Fluorimetric and Mass-Spectrometric Methods for Analysis of GTP-Converting Signal-Transducing Proteins and Enzymes

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie –
der Universität Regensburg

vorgelegt von
Christian Spangler
aus Hohenfels
2010

Fluorimetric and Mass-Spectrometric Methods for Analysis of GTP-Converting Signal-Transducing Proteins and Enzymes

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie –
der Universität Regensburg

vorgelegt von
Christian Spangler
aus Hohenfels
2010

Die vorliegende Arbeit entstand unter der Leitung von Herrn Prof. Dr. O. Wolfbeis im Zeitraum von November 2006 bis Januar 2009 am Institut für Analytische Chemie, Chemo- und Biosensorik der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie – der Universität Regensburg und unter der Leitung von Herrn Prof. Dr. R. Seifert zwischen Februar 2009 und September 2010 am Institut für Pharmakologie der Medizinischen Hochschule Hannover.

Das Promotionsgesuch wurde eingereicht am 14. Juli 2010.

Tag des Kolloquiums: 24. September 2010

Prüfungsausschuss:
Prof. Dr. Armin Buschauer (Vorsitzender)
Prof. Dr. Roland Seifert (Erstgutachter)
Prof. Dr. Otto Wolfbeis (Zweitgutachter)
Prof. Dr. Joachim Wegener (Drittprüfer)

Danksagung

Bei der Erstellung dieser Arbeit haben mir zahlreiche Personen mit (wissenschaflichem) Rat und Tat zur Seite gestanden und haben mir immer wieder neuen Antrieb gegeben:

An erster Stelle gilt mein besonderer Dank Herrn Prof. Dr. Roland Seifert für die stets fruchtbaren wissenschaftlichen Gespräche und Anregungen und das mir entgegengebrachte Vertrauen. Sie haben mich immer wieder von Neuem motiviert und mir große wissenschaftliche Freiheiten gelassen, wodurch ich viele wertvolle Erfahrungen machen konnte. Herzlichen Dank für Ihre Unterstützung.

Weiterhin möchte ich mich bei Herrn Prof. Dr. Otto Wolfbeis und Herrn Dr. Michael Schäferling für die hilfreichen wissenschafltichen Diskussionen und die Unterstützung bei der Entwicklung des Fluoreszenzassays danken. Vielen Dank für die Einblicke in die Welt der Fluoreszenzanalytik.

Herzlicher Dank gilt auch Herrn Prof. Dr. Volkhard Kaever für die Einführung in die Massenspektrometrie. Ich danke Dir für die vielen Ratschläge, Deine tatkräftige Unterstützung und das mir entgegengebrachte Vertrauen bei der Etablierung eines neuen Projektes. Vielen Dank.

Außerdem möchte ich der MS-Truppe Annette Garbe, Heike Burhenne und Kerstin Beste für die praktische Hilfe an den MS-Geräten danken. Ich danke Euch für die Geduld, die Ihr aufbringen musstet wenn ich Probleme an den Geräten hatte und die stets humorvolle und heitere Atmosphäre im MS-Labor.

Vielen Dank an Juliane von der Ohe für das Beibringen diverser mikrobiologischer Arbeitstechniken. Danke, dass Du so geduldig mit mir warst und mir den Laboralltag mit vielen hilfreichen Tips erleichtert hast. Ina Hackbarth danke ich für die Durchführung der Inhibitionsassays, Du hast mir auf der Zielgeraden den Abschluss der Arbeit sehr erleichtert. Mein Dank geht auch an Marina Golombek für die Durchführung der Gelelektrophoresen und die Hilfe bei der Proteinreinigung. Wir haben die Tücken des ÄKTA Purifiers gemeinsam ertragen.

Weiterhin gilt mein Dank Edeltraud Schmid und Annette Stanke, für die Zusammenarbeit bei der Bewältigung der unumgänglichen bürokratischen Herausforderungen und die notwendige Unterstützung in diversen Belangen des (Arbeits-)Alltags.

Besonderen Dank möchte ich auch an Herrn Prof. Dr. Urs Jenal, Herrn Dr. Alex Böhm, Herrn Dr. Sören Abel und Herrn Dr. Jacob Malone aussprechen, die mir durch eine umkomplizierte und sehr effektive Kooperation viele wissenschaftliche Möglichkeiten eröffnet haben. Herzlichen Dank für die gute Zusammenarbeit.

Außerdem geht mein Dank an Herrn Dr. Michael Spörner, der mir die verschiedenen Ras-Proteine zur Verfügung gestellt und mir bei der Etablierung des Ras-Assays entscheidende Hilfe gegeben hat.

Ich möchte mich bei Heike Mader, Mark-Steven Steiner, Doris Burger, Katrin Uhlmann und Ulrike Voigt für die gemütlichen Kaffeerunden und auch die vergnügsamen außerdienstlichen Aktivitäten bedanken. Ihr wart der Garant für eine stets heitere Laboratmosphäre und habt mich immer wieder motiviert neue Aufgaben anzugehen.

Des Weiteren bedanke ich mich bei allen Mitarbeitern des Instituts für Analytische Chemie, Chemo- und Biosensorik der Universität Regensburg und des Instituts für Pharmakologie der Medizinischen Hochschule Hannover für die fortwährende Unterstützung, die kollegiale Arbeitsatmosphäre und vor allem für die große Hilfsbereitschaft im Laboralltag. Danke an alle für die herzliche Aufnahme an beiden Instituten, die Arbeit mit Euch hat mir viel Spaß gemacht.

Weiterer Dank gilt der Deutschen Forschungsgemeinschaft (DFG) für die finanzielle Förderung bei der Entwicklung des Fluoreszenzassays.

Ganz besonders danken möchte ich meinen Eltern, Martha und Karl Spangler. Ihr habt mich den ganzen Weg bis hierher tapfer begleitet, habt mir große Freiheiten gelassen und mich stets in allen Belangen uneingeschränkt unterstützt. Ihr habt mir während des Studiums und der Promotion großen persönlichen Rückhalt gegeben auch, und vor allem dann, wenn schwierige Situationen zu meistern waren. Ohne Euch wäre das alles nicht möglich gewesen. Vielen Herzlichen Dank!

Mein ganz persönlicher Dank gilt meiner Frau Corinna, auf deren emotionale Unterstützung ich immer bauen konnte. Du hast mir auf dem langen Weg durch Studium und Promotion immer wieder neuen Mut zugesprochen und mich in meinen Entscheidungen bestärkt. Du hast mein häufiges Auf und Ab geduldig ertragen und es in einzigartiger Weise immer wieder geschafft, mich aufzumuntern. Ich danke Dir von ganzem Herzen!

Table of Contents

1. Introduction

Guanosine-5'-triphosphate (GTP) represents a major energy source for diverse biological processes and serves as key substrate in a large number of enzymatic reactions in both eukaryotes and prokaryotes. In the citric acid cycle, GTP is the only energy equivalent being produced after hydrolysis of succinyl-CoA to succinate by succinyl-CoA synthetase[1]. During protein biosynthesis, GTP bound to the elongation factor Tu (EF-Tu) is required as energy donor for the amino acid attachment to the developing polypeptide[2].

The central role of GTP, however, is its function as substrate for signal transducing enzymes. So far, GTP is known to be involved in four major signaling systems: (1) Small GTP-binding proteins constitute a large superfamily of low molecular mass proteins involved in the regulation of a wide variety of cellular functions[3]. (2) Heterotrimeric GTP-binding proteins represent an important class of high molecular mass proteins which transduce signals from G-protein-coupled receptors to intracellular effector systems[4]. (3) Guanylyl cyclases (GCs) synthesize the second messenger cyclic 3':5'-guanosine monophosphate (cGMP) which regulates very diverse processes such as cellular growth and contractility, cardiovascular homeostasis, inflammation, sensory transduction, and neuronal plasticity and learning[5]. (4) The bacterial second messenger cyclic 3':5'-di-guanosine monophosphate (c-di-GMP) is produced from GTP by di-guanylate cyclases (DGCs) and controls a range of functions including developmental transitions, biofilm formation, and the virulence of pathogens[6].

GTP-binding and -converting proteins have been extensively characterized during the last decades. Hence, the detailed and still growing knowledge about the molecular mechanisms reveals these proteins as major pharmacological targets for the development of novel drugs. Therefore, effective detection and quantitation methods for GTP turnover are essential and can offer a stable platform for the establishment of new therapies.

1.1 Small GTP-Binding Proteins

Small GTP-binding proteins comprise a superfamily of more than 100 members which have been identified in eukaryotes[7,8]. According to their respective cellular functions these members are classified into five subfamilies: Ras, Rho/Rac/Cdc42, Rab, Arf, and Ran[9]. Small GTP-binding proteins have molecular masses ranging from 20 to 40 kDa and show high structural homology which is characterized by conserved amino acid sequences essential for specific GTP- and GDP-binding as well as GTPase activity[10,11]. Small GTP-binding proteins are either found in the cytosol or attached to membranes. The Ras, Rho/Rac/Cdc42, and Rab subfamilies undergo posttranslational modifications at the C-terminus with lipophilic groups enabling interactions with membraneous structures necessary for their biological function[12].

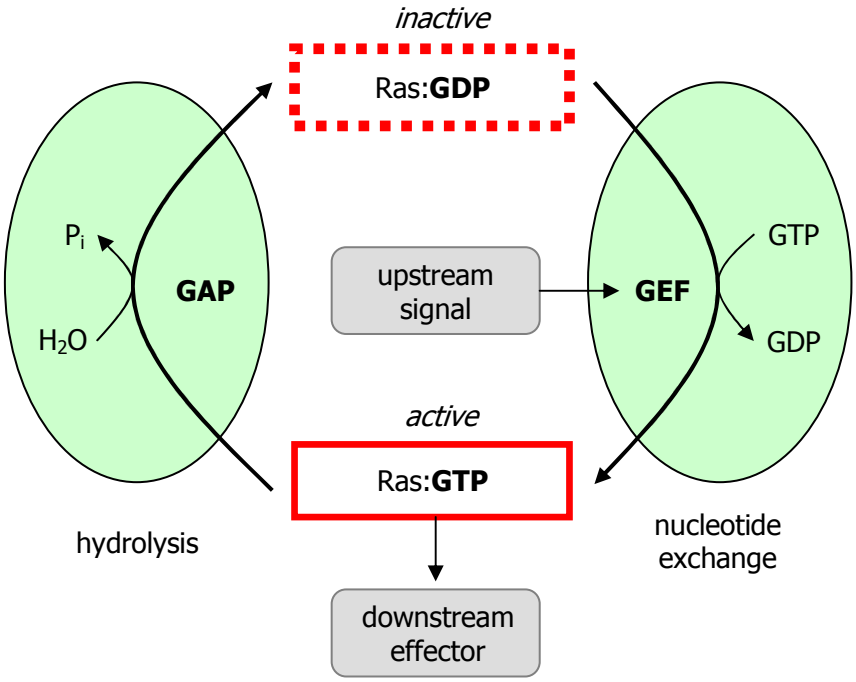


Fig. 1.1 Regualtion of the activity of small GTP-binding proteins. GAP: GTPase activating protein, GEF: guanine nucleotide exchage factor.

Small GTP-binding proteins act as molecular switches by cycling between an inactive, GDP-bound state and an active, GTP-bound form (see Fig. 1.1)[13]. The dissociation of GDP from the inactive form is induced by upstream signals and followed by GTP-binding. In the active, GTP-bound state, small GTP-binding proteins interact with downstream effectors *via* their downstream effector-binding regions and evoke diverse cellular responses. Inactivation of the GTP-bound state is achieved by GTP hydrolysis to GDP and P_i due to the intrinsic GTPase activity of small GTP-binding proteins. However,

both, the dissociation of GDP from the inactive state and GTP hydrolysis are intrinsically very slow. The GDP/GTP exchange process is stimulated by guanine nucleotide exchange factors (GEFs) the action of which is mostly regulated by upstream signals. The intrinsic GTPase activity is enhanced by GTPase activating proteins (GAPs). Most of the GEFs[14,15] and GAPs[14,16] are specific for each subfamily of small GTP-binding proteins.

1.1.1 Ras Proteins

The members of the Ras subfamily function mainly as regulators of gene expression. Fig. 1.2 indicates the mode of action of Ras proteins in gene expression. Activity of Ras proteins is triggered by various extracellular signals which mostly activate receptors with tyrosine kinase activity[17]. So-called adaptor proteins such as SHC and GRB2 bind to phosphotyrosines and than form complexes with SOS, a Ras-GEF. SOS activates membrane-bound Ras proteins by inducing GDP-GTP exchange. Gene expression by Ras proteins is subsequently initiated by a series of several kinases following the mitogen-activated protein (MAP) kinase cascade: activated Ras proteins directly bind to the Raf protein[18] which phosphorylates and activates MEK (MAPK/ERK kinase)[19] which in turn phosphorylates and activates ERK (extracellular regulated kinase)[20]. ERK translocates into the nucleus and activates transcription factors[21]. It is estimated that in about 30% of all human tumors mutated Ras oncogenes are present[22], emphasizing the great impact of abnormal Ras activity in carcinogenesis.

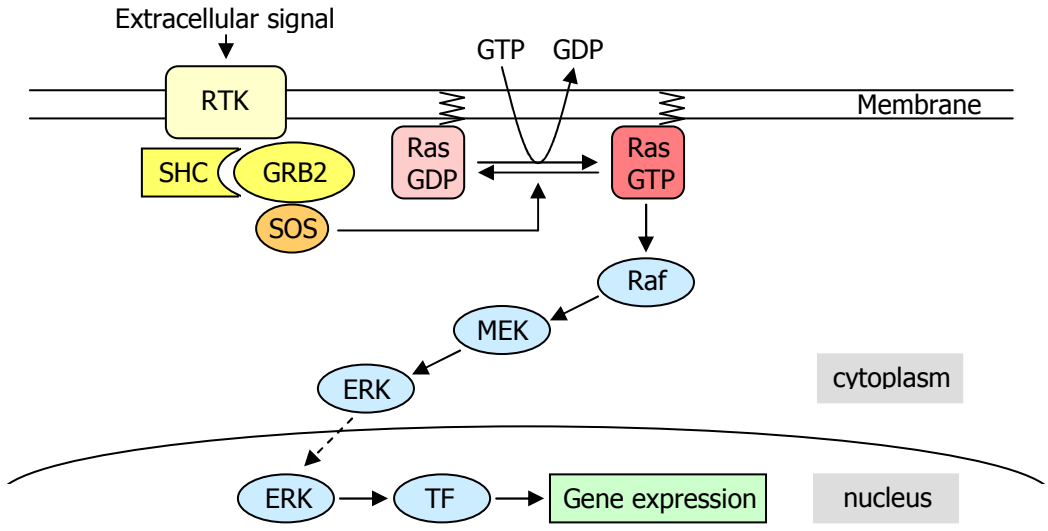


Fig. 1.2 Regulation of gene expression by Ras proteins (modified from Vojtek and Der[23]). RTK: receptor tyrosine kinase; SHC and GRB2: adapter proteins binding to phosphotyrosines; SOS: Son of sevenless (GEF); Raf: rapidly growing fibrosarcoma (protein kinase); MEK: MAPK/ERK kinase; ERK: extracellular regulated kinase; TF: transcription factor.

1.1.2 Rho/Rac/Cdc42 Proteins

The main function of Rho/Rac/Cdc42 proteins is the regulation of cytoskeletal reorganization in response to extracellular signals[24]. There are also hints for Rho/Rac/Cdc42 proteins to be involved in gene expression[25,26]. The actin cytoskeleton represents a network of actin filaments and specialized proteins located in the cytoplasm of cells[27]. Actin filaments can be structurally classified into three groups: (1) actin stress fibres consisting of bundles of actin filaments which traverse the cell and are linked to the extracellular matrix, (2) lamellipoda, a thin, two-dimensional actin mesh characterized by regularly occurring membrane ruffles found mostly at the edge of mobile cells and cultured fibroblasts, and (3) filopodia representing narrow, cytoplasmic projections containing bundles of cross-linked actin filaments found in motile cells and at neuronal growth cones (see Fig. 1.3). The formation of these three discrete structures is regulated by Rho[28], Rac[29], and Cdc42[30], respectively.

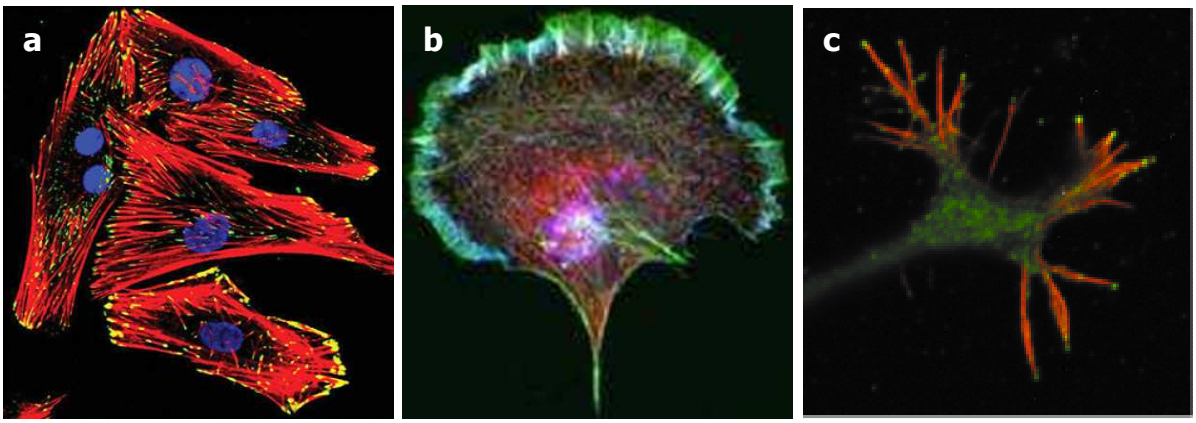


Fig. 1.3 Images of fluorescently labeled cells showing (**a**) stress fibres of rat aortic vascular smooth muscle cells (taken from Deshpande *et al.*[31]), (**b**) lamellipodium of human fibroblasts (cell line SV80) (http://www.umm.de/1425.0.html, 06-02-2010), and (**c**) filopodia at neuronal growth cone of neuroblastoma cells (cell line NG108-15) (taken from Nozumi *et al.*[32]).

In addition to GEFs and GAPs controlling the activity of Rho/Rac/Cdc42 proteins as described above, a third class of regulatory units plays an important role for Rho/Rac/Cdc42 protein activity: GDP dissociation inhibitors (GDIs) stabilize the inactive GDP-bound form *via* formation of stable complexes with Rho/Rac/Cdc42 proteins which have been posttranslationally modified with lipophilic groups. Hence, it is possible for Rho/Rac/Cdc42 proteins to remain soluble in the cytosol.

1.1.3 Rab Proteins

The Rab protein subfamily is the largest within the small GTP-binding protein superfamily. Rab protein activity is focused on the regulation of intracellular vesicle trafficking which is responsible for the transport of both transmembrane and secreted, soluble proteins between different membrane compartments. Essential cellular functions such as exocytosis, endocytosis, and cytokinesis are dependent on vesicle trafficking[33]. Intracellular vesicle trafficking is generally divided into four steps (see Fig. 1.4): (1) budding of vesicles from a donor membrane, (2) targeting and (3) docking of vesicles to an acceptor membrane, and (4) fusion with the acceptor membrane[3]. The budding process, however, is mainly regulated by Arf proteins (see section 1.1.4). Due to the interaction with membranous structures, it is necessary for Rab proteins to be modified with lipids. The most common posttranslational modification is the geranylgeranylation of cysteine residues at the highly variable C-terminal region which is essential for specific recognition of target membranes[34].

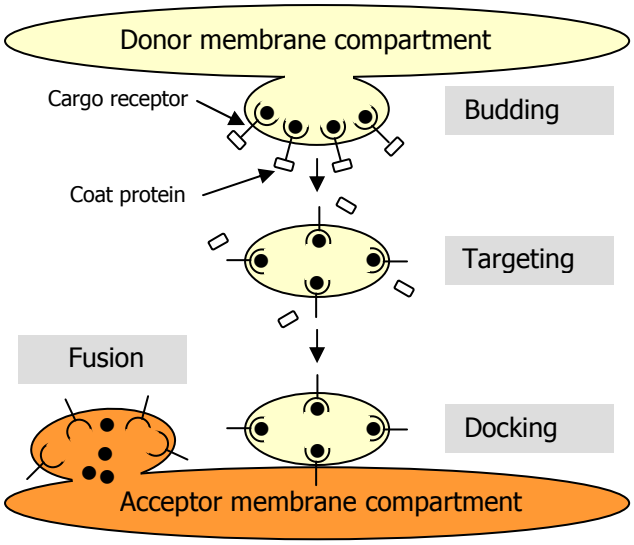


Fig. 1.4 Principle mechanism of intracellular vesicle trafficking. Modified from Takai *et al.*[3]

Rab protein activity is controlled by three regulatory units: GEF, GAP, and GDI (see Fig. 1.5). GDI interacts with GDP-bound Rab proteins which are maintained in the cytosol. After release of GDI, the GDP bound form is converted into the active GTP-bound state by the action of a GEF. The active Rab form interacts with effectors coupled to transport vesicles leading to recruitment of the vesicles to the target membrane. Inactivation occurs *via* action of a GAP and the GDP-bound form is stabilized in the cytosol by GDIs again[35]. Due to this membrane association/dissociation cycle 10-50% of a specific Rab protein are located in the cytosol.

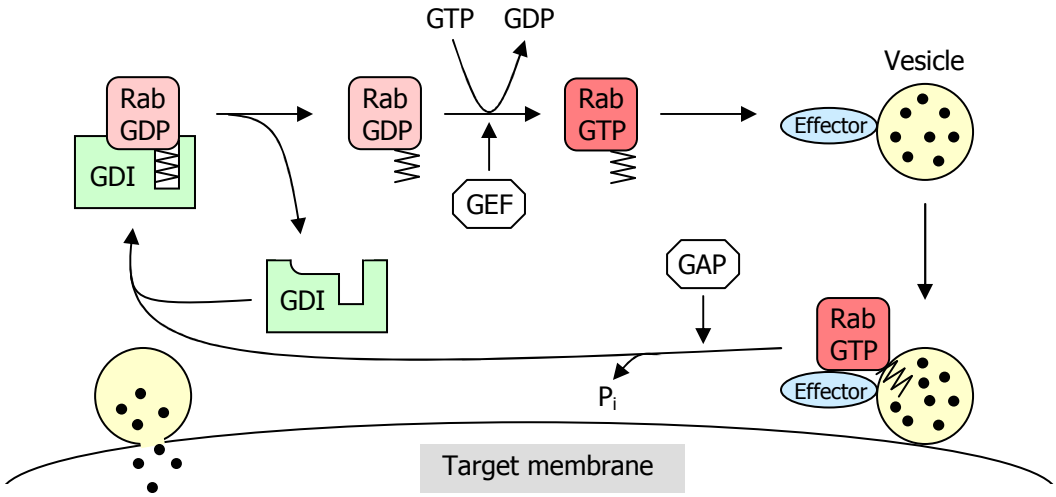


Fig. 1.5 Regulation of Rab protein activity and related translocation (modified from Takai *et al.*[3]). GDI: GDP dissociation inhibitor; GEF: Guanine nucleotide exchange factor; GAP: GTPase activating protein.

1.1.4 Arf Proteins

Arf (ADP-ribosylation factor) proteins play an important role in the budding process (see Fig. 1.4) of vesicle trafficking[36]. For this process, it is necessary that specific proteins coat the cytoplasmic face of a donor membrane. Such coat proteins are able to recognize distinct membrane receptors and mechanically support the formation of vesicle buds (see Fig. 1.6)[37]. Arf proteins are involved in the recruitment of coat proteins to the membrane. Their activity is regulated by specific GEFs and GAPs, but not GDIs. GDP-bound Arf proteins are present in the cytosol and undergo protein-membrane interaction after activation by GEFs. The membrane association is further stabilized by a conformational change of the N-terminal α-helix resulting in the interaction of several hydrophobic amino acid residues with the membrane. After vesicle budding, Arf proteins are deactivated by cytosolic GAPs which are recruited to the membrane[3]. Subsequent targeting, docking, and fusion steps are regulated by Rab proteins (see section 1.1.3).

