, skeletal muscle, cardiac muscle and lung [52].

After the inhibition, the vessel loses its patency due to an accumulation of fibrin and endothelial cells then undergoes apoptosis in about 2 days. A regression of these cells does not actually cause a hemorrhage thanks to the continuously decreasing patency, which precedes the cell death [51].

Even though cells related to vasculature are reduced, sleeves of basement membrane persist providing a scaffold for revascularization which rapidity differs in each organ (days to weeks) [51].

It was also show that blood vessels with numerous fenestrations and augmented expression of VEGFR-2 are especially sensitive to inhibitors of VEGF signaling [51].

1.2.4.3.6 Other Drug Targets

Matrix metalloproteases (MMPs) create space for newly formed blood vessels by a cleavage of extracellular matrix. Even though some researches showed a suppression of MMPs using inhibitors of metalloproteases to be a promising step in modifying tumour angiogenesis, the active substances usually failed at distinct phases of clinical trials for several reasons(including no improvement of survival or even a toxicity) [54-55]. More recently, it was observed that MMPs also cleave "protease-activated receptor", which consequently releases ligand activating G-protein in tumour cells. This initiates a cascade that finally enhances a tumour invasiveness [56].

An interesting fact may be a come-back of accursed Thalidomid, which is now indicated to patients with diagnose of multiple myeloma. Thalidomide is used since 2006 in combination with dexamethasone. However, the mechanism of action is not clear. Thalidomide was shown to inhibit a migration of endothelial cells in solid tumours. Such effects not seem to be in relation with its immunomodulatory activities. This drug is still a target of pharmaceutical research [57].

1.2.7 GCPII in tumour-associated vasculature

In 1999 Chang *et al.* showed quite surprisingly an expression of GCPII in tumour-associated neovasculature using five anti-GCPII antibodies that specifically recognized a malignant tissue vasculature. The GCPII localization on endothelium was confirmed with parallel CD34 (antigen of hematopoetic cells) immunohistochemistry [58]. Together with a fact that external domain-binding anti-GCPII antibodies showed markedly higher intensity, these experiments suggest the endothelial cells of tumour-associated neovasculature specifically express GCPII on their membrane. The immunohistochemistry also showed no GCPII positivity in benign tissues and **neoplastic** cells of vascular tumours. These facts raises a potential in specific mapping of tumour-associated vasculature and possible treatment of malignancies [58].GCPII was also shown not to be present in normal vasculature, therefore it is assumed its role is just related with the process of angiogenesis [59].

In 2011 it was shown by Liu *et al.* that tube formation was induced in human umbilical vein endothelial cells (HUVEC cells) cultured in tumour cells conditioned medium (highly metastatic breast cancer cells). At the same time the expression of GCPII was considerably increased and that such conditions even supported viability of HUVEC cells without substrate (Matrigel) in comparison with cells in VEGF-containing medium. The key factor secreted by breast cancer cell line and inducing GCPII expression in HUVEC cells is unknown to these days [60].

An effective and stylish therapeutic tactic was conducted successfully on rat model in 2002 by Liu *et al.* A soluble extracellular domain of **tissue factor** was coupled to a GCPII peptide active site inhibitor and was administered intravenously to rats. The treatment caused a microthrombosis and finally an infarction of tumours in tumour-bearing rats. To enhance a potential in reducing tumour mass, three infusions of STVT and liposomal doxorubicin were applied. There was no adverse effect observed on rats while their survival was extended in comparison with control group (only doxorubicin applied). An eradication of a majority of tumour mass showed that doxorubicin potentiated previous effects [61].

The **infarctive** therapy of tumours with low or no related adverse effects showed a great selectivity and potential of this approach. However, the GCPII expression pattern markedly differs in human and rat/mouse model [20]. GCPII expression was observed in numerous non-tumour tissues in human [20] and so the range of adverse effects may differ from mouse/rat model. These observations need to be considered before an introduction of related research on humans.

1.2.8 Does GCPII regulate angiogenesis?

Conway *et al.* published in 2006 that GCPII-null primary pulmonary endothelial cells had largely decreased abilities to penetrate Matrigel (imitating extracellular matrix) in comparison with wild-type cells. Also an invasiveness of HUVEC cells was limited by GCPII inhibitors or neutralizing antibody (GCPII antagonists) in dose-dependent manner [62].

GCPII was suggested to be involved in cell motility through specific integrin interaction. It was shown that cell ability to adhere to laminin was reduced in presence of GCPII inhibitors and antibodies. The results were confirmed by mRNA silencing [62].

As a response to integrin engagement, focal adhesion kinase (FAK), located in multiprotein structures linking extracellular matrix to cytoskeleton, is phosporylated [63]. Levels of FAK in cells treated with GCPII antagonists were significantly diminished. These cells showed morphological aberrations such as membrane **protrusions** and general loss of structural integrity [62].

The viability and fertility of GCPII knock-outs could implicate that GCPII is not essential for normal development [62]. On the other hand, other team presented completely different results concerning the knock-outs viability [64].

1.3 Dermcidin

Dermcidin is a constitutively expressed protein secreted in human sweat.

The gene of dermcidin (DCD, alternatively preproteolysin) is divided into five exons and is expressed as single transcript [65]. The whole protein consists of 110 amino acids and is constitutively expressed in human skin ecrine sweat glands while its expression was not detected in any of other analyzed tissues [65].

Dermcidin is proteolytically processed into two parts (Figure 6, page 21). C-terminal part is 47 amino acid long peptide later denominated as DCD-1, which was shown to be present in human sweat in concentration 1-10 μg/ml. DCD-1 has interesting antimicrobial and antifungicidal potential. Minimal inhibition concentration was set as 1 μg/ml for *E.coli*, *S.aureus*, *E. faecalis* and 10 μg/ml for *C.albicans*. This peptide has, unlike other antimicrobial proteins, negative net charge. The dynamics of its action is not clear [65].

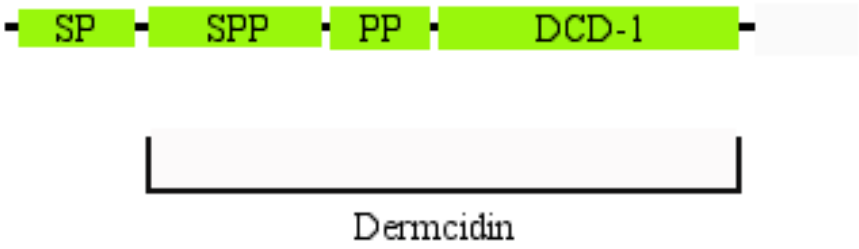

Figure 6: Dermcidin Sequence and Molecule Processing

Dermcidin is cleaved into 2 chains: survival-promoting peptide (SPP) and DCD-1 with antimicrobial activity. The full 110 amino acid sequence consists of signal peptide (SP), survival-promoting peptide, propeptide part (PP) and DCD-1.Created using data from UniProt database P81605.