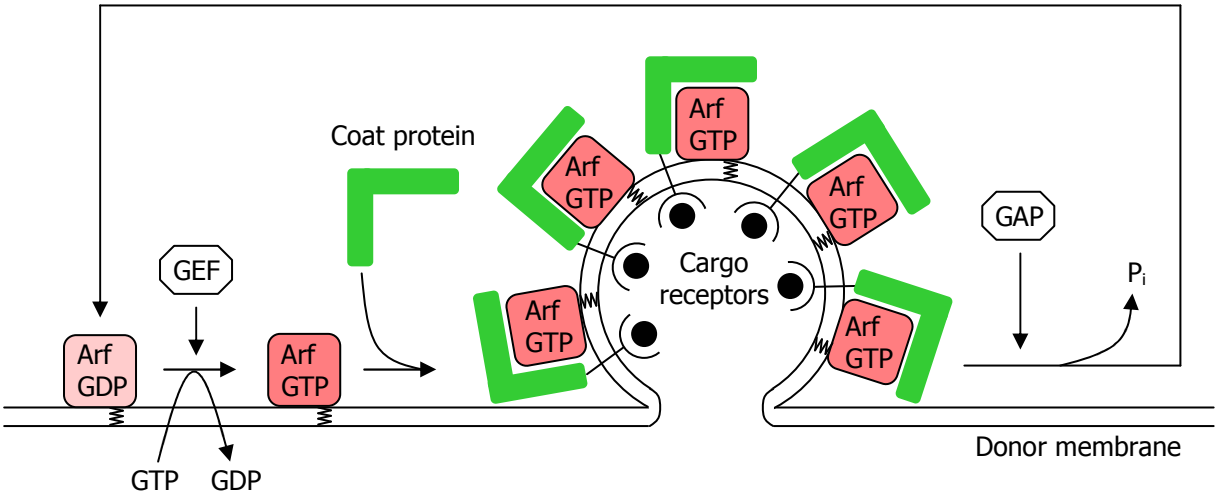


Fig. 1.6 Regulation of Arf protein activity and budding of vesicle from donor membrane (modified from Takai *et al.*[3]). GEF: guanine nucleotide exchange factor; GAP: GTPase activating protein.

1.1.5 Ran Proteins

Ran proteins (Ras-related nuclear proteins) represent the smallest subfamily of small GTP-binding proteins and play a central role in nucleocytoplasmic transport processes which are necessary for the transport of large molecules including proteins and RNAs[38]. Cargo molecules carrying the nuclear localization signal (NLS) (for import) or the nuclear export signal (NES) (for export) are recognized and bound by transport receptors, so called importins or exportins, respectively, and are transported through the nuclear pore complex (NPC) – a large protein complex spanning the nuclear envelope[39]. In some cases, the cargo-receptor interaction is not direct but mediated by adaptor molecules.

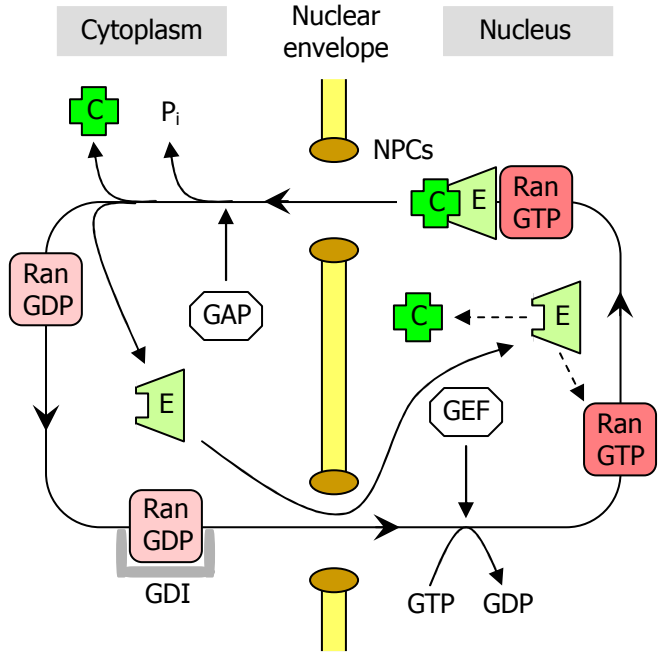


Fig. 1.7 Regulation of Ran protein activity and nucleocytoplasmic export process (modified from Takai *et al.*[3]).C: cargo molecule; E: exportin; NPCs: nuclear pore complexes; GAP: GTPase activating protein; GEF: guanine nucleotide exchange factor.

Ran proteins undergo activation and inactivation by GEFs and GAPs, respectively, (see Fig. 1.7) which are unevenly distributed in the nucleus and the cytoplasm. Hence, activated Ran

proteins are mostly located in the nucleus whereas inactive Ras proteins are found in the cytoplasm[3]. For export processes, GTP-bound Ran protein and the cargo molecule bind to the exportin and the resulting complex is transported through the NPC to the cytoplasm where the Ran protein is deactiveated by a GAP. As a consequence, dissociation of the transport complex is induced, the cargo molecule is released, and the free exportin is reimported to the nucleus again. Inactive Ran proteins are stabilized by GDIs which are necessary for the transport back into the nucleus. Nuclear import processes follow another mechanism where Ran proteins are not necessary for the transport itself but for the dissociation of the transport complex[40].

1.2 Heterotrimeric GTP-Binding Proteins

1.2.1 Signaling Mechanism of Heterotrimeric GTP-Binding Proteins

Heterotrimeric GTP-binding proteins (G-proteins) are central constituents of signal transduction and transduce extracellular information from ligand-binding to G-protein coupled receptors (GPCRs) into intracellular responses[41]. GPCRs form the initial part of the signal transduction cascade and consist of 7 transmembrane α-helices, an extracellular amino-terminal segment and an intracellular carboxy-terminal tail[42]. These receptors are the pharmacological targets of 50-60% of all existing drugs.

Active GPCRs specifically interact with G-proteins consisting of a Gα-subunit and a Gβ/γ heterodimer both of which are located on the cytosolic side of the membrane. The G-protein activation/inactivation cycle is shown in Fig. 1.8. In the inactive state, the Gα subunit is GDP-bound and associated with the G$\beta\gamma$ unit to form a heterotrimeric complex. After activation of the GPCR in response to agonist binding, the resulting conformational change induces the release of GDP from the Gα subunit and its replacement by GTP. Hence, the GPCR acts as guanine nucleotide exchange factor (GEF). Subsequent conformational changes of the Gα subunit in the active, GTP-bound form promote the separation of Gα subunit and G$\beta\gamma$ complex each of which can interact with specific effector proteins (see section 1.2.2). The intrinsic GTPase activity of the Gα subunit terminates function *via* hydrolysis of the terminal phosphate of bound GTP. Reassociation of the GDP-bound Gα subunit with the G$\beta\gamma$ complex completes the G-protein cycle[43]. G-proteins display a higher rate of GTP hydrolysis than small GTP-binding proteins, but it is still relatively low[44]. The GTPase activity is accelerated by regulators of

G-protein signaling (RGS), which act as GTPase activating proteins (GAPs) analoguously to small GTP-binding proteins (see section 1.1)[45].

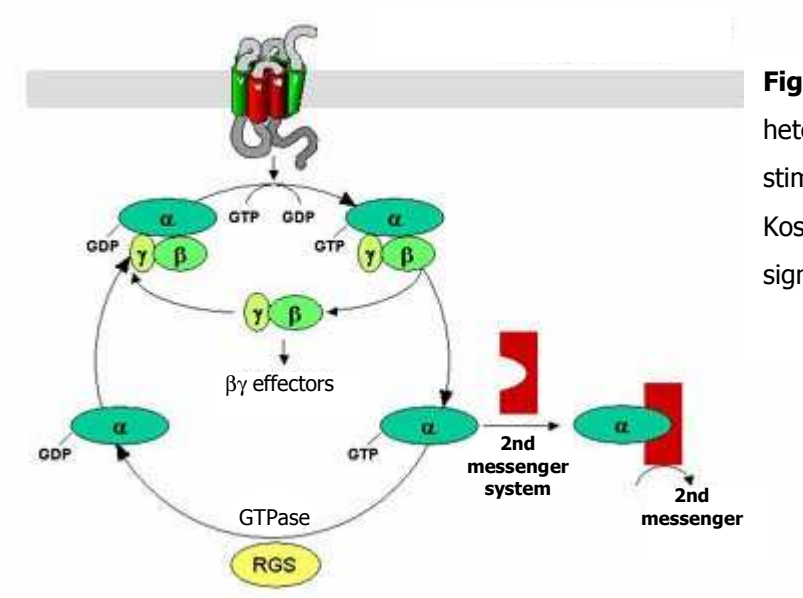


Fig 1.8 Activation/inactivation cylce of heterotrimeric G-proteins after GPCR stimulation (taken from Milligan and Kostenis[43]). RGS: regulator of G-protein signaling.

1.2.2 Structure and Function of Heterotrimeric GTP-Binding Protein-Subunits

Since the first steps for the identification of the three subunits of heterotrimeric GTP-binding proteins were made in the early 1980s by Rodbell and Gilman[46] who were awarded the Nobel Prize for Physiology or Medicine in 1994[47,48], structural and functional relationships of G-proteins have been intensively investigated.

The Gα subunits are a family of proteins with molecular masses ranging from 39-53 kDa and display an amino acid similarity of about 45-80%[49]. Gα subunits have been divided into four main classes according to their amino acid sequences[50]: (1) $G_s\alpha$ which mainly mediates hormonal stimulation of adenylyl cyclase, (2) $G_{i/o}\alpha$ involved in inhibition of adenylyl cyclase and opening and closing of K^+- and Ca^{2+}-channels, respectively, (3) $G_{q/11}\alpha$ whose members are primarily related to phospholipase C activity, and (4) $G_{12/13}\alpha$ whose function was only lately determined and shown to be involved in G-protein-linked signaling pathways and cell responses[51]. A detailed classification of Gα subunits and corresponding functions are shown in Table 1.1.

Gα proteins consist of two domains. The first domain, also referred to as Ras-like domain, contains a six-stranded β-sheet surrounded by five α-helices. It is responsible for guanine nucleotide binding and GTPase activity[52]. The second domain is completely α-helical and buries the GTP in the core of the protein[53]. Gα subunits are no transmembrane polypeptides and should therefore be soluble proteins. In fact, all Gα subunits are posttranslationally modified with lipids at or near the amino terminus[54].

They can be either myristoylated at an amino-terminal glycine residue or covalently modified with palmitate *via* a thioester linkage to a cysteine residue or both. Hence, Gα subunits can be present at the plasma membrane in order to interact with specific receptors.

Table 1.1 Heterotrimeric GTP-binding proteins: classification, main effectors, and related diseases (modified from Milligan and Kostenis[43]).

Family	*Subclass*	*Effectors*	*Related diseases*
$G_s\alpha$	$G_{s(S)}\alpha$ $G_{s(L)}\alpha$ $G_{s(XL)}\alpha$ $G_{olf}\alpha$	Adenylyl cyclases↑ ($G_{s,s(XL),\ olf}\alpha$) Maxi K channel↑ ($G_s\alpha$) Src tyrosine kinases↑ ($G_s\alpha$) GTPase of tubulin↑ ($G_s\alpha$)	$G_{s(XL)}\alpha$: brachydactyly, trauma-related bleeding tendency, neurological problems $G_s\alpha$: McCune-Albright syndrome, cholera, pseudohypoparathyroidism type Ia/b, testotoxicosis, adenomas of pituitary and thyroid
$G_{i/o}\alpha$	$G_{o1}\alpha$ $G_{o2}\alpha$ $G_{i1\text{-}i3}\alpha$ $G_z\alpha$ $G_{t1/2}\alpha$ $G_{gust}\alpha$	Adenylyl cyclase↓ ($G_{i,o,z}\alpha$) Rap1GAPII-dependent ERK/MAPkinase activation↑ ($G_i\alpha$) Ca^{2+} channels↓ ($G_{i,o,z,}\alpha$) K^+ channels↑ ($G_{i,o,z,}\alpha$) GTPase of tubulin↑ ($G_i\alpha$) Src tyrosine kinases↑ ($G_i\alpha$) cGMP-PDE↑ ($G_{t/gust}\alpha$)	$G_i\alpha$: whooping cough, adrenal and ovarian adenomas $G_t\alpha$: congenital cone dysfunction, night blindness
$G_{q/11}\alpha$	$G_q\alpha$ $G_{11}\alpha$ $G_{14}\alpha$ $G_{15}\alpha$ $G_{16}\alpha$	Phospholipase Cβ isoforms↑ p63-RhoGEF↑ ($G_{q/11}\alpha$) Bruton's tyrosine kinase↑ ($G_q\alpha$) K^+ channels↑ ($G_q\alpha$)	$G_{q/11}\alpha$: dermal hyperpigmentation and melanocytosis?
$G_{12/13}\alpha$	$G\alpha_{12}$ $G\alpha_{13}$	Phospholipase D↑ Phospholipase Cε↑ NHE-1↑ iNOS↑ E-cadherin-mediated cell adhesion↑ P115-RhoGEF PDZ-RhoGEF	Recent SNPs identified but no disease correlation yet
Gβ/γ	$\beta_{1\text{-}5}$ $\gamma_{1\text{-}12}$	PLCβs↑ Adenylyl cyclase I↓ Adenylyl cyclases II, IV, VII↑ PI-3 kinases↑ K^+ channels↑ Ca^{2+} channels↓	$G\beta_3$: atherosclerosis, hypertension, metabolic syndrome

The Gβγ unit is a tightly complexed dimer which only dissociates under denaturing conditions[55]. It interacts with the inactive Gα subunit and helps to increase its affinity for specific receptors. Although initially the Gβγ subunit was believed to only interact with Gα in order to control signaling and recruitment to the membrane due to its hydrophobic character, it has become clear that the Gβγ subunit itself interacts with a large number of effectors (see Table 1.1)[4]. Thus far, five Gβ and twelve Gγ subunits are known for which a high number of possible pairings is possible. However, only a small set of those Gβγ dimers is essentially formed.

The Gβ subunits are highly conserved sharing 50-83% identity with a molecular mass of 35-36 kDa. The amino-terminal region consists of an amphiphatic α-helix and the carboxy-terminus is composed of seven repeating units of 43 amino acids each[55]. The Gγ subunit is a much smaller group of proteins of 6-9 kDa. They are more diverse resulting from posttranslational modifications[56]. Gγ subunits undergo either farnesylation or gernanylgeranylation at the carboxy-terminus. In contrast to Gβ subunits, Gγ proteins only share 27-75% homology and are thus thought to account for the functional specificity of Gβγ complexes[55]. The tight interaction of the Gβ and Gγ subunit is based on a non-covalent mechanism and the association occurs in the cytosol prior to membrane attachment.

1.3 Guanylyl Cyclases

1.3.1 Structure and Regulation of Guanylyl Cyclases

In the late 1960s cyclic 3':5'-guanosine monophosphate (cGMP) was first identified as natural product of guanylyl cylcase (GC) activity[57]. It took until the mid-1970s to discover the existence of different forms of GCs[58]. Today, two types of GCs are known, soluble GC (sGC) and membrane-bound, particulate GC (pGC) which are partly homologous but strongly differ with respect to cellular localization, structure, and regulation[5]. Since the discovery of cGMP, a complex concept of cGMP signaling has been established which is still being expanded (see Fig. 1.9).

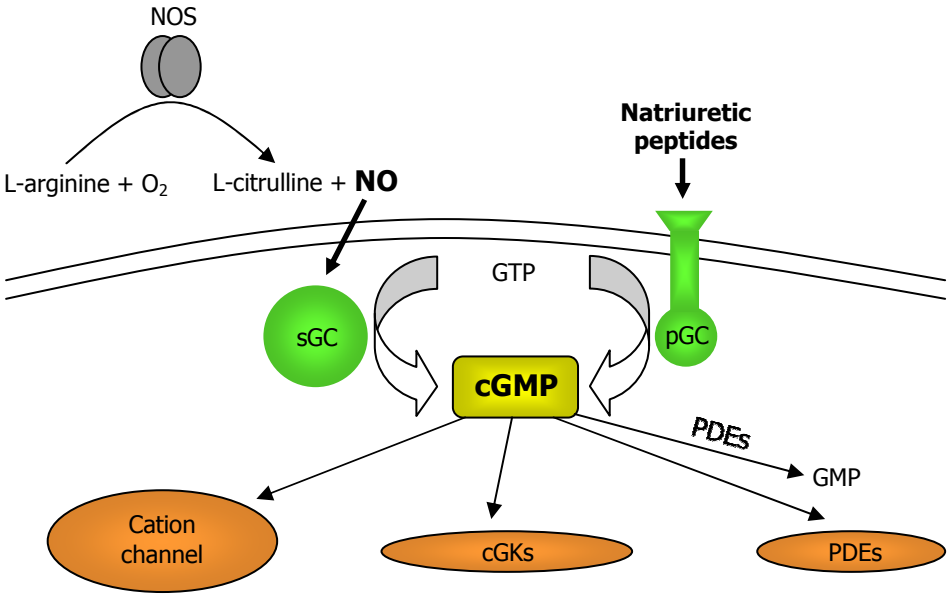


Fig. 1.9 Concept of cGMP signaling (modified from Feil and Kemp-Harper[59]). cGMP generators and effectors are shown in green and orange, respectively. NOS: nitric oxide synthase; sGC: soluble guanylyl cyclase; pGC: particulate guanylyl cyclase; cGKs: cGMP-dependent protein kinases; PDEs: phosphodiesterases.

sGC is a heterodimer which consists of two subunits, α and β and is stimulated by nitric oxide (NO) leading to a 200-fold increase in GC activity[60]. So far, four sGC subunits have been identified: α_1, α_2, β_1, and β_2. Only heterodimeric enzymes are active[61]. sGC exists as $\beta_1\,\alpha_1$ and $\beta_1\,\alpha_2$ isoform whereas isolated homodimers ($\alpha_1\,\alpha_1$ and $\beta_1\,\beta_1$) do not show any GC activity[62]. NO is synthesized by the nitric oxide synthase (NOS) from L-arginine. There are three forms of NOS: endothelial, neuronal, and inducible NOS (eNOS, nNOS, and iNOS)[63]. After NO synthesis, the stimulator rapidly diffuses across cell membranes and activates sGC. The sensitivity of sGC to NO is based on the presence of a heme group in the heterodimer. The heme group is bound to the amino-terminal region of the β chain of sGC *via* an imidazole residue (His-105 of β_1 subunit) coordinating the heme group in axial position[64]. Binding of NO to the central heme-iron yields the formation of a pentacoordinated complex and breaks the bond to the axial His-105 thereby activating the enzyme as a result of conformational changes[65]. The catalytic domain is located at the carboxy-terminal half of sGC and consists of components from both α and β subunit.

Besides sGC there are at least seven plasma membrane GCs (GC-A through GC-G) which are partly homologous to sGC but have a distinct topology. pGCs consist of an extracellular ligand-binding domain, a short transmembrane region, and an intracellular

domain that contains the catalytic region at its carboxy-terminus[66]. The intracellular region contains a protein kinase-homology domain (KHD), an amphiphatic α-helical or hinge region, and a carboxy-terminal cyclase-homology catalytic domain (see Fig. 1.10).

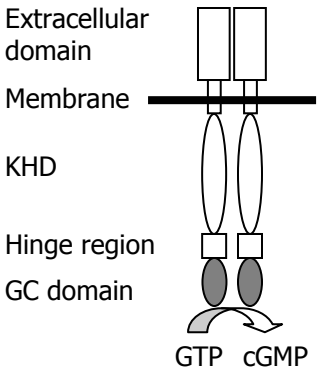


Fig 1.10 Predicted homodimeric topology of particulate guanylyl cyclases (modified from Kuhn[66]). KHD: kinase homology domain; GC: guanylyl cyclase.

Although the KHD has many amino acid residues conserved in the catalytic domain of protein kinases, no such activity has been detected yet. The KHD is known to modulate the GC activity of the cyclase-homology catalytic domain[67] and may serve as docking site for direct interactions between the pGC and other proteins[68]. The coiled-coil hinge region is responsible for oligomerization of pGCs. The cyclase catalytic domain is only activated after dimerization although each pGC peptide chain contains one catalytic domain. Specific ligands for the extracellular receptor domain have only been identified for four of the seven known pGCs, the other three forms are assumed to be orphan receptors[69]. In contrast to sGCs, pGCs are activated by various peptides[70]. The specific ligands and the functions of the different pGCs are displayed in table 1.2.

Table 1.2 Particulate guanylyl cyclases: specific ligands and main functions (modified from Schmidt *et al.*[70]). ANP: atrial natriuretic peptide; BNP: B-type natriuretic peptide; CNP: C-type natriuretic peptide.

Receptor	*Ligands*	*Functions*
GC-A	ANP, BNP	Decrease in arterial blood pressure and volume, inhibition of cardiomyocyte growth and cardiac fibrosis
GC-B	CNP	Inhibition of cardiomyocyte growth, vascular regeneration, endochondral ossification
GC-C	Heat-stable enterotoxins, guanylin, uroguanylin	Increased intestinal electrolyte and water transport, epithelial cell growth and differentiation, diuresis, natriuresis?
GC-D	Guanylin, uroguanylin	Pheromone detection?, salt and water homeostasis?
GC-E	Orphan	Vision, survival of cones
GC-F	Orphan	Vision
GC-G	Orphan	Renal protection?

1.3.2 Effectors of Cyclic 3':5'-Guanosine Monophosphate

cGMP synthesized by GCs can regulate cellular functions *via* interaction with different effectors (see Fig. 1.9): cyclic nucleotide-gated (CNG) channels, cGMP-dependent protein kinases (cGKs), and phosphodiesterases (PDEs).

CNG channels belong to the superfamily of pore-loop channels. The topology of CNG channel subunits is characterized by six transmembrane domains (S1-S6), a reentrant pore loop between S5 and S6, and cytosolic amino- and carboxy-termini. CNG channels exist as tetramers with four subunits arranged around a central pore[71]. CNG channels are ion channels which are activated by the binding of cGMP or cAMP to a cyclic nucleotide binding domain (CNBD). They translate changes of intracellular concentrations of cyclic nucleotides into changes of membrane potential and Ca^{2+} concentration. CNG channels have been originally discovered in photoreceptors and olfactory receptor neurons where they play a central role in sensory transduction[72]. For example, in rods and cones the CNG channel is kept in an open state in presence of a high cGMP concentration in the dark. The resulting influx of Ca^{2+} and Na^{+} depolarizes the photoreceptor. After activation of rhodopsin by light, cGMP is hydrolyzed by the action of a PDE. The lower cGMP concentration leads to the closure of the CNG channel, resulting in membrane hyperpolarization[73]. In contrast to phototransduction, olfactory transduction is significantly more diverse[74].

cGKs are serine/threonine kinases widely distributed in eukaryotes. cGKI and cGKII are encoded by the cGK genes *prkg1* and *prkg2*, respectively. In mammals, the two isozymes cGKIα and cGKIβ are present[75]. cGKs are composed of two domains, a regulatory (R) domain and a catalytic (C) domain. The R domain consists of the amino-terminal and the cGMP-binding domain. The latter is further divided into the high (cGMP I)- and low (cGMP II)-affinity binding pocket. Upon cGMP binding in both pockets, a pronounced change in secondary structure occurs resulting in a more elongated structure[76]. As a consequence, the inhibition of the catalytic domain containing the MgATP- and peptide-binding pockets is released and phosphorylation of serine and threonine residues in target proteins can take place. The main function of the amino-terminal region is dimerization of cGK monomers through a leucine zipper and the targeting of cGKs to specific subcellular localizations[77]. The identification of cGK substrates is essential in order to understand the underlying signaling mechanisms. Various cGK substrates have been found, and the knowledge of cGK effects in different

processes such as smooth and cardiac muscle relaxation and proliferation, inhibition of platelet function and hippocampal learning could be expanded[78].

Since the discovery of PDE activity in 1962, the PDE family has been expanded continuously. Currently, 11 gene families based on their sequence homologies and functional characteristics with a total of 21 genes are described[79]. On the one hand, PDEs hydrolyze cAMP or cGMP: PDE5, PDE6, and PDE9 selectively break down cGMP whereas PDE4, PDE7, and PDE8 hydrolyze cAMP. The remaining PDEs (PDE1, PDE2, PDE3, PDE10, PDE11) exhibit dual enzymatic specificity and accept both cyclic nucleotides as substrate. On the other hand, PDEs function as downstream effectors in the cGMP signaling system. Thereby, PDEs can be regulated either directly by binding of cGMP to regulatory domains or indirectly *via* phosphorylation events induced by interaction of cGMP with cGKs. Phosphorylation can induce both stimulation and inhibition of PDE activity. Moreover, cross-talk between cGMP and cAMP signaling can occur. The activity of PDEs catalyzing the hydrolysis of cAMP can be regulated in a cGMP-dependent manner or vice versa[80]. Thus, the levels of cyclic nucleotides can be fine-tuned and even small changes in cyclic nucleotide concentrations can be signaled. Diverse processes are related to cGMP signaling such as contraction of cardiac and smooth muscle, platelet aggregation, secretion, immune cell response and inflammation, neuronal excitability and synaptic plasticity[78].

1.4 Di-Guanylate Cyclases

1.4.1 Structure and Function of Di-Guanylate Cyclases

More than 20 years ago, the presence of cyclic 3':5'-di-guanosine monophosphate (c-di-GMP) in bacteria was discovered by Ross *et al.*[81] Since then, c-di-GMP has emerged as a ubiquitous second messenger in bacteria. c-di-GMP is synthesized from two GTP molecules by the action of di-guanylate cyclases (DGCs) and degraded by specific phosphodiesterases (PDEs) as indicated in Fig. 1.11. DGC and PDE activity are located in the GG(D/E)EF and EAL domain, respectively, which are named after highly conserved motifs within these domains[82]. EAL domain proteins degrade c-di-GMP to the linear intermediate pGpG which is further hydrolyzed to guanosine monophosphate (GMP) by so far unidentified PDEs. Apart from the EAL domain, a subgroup of the HD superfamily of metal-dependent phosphohydrolases, the so-called HD-GYP domain, was shown to

have c-di-GMP specific PDE-activity[83]. The HD-GYP domain has no sequence similarity to the EAL domain and, in contrast, readily hydrolyzes c-di-GMP to GMP. Regarding the different products of c-di-GMP degradation, one could speculate that pGpG is also an active signaling molecule providing additional complexity in cellular signaling. The number of GGDEF and EAL domain proteins encoded in bacterial genomes is highly variable ranging from none (e.g. *Helicobacter pylori*) to intermediate numbers (e.g. *Escherichia coli*: 19 GGDEF and 17 EAL domain proteins) up to 100 of these proteins in *Vibrio vulnificus*[6]. Obviously, GGDEF and EAL domain proteins are highly specific as the knock-outs of individual domain proteins result in distinct phenotypes despite the presence of other GGDEF, EAL, and/or HD-GYP domain proteins[84,85].

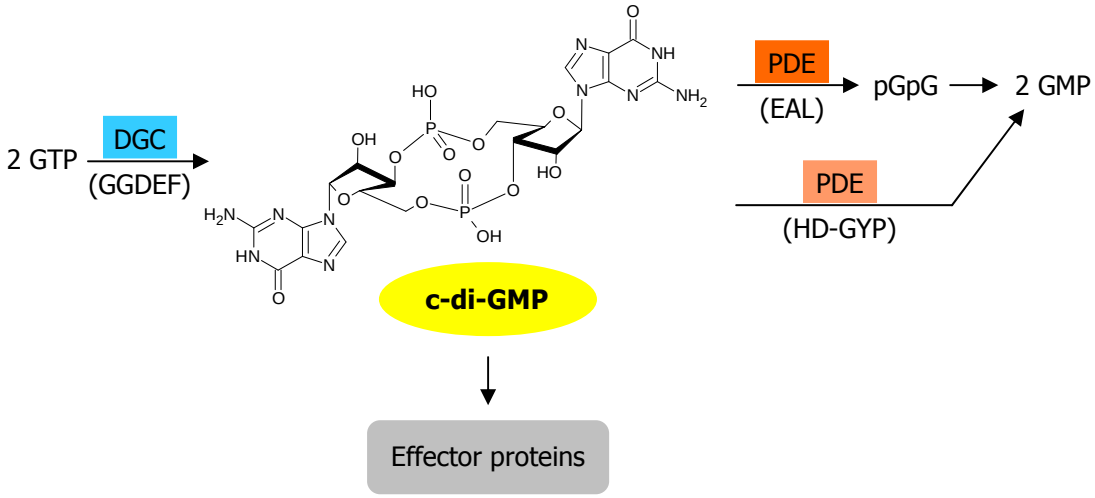


Fig. 1.11 c-di-GMP metabolism (modified from Tamayo *et al.*[86]). c-di-GMP is synthesized *via* condensation of two GTP molecules by GGDEF domain di-guanylate cyclases (DGCs) and hydrolyzed either by EAL domain phosphodiesterases (PDEs) to the linear intermediate pGpG which is further hydrolyzed by other PDEs to GMP or by HD-GYP domain PDEs which directly degrade c-di-GMP to GMP.

Detailed analyses of structure-function relationships of GGDEF domains showed that they have a pronounced secondary structure conservation with adenylyl cyclases (ACs) resulting in an AC-like fold and a similar catalytic mechanism[87]. As deduced from the crystal structure of the response regulator PleD, the DGC domain consists of a five-stranded central β-sheet surrounded by helices[88]. For the catalytic activity of DGCs, an anti-parallel arrangement of two substrate-loaded domains is required. Hence, DGCs function as dimers. Since c-di-GMP-metabolizing enzymes are central constituents of signal transduction, they are part of multi-domain proteins with one or more N-terminal signaling/receiving domains[89]. A combination of GGDEF and EAL domains is a frequently

found domain structure: more than one-third of the GGDEF and more than half of the EAL domains exist in this combination. A large variety of signals regulates the activity of DGCs and PDEs (see Fig. 1.12). A well-known characteristic of DGCs is the strong product inhibition resulting from c-di-GMP binding to the I-site located in close proximity to the catalytic A-site. Allosteric feedback inhibition of DGCs is thought to be a general regulatory principle of c-di-GMP signaling in bacteria.

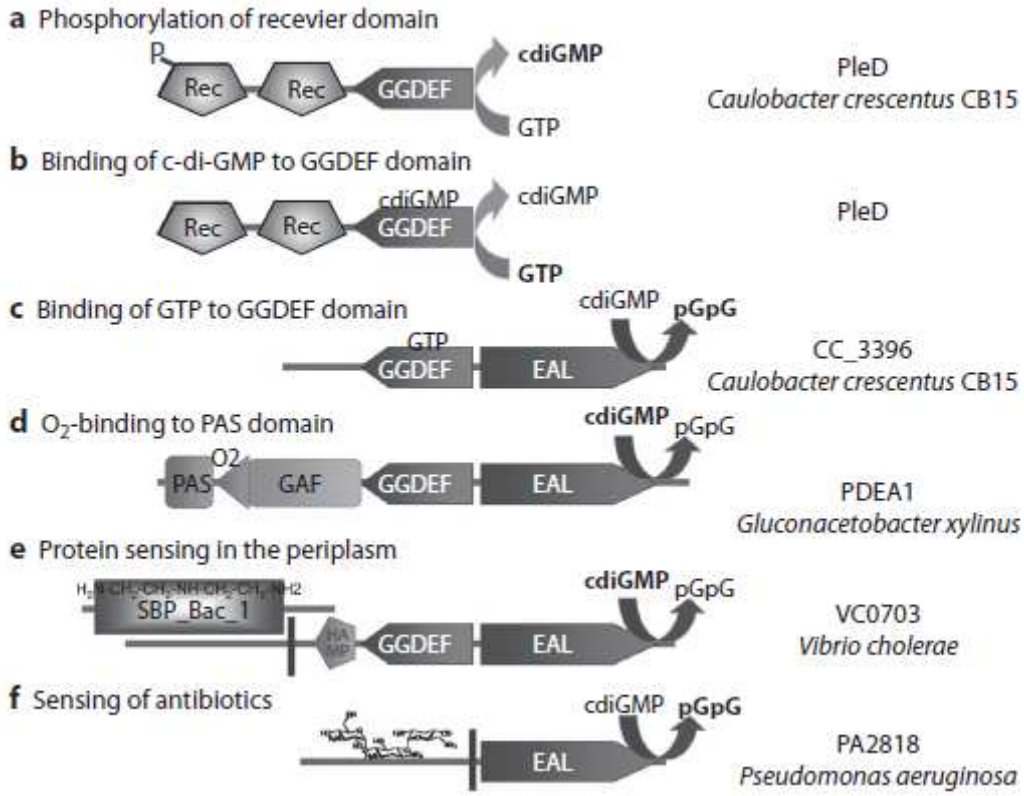


Fig. 1.12 Regulation of di-guanylate cyclases (DGCs) and phosphodiesterases (PDEs) (taken from Römling and Simm[90]). (**a**) Phosphorylation of the receiver domain (Rec) of response regulators activates the DGC function of GGDEF domains. (**b**) Binding of c-di-GMP to the I-site of the GGDEF domain leads to allosteric product inhibition of DGC activity. (**c**) Binding of GTP to a GGDEF domain with degenerate GGDEF motif activates the PDE function of EAL domains. (**d**) Oxygen binding to the PAS domain inhibits the PDE A1 in *Gluconacetobacter xylinus*. (**e**) Sensing of the substrate-loaded, putative norspermidine-binding protein by the GGDEF-EAL domain protein MbaA in *Vibrio cholerae* reduces the ability of MbaA to inhibit biofilm formation. (**f**) The EAL domain protein Arr in *Pseudomonas aeruginosa* senses the antibiotic tobramycin. GAF: nucleotide/nucleoside-binding domain; SBP_Bac_1: solute-binding protein family 1.

Environmental stimuli are transmitted by the c-di-GMP signaling network and, thereby, evoke specific responses of bacterial cells. So far, several direct targets for c-di-GMP have been identified. Particularly, the PilZ domain was the first c-di-GMP effector protein discovered and represents a family of c-di-GMP binding proteins combined with various N-terminal domains[91]. PilZ domains exhibit high sequence diversity and thus trigger diverse downstream physiological events: BcsA and YcgR are examples of PilZ domains representing a c-di-GMP-dependent bacterial cellulose synthase and a protein regulating flagellum-based motility, respectively[92]. The recently discovered PilZ domain protein PlzA from *Borrelia burgdorferi* is expressed during mammalian infection[93].

Moreover, c-di-GMP effectors other than PilZ domain proteins are being discovered consecutively: the transcriptional regulator FleQ[94] and the regulator of polysaccharide biosynthesis PelD[95], both encoded by *Pseudomonas aeruginosa*, and the c-di-GMP effector protein PopA[96] involved in cell cycle progression have been identified as c-di-GMP targets. The global regulator Clp from *Xanthonomas campestris* which positively regulates virulence factor production strongly interacts with c-di-GMP[97]. Eventually, c-di-GMP was found to bind to riboswitches in the untranslated regions of mRNAs and thus alters the expression of downstream genes[98].

During the last years of c-di-GMP research it has become obvious that c-di-GMP controls cellular functions at the transcriptional, translational and posttranslational level. However, researchers are just beginning to understand the complex c-di-GMP signaling network as accurately described by Römling and Simm: "Still, we have only scratched the surface in this new area of research."[90]

1.4.2 Formation and Characterization of Biofilms

During the last decades, it has become clear that most bacteria predominantly exist in complex communities, so-called biofilms[99]. The formation of biofilms is strongly influenced by intracellular c-di-GMP concentrations. In general, high c-di-GMP concentrations induce the formation of biofilms, whereas low concentrations favor a motile, planctonic lifestyle[100]. Biofilms can form in an enormous number of environments and can colonize on both biological and non-biological surfaces[101]. The formation of biofilms is a multi-step process specifically characterized by the formation of a robust exopolysaccharide (EPS) matrix as indicated in Fig 1.13[102].

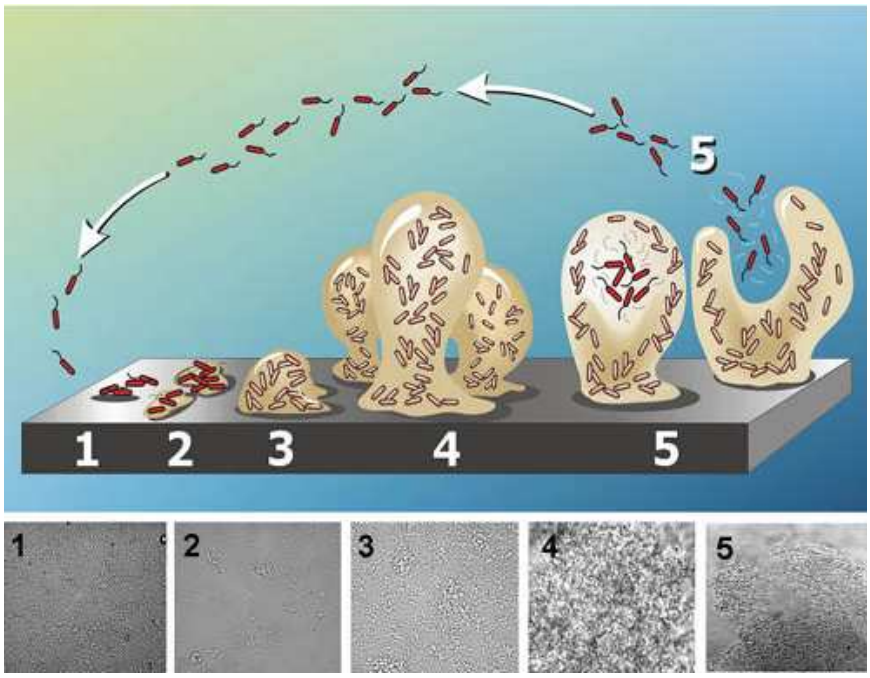


Fig 1.13 Development of a biofilm (http://www.genomenewsnetwork.org/articles/06_02/biofilms_image1.shtml, 06-10-2010). (1) Initial attachment of planctonic cells to the surface, (2) beginning production of exopolysaccharide (EPS) matrix and irreversible attachment to the surface, (3) development of biofilm architecture, (4) maturation of biofilm matrix, and (5) dispersion of single cells from the biofilm. The bottom photomicrographs show the five stages of biofilm formation of *Pseudomonas aeruginosa* grown on a glass substratum.

In natural environments, biofilms usually consist of numerous different bacterial species each of which finds its own niche within the biofilm[103]. The formation of biofilms is based on effective cell-to-cell communication, called quorum sensing, *via* extracellular signaling molecules[104]. The nature of these quorum sensing or autoinducer molecules is thought to be very diverse. Gram-negative bacteria are known to mainly use *N*-acyl-L-homoserine lactones (AHLs) to communicate with each other whereas furanosyl borate diesters are inter-species autoinducer molecules. Bacteria existing in robust biofilms cause persistent and chronic infections with inherent resistance to antibiotics[105]. Numerous human infections can be ascribed to the establishment of biofilms as indicated in Table 1.3. For example, the human pathogen *Pseudomonas aeruginosa* colonizes the airways of cystic fibrosis patients, thereby causing severe lung damages. Due to the biofilm architecture of the pathogen even long-term antibiotic therapy cannot eradicate the infection[106]. Moreover, biofilm forming bacteria can cause infections in patients with indwelling medical devices which is a serious problem for public health[107].

Table 1.3 Selection of human infections involving biofilms (modified from Costerton *et al.*[105]).

Infection or disease	*Common biofilm bacterial species*
Dental caries	Acidogenic Gram-positive cocci (e.g. *Streptococcus*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Non-typable strains of *Haemophilus influenzae*
Musculoskeletal infections	Gram-positive cocci (e.g. staphylococci)
Necrotizing fasciitis	Group A streptococci
Biliary tract infection	Enteric bacteria (e.g. *Escherichia coli*)
Osteomyelitis	Various bacterial and fungal species - often mixed
Bacterial prostatitis	*Escherichia coli* and other Gram-negative bacteria
Native valve endocarditis	Viridans group streptococci
Cystic fibrosis pneumonia	*Pseudomonas aeruginosa* and *Burkholderia cepacia*
Meloidosis	*Pseudomonas pseudomallei*

Facing the high number of biofilm-related infections and diseases it is necessary to elucidate the mechanisms of antibiotic resistance of bacteria in biofilms in order to develop new therapies. However, researchers are just beginning to find out the exact mechanisms[108,109]. A first hypothesis deals with slow or incomplete penetration of antibiotics into the biofilm. The antibiotic is more rapidly deactivated in the surface layer than it diffuses. Hence, its delivery into the core of the biofilm is retarded. Secondly, it is believed that biofilms have an altered microenvironment. Apart from anaerobic niches which can appear due to high oxygen comsumption, pronounced pH differences between the bulk fluid and the biofilm interior can be produced as a cause of local accumulation of waste products. These extreme environments can antagonize the action of antibiotics. As a result of nutrient depletion, bacteria within the biofilm are also believed to enter a non-growing state in which they cannot be attacked by antibiotics which often target cell-wall synthesis. The third and most speculative hypothesis is that bacteria in biofilms are able to differentiate into a protected phenotypic state, so-called persisters. Yet, as stated by Mah and O'Toole[110] "There is no one answer to the question of why and how bacteria growing in a biofilm develop increased resistance to antimicrobial agents."

1.5 Methods for the Analysis of GTP-Converting Proteins and Enzymes

The GTP-converting proteins and enzymes introduced in the previous sections are central constituents of specific signal transduction pathways. Since the discovery of the individual signaling systems, effective detection and quantitation methods for the respective signal molecules have been established. Facing the high complexity of signal transduction pathways, their detailed elucidation has challenged the scientific community. Today, a broad spectrum of methods for the analysis of GTP-based signaling systems is available. Table 1.4 provides an overview of the most applied methods and addresses corresponding advantages and disadvantages.

One of the first methods developed which is still used today, is based on radioactively labeled substrates ([^{32}P]- and [^{33}P]-labeled GTP) applied for *in vitro* enzyme assays[111,112,113]. Despite high sensitivity, the risks arising from the handling of radioactive material have led to the development of alternative methods. The introduction of column-chromatographic separation and purification techniques have been an important step resulting in the establishment of robust HPLC-UV detection methods which can be automated but have relatively high detection limits[114,115,116]. Fluorescently labeled nucleotides enabled the detection of the interaction of those nucleotides with specific proteins due to the sensitivity of fluorescent groups to their surrounding environment[117]. Coupling of HPLC separation to mass spectrometric analysis is very sensitive and allows simultaneous and highly specific detection and quantitation of signaling molecules[118]. Specifically for the detection of cGMP, competition-binding approaches are used: radio immuno assay (RIA)[119] and enzyme-linked immunosorbent assay (ELISA)[120]. These techniques became available after the devolpment of specific anti-cGMP antibodies. The read-out is based on the detection of either radioactively labeld cGMP (RIA) or the products of enzymatic reactions coupled to secondary antibodies.

Each analysis method has distinct advantages and disadvantages for a specific scientific question. However, as we elucidate more and more details of GTP signaling networks the analytical requirements continuously increase. Hence, there still is a great need for the development of more sensitive, robust, and reliable detection and quantitation methods in this field of research.

Table 1.4 Detection methods for GTP-converting proteins and enzymes. HPLC: high-performance liquid chromatography; MS: mass spectrometry; RIA: radio immuno assay; ELISA: enzyme-linked immunosorbent assay DGC: di-guanylate cyclase; GC: guanylyl cyclase.

Method	*Protein/enzyme*	*Advantages (+) and disadvantages (-)*
[^{32}P]/[^{33}P]-labeled substrates	GTP-binding proteins[112,121,122] DGCs[123,113] GCs[111,124]	+ high sensitivity + direct detection of nucleotides - radioactivity: demanding special training/facility - waste disposal
HPLC-UV	GTP-binding proteins[114] DGCs[116] GCs[115]	+ direct detection of nucleotide + non-radioactive - low sensitivity - slow sample processing
MS	DGCs[125,126,94,127] GCs[118]	+ high sensitivity + high specificity + direct detection of nucleotides - expensive equipment - slow sample throughput
Fluorescence	GTP-binding proteins[117,128]	+ high sensitivity + high sample throughput - derivatization necessary - interference with matrix components
RIA	GCs[119]	+ high sensitivity + high specificity - radioactivity: demanding special training/facility - laborious processing of samples prior to assay - cross-reactivity - antibody needed
ELISA	GCs[120]	+ high sensitivity + high specificity + non-radioactive - expensive kits - cross-reactivity - antibody needed

1.6 References

1 Nelson D, Cox M (2001) Lehninger Biochemie. *3rd Completely Revised Edition*, Springer-Verlag Berlin-Heidelberg-New York, 611-639

2 Nelson D, Cox M (2001) Lehninger Biochemie. *3rd Completely Revised Edition*, Springer-Verlag Berlin-Heidelberg-New York, 1126-1152

[3] Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. *Physiol Rev* **81:** 153-208

[4] Milligan G, Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* **147:** S46-S55

[5] Schmidt HHHW, Hofmann F, Stasch J-P (2009) cGMP: Generators, effectors and therapeutic implications. *Handbook of Experimental Pharmacology* **191**, Springer-Verlag Berlin-Heidelberg

[6] Jenal U, Malone J (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* **40:** 385-407

[7] Bourne HR, Sanders DA, McCormick F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* **348:** 125-132

[8] Hall A (1990) The cellular functions of small GTP-binding proteins. *Science* **249:** 635-640

[9] Garcia-Ranea JA, Valencia A (1998) Distribution and functional diversification of the ras superfamily in *Saccharomyces cerevisiae*. *FEBS Lett* **434:** 219-225

[10] Bourne HR, Sanders DA, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature* **349:** 117-127

[11] Valencia A, Chardin P, Wittinghofer A, Sander C (1991) The ras protein family: evolutionary tree and role of conserved amino acids. *Biochemistry* **30:** 4637-4648

[12] Willumsen BM, Christensen A, Hubbert NL, Papageorge AG, Lowy DR (1984) The p21 *ras* C-terminus is required for transformation and membrane association. *Nature* **310:** 583-586

[13] Wittinghofer A, Waldmann H (2000) Ras – a molecular switch involved in tumor formation. *Angew Chem Int Ed* **39:** 4192-4214

[14] Boguski MS, McCormick F (1993) Proteins regulating Ras and its relatives. *Nature* **366:** 643-654

[15] Wada M, Nakanishi H, Satoh A, Hirano H, Obaishi H, Matsuura Y, Takai Y (1997) Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. *J Biol Chem* **272:** 3875-3878

[16] Fukui K, Sasaki T, Imazumi K, Matsuura Y, Nakanishi H, Takai Y (1997) Isolation and characterization of a GTPase activating protein specific for the Rab3 subfamily of small G proteins. *J Biol Chem* **272:** 4655-4658

[17] Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363:** 45-51

[18] Van Aelst L, Barr M, Marcus S, Polverino A, Wigler M (1993) Complex formation between RAS and RAF and other protein kinases. *Proc Natl Acad Sci USA* **90:** 6213-6217

[19] Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, Avruch J (1992) Raf-1 activates MAP kinase-kinase. *Nature* **358:** 417-421

[20] Crews CM, Alessandrini A, Erikson RL (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* **258:** 478-480

[21] Marais R, Wynne J, Treisman R (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73:** 381-393

[22] Barbacid M (1987) *ras* genes. *Annu Rev Biochem* **56:** 779-827

[23] Vojtek AB, Der CJ (1998) Increasing complexity of the Ras signalling pathway. *J Biol Chem* **273:** 19925-19928

[24] Nobes CD, Hall A (1995) Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibres, lamellipodia, and filopodia. *Cell* **81:** 53-62

[25] Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81:** 1137-1146

[26] Perona R, Montaner S, Saniger L, Sánchez-Pérez I, Bravo R, Lacal JC (1997) Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev* **11:** 463-475

[27] Zigmond SH (1996) Signal transduction and actin filament organization. *Curr Opin Cell Biol* **8:** 66-73

[28] Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70:** 389-399

[29] Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70:** 401-410

[30] Kozma R, Ahmed S, Best A, Lim L (1995) The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol Cell Biol* **15:** 1942-1952

[31] Lyle AN, Deshpande NN, Taniyama Y, Seidel-Rogol B, Pounkova L, Du P, Papaharalambus C, Lassègue B, Griendling KK (2009) Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. *Circ Res* **105:** 249-259

[32] Nozumi M, Nakagawa H, Miki H, Takenawa T, Miyamoto S (2002) Differential localization of WAVE isoforms in filopodia and lamellipodia of the neuronal growth cone. *J Cell Science* **116:** 239-246

[33] Schimmöller F, Simon I, Pfeffer SR (1998) Rab GTPases, directors of vesicle docking. *J Biol Chem* **273:** 22161-22164

[34] Leung KF, Baron R, Seabra MC (2006) Geranylgeranylation of Rab GTPases. *J Lipid Res* **47:** 467-475

[35] Novick P, Brennwald P (1993) Friends and family: the role of the Rab GTPases in vesicular traffic. *Cell* **75:** 597-601

[36] Moss J, Vaughan M (1998) Molecules in the ARF orbit. *J Biol Chem* **273:** 21431-21434

[37] Schekman R, Orci L (1996) Coat proteins and vesicle budding. *Science* **271:** 1526-1533

[38] Melchior F, Paschal B, Evans J, Gerace L (1993) Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J Cell Biol* **123:** 1649-1659

[39] Sorokin AV, Kim ER, Ovchinnikov LP (2007) Nucleocytoplasmic transport of proteins. *Biochemistry* **72:** 1439-1457

[40] Ohno M, Fornerod M, Mattaj IW (1998) Nucleocytoplasmic transport: the last 200 nanometers. *Cell* **92:**327-336

[41] Hamm HE (1998) The many faces of G protein signaling. *J Biol Chem* **273:** 669-672

[42] Strader CD, Fong TM, Tota MR, Underwood D (1994) Structure and function of G protein-coupled receptors. *Annu Rev Biochem* **63:** 101-132

[43] Milligan G, Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Brit J Pharmacol* **147:** S46- S55