It was also shown that there are some other proteins derived from dermcidin in human sweat and that a lack of dermcidin or its decreased concentration are related with clinical complication with infections [66-67].

N-terminal part of dermcidin, 30 amino acid long peptide, is known as survival-promoting peptide, which also demonstrated phosphatase activity in presence of Mg^{2+} ions. The peptide significantly prolonged viability of neural cells facing oxidative stress. The mechanism is unknown. However, some researches suppose the survival-promoting peptide participates in a protection from glutamate toxicity (reviewed in [68]).

2 Objectives

- Clone tagged dermcidin in vector for expression in mammalian cells
- Express recombinant tagged dermcidin in mammalian cells
- Introduce new detection system based on a detection tag
- Investigate a possible interaction of dermcidin with recombinant glutamate carboxypeptidase II
- Further characterize the possible interaction

3 Materials and Methods

3.1 Chemicals

Biotium (Hayward, USA)

GelRed

Biotika (Prague,CZE)

ampicillin

Clontech (Mountain View, USA)

pTRE-Tight plasmid

Duchefa Biochemie (Haarlem, Netherlands)

3-(N-morpholino)propanesulfonic acid (MOPS)

Fluka (Buchs, Switzerland)

Tetramethylethylenediamine; N,N'-Methylenebisacrylamide

GE Healthcare (Little Chalfont, UK)

Protein G Sepharose 4 Fast Flow

Gibco (Carlsbad, USA)

10% fetal bovine serum (FBS); opti-MEM medium; L-glutamine; IMDM medium

Koh-i-noor Hardtmuth (České Budějovice, CZE)

96-well transparent microplate

Lach-Ner (Neratovice, CZE)

hydrochloric acid; sodium acetate; sodium carbonate; sodium hydroxide; sodium thiosulfate; sodium chloride; silver nitrate

New England BioLabs (Ipswich, USA)

restriction endonucleases ; T4 DNA-ligase

Penta (Prague,CZE)

methanol; acetic acid; acetone; formaldehyde; isopropylalcohol; ethanol; acetic acid; glycerol

Pierce (Rockford, USA)

D-biotin; SuperSignal West Dura Chemoluminiscence substrate; goat anti-mouse-IgG antibody conjugated with horseradish peroxidase (0,8 mg/ml)

Qiagen (Valencia, USA)

QIAprep Spin Miniprep Kit; QIAquick Gel Extraction Kit; 50 bp DNA marker; 500 bp DNA marker

ROCHE s.r.o. (Prague,CZE)

Streptavidin Mutein Matrix

Serva (Heidelberg, Germany)

Coomassie Brilliant Blue G-250; bromphenol blue; Triton X100; bovine serum albumine

Sigma-Aldrich (St. Louis, USA)

LB Broth; LB Agar; 2-mercaptoethanol; glycerol; EDTA; glycine; Tris (tris(hydroxymethyl)aminomethane); acrylamide; sodium dodecylsulphate; sacharose; Tween-20; Monoclonal Anti-FLAG M2-Peroxidase (HRP)

Thermo Scientific (Massachusetts, USA)

Casein blocker

3.2 Instrumentation

- pH-meter: 9450 pH meter, Unicam (USA)
- spectrophotometer: NanoDrop 1000, Thermo Scientific (USA)
- spectrophotometric reader: GENios, Tecan (Switzerland)
- autoclave: MLS-3020U Sanyo Labo Autoclave, Sanyo (Japan)
- transilluminator: Electronic Dualwave Transilluminator, Ultra-Lūm (USA)
- balance: HL-400, A&D Engineering, Inc. (USA)
  EK-400H, A&D Engineering, Inc. (USA)
- bath: Grant Instruments Ltd.(UK)
  Thermomix BUB.Braun (Germany)
- orbital incubator: Sanyo-Gallenkamp Orbital Incubator (UK)
- agarose electrophoresis: power supply: MP-500P, Major Science (Taiwan)
  apparatus: B1A Owl Separation Systems, Inc. (USA)
- vertical polyacrylamide electrophoresis: Sigma (USA)
- blotting machine: power supply: PowerPac HC, Bio-Rad (USA)
  apparatus: Trans-Blot SD, Bio-Rad (USA)
- incubators: TCH 100, Laboratorní přístroje Praha (CZE)
  MCO-17AI CO_2 Incubator, Sanyo (Japan)
- centrifuges: Biofuge Pico, Heraeus Instruments (Germany)
  Multifuge 3 S-R, Heraeus Instruments (Germany)
  Megafuge 2,0R, Heraeus Instruments (Germany)
  Centifuge 5415R, Eppendorf (Germany)
- microscopes: fluorescence microscope Olympus IX81, Tokio (Japan)
  optical microscope Nikon TMS (Japonsko)
- camera: SP-5000UZ, Olympus (Japan)
- CCD Camera: LAS-3000 CCD Camera, Fujifilm (Japan)
- laminar box: BSB4A Laminar Flow Box, Gelaire (Australia)
- sonicator: Soniprep 150, Sanyo (Japan)

3.3 Methods

3.3.1 DNA Digestion

The DNA digestion reactions were performed according to the manufacturer's (New England Biolabs) protocol in a volume of 30 μl.

Duration and a temperature of the DNA digestion were set according to the requirements of each reaction.

3.3.2 Horizontal Agarose Electrophoresis

Solutions:

Tris-acetate buffer: 40 mM Tris-HCl; 20 mM CH_3COOH; 1 mM EDTA; pH 8,0

Sample buffer: 40% sucrose; 0,1% bromphenol blue; 0,02% NaN_3

DNA was analyzed using horizontal gel electrophoresis in 1% agarose dissolved in Tris-acetate buffer containing GelRed fluorescent reagent in dilution 1:100 000.

DNA fragments were visualized with UV transiluminator Electronic Dualwave and the pictures were taken with SP-5000UZ camera with filter designed for ethidium bromide (Olympus).

For subsequent use the DNA fragments were isolated from the agarose gel and extracted using **QIAquick Gel Extraction Kit** according to the manufacturer's protocol.