[44] Wittinghofer A (1994) The structure of transducin $G_{\alpha t}$: more to view than just Ras. *Cell* **76:** 201-204

[45] Dohlmann HG, Thorner J (1997) RGS proteins and signaling by heterotrimeric G proteins. *J Biol Chem* **272:** 3871-3874

[46] Northup JK, Sternweis PC, Smigel MD, Schleifer LS, Ross EM, Gilman AG (1980) Purification of the regulatory component of adenylate cyclase. *Proc Natl Acad Sci USA* **77:** 6516-6520

[47] Rodbell M (1995) Signal transduction: evolution of an idea. *Biosci Rep* **15:** 117-133

[48] Gilman AG (1995) G-proteins and regulation of adenylyl cyclase. *Biosci Rep* **15:** 65-97

[49] Simon MI, Strathmann MP, Gautam N (1991) Diversity of G proteins in signal transduction. *Science* **252:** 802-808

[50] Rens-Domiano S, Hamm HE (1995) Structural and functional relationships of heterotrimeric G-proteins. *FASEB J* **9:** 1059-1066

[51] Riobo NA, Manning DR (2005) Receptors coupled to heterotrimeric G-proteins of the G_{12} family. *Trends Pharmacol Sci* **26:** 146-154

[52] Kjeldgaard M, Nyborg J, Clark BF (1996) The GTP binding motif: variations on a theme. *FASEB J* **10:** 1347-1368

[53] Noel JP, Hamm HE, Sigler PB (1993) The 2.2 Å crystal structure of transducin-α complexed with GTPγS. *Nature* **366:** 654-663

[54] Wedegaertner PB, Wilson PT, Bourne HR (1995) Lipid modifications of trimeric G proteins. *J Biol Chem* **270:** 503-506

[55] Hamm HE, Gilchrist A (1996) Heterotrimeric G proteins. *Curr Opin Cell Biol* **8:** 189-196

[56] Casey PJ (1995) Protein lipidation in cell signaling. *Science* **268:** 221-225

[57] Hardman JG, Sutherland EW (1969) Guanylyl cyclase, an enzyme catalyzing the formation of guanosine 3',5'-monophosphate from guanosine triphosphate. *J Biol Chem* **244:** 6363-6370

[58] Chrisman TD, Garbers DL, Parks MA, Hardman JG (1975) Characterization of particulate and soluble guanylate cyclases from rat lung. *J Biol Chem* **250:** 374-381

[59] Feil R, Kemp-Harper B (2006) cGMP signalling: from bench to bedside. *EMBO reports* **7:** 149-153

[60] Poulus TL (2006) Soluble guanylate cyclase. *Curr Opin Struct Biol* **16:** 736-743

[61] Harteneck C, Koesling D, Söling A, Schultz G, Böhme E (1990) Expression of soluble guanylyl cyclase: catalytic activity requires two enzyme subunits. *FEBS Lett* **272:** 221-223

[62] Zabel U, Häusler C, Weeger M, Schmidt HHHW (1999) Homodimerization of soluble guanylyl cyclase subunits: dimerization analysis using a glutathione *S*-transferase affinity tag. *J Biol Chem* **274:** 18149-18152

[63] Friebe A, Koesling D (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res* **93:** 96-105

[64] Wedel B, Humbert P, Harteneck C, Foerster J, Malkewitz J, Böhme E, Schultz G, Koesling D (1994) Mutation of His-105 in the β_1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc Natl Acad Sci USA* **91:** 2592-2596

[65] Wolin MS, Wood KS, Ignarro LJ (1982) Guanylate cyclase from bovine lung. A kinetic analysis of the regulation of the purified soluble enzyme by protoporphyrin IX, heme, and nitrosyl-heme. *J Biol Chem* **257:** 13312-13320

[66] Kuhn M (2003) Structure, regulation, and function of mammalian membrane guanylyl cyclase receptors, with a focus on guanylyl cylcase-A. *Circ Res* **93:** 700-709

[67] Chinkers M, Garbers DL (1989) The protein kinase domain of the ANP receptor is required for signaling. *Science* **245:** 1392-1394

[68] Kumar R, Grammatikakis N, Chinkers M (2001) Regulation of the atrial natriuretic peptide receptor by heat shock protein 90 complexes. *J Biol Chem* **276:** 11371-11375

[69] Kuhn M (2009) Function and dysfunction of mammalian membrane guanylyl cyclase receptors: lessons from genetic mouse models and implications for human diseases. *Schmidt HHHW, Hofmann F, Stasch J-P: cGMP: Generators, effectors and therapeutic implications. Handbook of Experimental Pharmacology* **191**, Springer-Verlag Berlin-Heidelberg, 47-48

[70] Kuhn M (2009) Function and dysfunction of mammalian membrane guanylyl cyclase receptors: lessons from genetic mouse models and implications for human diseases. *Schmidt HHHW, Hofmann F, Stasch J-P: cGMP: Generators, effectors and therapeutic implications. Handbook of Experimental Pharmacology* **191**, Springer-Verlag Berlin-Heidelberg, 48-51

[71] Higgins MK, Weitz D, Warne T, Schertler GFX, Kaupp UB (2002) Molecular architecture of a retinal cGMP-gated channel: the arrangement of the cytoplasmic domains. *EMBO J* **21:** 2087-2094

[72] Kaupp UB, Seifert R (2002) Cyclic nucleotide-gated ion channels. *Physiol Rev* **82:** 769-824

[73] Fu Y, Yau KW (2007) Phototransduction in mouse rods and cones. *Pflugers Arch – Eur J Physiol* **454:** 805-819

[74] Ma M (2007) Encoding olfactory signals via multiple chemosensory systems. *Crit Rev Biochem Mol Biol* **42:** 463-480

[75] Pfeifer A, Ruth P, Dostmann W, Sausbier M, Klatt P, Hofmann F (1999) Structure and function of cGMP-dependent protein kinases. *Rev Physiol Biochem Pharmacol* **135:** 105-149

[76] Wall ME, Francis SH, Corbin JD, Grimes K, Richie-Jannetta R, Kotera J, Macdonald BA, Gibson RR, Trewhella J (2003) Mechanisms associated with cGMP binding and activation of cGMP-dependent protein kinase. *Proc Natl Acad Sci USA* **100:** 2380-2385

[77] Hofmann F, Bernhard D, Lukowski R, Weinmeister P (2009) cGMP regulated protein kinases (cGK). *Schmidt HHHW, Hofmann F, Stasch J-P: cGMP: Generators, effectors and therapeutic implications. Handbook of Experimental Pharmacology* **191**, Springer-Verlag Berlin-Heidelberg, 139-141

[78] Hofmann F, Feil R, Kleppisch T, Schlossmann J (2006) Function of cGMP-dependent protein kinases as revealed by gene deletion. *Physiol Rev* **86:** 1-23

[79] Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* **58:** 488-520

[80] Kleppisch T (2009) Phosphodiesterases in the central nervous system. *Schmidt HHHW, Hofmann F, Stasch J-P: cGMP: Generators, effectors and therapeutic implications. Handbook of Experimental Pharmacology* **191**, Springer-Verlag Berlin-Heidelberg, 73-74

[81] Ross P, Weinhouse A, Aloni Y, Michaeli D, Weinberger-Ohana P, Mayer R, Braun S, de Vroom E, van der Marel GA, van Boom JH, Benziman M (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* **325:** 279-281

[82] Tal R, Wong HC, Calhoon R, Gelfand D, Fear AL, Volman G, Mayer R, Ross P, Amikam D, Weinhouse H, Cohan A, Sapir S, Ohana P, Benziman M, (1998) Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. *J Bacteriol* **180:** 4416-4425

[83] Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Cámara M, Williams P, Dow JM (2006) Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc Natl Acad Sci USA* **103:** 6712-6717

[84] Kirillina O, Fetherston JD, Bobrov AG, Abney J, Perry RD (2004) HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. *Mol Microbiol* **54:** 75-88

[85] Römling U, Rohde M, Olsén A, Normark S, Reinköster J (2000) *AgfD*, the checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways. *Mol Microbiol* **36:** 10-23

[86] Tamayo R, Pratt JT, Camilli A (2007) Roles of cylic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol* **61:** 131-148

[87] Pei J, Grishin NV (2001) GGDEF domain is homologous to adenylyl cyclase. *Protein Struct Funct Genet* **42:** 210-216

[88] Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T (2004) Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci* **101:** 17084-17089

[89] Römling U, Gomelsky M, Galperin MY (2005) c-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* **57:** 629-639

[90] Römling U, Simm R (2009) Prevailing concepts of c-di-GMP signaling. *Contrib Microbiol* **16:** 161-181

[91] Amikam D, Galperin MY (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* **22:** 3-6

[92] Ryjenkov DA, Simm R, Römling U, Gomelsky M (2006) The PilZ domain is a receptor for the second messenger c-di-GMP. The PilZ domain protein YcgR controls motility in enterobacteria. *J Biol Chem* **281:** 30310-30314

[93] Freedman JC, Rogers EA, Kostick JL, Zhang H, Iyer R, Schwartz I, Marconi RT (2010) Identification and molecular characterization of a cyclic-di-GMP effector protein, PlzA (BB0733): additional evidence for the existence of a functional cyclic-di-GMP

regulatory network in the Lyme disease spirochete, *Borrelia burgdorferi*. *FEMS Immunol Med Microbiol* **58:** 285-294

[94] Hickman JW, Harwood CS (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* **69:** 376-389

[95] Lee VT, Matewish JM, Kessler JL, Hyodo M, Hayakawa Y, Lory S (2007) A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Mol Microbiol* **65:** 1474-1484

[96] Duerig A, Abel S, Folcher M, Nicollier M, Schwede T, Amiot N, Giese B, Jenal U (2009) Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. *Genes Dev* **23:** 93-104

[97] Tao F, He YW, Wu DH, Swarup S, Zhang LH (2010) The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. *J Bacteriol* **192:** 1020-1029

[98] Sudarsan N, Lee ER, Weinberg Z, Moy RH, Kim JN, Link KH, Breaker RR (2008) Riboswitches in eubacteria sense the second messenger cyclic diGMP. *Science* **321:** 411-413

[99] Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15:** 167-193

[100] Simm R, Morr M, Kader A, Nimtz M, Römling U (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol* **53:** 1123-1134

[101] Costerton JW, Lewandowski Z (1995) Microbial biofilms. *Annu Rev Microbiol* **49:** 711-745

[102] Stoodley P, Sauer K, Davis DG, Costerton JW (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* **56:** 187-209

[103] Watnick P, Kolter R (2000) Biofilm, city of microbes. *J Bacteriol* **182:** 2675-2679

[104] Dickschat JS (2010) Quorum sensing and bacterial biofilms. *Nat Prod Rep* **27:** 343-369

[105] Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284:** 1318-1322

[106] Høiby N (1993) Antibiotic therapy for chronic infection of *Pseudomonas* in the lung. *Annu Rev Med* **44:** 1-10

[107] Donlan RM (2001) Biofilm formation: a clinically relevant microbiological process. *Healthcare Epidemiology* **33:** 1378-1392

[108] Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358:** 135-138

[109] Stewart PS (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* **292:** 107-113

[110] Mah TFC, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9:** 34-39

[111] White AA, Aurbach GD (1969) Detection of guanylyl cyclase in mammalian tissues. *Biochim Biophys Acta* **191:** 686-697

[112] Brandt DR, Ross EM (1986) Catecholamine-stimulated GTPase cycle. Multiple sites of regulation by β-adrenergic receptor and Mg^{2+} studied in reconstituted receptor-G_s vesicles. *J Biol Chem* **261:** 1656-1664

[113] Kumar M, Chatterji D (2008) Cyclic di-GMP: a second messenger required for long-term survival, but not for biofilm formation, in *Mycobacterium smegmatis*. *Microbiology* **154:** 2942-2955

[114] Schweins T, Geyer M, Scheffzek K, Warshel A, Kalbitzer HR, Wittinghofer A (1995) Substrate-assisted catalysis as a mechanism for GTP hydrolysis of $p21^{ras}$ and other GTP-binding proteins. *Nat Struct Biol* **2:** 36-44

[115] Pietta PG, Mauri PL, Gardana C, Benazzi L (1997) Assay of soluble guanylate cyclase activity by isocratic high-performance liquid chromatography. *J Chromatogr B* **690:** 343-347

[116] Schmidt AJ, Ryjenkov DA, Gomelsky M (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol* **187:** 4774-4781

[117] Neal SE, Eccleston JF, Webb MR (1990) Hydrolysis of GTP by $p21^{NRAS}$, the *NRAS* protooncogene product, is accompanied by a conformational change in the wild-type protein: use of a single fluorescent probe at the catalytic site. *Proc Natl Acad Sci USA* **87:** 3562-3565

[118] Lorenzetti R, Lilla S, Donato JL, de Nucci G (2007) Simultaneous quantification of GMP, AMP, cyclic GMP and cyclic AMP by liquid chromatography coupled to tandem mass spectrometry. *J Chromatogr B* **859:** 37-41

[119] Steiner AL, Wehmann RE, Parker CW, Kipnis DM (1972) Radioimmunoassay for the measurement of cyclic nucleotides. *Adv Cyclic Nucleotide Res* **2:** 51-61

[120] Wellard J, Blind B, Hamprecht B (2004) An enzyme-linked immunosorbent assay for the rapid quantification of intracellular and extracellular guanosine 3',5'-cyclic monophosphate in cultured glial cells. *Neurochem Res* **29:** 2177-2187

[121] Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA (1990) Stimulation of p21*ras* upon T-cell activation. *Nature* **346:** 719-723

[122] Rubio I, Pusch R, Wetzker R (2004) Quantification of absolute Ras-GDP/GTP levels by HPLC separation of Ras-bound [^{32}P]-labelled nucleotides. *J Biochem Biophys Methods* **58:** 111-117

[123] Paul R, Abel S, Wassmann P, Beck A, Heerklotz H, Jenal U (2007) Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. *J Biol Chem* **282:** 29170-29177

[124] Krishnan N, Krishna G (1976) A simple and sensitive assay for guanylate cyclase. *Anal Biochem* **70:** 18-31

[125] Simm R, Morr M, Remminghorst U, Andersson M, Römling U (2009) Quantitative determination of cyclic diguanosine monophosphate concentrations in nucleotide extracts of bacteria by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *Anal Biochem* **386:** 53-58

[126] Thormann KM, Duttler S, Saville RM, Hyodo M, Shukla S, Hayakawa Y, Spormann AM (2006) Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. *J Bacteriol* **188:** 2681-2691

[127] Waters CM, Lu W, Rabinowitz JD, Bassler BL (2008) Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of *vpsT*. *J Bacteriol* **190:** 2527-2536

[128] Jameson EE, Cunliffe JM, Neubig RR, Sunahara RK, Kennedy RT (2003) Detection of G proteins by affinity probe capillary electrophoresis using a fluorescently labeled GTP analogue. *Anal Chem* **75:** 4297-4304

2. Aim of Work

Fluorescence Assay for Ras

The development of cancer still poses a high risk for public health and concerns both children and adults. Most forms of cancer are very aggressive and have high morbidity rates. Successful therapies often depend on early recognition of the disease but at later stages effective treatment is difficult. The development of cancer is often characterized by the presence of mutated Ras proteins. In about 30% of all human tumors, mutated Ras oncogenes are present. Hence, much attention has been addressed to the elucidation of the mode of action and regulation of these small GTP-binding proteins, particularly with regard to the influence of various Ras regulators and their potential of altering Ras protein activity. A fast, simple and reliable detection method is a prerequisite for the examination of the effects of such regulators on Ras proteins. Hence, this work presents the development of a suitable, fluorescence-based *in vitro* assay for monitoring the activity of Ras protein mutants which can serve as robust platform for high-throughput screening experiments.

HPLC-MS/MS Assay for Di-Guanylate Cyclases

The establishment of chronic infections in different organs or tissues resulting in severe and partly lethal courses of disease also represents a major risk for public health. Acute infections often can be effectively treated by the application of antibiotics whereas chronic infections such as in cystic fibrosis patients are highly persistent and antibiotic treatment has only negligible success. Chronic infections are characterized by the formation of bacterial biofilms whose development is regulated by the concentration of the second messenger c-di-GMP. The detailed characterization of the c-di-GMP-signaling network is a prerequisite to understand the exact correlation of c-di-GMP concentration and biofilm formation. In this thesis, a very sensitive and highly specific HPLC-MS/MS quantification method for c-di-GMP is presented which is applicable to both *in vitro* and *in vivo* detection.

The development of effective antibiotics against biofilm formation is stagnant. Di-guanylate cyclases (DGCs) can serve as pharmacological targets in order to interfere with the c-di-GMP-signaling network in bacteria, and hence, the formation of persistent biofilms. Thus the interaction of a DGC with possible inhibitors based on a simple fluorescent assay and the HPLC-MS/MS method is presented.

3. Kinetic Determination of the GTPase Activity of Ras Proteins by Means of a Luminescent Terbium Complex

3.1 Abstract

Guanine nucleotide binding proteins, such as Ras proteins, play a pivotal role in maintaining the regular life cycle of cells. The involvement of Ras mutants in the progress of cancer has attracted many efforts to find detection methods for Ras activity. In this study we present a luminescent microwell plate assay for monitoring GTPase activity of Ras proteins. The luminescence intensity of the Tb-norfloxacin complex is influenced by nucleoside phosphates as well as by inorganic phosphates. Real-time kinetics of the GTPase activity of wildtype Ras and Ras mutants can be monitored online. The effect of a GTPase activating protein as well as of a downstream effector (Ras-binding domain of human Raf-1) on the GTPase activity of different Ras mutants is examined. In contrast to other methods, this assay does not require the use of radioactively labeled substrates or chromatographic separation steps. Moreover, the application of fluorescently labeled GTP substrates which often interfere with enzymatic activity can be avoided. This *in vitro* assay can serve as a model system for the screening of regulators affecting the GTPase activity of Ras proteins.

3.2 Introduction

Guanine nucleotide binding proteins of the Ras superfamily consist of several subunits with about 100 different proteins. One of the subunits comprises those of the so-called Ras (rat sarcoma) family. These proteins are central constituents of the signal transduction pathway, ranging from membrane receptors to downstream intracellular effectors, and thereby control cell growth, differentiation, and apoptosis[1]. Nowadays, it is estimated that in up to 30% of all human tumors mutated Ras oncogenes are present[2]. Ras GTPases act as binary switches by cycling between a GDP-bound "off" state and a GTP-bound "on" state. The intrinsic GTPase activity converts GTP to GDP and phosphate, the latter being released. Mg^{2+}-GDP and Mg^{2+}-GTP are coordinated by the active center of Ras in presence of an excess of Mg^{2+}. It is essential for Ras to be localized in the plasma membrane in order to accomplish its biological function. Therefore, several

posttranslational modification processes are necessary in which lipids are covalently attached to the carboxy-terminus of Ras. Ras proteins interact with downstream effectors such as Raf (rapidly growing fibrosarcoma) in the active, GTP-bound form. They activate specific signaling pathways such as the mitogen-activated protein kinase cascade. Since the intrinsic GTPase activity is only low and the associated GDP-GTP exchange process is intrinsically very slow, regulatory units determine Ras activity. Guanine nucleotide exchange factors (GEFs) activate the inactive, GDP-bound Ras by a fast GDP-GTP exchange process, whereas GTPase activating proteins (GAPs) accelerate GTP hydrolysis[3].

Mutated Ras proteins are widely involved in the development of cancer. This has aroused interest for Ras regulators as potential antitumor drugs. Mainly, four different approaches are pursued which aim at controlling Ras activity[4,5,6]: (1) Inhibition of Ras activation by preventing GDP-GTP exchange via GEFs, (2) deactivation of Ras by stimulating the intrinsic GTPase activity thereby avoiding interactions of the active, GTP bound form of Ras with its downstream effectors, (3) inhibition of Ras posttranslational modification by farnesylation, and (4) antagonizing Ras interactions with its downstream effectors. Hence, fast and straightforward methods for monitoring Ras activity and the influence of possible drugs are sought after.

Analytical techniques for the detection of Ras activity are based on the determination of the GTPase substrate and/or reaction products. Mainly, [^{32}P]-labeled nucleotides are applied for Ras activity assays. After immunopurification, Ras-bound nucleotides are eluted and a separation of GDP/GTP by thin layer chromatography (TLC) is carried out[7]. Scintillation counting is required for quantitative analysis. This procedure is very prone to errors and time-consuming. A more rapid analysis of absolute GDP/GTP levels is achieved by using high-performance liquid chromatography (HPLC). For this purpose, the radioactively labeled nucleotides are eluted after immunoprecipitation of Ras and separated on a reversed phase C-18 column. The eluted nucleotides are detected online by Čerenkov counting[8].

The GDP/GTP ratio can also be accessed by direct UV absorption of the nucleotides after HPLC separation with preceding denaturation of Ras as presented in "Materials and Methods". This method renders the use of labeled nucleotides redundant but is limited in terms of time resolution. Other non-radioactive techniques have been described which take advantage of the higher affinity of the Ras-binding domain (RBD) of Ras-effectors for the active, GTP-bound conformation of Ras[9,10]. A recombinant RBD is

added to cell extracts, thereby precipitating only Ras-GTP owing to a three orders of magnitude higher affinity compared to Ras-GDP. Ras is detected by Western blotting. However, this method can only reflect semi-quantitative results due to the high number of experimental steps necessary.

Moreover, fluorescent guanine nucleotide derivatives, namely 3'-*O*-(*N*-methylanthraniloyl (MANT)[11] and boron-dipyrromethene (BODIPY)[12] have been developed. MANT has been used for investigating basal and GAP-stimulated GTPase activity[13,14,15] as well as for studying interactions with GEFs[16,17] and effectors[18,19,20] whereas BODIPY was applied to Ras-like proteins to monitor guanine nucleotide exchange[21,22]. These approaches demonstrate the importance of fluorescence analysis and underline the efforts made in designing fluorescent probes for particular applications. Since fluorescence is very sensitive, many probes have been and still are being developed, especially for nucleoside phosphates and inorganic phosphates, which are most ubiquitous in biological systems. While numerous fluorescent probes for phosphate and pyrophosphate as well as for adenine nucleotides are available[23], probes for guanine nucleotides are rather rare[24]. Most probes cannot be used under physiological conditions which restricts their applicability.

Various lanthanide complexes have been reported to respond to phosphates and/or nucleotides, particularly Eu^{3+} [25,26,27] and Tb^{3+} [28,29] chelates. The great interest in lanthanide ions originates from their distinct spectral properties, such as line-like emission, long luminescence lifetime and a large Stokes shift. Most often, so-called antenna ligands are used for the coordination to overcome the small absorptivity of the ions themselves. The use of the pure lanthanide ion as a luminescent probe is rather rare[30]. A non-radiative energy transfer from the excited organic ligand to the central lanthanide ion results in a long wave emission although the complex is excited in the UV. Tb^{3+}-norfloxacin (Tb-Nflx) (with a stoichiometry of 4.5:1) was reported to respond to ATP by a strong luminescence enhancement[31]. The effect of different nucleotides as well as of phosphate and pyrophosphate was examined and an assay for the determination of adenylyl cyclase activity was established[32,33]. No interferences occur upon addition of physiological levels of Ca^{2+} and Mg^{2+} or other proteins.

In this work, the response of Tb-Nflx to GTP, GDP and P_i was used to set up an assay for GTPase activity. We used Tb-Nflx as luminescent probe to monitor Ras activity at an emission wavelength of 545 nm. Thereby, the release of phosphate due to hydrolysis of GTP is indicated in real-time by means of luminescence quenching. The

kinetics of several Ras mutants with different activities are compared and evaluated by the determination of hydrolysis constants k_{hyd}. The influence of both a GAP and a downstream effector (RBD of human Raf-1, Raf-RBD) on the activity of different mutants of Ras proteins can be monitored with this method.

3.3 Materials and Methods

3.3.1 Protein Purification

Wildtype proteins of human *H*-Ras [Ras(wt)] and mutant proteins of human *H*-Ras (amino acids 1 to 189) were expressed in *Escherichia coli* and purified as described before[34]. The final purity of the protein was > 95% as judged by sodium dodecyl sulfate polyacrylamid gel electrophoresis. Nucleotide exchange to GTP was performed incubating 200 µM of Ras with 50 mM tris(hydroxymethyl)aminomethane (pH 7.5), 30 mM EDTA, 10 mM GTP, and 2 mM 1,4-dithioerythritol for 5 h or 18 h at 5 °C. Afterwards, EDTA and free nucleotides were completely removed by gel filtration. The concentration of active nucleotide bound Ras was determined via the nucleotide concentration by C18-reversed phase chromatography using 100 mM K_2HPO_4/KH_2PO_4 pH 6.5/10 mM tetrabutyl-ammonium bromide/7% (v/v) acetonitrile as the buffer and a calibrated detector system. Finally, 80-85% of Ras was bound to GTP and 15-20% to GDP. Raf-RBD (amino acids 51 to 131) was expressed in *Escherichia coli* and purified as described before[35]. Neurofibromin 1-333 was expressed as glutathione-*S*-transferase fusion protein, followed by the cleavage using thrombin on a glutathione-Sepharose column. Afterwards, a gel filtration step was performed to remove the protease.