3.3.3 DNA Ligation

The ligation reaction was performed according to the manufacturer's protocol at 16 °C, for 2-16 hours. The total volume of the ligation reaction was 10 μl, the ratio of plasmid: insert was set up approximately 1:7 and ligation was performed with 1 μl (400 units) of T4-DNA ligase (New England BioLabs, USA).

3.3.4 Transformation of Competent *E. Coli* DH5α cells

10 μl of ligation reaction mixture was mixed with 40 μl of competent *E. Coli* DH5α cells and incubated 25 min on ice. Afterwards, cells were incubated for 90 seconds at 42 °C and immediately cooled on ice for 30 sec. Samples were mixed with 300 μl of LB medium and cultivated at 37 °C. After one hour 200 μl of cell suspension was spread out onto LB-agar (Sigma) plates containing ampicilin (100 μg/ml, Biotika). Cells were incubated at 37°C overnight.

3.3.5 Minipreparation of Plasmid DNA

12 ml of LB medium (Sigma) containing ampicillin (100 μg/ml, Biotika) was inoculated with a colony from an LB-agar plate and incubated at 37 °C overnight, shaking 230 rpm. The bacterial suspension was centrifuged 3360x g for 10 min, at 4 °C. Supernatant was discarded and the plasmid DNA from the pellet was isolated using QIAprep Spin Miniprep Kit according to the recommendation of manufacturer.

3.3.6 Transient Transfection of Mammalian Cells

Mammalian cells HEK293offA2 derived from HEK293 cell line (established in our laboratory) were used for transient transfection using polyethylenimine as a transfection reagent. Cell line HEK293offA2 enables regulated expression of proteins in dependence on presence or absence of doxycycline.

Cells were cultivated in 60 mm dish in IMDM medium (Gibco) and reached 60% confluence on the day of transfection. 7 μg of DNA was diluted into reduced serum medium Opti-MEM (Gibco) to final volume 350 μl. 21 μl of transfection agent polyethylenimine was added to the mixture and the tube was incubated 20 min at 20 °C. Subsequently, this solution was add by drops to cells, gently agitated and cells were incubated 24 hours at 37°C in 5% CO_2. After 48 hours cells were harvested and washed twice by 1ml of PBS and immediately either lysated or stored until further use at -20 °C.

3.3.7 Cell Lysis

The harvested cells were resuspended in cold TBS and sonicated three times for 5 seconds on ice, using Soniprep 150 Sonicator (5 μm amplitude). Mixtures were let on ice for 30 min, and then centrifuged 16100 x g for 10 min. Supernatant was aspired and stored at -20 °C.

3.3.8 Bradford Protein Assay

1 μl of the cell lysate was mixed with 200 μl of dye reagent solution. Following one-minute incubation at room temperature, the absorbance was measured at 595 nm. Protein concentration was determined from a calibration curve constructed using bovine serum albumin as a standard.

3.3.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Solutions:

Stacking gel (5% acrylamide):	250 mM Tris-HCl (pH 6,8); 5,5% acrylamide, 0,15% N,N'-methylene-bisacrylamide; 0,1% sodium dodecylsulfate (SDS); 0,005% tetramethylethylendiamine; 0,1% ammonium persulfate
Separating gel (11% acrylamide):	313 mM Tris-HCl (pH 8,8); 9,25% acrylamide, 0,25% N,N'-methylene-bisacrylamide; 0,1% sodium dodecylsulfate (SDS); 0,001% tetramethylethylendiamine; 0,1% ammonium persulfate
Separating gel (18% acrylamide):	313 mM Tris-HCl (pH 8,8); 14,8% acrylamide, 0,7% N,N'-methylene-bisacrylamide; 0,1% sodium dodecylsulfate (SDS); 0,001%

	tetramethylethylendiamine; 0,1% ammonium persulfate
Running buffer (5x):	125 mM Tris-HCl; 1,25 M glycine; 0,5% sodium dodecylsulfate (SDS); pH 8,8
Sample buffer (6x):	50 mM Tris-HCl (pH 6,8); 30% glycerol; 10% sodium dodecylsulfate (SDS); 6% 2-mercaptoethanol; 0,012% bromphenol blue

Before the loading onto gel, the sample were mixed with the sample buffer and boiled for 3-4 min. Gels were prepared, let to polymerize and then placed into a vertical electrophoresis apparatus. The upper and lower reservoirs of the apparatus were filled with the running buffer. Electrophoresis was run at 145 V until the bromphenol blue dye reached the bottom of the running gel. Proteins in the gel were then either visualized by silver staining or subjected to Western blotting.

3.3.10 Silver Staining of SDS-PAG

Solutions:

Solution 1: 12% acetic acid; 50% methanol; 0,02% formaldehyde

Solution 2: 50% methanol

Solution 3: 0,02% $Na_2S_2O_3 \cdot 5H_2O$

Solution 4: 0,2% $AgNO_3$; 0,02% formaldehyde

Solution 5: 566 mM Na_2CO_3, 16 µM $Na_2S_2O_3 \cdot 5H_2O$; 0,02% formaldehyde

Solution 6: 12% acetic acid, 50% methanol

Proteins resolved by SDS-PAGE were visualized by silver staining. The gel was incubated for at least 20 min in Solution 1 and then washed three times in Solution 2 for 5 min. Subsequently, the gel was incubated in Solution 3 for 1 min, washed with water three

times and incubated in Solution 4 for 20 min. The gel was then rinsed with water three times and the silver staining was developed by addition of Solution 5. When the protein bands were clearly visible, the gel was washed with water three times and the developing process was stopped by addition of Solution 6. The gel was then stored in Solution 2.

3.3.11 Western Blotting

Solutions:

Transfer buffer: 92 mM glycine; 25 mM Tris-HCl; 10% methanol; 0,1% sodium dodecylsulfate (SDS); (pH not adjusted)

PBS: 137 mM NaCl; 2,7 mM KCl; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4; pH 7,4

After electrophoretic separation of proteins, the gel and a nitrocellulose membrane were equilibrated in the blotting buffer for 5 min. Proteins were electroblotted at 12 V for 12 min (in case of smaller proteins 15V and only 10 min). The membrane was then incubated with 5 ml of Casein Blocker (Thermo Sc.) for 1 hour at 4°C to block nonspecific sites. Subsequently, an appropriate antibody was added (dilution depended on a type of an antibody; for commercial antibodies recommended dilution was used) and membrane was incubated overnight at 4°C. Then, the membrane was washed three times with PBS + 0,05% Tween-20 for 5 minutes. If needed, a secondary goat anti-mouse antibody conjugated with horse-radish peroxidase was added and incubated for 1 hour at 4°C (concentration 320 ng/ml). Membrane was washed again three times with PBS + 0,05% Tween-20 to remove free secondary antibody and 0,4 ml of the Luminol/Enhancer Solution (SuperSignal West Dura Chemiluminiscence Substrate, Pierce) and the blot was incubated with the mixture for 5 min while gentle agitated. The membrane was then dried between two sheets of filter paper and placed in trasparent plastic foil. The chemiluminiscent signal was detected using CCD camera (LAS-1000, Fujifilm, Tokyo, Japan).

3.3.12 GCPII Pull-down Using Streptavidin Mutein Matrix

Solutions:

Washing buffer:	100 mM Tris-HCl (pH 7.2)
	150 mM NaCl
Elution buffer :	100 mM Tris-HCl (pH 7.2)
	150 mM NaCl
	2 mM D-biotin
Resin:	Streptavidin Mutein Matrix (Roche)

Resin (50 μl per sample) was washed three times with 3 ml of cold washing buffer.

Equilibrated resin (50 μl per sample) was incubated with 3 μg (i.e. 25 μl) per sample of purified biotinylated extracellular portion of GCPII (prepared by Jan Tykvart in laboratory of Doc. Jan Konvalinka) for 5 hours at 4°C. As negative control, equilibrated resin was incubated with 100 μl of washing buffer containing no GCPII.

Both tubes were centrifuged at 540 x g at 4°C and the supernatant was discarded. After that resin with immobilized GCPII was mixed with 50 μl of cell lysate containing tested protein (total protein concentration about 2 μg/μl). The same procedure was performed for negative control and mixtures were incubated overnight at 4°C or 37°C.

Next day samples were centrifuged (2000 x g; 4°C) and the supernatant (flow-through fraction) was aspired. After that resin was washed three times (200, 200, 100 μl) with washing buffer.

Finally, proteins were eluted by elution buffer at 4 °C. Elution fraction 1 (100 μl) incubated 1 hour, elution fraction 2 (200 μl) incubated half an hour.

All fractions were analyzed on either silver-stained SDS-PAGE gels or western blots.

4 Results

Preliminary experiments based on affinity pull-down experiment in our laboratory revealed several proteins potentially interacting with GCPII. We chose one of them- human dermcidin and decided to clone it with FLAG-tag on its terminus. As dermcidin consists of only 110 amino acids we cloned also a fusion protein of dermcidin and enhanced green fluorescent protein (EGFP). This approach was supposed to have three advantages: it might be easier to work with a larger protein, we were able to verify the expression *in vivo* and we could use EGFP fluorescence for characterization of the possible interaction.

4.1 Dermcidin Cloning

The dermcidin DNA coding sequence was synthetised by RNDr. Marek Ingr, Ph.D. at Faculty of Sciences, Charles University. The plasmids pTRE-dermcidin and pTRE-dermcidin-EGFP encoding dermcidin and dermcidin-EGFP fusion protein were already prepared in our laboratory by Tomáš Knedlík.

The DNA sequences encoding dermcidin and dermcidin-EGFP were cleaved off by restriction endonucleases *KpnI* and *BsmI* and consequently inserted into pTRE plasmid with FLAG-tag (prepared by Pavel Šácha) using T4 ligase. The sequences of resulting plasmids pTRE-FLAG-dermcidin-EGFP and pTRE-FLAG-dermcidin were verified by a control digestion with restriction endonucleases *KpnI* and *BsmI* (Figure 7, page 33).

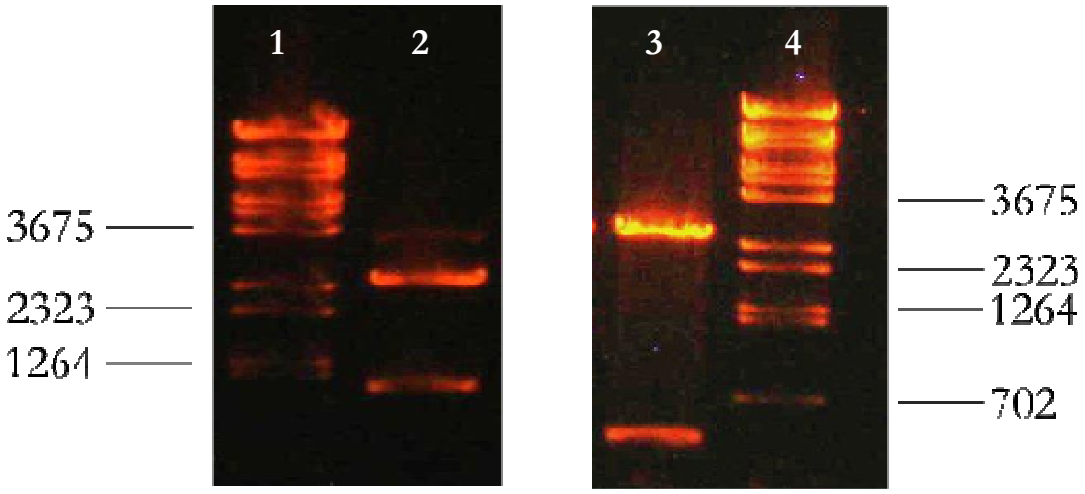

Figure 7: Control Digestion of pTRE-FLAG-dermcidin and pTRE-FLAG-dermcidin-EGFP plasmids

Plasmid pTRE-FLAG-dermcidin-EGFP (lane 2) digested with *KpnI* and Bsm*I* was supposed to provide two fragments of 2472 and 1183 bp. Plasmid pTRE-FLAG-dermcidin (lane 3) digested with *KpnI* and Bsm*I* was supposed to provide also two fragments of 2472 and 497 bp. Constructs were analyzed by 1% agarose electrophoresis. The apparent sizes of the fragments were according to the expectation. Molecular markers are in lanes 1 and 4.

Plasmids pTRE-FLAG-dermcidin and pTRE-FLAG-dermcidin-EGFP are reviewed in Figure 8, page 34.