3.3.2 Microwell Plate-Based Luminescence Assay

Luminescence intensities (time-resolved) were acquired by using a BMG FLUOstar OPTIMA microplate reader (Jena, Germany). The excitation/emission filters were set to 337 and 545 nm, respectively. All dilution series and calibration plots were obtained in 96-microwell plates with four replicates. Enzyme assays were performed with 3 replicates applying time-gated detection at a time lag of 60 µs after the excitation pulse and a signal-integration time of 60 µs. For detailed description of time-gated measurements see Ref. 36. All experiments were performed at 37 °C, unless otherwise stated. The 96-microwell plates were obtained from Greiner Bio-One (Frickenhausen, Germany).

3.3.3 Reagents

All chemicals were of analytical grade. Magnesium dichloride hexahydrate and tri-sodiumphosphate dodecahydrate were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) (≥ 96%, essentially fatty acid free), $TbCl_3 \cdot 6H_2O$, norfloxacin, guanosine-5´-triphosphate sodium salt hydrate (≥ 95%, HPLC), guanosine-5'-diphosphate sodium salt (≥ 96%, HPLC) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (≥ 99.5 % titration) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

Buffered solutions contained 40 mM HEPES at pH 7.4. Tb-Nflx solutions contained 100 µM $TbCl_3 \cdot 6H_2O$ and 22.22 µM norfloxacin. The ratio Tb^{3+} to norfloxacin was 4.5:1 as reported in the literature. A 100 µM norfloxacin solution was prepared by dissolving norfloxacin in a small amount of 1 mM hydrochloric acid followed by dilution with HEPES-buffered solution to the appropriate volume. Five hundred microliters of a 1 mM Tb^{3+} solution and 1,111 µL of a 100 µM norfloxacin solution were combined and made up to 5 mL. The amount of Tb-Nflx in solution is represented by the concentration of Tb^{3+}.

3.3.4 Enzyme Activity Assay

The assays were recorded in 96-well microplates with each well containing a final volume of 100 µL at final concentrations of 0.5 mM Mg^{2+} and 0.1 % (m/v) BSA. The Tb-Nflx concentration was 20 µM (relating to Tb^{3+}). The amount of Ras added to the single kinetics is represented by the corresponding GTP concentration which is determined subject to the extent of GTP loading of each Ras mutant. Hence, all kinetics are referred to the initial GTP rather than the initial Ras concentration. Initial GTP concentrations were varied from 6 to 24 µM.

The effect of neurofibromin 1 (NF1) on the enzyme kinetics was examined by adding 4 µL of a 1:10 diluted NF1 stock solution to each well, independent of the applied enzyme concentration resulting in a high stoichiometric deficiency of NF1 compared to Ras of roughly 1:100. Raf-RBD was added in a 1.5 molar excess compared to the initial GTP concentration used.

The assay solution was incubated at 37 °C for 30 min before the addition of Ras proteins and their additional regulators, all of which were stored on ice until their addition to the assay solution. After addition of the enzyme, the measurements were started immediately.

3.4 Results

3.4.1 Calibration Plots for Guanine Nucleotides, Inorganic Phosphate, and GTPase Reaction

The luminescence spectrum of Tb-Nflx has been presented previously[31]. This assay used the change in intensity of the Tb^{3+} main emission band at a wavelength of 545 nm which corresponds to the $^5D_4 \rightarrow {}^7F_5$ electronic transition. The ligand was excited at 337 nm throughout all experiments. The effect of GTP, GDP and inorganic phosphate on the emission of the complex is displayed in Fig. 3.1. The concentration of Tb-Nflx was kept constant at 20 µM (relating to Tb^{3+}) throughout, unless otherwise stated. GDP and GTP had different effects on the emission intensity. GDP enhanced the luminescence more than twofold, whereas GTP induced only a weak increase of about 30%. Phosphate quenched the emission of Tb-Nflx to 10% of its initial value. All three ligands seemed to form a 1:1 complex with Tb^{3+}. Tb-Nflx was able to clearly discriminate between GDP and GTP. This was in contrast to ADP and ATP which both induced the same luminescence enhancement of Tb-Nflx[32]. This turned Tb-Nflx into a promising probe for monitoring GTPase activity which converts GTP to GDP and phosphate.

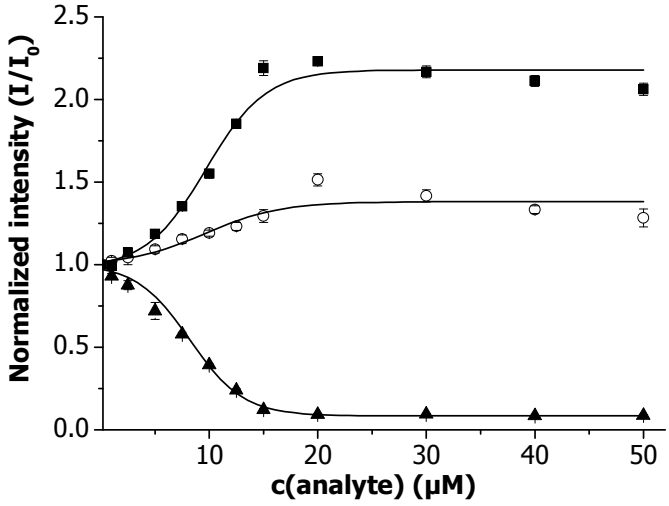
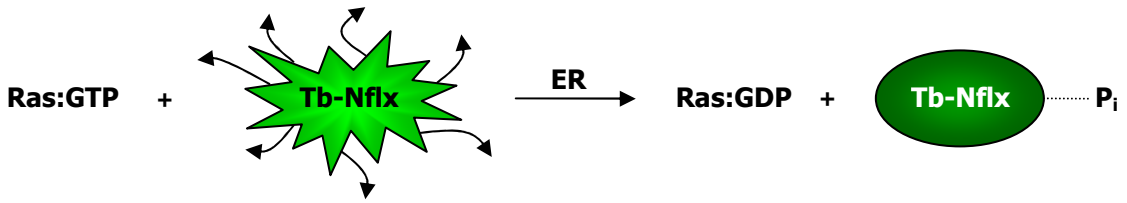


Fig. 3.1 Normalized luminescence intensity I/I_0 of the Tb-Nflx complex (20 µM) in presence of various concentrations of GDP (squares), GTP (circles) and phosphate (triangles) at 25 °C. The single response curves are fitted sigmoidally. Error bars are standard deviations of the mean.



Scheme 3.1 Enzymatic conversion of GTP to GDP and phosphate (P_i) by Ras accompanied by a significant decrease in lanthanide luminescence of Tb-Nflx due to strong quenching by P_i. ER: enzymatic reaction.

In the course of the GTPase reaction of Ras proteins, GTP is converted to GDP and phosphate (Scheme 3.1). GDP remains bound to Ras, whereas phosphate is released. The specific response of Tb-Nflx to phosphate under assay conditions (see "Materials and Methods") is illustrated in Fig. 3.2. The referenced emission signal decreased with increasing phosphate concentration until it reached a constant level of about 0.15 at a phosphate concentration of 20 µM. This indicated the formation of a 1:1 complex also under assay conditions. The linear range was between 1 and 12 µM of phosphate at a Tb-Nflx concentration of 20 µM.

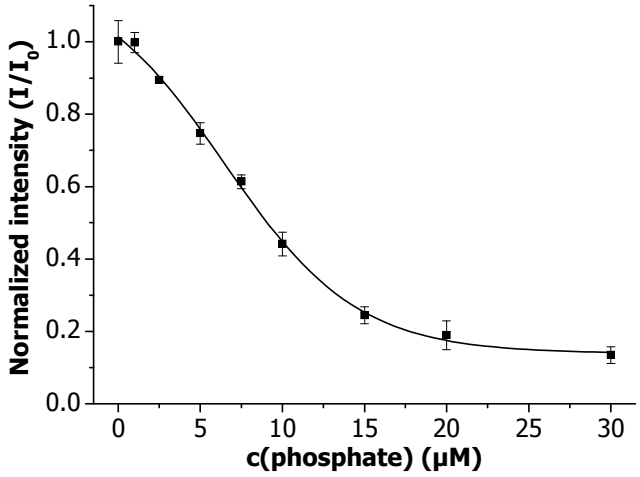


Fig. 3.2 Normalized luminescence intensity I/I_0 of the Tb-Nflx complex (20 µM) with sigmoidal fit in presence of phosphate under assay conditions (see "Materials and Methods"). Error bars are standard deviations of the mean.

3.4.2 Monitoring of GTPase Reaction

Ras(wt) served as model enzyme for the GTPase assay. Prior to recording the different kinetics, we added inactive, GDP-bound Ras(wt) [Ras(wt):GDP] to Tb-Nflx to assess unspecific interactions of the protein with the lanthanide complex. Its luminescence emission was only slightly enhanced in presence of increasing Ras(wt):GDP concentrations. After an addition of 25 µM Ras(wt):GDP, an increase of only 15% was detected. This unspecific luminescence enhancement can be induced by interactions of

nucleophilic groups (e.g. hydroxyl, carboxylic acid or thiol groups) in the amino acid side chains on the outer surface of the Ras protein with the terbium ion. However, this weak unspecific interference of Ras with the luminescence of Tb-Nflx was not significant since all data were referenced as I/I_0, where I_0 represents the luminescence intensity at t=0 directly after addition of Ras.

The Ras(wt) protein was loaded with GTP [Ras(wt):GTP]. Its intrinsic GTP hydrolysis activity was enhanced with increasing temperature, and was measured at 37 °C throughout all experiments. GTP was converted to GDP which remained bound to Ras(wt) and phosphate was released. GEFs or GAPs were not used because the intrinsic activity of Ras(wt) at a concentration of 25 µM was sufficient to be monitored in the assay within a reasonable timescale. A 30 minute tempering of the assay solution at 37 °C was necessary prior to the addition of Ras(wt):GTP to ensure a stable emission signal of the lanthanide complex which exhibited a significant thermal quenching effect. The luminescence intensity of Tb-Nflx was decreased by 30% when the temperature of the sample solution was raised from 25 to 37 °C. The emission of Tb-Nflx at 545 nm decreased after addition of Ras(wt):GTP to the assay mixture (Fig. 3.3). Ras was stored at 4 °C prior to the addition. The rate of decrease was dependent on the initial GTP concentration. All kinetic measurements were referred to the initial GTP concentration and not to the specific enzymatic activity of Ras because it was not possible to completely load the Ras samples with GTP. The luminescence response of Tb-Nflx to phosphate was one order of magnitude faster than the timescale of the enzyme kinetics. Therefore, real-time kinetics of the intrinsic GTPase activity of Ras were recorded.

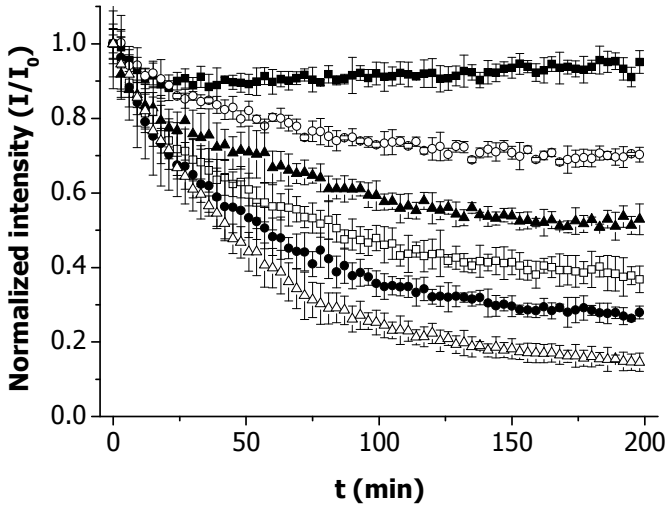


Fig. 3.3 Kinetics of different concentrations of initial GTP in wildtype protein of human *H*-Ras [Ras(wt)]: 6 µM (open circles), 12 µM (closed triangles), 15 µM (open squares), 18 µM (closed circles), 24 µM (open triangles), and Tb-Nflx (20 µM) as a reference (closed squares). Error bars are standard deviations of the mean.

The kinetics of Ras(wt):GTP displayed in Fig. 3.3 required referencing to the initial GTP concentration in order to assess the specific hydrolysis constant (k_{hyd}). A slight shift in the reference signal occured in the first minutes due to temperature effects that had an impact on the luminescence of the probe as indicated by the reference in Fig. 3.3. After about 20 to 25 minutes the reference signal had stabilized. Thus, k_{hyd} was determined in a subsequent time period from 25 to 50 minutes: The absolute value of the slope of the linear range between 25 and 50 minutes of each kinetic trace was divided by the corresponding initial GTP concentration according to Equation 1. The extent of GTP loading of the individual Ras mutants differed owing to the preparation procedure of the Ras:GTP complex. Only by applying the same initial GTP concentration, it was feasible to compare different kinetic runs. Hence, it was favored to calculate a specific hydrolysis constant by referencing to the initial GTP concentration and not to the associated amount of enzyme. An average specific hydrolysis constant of 360 ± 116 L/(min mol) was determined for Ras(wt):GTP.

$$k_{hyd} = \frac{|\text{slope}|\,[1/\text{min}]}{c(\text{GTP})\,[\text{mol/L}]} \qquad \textbf{Equation 1}$$

The kinetics of Ras(wt):GTP were compared with those of several Ras mutants with different GTPase activities, namely, Ras(T35S):GTP, Ras(Y32R):GTP, and the oncogene Ras(G12V):GTP. The GTPase activity decreased in the following order: Ras(Y32R):GTP > Ras(wt):GTP > Ras(T35S):GTP > Ras(G12V):GTP (Spoerner M, Hosza C, Poetzl J, Reiss K, Ganser P, and Kalbitzer HR, unpublished results). The shape of the kinetic traces of GTP hydrolysis for Ras(Y32R):GTP and Ras(T35S):GTP were comparable to those of Ras(wt):GTP (Fig. 3.4), while the slopes between 25 and 50 minutes varied due to the different intrinsic GTPase activities. GTP hydrolysis by Ras(G12V):GTP was very slow. Hence, only a slight decrease of the emission signal was observed even when using high initial GTP concentrations. Ras(G12V):GTP activity was, therefore, not evaluated by the calculation of k_{hyd} but served as negative control of an inactive Ras mutant. The active Ras mutants had different activities but in all cases the same referenced emission signal level ($I/I_0 \sim 0.2$) was obtained after 200 minutes. This coincided with the use of the same initial GTP concentration and, hence, the same amount of released phosphate. This had to be accompanied by an identical endpoint of the luminescence decrease for each Ras mutant. Furthermore, the final emission signals

of the various kinetic determinations agreed with the signal obtained from the calibration plot (see Fig. 3.2) at a phosphate concentration of 18 µM. Hence, instead of I/I_0 the calculated concentration of released phosphate can be plotted versus time.

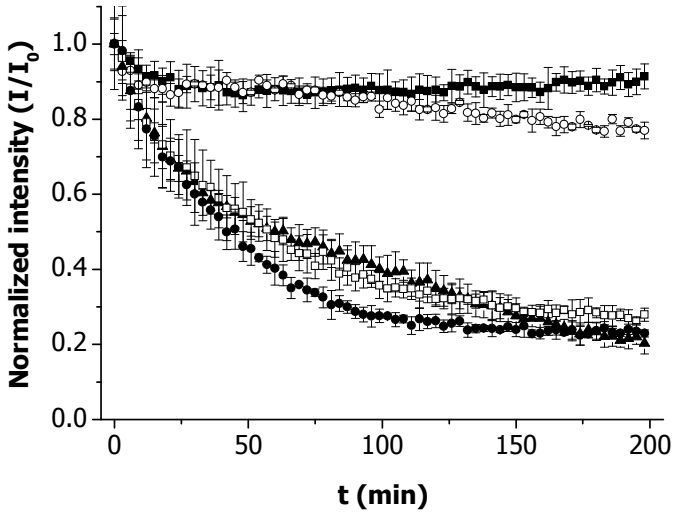


Fig. 3.4 Kinetics of Ras(G12V):GTP (open circles), Ras(T35S) (triangles), Ras(wt):GTP (open squares), and Ras(Y32R):GTP (closes circles) at an initial GTP concentration of 18 µM and Tb-Nflx (20 µM) as a reference (closed squares). Error bars are standard deviations of the mean.

Table 3.1 compares the calculated specific hydrolysis constants of the Ras mutants at an initial GTP concentration of 18 µM. Ras(T35S):GTP displayed a smaller k_{hyd} compared with Ras(wt):GTP, while Ras(Y32R):GTP had a higher k_{hyd} than expected. The GTPase activity of the different Ras mutants was monitored at several initial GTP concentrations as illustrated above for Ras(wt):GTP. The standard deviations obtained had the same dimension as presented for Ras(wt):GTP. However, for some kinetic runs the k_{hyd} values may deviate to a greater extent, particularly in case of lower Ras:GTP concentrations. Thus, it was important to define standard conditions for the comparison of different Ras mutants and the potency of regulators. The use of approximately equimolar amounts of initial GTP to Tb^{3+} is recommended as maximum quenching was obtained in case of a 1:1 complex of Tb^{3+} with phosphate (Fig. 3.2). For this reason, only kinetics at an initial GTP concentration of 18 µM are presented exemplarily in the following, even though they were also recorded for different concentrations from 6 to 24 µM. Table 3.1 shows that it was possible to monitor GTP hydrolysis by Ras proteins due to the detection of phosphate release by Tb-Nflx. The activities of different Ras mutants were accessible by means of the determination of hydrolysis constants derived from the linear range of the kinetics. The stated hydrolysis constants are mean values

from three separate kinetic runs. Standard deviations of around 20% for all selected Ras mutants were found.

The conditions of the assay had to be chosen carefully. The dynamic range for phosphate determination by Tb-Nflx was strongly dependent on the concentration of the probe, because phosphate undergoes 1:1 binding with Tb^{3+} as can be deduced from Fig. 3.1. The same initial GTP concentration using Ras(wt):GTP can result in a completely different extent of quenching at different Tb^{3+} concentrations.

Table 3.1 Hydrolysis constants (k_{hyd}) (L/(min mol)) for different Ras mutants at an initial GTP concentration of 18 µM.

	Ras(wt):GTP	Ras(T35S):GTP	Ras(Y32R):GTP	*Ras(G12V):GTP*
k_{hyd}	353 ± 88	257 ± 58	398 ± 54	Inactive

3.4.3 Effect of GAP and Raf-RBD on Ras Activity

The GTPase activity of Ras proteins is regulated by GAPs and/or GEFs. Furthermore, active, GTP-bound Ras proteins interact with downstream effectors and thereby initialize signaling cascades. In this assay, the effect of a GAP (NF1) and a downstream effector (Raf-RBD) on the GTPase activity of various Ras mutants was investigated. In the following the influence of NF1 and/or Raf-RBD on Ras mutants is exemplarily presented for an initial GTP concentration of 18 µM, throughout.

NF1 is known to increase the activity of Ras(wt) and of Ras(T35S)[37]. The kinetics of Ras(wt):GTP and Ras(T35S):GTP in presence of NF1 are shown in Figs. 3.5a and b. In case of Ras(wt):GTP, the GTP hydrolysis was completed within about 75 minutes in presence of NF1 as indicated by the constant emission signal of 0.25. The signal without NF1, however, reached the same level only after 200 minutes. The Ras(T35S):GTP kinetics also displayed higher GTPase activity after addition of NF1 compared with the kinetics without GAP. GTP hydrolysis was almost complete after about 50 minutes. The increase of Ras activity was evaluated by the determination of k_{hyd} as introduced earlier (see Equation 1). k_{hyd} values were higher in presence of NF1 for both Ras(wt):GTP and Ras(T35S):GTP as indicated in Table 3.2.

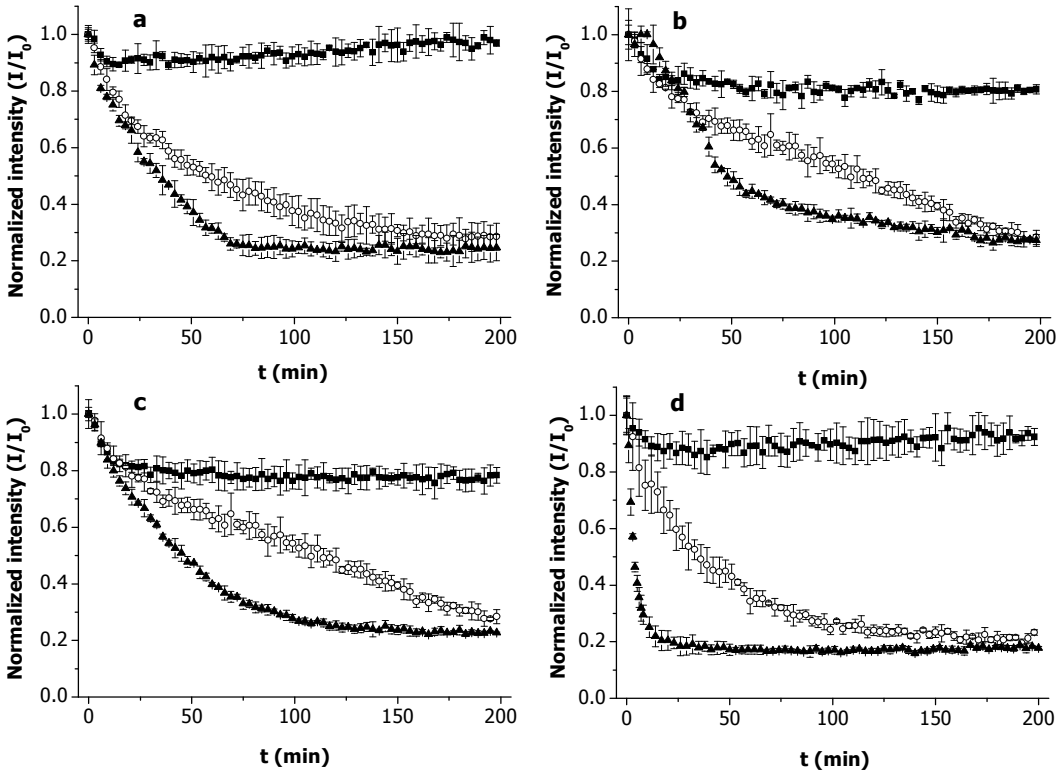


Fig. 3.5 Kinetics of various Ras mutants at an initial GTP concentration of 18 µM in presence of different regulators with Tb-Nflx (20 µM) as a reference (squares). (**a**) Ras(wt):GTP and (**b**) Ras(T35S):GTP in absence (circles) and presence (triangles) of neurofibromin 1 (NF1), (**c**) Ras(T35S):GTP and (**d**) Ras(Y32R):GTP in absence (circles) and presence (triangles) of the Ras-binding domain of human Raf-1. Error bars are standard deviations of the mean.

Table 3.2 Hydrolysis constants (k_{hyd}) (L/(min mol)) for different Ras mutants in presence of neurofibromin 1 (NF1) or the Ras-binding domain of human Raf-1 (Raf-RBD) at an initial GTP concentration of 18 µM.

Ras-binding protein	Ras(wt)	Ras(T35S)	Ras(Y32R)
-	353	257	398
NF1	446	801	527
Raf-RBD	-	411	6,167[a]

[a] Calculated between 0 and 5 minutes

To examine the influence of a downstream effector on Ras activity we used Raf-RBD. Downstream effectors usually do not alter the GTPase activity of Ras proteins. However, the rate of GTP hydrolysis can be accelerated in mutated forms of Ras(wt). Figs. 3.5c and d show the kinetics of Ras(T35S):GTP and Ras(Y32R):GTP in absence and presence of Raf-RBD. GTP hydrolysis by Ras(T35S):GTP after addition of Raf-RBD was complete after 100 minutes, whereas it only took about 25 minutes in case of Ras(Y32R):GTP which was strongly effected by Raf-RBD. The k_{hyd} values in presence of Raf-RBD are given in Table 3.2 and were higher compared with k_{hyd} without the effector

for both Ras(T35S):GTP and Ras(Y32R):GTP. The specific hydrolysis constant of Ras(Y32R):GTP was calculated between 0 and 5 minutes due to the extremely fast GTP hydrolysis in presence of Raf-RBD resulting in a complete turnover after 25 minutes.