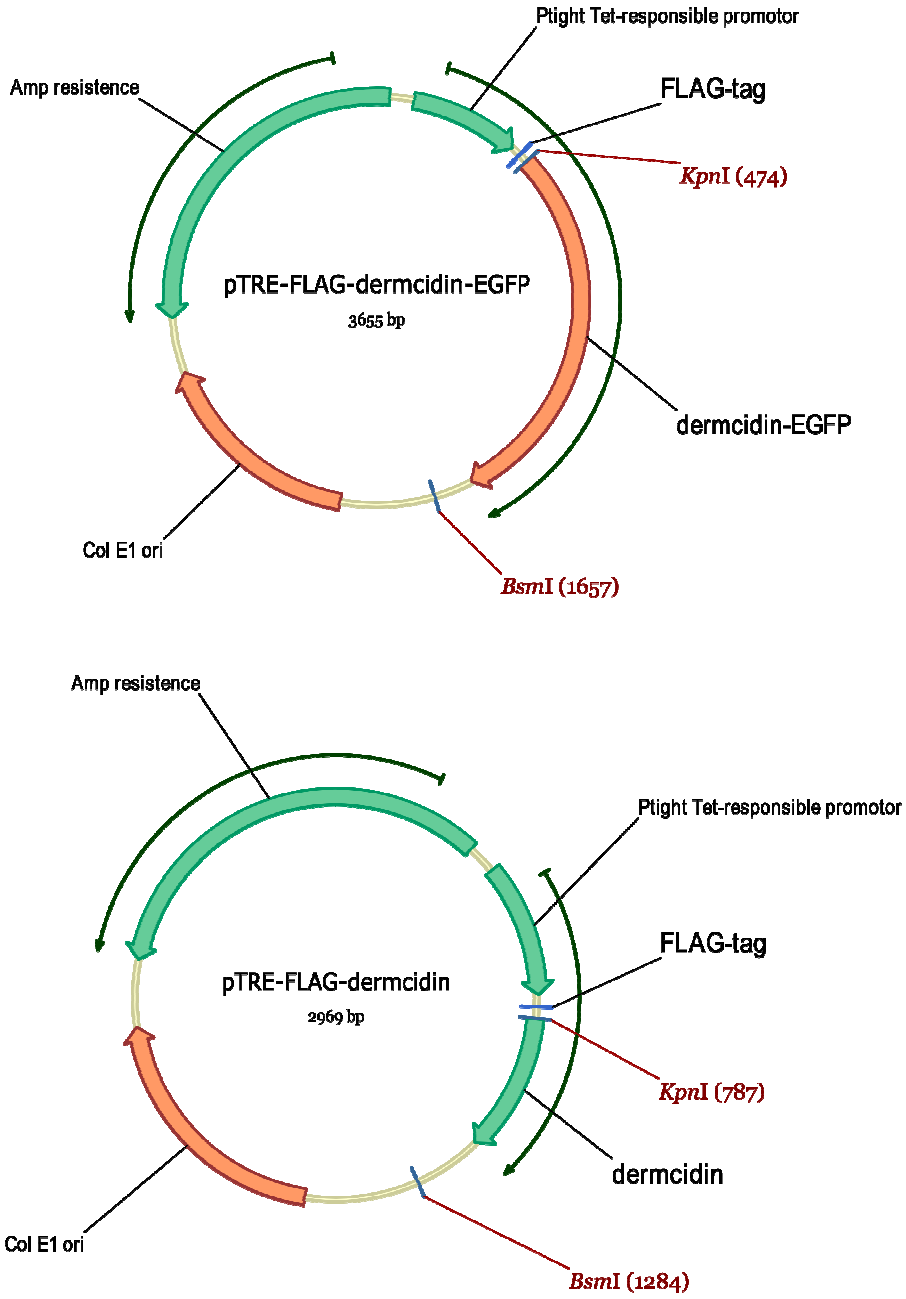

Figure 8: The Structure of Plasmids Encoding FLAG-dermcidin-EGFP and FLAG-dermcidin

Tagged proteins were expressed under Ptight Tet-responsible promotor in plasmids possessing ampicillin resistance. External green arrows show two longest open-reading frames. Col E1 ori marks the origin of replication of the plasmid in *E.coli*. The structures were created in Vector NTI® by Invitrogen.

In Summary, using plasmids pTRE-FLAG-dermcidin, pTRE-FLAG-dermcidin-EGFP and pTRE-FLAG-GCPII we prepared these recombinant proteins: dermcidin and its fusion with either FLAG peptide alone (FLAG-dermcidin) or EGFP and FLAG peptide on the N-terminus (FLAG-dermcidin-EGFP).

FLAG-tag is a synthetic octapeptide easily recognized by several specific, commercially available monoclonal antibodies. Finally, glutamate carboxypeptidase II (GCPII) fusion with the FLAG peptide was also prepared (FLAG-GCPII) (Figure 9).

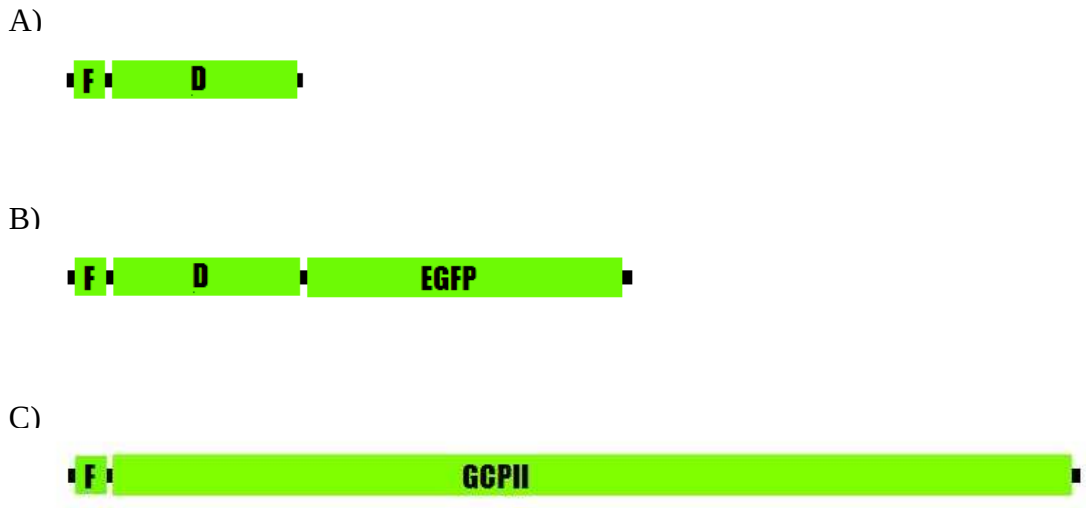

Figure 9: An overview of recombinant proteins prepared by expression of plasmids pTRE-FLAG-dermcidin, pTRE-FLAG-dermcidin-EGFP and pTRE-FLAG-GCPII in mammalian cells: A)FLAG-dermcidin (F- FLAG, D- dermcidin); B) FLAG-dermcidin-EGFP; C) FLAG-GCPII. Proteins are depicted with N-terminus on the left.

4.2 Expression of FLAG-dermcidin and FLAG-dermcidin-EGFP in mammalian cells

Plasmids pTRE-FLAG-dermcidin, pTRE-FLAG-dermcidin-EGFP and pTRE-FLAG-GCPII coding proteins FLAG-dermcidin, FLAG-dermcidin-EGFP and FLAG-GCPII, respectively (reviewed in Figure 9) were used for transfection of mammalian cells HEK293offA2 (enabling an inducible expression of proteins in dependence on presence or absence of doxycycline). The transfection was successful in case of fusion protein FLAG-dermcidin-EGFP because we could observe an EGFP fluorescence of the cells *in vivo* (Figure 10, page 36). The presence of FLAG-dermcidin, on the other hand, could not be

approved in this way. We were not able to detect any FLAG-dermcidin protein expressed in mammalian cells by specific anti-FLAG antibody (data not shown).

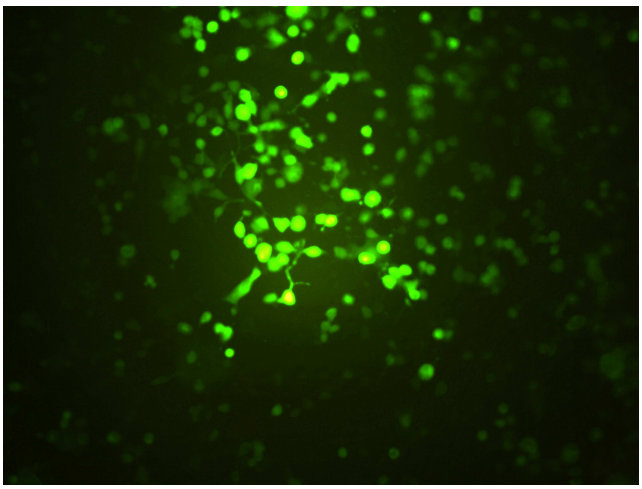

Figure 10: The Expression of FLAG-dermcidin-EGFP in Mammalian Cells
The mammalian cells HEK293offA2 were transiently transfected with plasmid pTRE-Tight-FLAG-dermcidin-EGFP. The expression of the protein FLAG-dermcidin-EGFP was verified by EGFP fluorescence

4.3 Selectivity and Sensitivity of FLAG Detection System

To determine selectivity of anti-FLAG M2-antibody conjugated with horseradish peroxidase (anti-FLAG-HRP; Sigma), its cross-reactivity with other proteins present in different cell lysates was investigated. Proteins were electroblotted from gel onto a membrane that was probed with anti FLAG-HRP antibody. A cross-reactivity of anti-FLAG antibody with EGFP was observed but the overall selectivity was more than sufficient (data not shown).

For determination of anti-FLAG-HRP antibody sensitivity (on western blots), an equal amount of FLAG-GCPII was probed with both GCP-04 and anti-FLAG-HRP antibodies (GCP-04 is a specific monoclonal antibody recognizing denaturated GCPII, which was prepared in laboratory of Prof. Hořejší) (Figure 11, page 37). The chemiluminiscence signals of bands with similar intensity were densitometrically compared. The sensitivity of anti-FLAG-HRP antibody was approximately one order of magnitude lower than the sensitivity of GCP-04 monoclonal antibody.

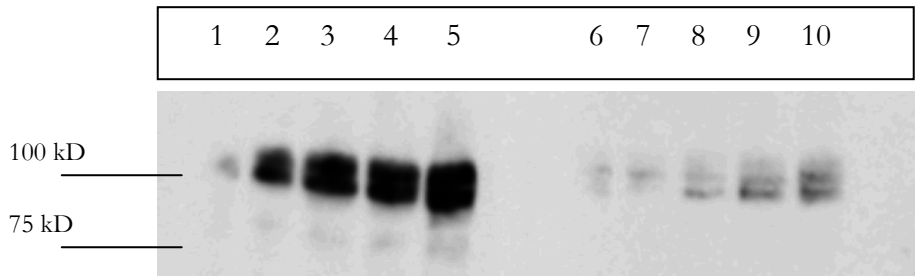

Figure 11: Western Blot Showing Equal Amounts of FLAG-GCPII Were Visualized via GCP-04 or Anti-FLAG-HRP Antibody

For quantification we used 2,5 ng (lane 1,6); 3,5 ng (2,7); 5 ng (3,8); 7 ng (4.9) and 10,5 ng (5,10) of FLAG-GCPII and visualized them via GCP-04 (lanes 1-5) and anti-FLAG-HRP (5-10) A densitometrical comparison of signals of most similar intensity (lanes 1,2 and 9,10) showed that GCP-04 antibody is about one order of magnitude more sensitive than anti-FLAG-HRP antibody.

4.4 Interaction of GCPII with Dermcidin *In Vitro*

To examine the possible interaction of GCPII with dermcidin and dermcidin-EGFP we immobilized GCPII via biotin and mutated streptavidin interaction (GCPII is expressed in insect cells with a peptide tag enabling its *in vivo* biotinylation during expression. The method was established by Jan Tykvart in laboratory of Doc. Jan Konvalinka (paper in preparation). Immobilized GCPII was used to isolate FLAG-dermcidin or FLAG-dermcidin-EGFP from cell lysates of cells transfected with appropriate plasmids. Experiments were carried out either at 4°C (FLAG-dermcidin-EGFP) or at both 4°C and 37°C (FLAG-dermcidin) (see Methods).

After the incubation of GCPII with the cell lysates, the resin was washed and GCPII was eluted from the streptavidin resin with excess of biotin. The possible complex of GCPII with FLAG-dermcidin or FLAG-dermcidin-EGFP was disrupted by boiling in reducing conditions in SDS. The resulting proteins were resolved by electrophoresis, transferred onto membrane and FLAG-dermcidin-EGFP was visualized by anti-FLAG-HRP antibody (Figure 12, page 38).