It is known that neither NF1 nor Raf-RBD effects the activity of Ras(G12V):GTP and that Raf-RBD as a downstream effector does not alter the GTPase activity of Ras(wt):GTP (Spoerner M, Hosza C, Poetzl J, Reiss K, Ganser P, and Kalbitzer HR, unpublished results). The selectivity of NF1 and/or Raf-RBD for certain Ras mutants and the corresponding increase of GTPase activity was examined by performing negative control kinetic experiments. The effect of NF1 and Raf-RBD on Ras(G12V):GTP was examined in separate kinetic runs. Both Ras binding proteins did not alter the GTPase activity of Ras(G12V):GTP. Furthermore, Raf-RBD did not influence the activity of Ras(wt):GTP (results not shown). These results confirm the selective effects of NF1 and Raf-RBD for specific Ras mutants (Fig. 3.5) and underline the applicability of this assay to investigate the influence of various regulators on different Ras mutants.

Ras(Y32R):GTP is activated by Raf-RBD as presented above, but it is not known so far whether its activity can be influenced by NF1. Fig. 3.6 shows that NF1 increased the GTPase activity of Ras(Y32R):GTP. GTP hydrolysis was completed within 75 minutes in presence of NF1. Hence, this assay reveals for the first time that NF1 has an activating effect on the Ras(Y32R):GTP mutant. This was confirmed by the determination of a corresponding k_{hyd} value of 527 L/(min mol) which was higher than in absence of NF1 with 398 L/(min mol)). Thus, this assay indicates the enhancement of Ras(Y32R):GTP and Ras(T35S):GTP activity by both NF1 and Raf-RBD.

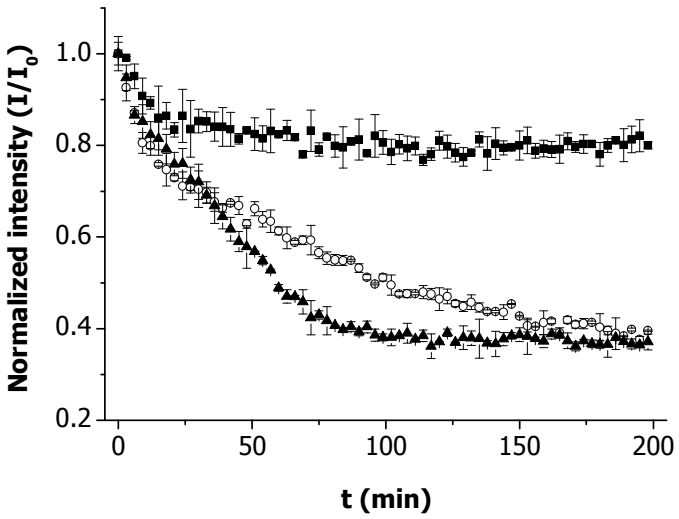


Fig. 3.6 Kinetics of Ras(Y32R):GTP in absence (circles) and presence (triangles) of neurofibromin 1 (NF1) at an initial GTP concentration of 15 µM and Tb-Nflx (20 µM) as a reference (squares). Error bars are standard deviations of the mean.

3.5 Discussion

The routinely applied radioassays for the monitoring of enzymatic activity have the obvious disadvantages of handling harmful radioactive material including expensive waste disposal and the risk for the lab personal. Moreover, the assays are rather complex, and require chromatographic separation steps. However, the well established precipitation assay using a charcoal suspension is very sensitive and is successfully applied to the detection of the GTPase activity of heterotrimeric GTP-binding proteins[38]. For other non-radioactive assays, often complex immunoprecipitation steps are necessary. None of these detection methods enable a direct online monitoring of GTPase activity. The luminescent assay presented here allows for the first time the determination of real-time kinetics by rapid detection of phosphate released by GTP hydrolysis. However, the assay requires large amounts of protein which are not always easy to access. The assay can be easily applied to a microplate format which paves the way for the simultaneous performance of multiplex kinetic experiments under identical conditions. The microplate format enables the effect of various effectors and/or activators to be screened in parallel with short analysis times and at low costs.

The presented luminescent assay offers the possibility of examining the impact of newly discovered regulators on the GTPase activity of Ras proteins and/or their GAPs, GEFs, and downstream effectors. Moreover, the effect of Ras regulators (GAPs, GEFs, downstream effectors) on various Ras mutants can be investigated in a high-throughput manner. This study revealed that also NF1, a GAP, further stimulates the GTPase activity of the Ras(Y32R):GTP mutant.

It is aimed to extend this assay by the addition of GEFs. This will allow the introduction of Ras:GDP to the assay in the place of Ras:GTP. In this case, the reaction is started by activation of inactive, GDP bound Ras by GEFs via a fast GDP-GTP exchange process. Thereby, the change of the ratio between GTP and GDP/phosphate in the course of the GTPase reaction can be monitored.

3.6 Conclusion

In this study, we demonstrated the application of the Tb-Nflx complex as luminescent probe for the monitoring of GTPase activity of Ras proteins. The different activities of Ras(wt) and the mutants Ras(G12V), Ras(T35S), and Ras(Y32R) were detected in a straightforward microwell plate assay. Hydrolysis constants for these different Ras

mutants were determined. Furthermore, the effects of NF1 and Raf-RBD on the GTPase activity of these Ras mutants were evaluated. The determination of the corresponding hydrolysis constants enables the comparison of GTPase activities in absence and presence of GAP or downstream effectors. It was shown that Tb-Nflx is a sensitive probe for various nucleoside phosphates and inorganic phosphates. Its robustness and tolerance towards various cationic cofactors and other proteins such as BSA allows the adjustment to appropriate reaction conditions required for this kind of enzyme. This assay is cheap, straightforward, and fast to accomplish.

3.7 References

1 Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ (1998) Increasing complexity of Ras signaling. *Oncogene* **17:** 1395-1413

2 Barbacid M (1987) *ras* genes. *Annu Rev Biochem* **56:** 779-827

3 Wittinghofer A, Waldmann H (2000) Ras – a molecular switch involved in tumor formation. *Angew Chem Int Ed* **39:** 4192-4214

4 Buolamwini JK (1999) Novel anticancer drug discovery. *Curr Opin Chem Biol* **3:** 500-509

5 Waddick KG, Uckun FM (1998) Innovative treatment programs against cancer: I. Ras oncoprotein as a molecular target. *Biochem Pharmacol* **56:** 1411-1426

6 Levitzki A (1994) Signal-transduction therapy. A novel approach to disease management. *Eur J Biochem* **226:** 1-13

7 Vaillancourt RR, Harwood AE, Winitz S (1994) Analysis of guanine nucleotides associated with protooncogene ras. *Methods Enzymol* **238:** 255-258

8 Rubio I, Pusch R,Wetzker R (2004) Quantification of absolute Ras-GDP/GTP levels by HPLC separation of Ras-bound [^{32}P]-labelled nucleotides. *J Biochem Biophys Methods* **58:** 111-117

9 Taylor SJ, Shalloway D (1996) Cell cycle-dependent activation of Ras. *Curr Biol* **6:** 1621-1627

10 De Rooij J, Bos JL (1997) Minimal Ras-binding domain of Raf-1 can be used as an activation-specific probe for Ras. *Oncogene* **14:** 623-625

[11] Hiratsuka T (1983) New ribose-modified fluorescent analogs of adenine and guanine nucleotides available as substrates for various enzymes. *Biochem Biophys Acta* **742:** 496-508

[12] McEwen DP, Gee KR, Kang HC, Neubig RR (2001) Fluorescent BODIPY-GTP analogs: real-time measurement of nucleotide binding to G proteins. *Anal Biochem* **291:** 109-117

[13] Moore KJM, Webb MR,Eccleston JF (1993) Mechanism of GTP hydrolysis by $p21^{N\text{-}ras}$ catalyzed by GAP: studies with a fluorescent GTP analog. *Biochemistry* **32:** 7451-7459

[14] Klebe C, Bischoff FR, Ponstingl H, Wittinghofer A (1995) Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and RanGAP1. *Biochemistry* **34:** 639-647

[15] Ahmadian MR, Hoffman U, Goody RS, Wittinghofer A (1997) Individual rate constants for the interaction of Ras proteins with GTPase-activating proteins determined by fluorescence spectroscopy. *Biochemistry* **36:** 4535-4541

[16] Lenzen C, Cool RH, Prinz H, Kuhlmann J, Wittinghofer A (1998) Kinetic analysis by fluorescence of the interaction between Ras and the catalytic domain of the guanine nucleotide exchange factor $Cdc25^{Mm}$. *Biochemistry* **37:** 7420-7430

[17] Klebe C, Prinz H, Wittinghofer A, Goody RS (1995) The kinetic mechanism of Ran-nucleotide exchange catalyzed by RCC1. *Biochemistry* **34:** 12543-12552

[18] Thompson G, Owen D, Chalk PA, Lowe PN (1998) Deliniation of the Cdc42/Rac-binding domain of p21-activated kinase. *Biochemistry* **37:** 7885-7891

[19] Kuhlmann J, Macara I, Wittinghofer A (1997) Dynamic and equilibrium studies on the interaction of Ran with its effector RanBP1. *Biochemistry* **36:** 12027-12035

[20] Gorman C, Skinner RH, Skelly JV, Neidle S, Lowe PN (1996) Equilibrium and kinetic measurements reveal rapidly reversible binding of Ras to Raf. *J Biol Chem* **271:** 6713-6719

[21] Korlach J, Baird DW, Heikal AA, Gee KR, Hoffman GR, Webb WW (2004) Spontaneous nucleotide exchange in low molecular weight GTPases by fluorescently labeled γ-phosphate-linked GTP analogs. *Proc Natl Acad Sci USA* **101:** 2800-2805

[22] Jameson EE, Cunliffe JM, Neubig RR, Sunahara RK, Kennedy RT (2003) Detection of G proteins by affinity probe capillary electrophoresis using a fluorescently labeled GTP analogue. *Anal Chem* **75:** 4297-4304

[23] Spangler C, Schaeferling M, Wolfbeis OS (2008) Fluorescent probes for microdetermination of inorganic phosphates and biophosphates. *Microchim Acta* **161:** 1-39

[24] Kwon YJ, Singh NJ, Kim HN, Kim SK, Kim KS, Yoon J (2004) Fluorescent GTP-sensing in aqueous solution of physiological pH. *J Am Chem Soc* **126:** 8892-8893

[25] Duerkop A, Turel M, Lobnik A, Wolfbeis OS (2006) Microtiter plate assay for phosphate using a europium-tetracycline complex as a sensitive luminescent probe. *Anal Chim Acta* **555:** 292-298

[26] Schäferling M, Wolfbeis OW (2007) Europium tetracycline as a luminescent probe for nucleoside phosphates and its application to the determination of kinase activity. *Chem Eur J* **13:** 4342-4349

[27] Hou F, Miao Y, Jiang C (2005) Determination of adenosine disodium triphosphate (ATP) using oxytetracyline-Eu^{3+} as a fluorescence probe by spectrofluorimetry. *Spectrochim Acta Part A* **61:** 2891-2895

[28] Ci Y, Li Y, Chang W (1992) Fluorescence enhancement of terbium(III) by nucleotides and polyhomonucleotides in the presence of phenanthroline. *Fresen J Anal Chem* **342:** 91-94

[29] Wang DY, Zhao Y, Xu JG, Guo XQ (1997) Sensitive determination of nucleotides and polynucleotides based on the fluorescence quenching of the Tb^{3+}-Tiron complex. *Fresen J Anal Chem* **358:** 514-518

[30] Malcolm ADB (1977) A simple fluorimetric assay for guanosine nucleotides. *Anal Biochem* **77:** 532-535

[31] Miao Y, Liu J, Hou F, Jiang C (2006) Determination of adenosine disodium triphosphate (ATP) using norfloxacin-Tb^{3+} as a fluorescence probe by spectrofluorimetry. *J Luminesc* **116:** 67-72

[32] Spangler CM, Spangler C, Schäferling M (2008) Luminescent lanthanide complexes as probes for the determination of enzyme activities. *Ann N Y Acad Sci* **1130:** 138-148

[33] Spangler CM, Spangler C, Göttle M, Shen Y, Tang WJ, Seifert R, Schäferling M (2008) A fluorimetric assay for real-time monitoring of adenylyl cyclase activity based on terbium norfloxacin. *Anal Biochem* **381:** 86-93

[34] Tucker J, Sczakiel G, Feuerstein J, John J, Goody RS, Wittinghofer A (1986) Expression of p21 proteins in *Escherichia coli* and stereochemistry of the nucleotide-binding site. *EMBO J* **5:** 1351-1358

[35] Herrmann C, Martin GA, Wittinghofer A (1995) Quantitative analysis of the complex between p21^{ras} and the Ras-binding domain of the human Raf-1 protein kinase. *J Biol Chem* **270:** 2901-2905

[36] Soini E, Lövgren T, Reimer CB (1987) Time-resolved fluorescence of lanthanide probes and applications in biotechnology. *Crit Rev Anal Chem* **18:** 105-154

[37] Spoerner M (2002) Konformationelle Dynamik des Ras-Proteins und ihre Bedeutung für die Effektor-Wechselwirkung. *PhD thesis*, University of Regensburg

[38] Brandt DR, Ross EM (1986) Catecholamine-stimulated GTPase cycle. Multiple sites of regulation by β-adrenergic receptor and Mg^{2+} studied in reconstituted receptor-G_s vesicles. *J Biol Chem* **261:** 1656-1664

4. A Liquid Chromatography-Coupled Tandem Mass Spectrometry Method for Quantitation of Cyclic Di-Guanosine Monophosphate

4.1 Abstract

Cyclic 3':5'-di-guanosine monophosphate (c-di-GMP) represents an important ubiquitous second messenger in bacteria. It controls the transition between a sessile and a motile lifestyle of bacteria and, hence, affects the formation of biofilms which are highly resistant to antimicrobial treatment. c-di-GMP is synthesized by di-guanylate cyclases (DGCs) and degraded by specific phosphodiesterases (PDEs), two highly abundant protein families in bacteria. We have established a robust, and highly sensitive high performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS) based method for the quantitation of c-di-GMP and investigated various method performance parameters such as limit of detection (LOD), lower limit of quantitation (LLOQ), linearity, accuracy, recovery and analyte stability. As a proof of principle we used this method to accurately measure the activity of the prototype DGC PleD* from *Caulobacter crescentus in vitro*. In addition, the new HPLC-MS/MS method was successfully applied to determine *in vivo* levels of c-di-GMP in bacterial extracts of *Escherichia coli* at different stages of bacterial growth. This demonstrates that our method is suitable for the sensitive and specific quantitation of c-di-GMP in bacterial cell extracts.

4.2 Introduction

Second messengers function as universal signaling molecules and play a pivotal role in processing extracellular information. Cyclic 3':5'-di-guanosine monophosphate (c-di-GMP), originally reported as a positive allosteric regulator of cellulose synthase in *Gluconacetobacter xylinus*[1], has emerged as a ubiquitous bacterial second messenger in the last two decades. c-di-GMP affects bacterial physiology by binding to a number of specific c-di-GMP binding effector components. Specifically, the PilZ domain was discovered as the first c-di-GMP effector[2]. Furthermore, the transcription factor FleQ and the putative inner membrane protein PelD from *Pseudomonas aeruginosa*[3] as well as the

newly discovered protein PopA from *Caulobacter crescentus*[4] were shown to act as c-di-GMP effectors. Finally, c-di-GMP binding to a class of riboswitches found in the untranslated regions of different mRNAs in several bacterial species was reported to induce structural changes which alter the expression of downstream genes[5]. Thus, despite the low number of identified c-di-GMP target structures, it has already become clear that c-di-GMP controls cellular functions at the transcriptional, translational and posttranslational level.

c-di-GMP is produced *via* the condensation of two GTP molecules and hydrolysed to GMP *via* the linear intermediate pGpG. The synthesis and degradation of c-di-GMP is regulated by di-guanylate cyclases (DGCs) and phosphodiesterases (PDEs), respectively. DGCs harbor GGDEF domains while PDEs contain EAL domains[6,7]. These c-di-GMP-metabolizing proteins are widely distributed in eubacteria, and in most cases, a single bacterial genome encodes many different members of these protein families[8]. c-di-GMP levels regulate the transition from a motile, planctonic lifestyle to a sessile, biofilm-forming state in numerous bacterial species (for review see Ref. 9). Biofilms are adherent bacterial communities which can be formed on biotic and abiotic surfaces, including human tissues. This results for example in chronic infections by *Pseudomonas aeruginosa* in the airways of patients with cystic fibrosis[10]. Biofilms are highly resistant to antibiotic treatment and therefore contribute to bacterial persistence in chronic infections.

The enzymatic function of proteins involved in c-di-GMP metabolism and biofilm formation has been revealed by *in vitro* biochemical analyses. However, *in vitro* assays cannot give any insight in the *in vivo* cellular c-di-GMP levels due to the high number of c-di-GMP metabolizing enzymes encoded by a single bacterial genome. Thus, there is a need for the establishment of a reliable quantitation method for c-di-GMP in bacteria.

Analytical techniques for the detection of c-di-GMP usually aim at the assessment of either *in vitro* DGC- and PDE-activities or at the determination of *in vivo* levels of c-di-GMP in bacterial extracts. Frequently, [^{33}P]- or [^{32}P]-labeled nucleotide substrates are applied for both DGC[11,12] and PDE[13,14] activity assays. The reaction products are subsequently separated by thin-layer chromatography (TLC) and quantified *via* the radioactive intensities of the resulting spots. PDE assays require complex enzymatic synthesis and chromatographic purification of the radioactively labeled c-di-GMP substrate which is not commercially available.

The ratio of substrate to product can also be assessed by direct UV absorption at 254 nm after HPLC separation[7]. This method avoids the use of radioactively labeled substrates but suffers from low sensitivity and requires good peak separation.

The amount of c-di-GMP after nucleotide extraction from bacterial cell cultures can commonly only be determined in DGC overexpression studies[15] and, therefore, does not relate to *in vivo* c-di-GMP levels responsible for phenotypic characteristics. Low c-di-GMP concentrations can be determined using mass spectrometry methods which often provide sufficient sensitivities. Recently, c-di-GMP concentrations in bacterial species have been measured by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry[16]. However, this method requires an elaborate chromatographic work-up step prior to the sample preparation for the final MALDI-TOF analysis and suffers from a poor linearity range.

Some high performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS) based methods for the quantitation of c-di-GMP levels have been reported[17,3,18]. However, these methods exhibit various limitations, i.e. long analysis time, lack of internal standard, or inadequate validation.

The biochemical analysis of enzymes and the determination of *in vivo* c-di-GMP levels in bacteria require the establishment of a reliable and sensitive quantitation method in order to better understand the connection between c-di-GMP metabolism and the corresponding physiological output. In this report, we present the development and validation of a new HPLC-MS/MS method for the sensitive quantitation of c-di-GMP following a suitable nucleotide extraction procedure. The method was evaluated by measuring the *in vitro* activity of the constitutively active DGC PleD*. Application of the method to monitor the change of c-di-GMP concentration along the growth curve of *Escherichia coli* revealed that this bacterium displays increased c-di-GMP levels in stationary phase.

4.3 Materials and Methods

4.3.1 Chemicals

Solvents used for extraction and in HPLC analysis were water, methanol and acetonitrile (HPLC-gradient grade, J. T. Baker, Deventer, The Netherlands). Isopropyl β-D-1-thiogalactopyranoside (IPTG), ammonium acetate, bovine serum albumin (BSA) and guanosine 5'-triphosphate (GTP) were purchased from Sigma Aldrich (Steinheim,

Germany), sodium hydroxide, tris(hydroxymethyl)-aminomethane and magnesium chloride hexahydrate were obtained from Merck (Darmstadt, Germany) and acetic acid was from Riedel-de Haen (Hannover-Seelze, Germany). Ammonium acetate and acetic acid were added to the aqueous eluent A. Cyclic 3':5'-di-guanosine monophosphate (c-di-GMP) was kindly provided by BioLog (Bremen, Germany) and cyclic 3':5'-xanthosine monophosphate (cXMP), used as internal standard, was from Sigma Aldrich (Steinheim, Germany). c-di-GMP, cXMP and GTP were used in aqueous stock solutions at a concentration of 0.1 mg/mL, 1 mg/mL and 13 mg/mL, respectively, and stored at -20 °C.

4.3.2 Expression and Purification of PleD*

BL21(λDE3)pLys cells carrying the His_6-pleD* expression plasmid were grown in LB medium supplemented with ampicillin (100 µg/mL) and chloramphenicol (80 µg/mL). Expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (0.5 mM) for 3 hours at 25 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (10 mM Tris-HCl at pH 7.9, 300 mM NaCl, 10 mM benzamidine, 0.5 mM phenylmethanesulphonylfluoride, 10 µg/mL leupeptine, 5 mM imidazole) and lysed by passage through a French press cell. The resulting suspension was clarified by centrifugation (13.000 x g). The supernatant fluid was loaded onto Ni-NTA affinity resin (HiTrap Chelating HP, GE Healthcare, Munich, Germany), washed with starting buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and high-salt buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Eluted fractions were examined for purity by SDS-PAGE and fractions containing pure protein were pooled. Desalting and buffer exchange were performed on a Sephadex G-25 column (PD-10 Desalting, GE Healthcare, Munich, Germany). Final protein concentration was determined with a Bradford assay (Carl Roth, Karlsruhe, Germany).

4.3.3 Di-Guanylate Cyclase Assay

The standard reaction mixture contained 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 0.1% (m/v) BSA and 100 µM GTP in a 1.5 mL volume at 30 °C. The assay was initiated by addition of 100 nM PleD*. Aliquots (70 µL) were withdrawn at different time points and the enzymatic reaction was stopped by heating to 95 °C for 5 minutes. The suspension was then centrifuged at maximum speed (20.000 x g) for 5 minutes in order to pellet denatured protein. The supernatant fluid was analyzed by reversed phase-HPLC-MS/MS. Assays were performed in triplicate.

4.3.4 Extraction of c-di-GMP

Escherichia coli K-12 wildtype MG1655 was cultivated in TB-medium at 37 °C with aeration. Cells were harvested (5 mL culture volume) at the indicated optical density (OD_{600}) by quick centrifugation at 4 °C and the resulting cell pellet was extracted immediately.

The nucleotide extraction method reported by Rabinowitz and Kimball[19] was modified for the extraction of c-di-GMP. The extraction solvent was a mixture of acetonitrile/methanol/water (40/40/20, v/v/v). The cell pellet was resuspended in 300 µL of ice-cold extraction solvent containing the internal standard cXMP to quench metabolism and initiate the extraction process followed by a 15 minutes incubation step at 4 °C. The cell suspension was then heated to 95 °C for 10 minutes. After cooling, the suspension was centrifuged at maximum speed (20.000 x g) for 5 minutes in order to separate insoluble material from the extracted nucleotides. The extraction of the resulting pellet was repeated twice with 200 µL of extraction solvent (without cXMP) at 4 °C omitting the heating step. The solvent of the combined supernatants (700 µL) was then evaporated until dryness at 40 °C under a gentle stream of nitrogen gas. The residue was resuspended in 200 µL of water under vigorous vortexing and then analyzed by reversed phase-HPLC-MS/MS. Extractions were performed in triplicate with two independent bacterial cultures as biological duplicate.

Protein content was determined by dissolving the cell pellet of 1 mL aliquots of the bacterial cultures in 800 µL of 0.1 M NaOH by heating for 15 minutes at 95 °C and subsequently performing a protein determination with a BCA (bicinchoninic acid) assay (Thermo Scientific, Rockford, IL, USA). Measurements were repeated in triplicate and final c-di-GMP concentrations were expressed as pmol/mg of bacterial protein.

4.3.5 Quantitation of c-di-GMP by HPLC-MS/MS

The chromatographic separation was performed on a Series 200 HPLC system (Perkin Elmer Instruments, Norwalk, CT, USA) equipped with a binary pump system and a 200 µL sample loop. A combination of Supelco Column Saver (2.0 µm filter, Supelco Analytical, Bellafonte, CA, USA), Security Guard Cartridge (C18, 4 x 2 mm) in an Analytical Guard Holder KJO-4282 (Phenomenex, Aschaffenburg, Germany) and an analytical NUCLEODUR C18 Pyramid RP column (50 x 3 mm, 3 µm particle size, Macherey-Nagel, Düren, Germany) temperature-controlled by a convenient HPLC column oven (Series 200 Peltier column oven, Perkin Elmer Instruments) at 30 °C was used.

Eluent A consisted of 10 mM ammonium acetate and 0.1% (v/v) acetic acid in water and eluent B was methanol. The injection volume was 50 µL and the flow rate was 0.4 mL/min throughout the chromatographic run. Eluent A (100%) was used from 0 to 5 minutes followed by a linear gradient from 100% A to 70% A until 9 minutes. The internal standard cXMP and c-di-GMP were eluted during this gradient phase with retention times of 6.1 and 8.6 minutes, respectively. Re-equilibration of the column was achieved by constantly running 100% A from 9 to 13 minutes.

The analyte detection was performed on an API 3000 triple quadrupole mass spectrometer equipped with an electro spray ionization (ESI) source (Applied Biosystems Inc, Foster City, CA, USA) using selected reaction monitoring (SRM) analysis in positive ionization mode. The following SRM transitions using a dwell time of 40 ms were detected: cXMP: +347/153 (quantifier), +347/136 (qualifier); c-di-GMP: +691/152 (quantifier), +691/135 and +691/248 (qualifier). The SRM transitions labeled as "quantifier" were used to quantify the compound of interest whereas "qualifier" SRM transitions were monitored as confirmatory signals. The quantifier SRM transitions were most intense and were therefore used for quantitation. The same quantifier (+691/152) and one of the two qualifier (+691/248) SRM transitions for c-di-GMP were also reported by Hickman and Harwood, 2008. The mass spectrometer parameters were as follows: IS voltage: 5500 V, temperature: 350 °C, nebulizer gas: 6 psi, curtain gas: 15 psi. MS/MS was performed using nitrogen as collision gas. The following collision energies were applied: 29 eV (+347/153), 59 eV (+347/136), 61 eV (+691/152), 123 eV (+691/135), 39 eV (+691/248).

4.4 Results

4.4.1 HPLC-MS/MS Method Performance

We have developed an HPLC-MS/MS method for the quantitation of c-di-GMP which was validated regarding various method performance parameters (see Table 4.1): limit of detection (LOD, signal-to-noise (S/N) ratio 3:1), lower limit of quantitation (LLOQ), squared correlation coefficient (R^2), intra- and inter-day precision by means of relative standard deviation (RSD), accuracy, extraction recovery and 24 h stability of c-di-GMP. These performance characteristics were determined according to the Guidance for Industry, Bioanalytical Method Validation[20].

Table 4.1 Method performance parameters determined by HPLC-MS/MS for three c-di-GMP standards.

Retention time (min)	8.6		
LOD (ng/mL)	1.2		
LLOQ (ng/mL)	4.1		
Linearity[a] (R^2)	0,9999		
	c-di-GMP standards (ng/mL)		
	10	50	500
RSD intra-day[b] (%)	10.9	7.8	2.7
RSD inter-day[b] (%)	11.2	6.4	5.9
Accuracy (%)	85-109	89-109	96-101
Recovery[c] (%)	76.4	74.2	108.4
Drug stability[d] (%)	73.7	74.8	91.5

[a] c(c-di-GMP) = 4-1000 ng/mL
[b] n = 5
[c] nucleotide extraction recovery, n = 5
[d] room temperature for 24 h, n = 5

The extraction and quantitation of c-di-GMP in bacteria is shown exemplarily for *Escherichia coli* cultivated in TB, and was analogously applied for other bacterial strains in various liquid culture media (data not shown). Hence, the validation was performed in water (except for extraction recovery experiments) in order to ascertain the performance parameters in a standard matrix. c-di-GMP possessed a retention time of 8.6 minutes in the established HPLC procedure. LOD and LLOQ were in the low nanogram/mL range (Table 4.1). Standard calibration curves for the detection of validation parameters were acquired ranging from 4 to 1,000 ng/mL with R^2 values close to 1. The method was found to be linear up to 5,000 ng/mL. Intra- and inter-day precisions as well as accuracies for the different standard concentrations examined were within the required range (< 15%) according to the Guidance for Industry, Bioanalytical Method Validation[20]. Furthermore, acceptable recovery rates have been determined and the 24 h stability tests of c-di-GMP at room temperature showed slight degradation which was more pronounced at lower c-di-GMP concentrations.

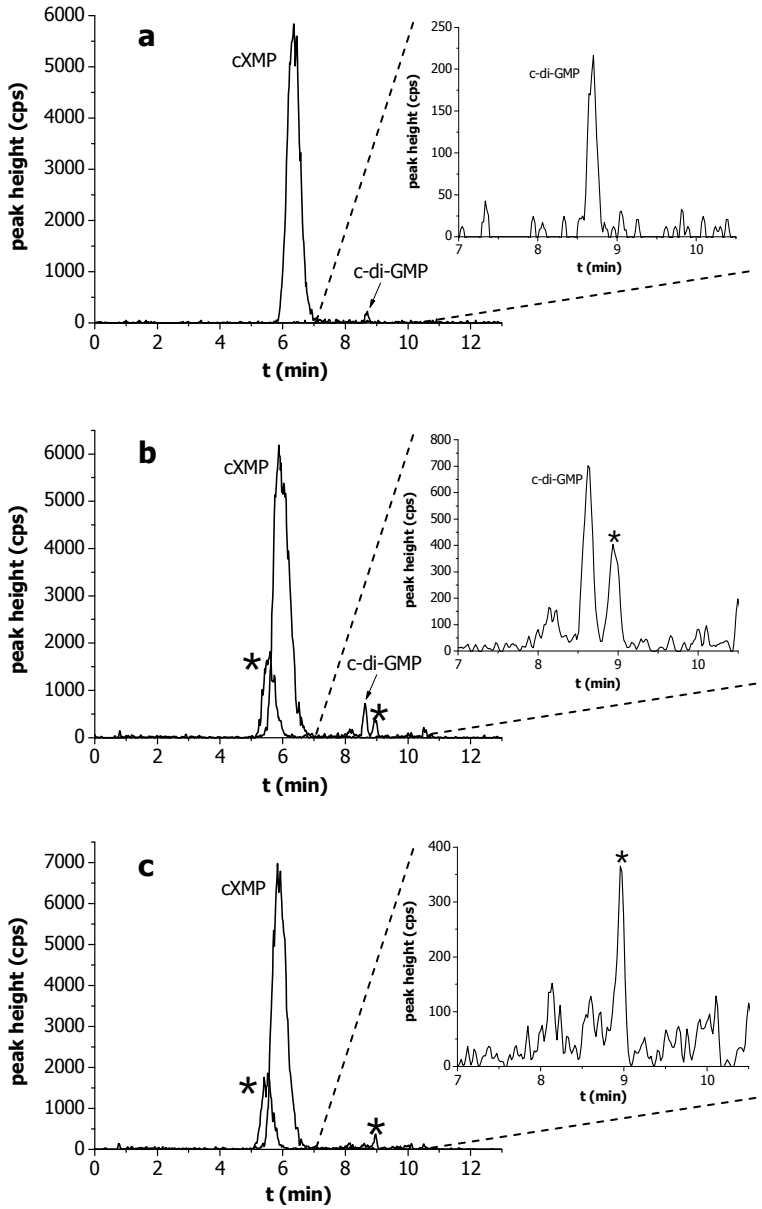


Fig. 4.1 Detection of c-di-GMP in different matrices with the internal standard cXMP (200 ng/mL) by HPLC-tandem mass spectrometry displaying the SRM transition 691 → 152: Standard sample of c-di-GMP at the LLOQ (4.1 ng/mL) in water (**a**), c-di-GMP in a bacterial extract of *Escherichia coli* at an OD_{600} of 1.42 (**b**) and 0.5 (c(c-di-GMP) < LOD) (**c**). The peaks labeled with an asterisk represent interfering endogenous metabolites in the bacterial extract of *Escherichia coli*. The inserts represent enlargements of the low intensity peaks.

The HPLC-MS/MS method was applied to the detection and quantitation of c-di-GMP in bacterial extracts. Fig. 4.1 displays chromatographic runs with the internal standard cXMP and c-di-GMP both in water and in extraction matrix (bacterial extracts of *Escherichia coli* at different optical densities). The c-di-GMP peak in the bacterial extract (OD_{600} 1.42) was clearly identified by its retention time and its distinct SRM transitions (see "Materials and Methods"). Additional endogenous metabolites having the same

quantifier SRM transition as c-di-GMP (+691/152) appeared at 5.6 and 8.9 minutes (see Fig. 4.1), the latter being base-line separated from the c-di-GMP peak (8.6 minutes). The unidentified metabolites and c-di-GMP also had one qualifier SRM transition in common (+691/135) whereas the second qualifier SRM transition (+691/248) was unique to c-di-GMP. The presented method can also be used for the detection and quantitation of cyclic 3':5'-di-adenosine monophosphate (c-di-AMP) with a retention time of 9.3 min (data not shown).

4.4.2 Di-Guanylate Cyclase Assay with PleD*

Biochemical analysis of purified enzymes is a major tool in order to identify and characterize enzymatic activities. The family of di-guanylate cyclases comprises key enzymes of second messenger signaling in bacteria. DGCs require dimerization for their catalytic activity which is responsible for the condensation of two GTP molecules into c-di-GMP. For this study, we extracted and purified the 50 kDa DGC PleD* as a model enzyme which is a constitutively active mutant of the *Caulobacter crescentus* DGC PleD[6]. We applied the newly developed reversed phase HPLC-MS/MS method to monitor the turnover of GTP to c-di-GMP by PleD* in an *in vitro* assay (Fig. 4.2). Under the chosen conditions, c-di-GMP production was linear up to 7 µg/mL (10 µM). The hyperbolic shape of the kinetic is presumably due to allosteric product inhibition at higher c-di-GMP concentrations[21] although k_i values have not yet been determined.

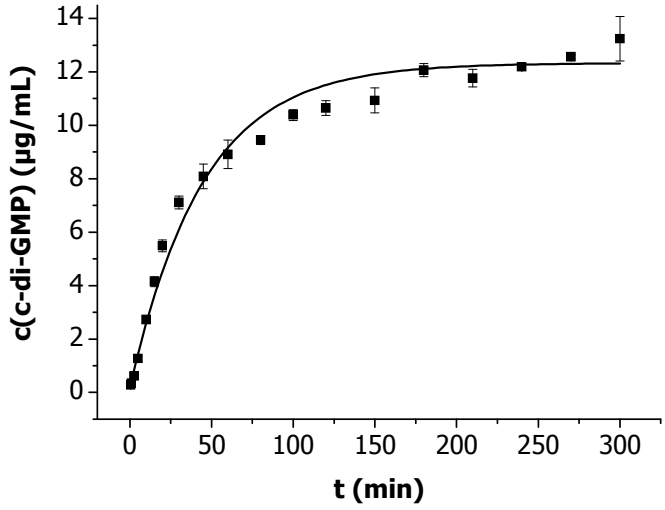


Fig 4.2 Turnover of GTP to c-di-GMP by the constitutively active di-guanylate cyclase PleD* (for assay conditions see "Materials and Methods"). The response curve shows monophasic saturation and is fitted sigmoidally (R^2 = 0.996). Error bars are standard deviations of the mean. Calculations and fit were conducted with Origin 8.

4.4.3 c-di-GMP Concentration along Growth Curve of *Escherichia coli*

Changes of c-di-GMP levels along the growth curve were determined for the *Escherichia coli* wildtype strain MG1655. The experiment was performed in duplicate with two independent bacterial cultures. c-di-GMP was extracted at an optical density (OD_{600}) of 0.5 followed by extractions in one-hour intervals for six more hours. Corresponding OD_{600} values were measured at the time of extraction. The course of c-di-GMP concentration (mean values of biological duplicates) is displayed in Fig. 4.3. The c-di-GMP level rised from levels below the limit of detection during exponential growth, to a maximum concentration of 2.1 pmol/mg of bacterial protein at entry into stationary phase (OD_{600} of 1.34) and decreased again in latter stages of stationary phase. These results suggest that c-di-GMP levels are controlled in a growth phase-dependent manner.

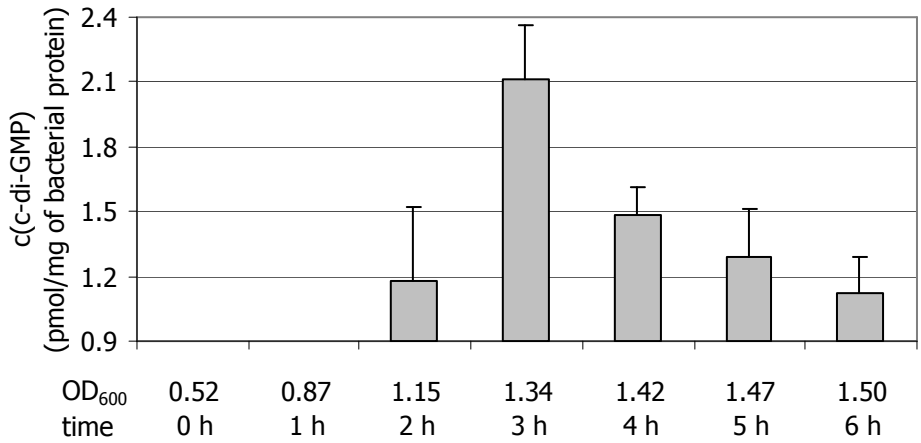


Fig. 4.3 Change of c-di-GMP concentration along growth curve of *Escherichia coli*. (LOD = < 0.9 pmol/mg of bacterial protein). Bars represent c-di-GMP concentrations with standard deviations of the mean. Error bars were calculated using Excel.

4.5 Discussion

The bacterial second messenger c-di-GMP regulates the transition from a motile planctonic lifestyle to a sessile biofilm-forming state in a wide range of species and is responsible for the switch between virulence and persistence. For a detailed understanding of the regulatory mechanisms of the c-di-GMP signaling system, a reliable and sensitive c-di-GMP quantitation method for *in vitro* and *in vivo* experiments is essential. Our study presents the development and validation of a suitable reversed phase-HPLC-MS/MS method.

The method as presented here possesses several advantages compared to existing HPLC or mass spectrometry based methods. For example, detection of c-di-GMP by UV absorption at 254 nm after HPLC separation requires complete peak separation and is, thus, not ideal to quantify c-di-GMP in cellular extracts containing high concentrations of related nucleotides such as GTP and ATP. As a consequence, HPLC-UV analysis is only suitable to determine c-di-GMP when DGCs are overexpressed[22].

c-di-GMP levels in bacterial extracts have been measured by using MALDI-TOF mass spectrometry[16]. This method requires a separate chromatographic work-up for the isolation of c-di-GMP. Successively collected 1 mL elution fractions have to be separately processed and individually subjected to mass spectrometric analysis. Moreover, the varying quality of the sample spot, the uneven matrix crystallization and unequal spreading of matrix on the template are unsolved technical problems associated with MALDI-TOF technology. Hence, MALDI-TOF mass spectrometry is limited in terms of reliability and reproducibility. In contrast, the direct coupling of chromatographic separation to the tandem mass spectrometer via electrospray ionization (ESI) as described here facilitates the quantitation of c-di-GMP with high sensitivity which is comparable to the described MALDI-TOF method.

Some liquid chromatography tandem mass spectrometry methods for the detection of c-di-GMP have been reported before[17,3,18]. However, the published methods have several drawbacks. A validation supporting their reliability has not yet been performed. In some cases, the individual chromatographic runs are time-consuming (up to 45 minutes) which is disadvantageous when high sample throughput is required. Moreover, a reliable quantitation by mass spectrometry requires the inclusion of an adequate internal standard (IS). We use cyclic 3':5'-xanthosine monophosphate (cXMP) which (a) is structurally related to the analyte due to its nucleotide character and (b) is absent from bacteria and hence does not interfere with the extraction of endogenous nucleotides.

Tandem mass spectrometers are highly selective due to the detection of specific molecular fragments. Depending on the instrumentation and the used ion source, a so-called "in-source fragmentation" can occur. Thereby, the analyte molecule decomposes into smaller fragments during the ionization process directly at the ion source even before reaching the collision cell or any of the mass discriminating fields of the mass spectrometer. c-di-GMP can undergo decomposition to cyclic 3':5'-guanosine monophosphate (cGMP). This has to be considered and corrected for if the extent of in-

source fragmentation is significantly high. However, HPLC-coupled ESI-MS/MS analysis still displays higher specificity compared to single-MS or non-MS methods.

The validation performance parameters (see Table 4.1) of the presented reversed phase-HPLC-MS/MS method are within the required ranges (Guidance for Industry, Bioanalytical Method Validation[20]), which renders the method useful for the detection of c-di-GMP in biological assays. The extraction recovery rates (see Table 4.1) for low concentration standards (10 and 50 ng/mL) are smaller than for the high concentration standard (500 ng/mL). Losses during the extraction process are more pronounced in samples spiked with low, compared to high, amounts of c-di-GMP. This accounts for the different recovery rates of the three c-di-GMP standards. Furthermore, the 24 h stability of c-di-GMP at room temperature (see Table 4.1) is superior at increased c-di-GMP concentrations. This finding can be possibly attributed to the formation of dimers or even tetramers of c-di-GMP as proposed in an earlier study[23]. Supposedly, c-di-GMP is stabilized in these aggregates at higher concentrations.

The application of both detection and quantitation methods to biological samples can suffer from interfering substances in the biological matrix. In a previous study, an unknown compound was identified by HPLC-MS/MS analysis in extracts of *Vibrio cholerae* with a retention time and molecular mass close to c-di-GMP[18]. These findings illustrate that chromatographic conditions have to be highly optimized for the identification and separation of analyte peaks, not only in standard samples but particularly in biological matrices. In bacterial extracts of *Escherichia coli* we found two matrix peaks at 5.6 and 8.9 minutes (see Fig. 4.1), the latter being base-line separated from the c-di-GMP peak at 8.6 minutes. Only two of the three SRM transitions for c-di-GMP can be found for the peak at 8.9 minutes. Moreover, both unknown peaks appear with the same intensity in both samples with and without quantifiable amounts of c-di-GMP as indicated in Fig. 4.1. In extracts of different bacterial species, a similar peak was observed close to the retention time of c-di-GMP (data not shown). In the *in vitro* assay with the constitutively active DGC PleD* (see Fig. 4.2) the additional peak close to c-di-GMP did not appear. Hence, the unknown peaks in the bacterial extracts of *Escherichia coli* seem to be interfering endogenous bacterial metabolites which can be clearly discriminated from c-di-GMP with our method and do not interfere with its quantitation.

Apart from the direct determination of c-di-GMP levels in bacterial cells, the identification and characterization of protein domains participating in c-di-GMP metabolism is of basic interest. The activities of GGDEF and EAL domains have been

determined by *in vitro* biochemical analyses. We applied our HPLC-MS/MS method for the establishment of an *in vitro* DGC assay with the constitutively active DGC PleD*. The method can serve as a simple, reliable and very sensitive approach for the analysis of newly discovered c-di-GMP producing enzymes and thus contributes to the elucidation of their role in the c-di-GMP regulatory system.

The virulence and persistence of many bacterial pathogens is strongly dependent on their lifestyle. The expression of virulence factors is favored in the planctonic state, whereas chronic infections are often characterized by the formation of robust biofilms which persist despite attacks by the immune system and antimicrobial treatment. The second messenger c-di-GMP plays a key role in the adaptation process of bacteria to their environment. We measured the change of c-di-GMP levels in liquid cultures of *Escherichia coli* along the growth curve and observed clear fluctuations dependent on the optical density. An increase in c-di-GMP concentrations during the exponential growth phase with a maximum at an OD_{600} of 1.34 at the entry into the stationary phase is followed by a smooth decrease in the stationary phase. This implies that the *Escherichia coli* c-di-GMP signaling network clearly responds to environmental conditions via up- or down-regulation of c-di-GMP metabolism. The variation of *in vivo* c-di-GMP levels of bacterial communities during colonization and biofilm formation on suitable templates remains to be determined. A modification of the presented extraction procedure for bacterial liquid cultures can offer the possibility to extract c-di-GMP from biofilms grown on solid templates as described in another study for cultures grown on filter templates[19]. The study of c-di-GMP levels in biofilms could foster the development of strategies which help to avoid the formation of persistent bacterial communities and the resulting antibiotic resistance. The determination of overall c-di-GMP levels in intact bacteria is an important step towards the understanding of c-di-GMP metabolism concerning the cooperative effects of c-di-GMP metabolizing enzymes. However, there are hints that enzymes involved in c-di-GMP metabolism are targeted to specific sites in the bacterial cell[6] possibly leading to only locally elevated c-di-GMP levels. Hence, the identification and quantitation of local c-di-GMP pools is the great challenge for the future.

In conclusion, our study shows that the novel reversed phase-HPLC-MS/MS method combined with an efficient nucleotide extraction procedure serves as a robust, specific, and sensitive tool for both the *in vitro* biochemical analysis of c-di-GMP-generating or -degrading enzymes and the determination of *in vivo* c-di-GMP

concentrations. Hence, our method can contribute to better understand the complex c-di-GMP signaling network in bacteria. The described method has been successfully applied to two studies aimed at the identification and characterization of novel c-di-GMP regulatory systems[24,25].

4.6 References

1 Ross P, Weinhouse H, Aloni Y, Michaeli D, Weinberger-Ohana P, Mayer R, Braun S, de Vroom E, van der Marel GA, van Boom JH, Benziman M (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* **325:** 279-281

2 Amikam D, Galperin MY (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* **22:** 3-6

3 Hickman JW, Harwood CS (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* **69:** 376-389

4 Duerig A, Abel S, Folcher M, Nicollier M, Schwede T, Amiot N, Giese B, Jenal U (2009) Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. *Genes Dev* **23:** 93-104

5 Sudarsan N, Lee ER, Weinberg Z, Moy RH, Kim JN, Link KH, Breaker RR (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* **321:** 411-413

6 Paul R, Weiser S, Amiot NC, Chan C, Schirmer T, Giese B, Jenal U (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev* **18:** 715-727

7 Schmidt AJ, Ryjenkov DA, Gomelsky M (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol* **187:** 4774-4781

8 Galperin MY (2005) A census of membrane-bound and intracellular signal transduction proteins in bacteria: Bacterial IQ, extroverts and introverts. *BMC Microbiology* **5:** 35-53

9 Jenal U, Malone J (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* **40:** 385-407

[10] Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413:** 860-864

[11] Paul R, Abel S, Wassmann P, Beck A, Heerklotz H, Jenal U (2007) Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. *J Biol Chem* **282:** 29170-29177

[12] Kumar M, Chatterji D (2008) Cyclic di-GMP: a second messenger required for long-term survival, but not for biofilm formation, in *Mycobacterium smegmatis*. *Microbiology* **154:** 2942-2955

[13] Christen M, Christen B, Folcher M, Schauerte A, Jenal U (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* **280:** 30829-30837

[14] Tamayo R, Tischler AD, Camilli A (2005) The EAL domain protein VieA is a cyclic diguanylate phosphodiesterase. *J Biol Chem* **280:** 33324-33330

[15] Simm R, Morr M, Kader A, Nimtz M, Römling U (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol* **53:** 1123-1134

[16] Simm R, Morr M, Remminghorst U, Andersson M, Römling U (2009) Quantitative determination of cyclic diguanosine monophosphate concentrations in nucleotide extracts of bacteria by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *Anal Biochem* **386:** 53-58

[17] Thormann KM, Duttler S, Saville RM, Hyodo M, Shukla S, Hayakawa Y, Spormann AM (2006) Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. *J Bacteriol* **188:** 2681-2691

[18] Waters CM, Lu W, Rabinowitz JD, Bassler BL (2008) Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of *vpsT*. *J Bacteriol* **190:** 2527-2536

[19] Rabinowitz JD, Kimball E (2007) Acidic acetonitrile for cellular metabolome extraction from *Escherichia coli*. *Anal Chem* **79:** 6167-6173

[20] Guidance for Industry, Bioanalytical Method Validation (2001) U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM)

[21] Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T (2004). Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci USA* **101:** 17084-17089

[22] Kulesekara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S, Lee DG, Neely AN, Hyodo M, Hayakawa Y, Ausubel FM, Lory S (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc Natl Acad Sci* **103:** 2839-2844

[23] Liaw YC, Gao YG, Robinson H, Sheldrick GM, Sliedregt LAJM, van der Marel GA, van Boom JH, Wang AHJ (1990) Cyclic diguanylic acid behaves as a host molecule for planar intercalators. *FEBS Lett* **264:** 223-227

[24] Boehm A, Kaiser M, Li H, Spangler C, Kasper CA, Ackermann M, Kaever V, Sourjik V, Roth V, Jenal U (2010) Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141:** 107-116

[25] Malone JG, Jaeger T, Spangler C, Ritz D, Spang A, Arrieumerlou C, Kaever V, Landmann R, Jenal U (2010) YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. *PLoS Pathog* **6(3):** e1000804

5. Interaction of the Di-Guanylate Cyclase YdeH of *Escherichia coli* with 2′,(3′)-Substituted Purine and Pyrimidine Nucleotides

5.1 Abstract

Di-guanylate cyclases (DGCs) synthesize the bacterial intracellular second messenger cyclic 3′:5′-di-guanosine monophosphate (c-di-GMP) which is degraded by specific phosphodiesterases (PDEs). c-di-GMP levels control the transition of bacteria from a motile to a biofilm forming lifestyle. These bacterial communities are highly resistant to antibiotic treatment and represent the predominant lifestyle in most chronic infections. Hence, DGCs serve as starting-point for the development of novel therapeutics interfering with the second messenger signaling network in bacteria. In previous studies we showed that 2′(3′)-*O*-(*N*-methylanthraniloyl) (MANT)- and 2′,3′-*O*-(2,4,6-trinitrophenyl) (TNP)-substituted nucleotides are potent adenylyl and guanylyl cyclase inhibitors. The catalytic domain of DGCs is homologuous to the mammalian adenylyl cyclase catalytic domain. Therefore, we investigated the interaction of various MANT purine and pyrimidine nucleotides with the model DGC YdeH from *Escherichia coli*. We observed strong fluorescence resonance energy transfer (FRET) between tryptophan and tyrosine residues of YdeH and the MANT-group of MANT-NTPs (MANT-ATP, -CTP, -GTP, -ITP, -UTP, and -XTP) and an enhanced direct MANT fluorescence upon interaction with YdeH. We assessed the affinity of MANT-NTPs to YdeH by performing competition assays with NTPs. We conducted an amino acid alignment of YdeH with the earlier crystallized *Caulobacter crescentus* DGC PleD and found high similarities in the nucleotide binding site of PleD. *In vitro* mass-spectrometric activity assays with YdeH resulted in the identification of new MANT/TNP nucleotide-based inhibitors of DGC activity. Collectively, the analysis of interactions between MANT/TNP nucleotides and YdeH provided a new basis for the identification and development of DGC inhibitors and allows insights into nucleotide-protein interactions.