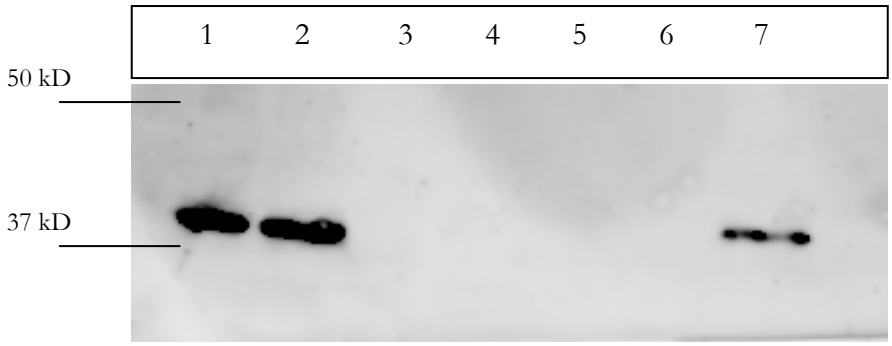

Figure 12: Western Blot Showing FLAG-tagged Dermcidin-EGFP in Fractions from Affinity Pull-down Experiment

FLAG-tagged dermcidin-EGFP present in fractions from FLAG-dermcidin-EGFP pull-down experiment was visualized via anti-FLAG-HRP antibody. Cell lysate from HEK293offA2 cells transfected with FLAG-dermcidin-EGFP was incubated either with immobilized GCPII or streptavidin mutein beads alone (negative control), washed and eluted with biotin (for details see Methods). Fractions: lane 1- cell lysate (15 µl); 2- flow-through (15 µl); 3- wash3 (15 µl); 4- elution1 (15 µl); 5- elution1 negative control (15 µl); 6- wash3 negative control (15 µl); 7- flow-through negative control (15 µl). A presence of tagged protein in the cell lysate confirms that FLAG-tagged dermcidin-EGFP is expressed by the cells and therefore is present in the cell lysate. No signal in the elution fraction lane indicates that it was not pulled down from the solution.

We observed no direct interaction of the fusion protein FLAG-dermcidin-EGFP with GCPII. The fusion protein was present in cell lysate and flow-through fractions (Figure 12; lanes 1,2 and 7), that confirms that the protein was expressed by the cells. However, no presence of FLAG-tagged dermcidin-EGFP in the elution lane shows that it does not interact with GCPII in this experimental set-up.

As for tagged dermcidin alone, the results were not reproducible. We were either not able to detect it or we detected very faint signal in lanes of cell lysate and flow-through but could not detect them in flow-through of negative control (both temperatures, data not shown).

5 Discussion

In the theoretical part of the thesis we presented GCPII, highlighted experimental facts suggesting its possible receptor function and discussed the process of both physiological and pathological angiogenesis. In this way we attempted to outline the hypothetical involvement of GCPII in the molecular process of formation of blood vessels.

There are several reports in the literature describing an existence of potential protein partner of GCPII. Some possible interacting proteins were also identified in the laboratory of Doc. Jan Konvalinka by experiments with set-up similar to GCPII affinity pull-down with streptavidin mutein matrix we presented in Methods. GCPII was immobilized on a resin via biotin- streptavidin interaction and the suspension was incubated with LNCaP cells lysate (Lymph Node Carcinoma of the Prostate Cells). The complex of GCPII with unknown proteins was eluted from the resin with biotin, denaturated and analyzed by electrophoresis. Some of the bands in the elution were analyzed by mass spectrometry, which revealed several proteins possibly interacting with GCPII. One of these proteins was dermcidin, a secreted protein from human sweat glands with interesting antimicrobial and neuroprotective activities. The experimental part of the thesis analyzes a potential interaction of GCPII with recombinant dermcidin.

Originally the plasmids coding the two proteins were synthesized without any tag. As the commercial antibodies against dermcidin did not recognize it at all (previous unpublished data), we decided to clone the proteins with a tag appropriate for their visualization. FLAG-tag is a synthetic hydrophilic octapeptide (N-DYKDDDDK-C) originally designed for immunoaffinity chromatography [69]. Several commercially available monoclonal antibodies are allegedly very sensitive and recognize it selectively. The hydrophilicity of FLAG-tag facilitates to work in native conditions, making it less likely to cause any non-specific interaction with the tagged protein.

We managed to clone dermcidin and a fusion protein of dermcidin with enhanced green fluorescent protein (EGFP) tagged with FLAG-tag on their N-termini. We decided to express these proteins in a mammalian expression system, because dermcidin was shown to be secreted and processed [65]. For transfection we chose HEK293offA2 cells because this cell line is well-established in our laboratory and is easy to transfect and to work with. Moreover, this cell line enables inducible expression of proteins under pTRE-promotor. This could be later used for experiments investigating the possible interaction *in vivo*.

The sensitivity of the anti-FLAG antibody was verified by a visualization of FLAG-GCPII using α-FLAG-HRP and GCP-04 antibodies. The sensitivity of antibody against FLAG peptide was one order of magnitude lower than that of antibody for GCPII, commonly used in our laboratory. We assumed that it is still appropriate for our purpose. The selectivity was determined by testing a cross-reactivity with distinct cell lysates.The selectivity was high except for a little cross-reactivity with EGFP.

The expression of the fusion protein FLAG-dermcidin-EGFP was documented by an EGFP fluorescence of transfected cells and western-blot of the cell lysate sample. The interaction of FLAG-dermcidin-EGFP with GCPII was examined by a pull-down assay, where we tried to pull FLAG-dermcidin-EGFP down from the cell lysate by immobilized GCPII. The interaction of GCPII with FLAG-dermcidin-EGFP was not confirmed by a presence of FLAG-dermcidin-EGFP in the fraction of GCPII eluted from the resin (Figure 11, page 37).

We were not able to document an expression of FLAG-dermcidin alone that we cloned and tagged as well as FLAG-dermcidin-EGFP. Its detection via anti-FLAG-HRP antibody failed or was not reproducible. As the expression of FLAG-dermcidin-EGFP was attested, we had no relevant reason to presume FLAG-dermcidin alone was not expressed, because the conditions of transfection and expression were identical for both proteins.

Since we did not observe any reproducible signal for FLAG-dermcidin nor in a FLAG-dermcidin cell lysate, we considered following options: First, the protein was not even expressed; second, the protein was not transferred onto the membrane successfully and third, the tag was removed in certain step of the procedure probably proteolytically.

An explication of the inability to visualize FLAG-dermcidin might be that FLAG-tag is proteolytically degraded. Since GCPII exhibits only a exopeptidase activity with high substrate specifity [70], FLAG-tag could be digested only by another peptidase present in the cell lysate. However, there was no such degradation observed in case of FLAG-dermcidin-EGFP which was treated under the same conditions. Therefore we do not have clear explanation for this result.

The experiments with a fusion protein FLAG-dermcidin-EGFP showed the GCPII pull-down assay using streptavidin matrix to be a convenient method for verification of protein-protein interaction. Due to the fact we are not able to detect the pressumed interaction in this system, we were not able to further analyze the strength of the interaction. Nevertheless, this method can be used also in principal for a characterization of some other cases of protein-protein interaction by changing conditions of the incubation.

An analysis of chemiluminiscence signals and consecutive densitometrical measurement may provide a semi-quantitaive information that can be used to describe the interaction.

The data gathered during our experiments do not support the original hypothesis that dermcidin serves as a ligand for GCPII. We will look for other possible protein partners of GCPII employing methods we introduced.

6 Conclusion

- We managed to clone recombinant FLAG-tagged dermcidin and fusion protein dermcidin-EGFP into vectors for expression in mammalian cells

- We expressed FLAG-tagged dermcidin-EGFP in mammalian cells

- We introduced new FLAG-tag detection system

- Using novel affinity pull-down system with biotinylated GCPII, we did not detect direct interaction of GCPII with dermcidin *in vitro*

- Described system will be used in further protein-protein interaction studies in our laboratory

List of Abbreviations

AP-2	Adaptor Protein-2
APS	Ammonium Persulfate
bFGF	Basic Fibroblast Growth Factor
CD34	Antigen of Hematopoetic Cells
DCD	Dermcidin (Protein)
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced Green Fluorescence Protein
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GCP-04	Mouse Anti-GCPII antibody
GCPII	Glutamate carboxypeptidase II
HEK293offA2	Human Embryonic Kidney Cells with Doxycycline-Regulated Expression
HUVEC cells	Human Umbilical Vein Endothelial Cells
IUBMB	International Union of Biochemistry and Molecular Biology
LNCaP	Lymph Node Carcinoma of the Prostate Cells
MMP	Matrix metalloprotease
NAAG	N-Acetyl-L-Aspartyl-L-Glutamate
NAALADase	N-Acetylated Alpha-Linked Acidic Dipeptidase
PDGFR	Platelet-Derived Growth Factor Receptor
PECAM-1	Platelet Endothelial Cell Adhesion Molecule (CD31)
PEI	Polyethyleneimine
PGF	Placental Growth Factor
PSMA	Prostate-Specific Membrane Antigen
RIP-Tag2	Pancreatic Islet-Derived Tumour
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TBS	Tris-Buffered Saline
TEM	Tumour Endothelial Marker
TEMED	Tetramethylethylenediamine
TfR1	Transferrin Receptor 1
TGF- β	Transforming Growth Factor-β
Tris	Trishydroxymethylaminomethane
US FDA	United States Food and Drug Administration
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
α-SMA	α-Smooth Muscle Actin

Terms Dictionary

angiogenesis	a process of formation of new blood vessels in general
angiogenic switch	a process of transformation of tumour in avascular phase into a tumour in vascular phase
avascular phase	one of two stages of tumour growth during which the tissue is provided with oxygen and nutrients via diffusion
basement membrane	a thin sheet of amorphous extracellular material that covers a surface, lines a cavity, or divides a space or organ. The basal surfaces of epithelial cells rest upon the basement membrane
bioavailability	the fraction (percent) of an administered dose of a drug that reaches the systemic circulation
biphasic response	two responses that are separated in time
cardiac ischemia	(also myocardial ischemia) an insufficient supply of blood to a hearth muscle
cartilaginous	(also chondral) consisting of cartilage or having the nature of cartilage
dose response curve	the change in effect on an organism or organ caused by differing levels of exposure to a given drug
dynamic	describing mechanics of the action of a drug by investigating movements of key molecules as single individuals (in contrast to "kinetic" that describes movements and conversions of a set of molecules depending up the time) e.g.: pharmacodynamics and pharmacokinetics
endothelial cells	cells lining the lumen of blood vessels
endothelial fenestrations	a presence of circular fenestrae or pores that penetrate the endothelium
extravasation	a leakage of a fluid or cell out of the blood vessel
filopodia	cytoplasmic projections extending beyond the leading edge of a cell
hemorrhage	bleeding
infarct	an area of tissue that undergo a necrosis due to a local lack of oxygen caused by an obstruction of blood supply
interstitial pressure	a hydrostatic pressure in parts or interspaces of a tissue
intussusception	an increase of a number of capillaries by splitting and rearrangement of endothelial cells
lectins	are a group of hemagglutinating proteins, which bind specifically to the branching sugar molecules of glycoproteins and glycolipids on the surface of cells
low doses regiment	an administration of low or lower than therapeutic doses of a drug over a longer period of time

maximal tolerated dosing	using of the highest dose of a radiological or pharmacological treatment that will produce the desired effect without unacceptable toxicity
metronomic therapy	a continuous or frequent treatment with low doses of drug, often combined with other methods of therapy
neoplasia	a process of uncontrolled and abnormal growth of cells
neoplasm	(also tumour) an abnormal mass of tissue resulting of neoplasia
neovascularization	(also vasculogenesis, vasculoneogenesis) is a process of forming of new blood vessels when there is no pre-existing one
patency	an opened or unobstructed state of a passage
pericyte	(also adventitial cell) a little, elongated, contractile, relatively undifferentiated, connective tissue cell that occurs about capillaries or other small blood vessels outside the basement membrane
protrusions	irregular projections of plasma membrane
RAS/RAF/MEK/ERK	a signaling pathway of selective protein-kinases that facilitates a transduction of signal from growth factor receptor to a nucleus consequently regulating transcription and translation
rubor, **tumour**, **calor** and **dolor**	redness, swelling, heat, and pain, respectively
tissue factor	(also platelet tissue factor, factor III, thrombokinase, or CD142) a primary cellular initiator of blood coagulation that activates the coagulation protease cascade
tissue hypoxia	a condition in which tissue cells experience inadequate oxygen utilization usually due to a decreased partial pressure of oxygen in this given tissue
vascular phase	one of two stages of tumour growth during which the tissue is provided with oxygen and nutrients via blood flow

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Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a příjmení S adresou	Číslo OP	Datum vypůjčení	Poznámka