5.2 Introduction

Bacteria universally form communities by attachment and aggregation on a surface which may take many forms such as inert solid materials, living tissue or boundary surfaces in aquatic systems[1]. These biofilms are characterized by the formation of an exopolysaccharide matrix in which the microorganisms are encased[2]. One or more different bacterial species can accumulate in one single biofilm. For example dental biofilms are estimated to contain more than 500 bacterial species[3]. Biofilm-grown cells are highly persistent and, in contrast to planctonic cells, display increased resistance to antimicrobial treatment and host defense. Most chronic bacterial infections result from the formation of stable biofilms. In case of cystic fibrosis, the airways of patients are infected by *Pseudomonas aeruginosa*[4]. Biofilm formation on diagnostic or surgical medical devices also poses a serious problem for public health[5]. The mechanisms of antibiotic resistance are only partially understood (for review see Refs. 6 and 7). Hence, the development of effective antibiotics is stagnant. Only few chemical compounds capable of affecting biofilm formation have been identified[8,9].

The transition from a motile, planctonic lifestyle to a sessile, cooperative lifestyle is regulated by the bacterial second messenger cyclic 3':5'-di-guanosine monophosphate (c-di-GMP) (for review see Ref. 10). In general, elevated levels of c-di-GMP account for increased biofilm formation. c-di-GMP is synthesized by di-guanylate cyclases (DGCs) *via* the condensation of two GTP molecules and degraded by specific phosphodiesterases (PDEs) to GMP *via* the linear intermediate pGpG. These two highly abundant protein families in bacteria contain the conserved GGDEF and EAL domains, respectively[11,12]. In most cases, a single bacterial genome encodes many different members of these protein families[13]. Hence, c-di-GMP-metabolizing enzymes, especially DGCs as key enzymes of second messenger signaling in bacteria, constitute a pharmacological target for the developement of possible inhibitors capable of affecting c-di-GMP biosynthesis and biofilm formation.

In previous studies, we identified various 2'(3')-*O*-(*N*-methylanthraniloyl) (MANT)-substituted nucleotides as potent inhibitors of the bacterial adenylyl cyclase (AC) toxin edema factor (EF) and of mammalian membrane ACs (mACs) by both enzymatic and fluorescence spectroscopy methods[14,15]. Moreover, 2',3'-*O*-(2,4,6-trinitrophenyl) (TNP)-substituted nucleotides are potent inhibitors of various AC isoforms and soluble guanylyl cyclase (GC)[16]. Sequence similarity between the GGDEF domain and mAC catalytic domain has been detected[17]. From this homology, it was deduced that the fold of the

GGDEF domain is similar to the mAC catalytic domain. This finding prompted us to investigate the interaction of different MANT-substituted nucleotides with a model DGC. For this purpose, we used the DGC YdeH from *Escherichia coli*. The YdeH gene has been identified as target for the carbon storage regulator CsrA, an RNA-binding protein which, amongst others, controls biofilm formation[18]. Later, it was shown that YdeH possesses *in vitro* DGC activity[19]. However, in a previous study it was shown that despite the similar fold of the DGC domain and the mAC domain, the nucleotide-binding mode in the DGC PleD from *Caulobacter crescentus* is substantially different[20].

In this study, we investigated the interaction between MANT-/TNP-substituted nucleotides and the DGC YdeH by fluorimetric and mass-spectrometric means. In a first approach, we evaluated the affinity of MANT nucleotides to YdeH by performing fluorescence competition assays with unsubstituted nucleotides and assessed structural aspects of nucleotide binding by YdeH *via* amino acid alignment with the DGC PleD. The DGC activity of YdeH was determined *in vitro* using sensitive HPLC-coupled tandem mass spectrometry. The *in vitro* analysis of a series of MANT- or TNP-substituted nucleotides for their potential inhibitory effect on DGC activity of YdeH resulted in the identification of three GTP-based YdeH inhibitors.

5.3 Materials and Methods

5.3.1 Chemicals

Solvents used in HPLC analysis were water, methanol, and acetonitrile (HPLC-gradient grade, J. T. Baker, Deventer, The Netherlands). Isopropyl β-D-1-thiogalactopyranoside (IPTG), ammonium acetate, bovine serum albumin (BSA), L-glutamic acid, L-arginine, adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), inosine 5′-triphosphate (ITP), uridine 5′-triphosphate (UTP), and guanosine-$^{13}C_{10}$,$^{15}N_5$ 5′-triphosphate sodium salt were purchased from Sigma Aldrich (Steinheim, Germany). Cytidine 5′-triphosphate (CTP), xanthosine 5′-triphosphate (XTP), MANT-ATP, MANT-GTP, MANT-XTP, MANT-GTPγS, and TNP-GTP were from Jena Bioscience (Jena, Germany). Sodium chloride, sodium hydroxide, tris(hydroxymethyl)-aminomethane, magnesium chloride hexahydrate, and sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany), acetic acid was from Riedel-de Haen (Hannover-Seelze, Germany), and imidazole from Carl Roth (Karlsruhe, Germany). Complete, EDTA-

free protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany). Cyclic 3':5'-di-guanosine monophosphate (c-di-GMP) was kindly provided by BioLog (Bremen, Germany). MANT-CTP, MANT-ITP, and MANT-UTP were synthesized as described[14].

5.3.2 Expression and Purification of YdeH

C-terminally His_6-tagged YdeH was expressed from a pET28 vector in the *Escherichia coli* Rosetta strain kindly provided by Drs. U. Jenal and A. Böhm, Molecular Microbiology Division, Biozentrum, University of Basel, Switzerland. Cells were grown in LB medium supplemented with kanamycin (50 µg/mL) and chloramphenicol (30 µg/mL) and expression of YdeH was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (1 mM) for 3 hours at 30 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM NaH_2PO_4 pH 7.5, 200 mM NaCl, 10 mM imidazole, 50 mM L-glutamic acid, 50 mM L-arginine) containing EDTA-free protease inhibitor cocktail (1 tablet/50 mL buffer) and lysed by ultrasonic treatment on ice using seven 30 second burst periods at 250 W with 30 second cooling periods in between. The lysate was cleared by centrifugation (20.000 x g) and the supernatant fluid was filtered (0.22 µm). YdeH was purified by Ni-NTA affinity chromatography using a 5 mL HisTrap FF column (GE Healthcare, Munich, Germany). After washing the column with lysis buffer (without protease inhibitors), YdeH was eluted with a linear gradient of imidazole from 10 to 500 mM. Desalting and buffer exchange of pooled fractions were performed on a 5 mL HiTrap Desalting column (GE Healthcare). Final protein concentration was determined with a Bradford assay (Carl Roth, Karlsruhe, Germany).

5.3.3 Fluorescence Experiments for Monitoring MANT-Nucleotide Binding to YdeH

Fluorescence experiments were performed using a quartz UV ultra-microcuvette from Hellma (Müllheim, Germany, type 105.251-QS) in a Varian Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, CA, USA). Reaction mixtures contained 5 mM $MnCl_2$ and 50 mM NaCl in 50 mM TRIS-HCl pH 8.0, followed by sequential addition of MANT nucleotides (1 µM final concentration) and YdeH (5 µM final concentration) in a total assay volume of 100 µL. Steady-state fluorescence emission spectra of MANT nucleotides were recorded at low-speed in the scan mode. In fluorescence resonance energy transfer (FRET) experiments, the excitation wavelength was λ_{ex} = 280 nm with

λ_{em} = 305-540 nm, whereas in direct fluorescence experiments, the MANT group was excited at λ_{ex} = 350 nm, and the emission was recorded at λ_{em} = 380-540 nm. In kinetic competition experiments, MANT nucleotides where displaced from YdeH by sequential addition of increasing concentrations of nucleotides to the assay mixture. Direct fluorescence emission of MANT nucleotides was recorded at λ_{em} = 440 nm after excitation at λ_{ex} = 350 nm.

5.3.4 YdeH *in vitro* Activity Assay

In DGC activity assays, 10 nM YdeH was used. MgGTP concentrations were varied between 100 nM and 1 mM. The standard reaction mixture contained 50 mM TRIS-HCl pH 8.0, 5 mM $MgCl_2$, 0.1% (m/v) BSA in a total assay volume of 50 µL. The temperature was set to 30 °C. The reaction was initiated by the addition of YdeH and was stopped after 30 minutes by heat-inactivation at 95 °C for 5 minutes. The resulting suspension was centrifuged (20.000 x g) in order to remove denatured protein. c-di-GMP concentration was determined in the supernatant by high performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS) as described[21] except for the use of the enzymatically synthesized internal standard $^{13}C_{20}$,$^{15}N_{10}$ cyclic 3':5'-di-guanosine monophosphate ($^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP) (synthesis protocol see below) instead of cyclic 3':5'-xanthosine monophosphate (cXMP). $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP was present in a final concentration of 200 ng/mL and was detected using selected reaction monitoring (SRM) analysis in positive ionization mode with an SRM transition of +721/162 and a collision energy of 61 eV.

Assay mixtures for the inhibition experiment of YdeH with MANT-GTP contained MANT-GTP at final concentrations of 100 nM, 300 nM, 1 µM, 3 µM, and 10 µM. Inhibition experiments with MANT-GTPγS and TNP-GTP additionally contained the inhibitors at final concentrations of 10 nM and 30 nM. In screening experiments for potential DGC inhibitors, MANT- or TNP-substituted nucleotides were present at a concentration of 10 µM. Inhibition experiments were performed under conditions as described above with 5 µM Mg/GTP as substrate and an incubation time of 15 minutes. All assays were performed in triplicate.

5.3.5 Enzymatic Synthesis of $^{13}C_{20}$,$^{15}N_{10}$ Cyclic Di-Guanosine Monophosphate

For the synthesis of $^{13}C_{20}$,$^{15}N_{10}$ cyclic 3′:5′-di-guanosine monophosphate ($^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP) 2 µM YdeH was incubated with 500 µM guanosine-$^{13}C_{10}$,$^{15}N_5$ 5′-triphosphate in 50 mM TRIS-HCl pH 8.0, 5 mM $MgCl_2$, and 0.1% (m/v) BSA for 18 hours at 30 °C. From an identical experiment using unlabeled GTP substrate it can be deduced that substrate turnover is complete under these conditions. The reaction was stopped by heating to 95 °C for 15 minutes and the suspension was clarified by centrifugation (20.000 x g). The concentration of $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP in the supernatant fluid was determined by measuring the absorption at 254 nm (ε_{254} = 23.700 M^{-1} cm^{-1}). Further purification steps were not necessary for the use of $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP as internal standard and were therefore omitted.

5.4 Results

5.4.1 Interaction of MANT Nucleotides with YdeH in Steady State Fluorescence Experiments

Tryptophan and tyrosine residues represent intrinsic fluorophores in proteins and can be excited at λ_{ex} = 280 nm resulting in endogenous fluorescence with a maximum at λ_{em} = 350 nm as indicated for YdeH in Figs. 5.1a-f. MANT nucleotides exhibited only minimal endogenous fluorescence after excitation at λ_{ex} = 280 nm. After addition of YdeH to all MANT nucleotides investigated, distinct new fluorescence signals appeared in presence of Mn^{2+} with a maximum ranging from 425 to 435 nm (Figs. 5.1a-f). These peaks are ascribed to fluorescence resonance energy transfer (FRET) from tryptophan and tyrosine residues to the MANT group. The extent of FRET was comparable for all examined MANT nucleotides. In presence of Mg^{2+}, FRET signals were smaller or even disappeared (data not shown). Lower FRET intensities with MANT nucleotides using Mg^{2+}- compared to Mn^{2+}-containing buffers have also been detected earlier in experiments with mACs[22]. Although Mg^{2+} is the physiologically relevant cation, all fluorescence studies were conducted with Mn^{2+}. The reversibility of MANT nucleotide binding to YdeH was examined by the addition of 10 µM GTP. As a consequence, the FRET signals considerably decreased. In case of MANT-GTP, only a very small effect was observed. With the applied GTP concentration, the FRET signals did not disappear completely as indicated by the remaining shoulders between 400 and 500 nm.

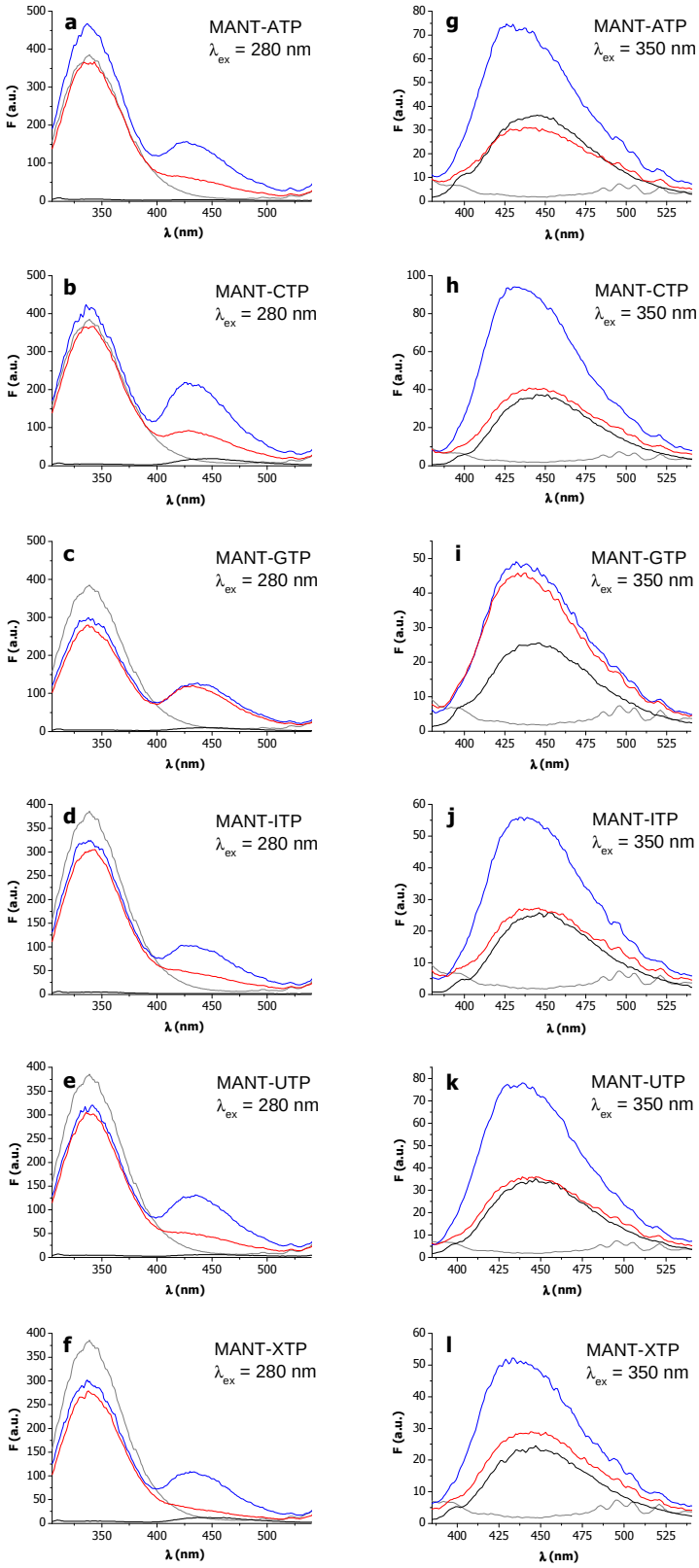


Fig. 5.1 Analysis of MANT-NTP (NTP = ATP, CTP, GTP, ITP, UTP, XTP) interaction with YdeH by performing both FRET and direct fluorescence studies as described under "Materials and Methods". The following components were added consecutively: MANT nucleotide, 1 µM (black); YdeH, 5 µM (blue); GTP, 10 µM (red). YdeH alone, 5 µM, is represented in grey. Representative FRET experiments (λ_{ex} = 280 nm) and direct fluorescence experiments (λ_{ex} = 350 nm) are shown in panels **a**-**f** and **g**-**l**, respectively.

We additionally studied the changes of direct MANT nucleotide fluorescence after interaction with YdeH at an excitation wavelength of λ_{ex} = 350 nm. MANT nucleotides exhibited strong endogenous fluorescence peaking at λ_{em} = 450 nm (Figs. 5.1g-l). In presence of YdeH, the fluorescence intensity was enhanced by a factor of 2 to 2.5 accompanied by a shift of the emission maximum to shorter wavelengths (blue-shift) by about 10-20 nm, depending on the MANT nucleotide studied. This blue-shift has been observed in binding studies of MANT nucleotides to bacterial and mammalian ACs[16,22] and is ascribed to the movement of the MANT group into a more hydrophobic environment[23]. The addition of 10 µM GTP reduced the fluorescence of the MANT nucleotides to almost the endogenous fluorescence intensities in absence of YdeH, except for MANT-GTP whose fluorescence was only marginally decreased. This goes along with the before-mentioned small reduction of FRET signal in case of MANT-GTP.

5.4.2 Competition Studies Between MANT Nucleotides and NTPs for Binding to YdeH

We examined the reversibility of MANT nucleotide fluorescence enhancement after interaction with YdeH in competition assays with different NTPs (ATP, CTP, GTP, ITP, and UTP) by monitoring changes in direct MANT fluorescence. Fig. 5.2 exemplarily shows the kinetics of MANT-ATP (Figs. 5.2a-c) and MANT-GTP (Figs. 5.2d-e) fluorescence at λ_{em} = 440 nm after excitation at λ_{ex} = 350 nm. In presence of YdeH, a pronounced fluorescence increase for both MANT nucleotides was observed. The response time of MANT nucleotide florescence was on a time scale of a few seconds at the most. The sequential addition of increasing concentrations (0.1 µM to 50 µM) of GTP or ATP resulted in differently pronounced fluorescence decreases.

After addition of GTP and ATP, the fluorescence intensity of MANT-ATP was reduced to the original level in absence of YdeH (Figs. 5.2a and b). Very similar results were obtained after addition of CTP, ITP, and UTP (data not shown). The endogenous MANT-ATP fluorescence remained constant over time in control experiments (with all NTPs examined) where buffer was added instead of YdeH at time point (1) (Fig. 5.2c: addition of ATP).

The emission intensity of MANT-GTP was decreased to a lesser extent even in presence of high concentrations of NTPs (50 µM). The five NTPs examined showed slightly different potencies of reducing YdeH-induced MANT-GTP fluorescence: GTP > ITP > ATP/UTP > CTP. Figs. 5.2d-f exemplarily show changes in MANT-GTP

fluorescence after addition of GTP and ATP (data for CTP, ITP, and UTP not shown) and a representative control experiment with ATP where YdeH was replaced by buffer. Controls were performed for all NTPs examined. No changes in MANT-GTP fluorescence were detected under these conditions.

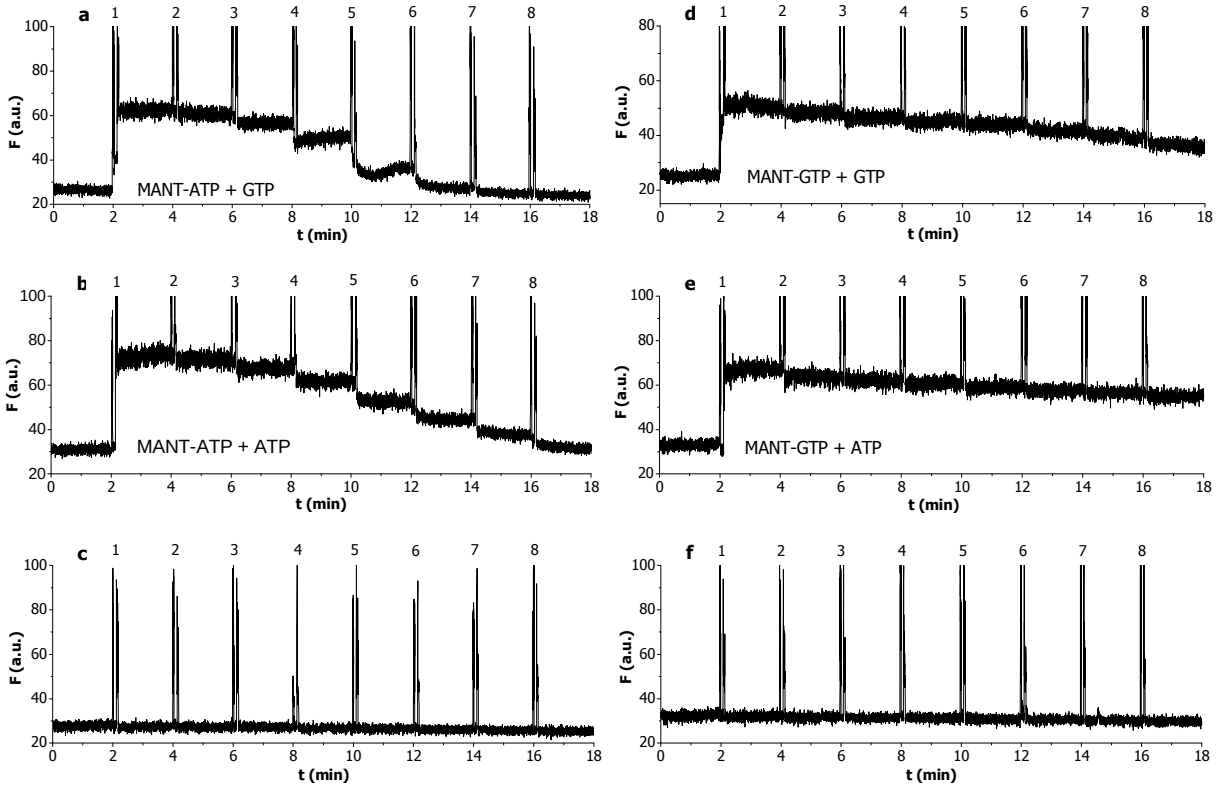


Fig. 5.2 Kinetic analysis of the competition of MANT nucleotides and NTPs for binding to YdeH in fluorescence experiments. The following components were added consecutively to cuvettes containing MANT nucleotide (1 µM; **a**-**c**: MANT-ATP, **d**-**f**: MANT-GTP): (1) YdeH (5 µM; panels **c** and **f**: buffer instead of YdeH as control); NTPs: (2) 0.1 µM, (3) 0.5 µM, (4) 1 µM, (5) 2.5 µM, (6) 5 µM, (7) 10 µM, (8) 50 µM; panels **a** and **d**: GTP, panels **b**, **c**, **e**, **f**: ATP. Excitation wavelength was set to λ_{ex} = 350 nm and emission of MANT nucleotides was detected at λ_{em} = 440 nm over time.

5.4.3 YdeH *in vitro* Activity Assay

Assays monitoring c-di-GMP production by YdeH were performed using reversed-phase high performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS)[21]. The reported method was modified in terms of using a newly synthesized internal standard (IS), $^{13}C_{20}$,$^{15}N_{10}$ cyclic 3′:5′-di-guanosine monophosphate ($^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP), instead of cyclic 3′:5′-xanthosine monophosphate (cXMP). $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP was enzymatically produced using YdeH. Although DGCs all exhibit strong product inhibition, YdeH has a comparably high residual DGC activity[19] which is sufficient for the production of $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP from guanosine-$^{13}C_{10}$,$^{15}N_5$ 5′-triphosphate.

Fig. 5.3 shows a representative chromatographic run with $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP and unlabeled c-di-GMP. The retention time of $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP was identical in comparison with unlabeled c-di-GMP (8.6 minutes). In contrast, the formerly used IS cXMP eluted earlier than unlabeled c-di-GMP at 6.1 minutes. Due to the structural identity and equal chromatographic behavior compared to unlabeled c-di-GMP, $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP was used as IS for all mass spectrometric assays.

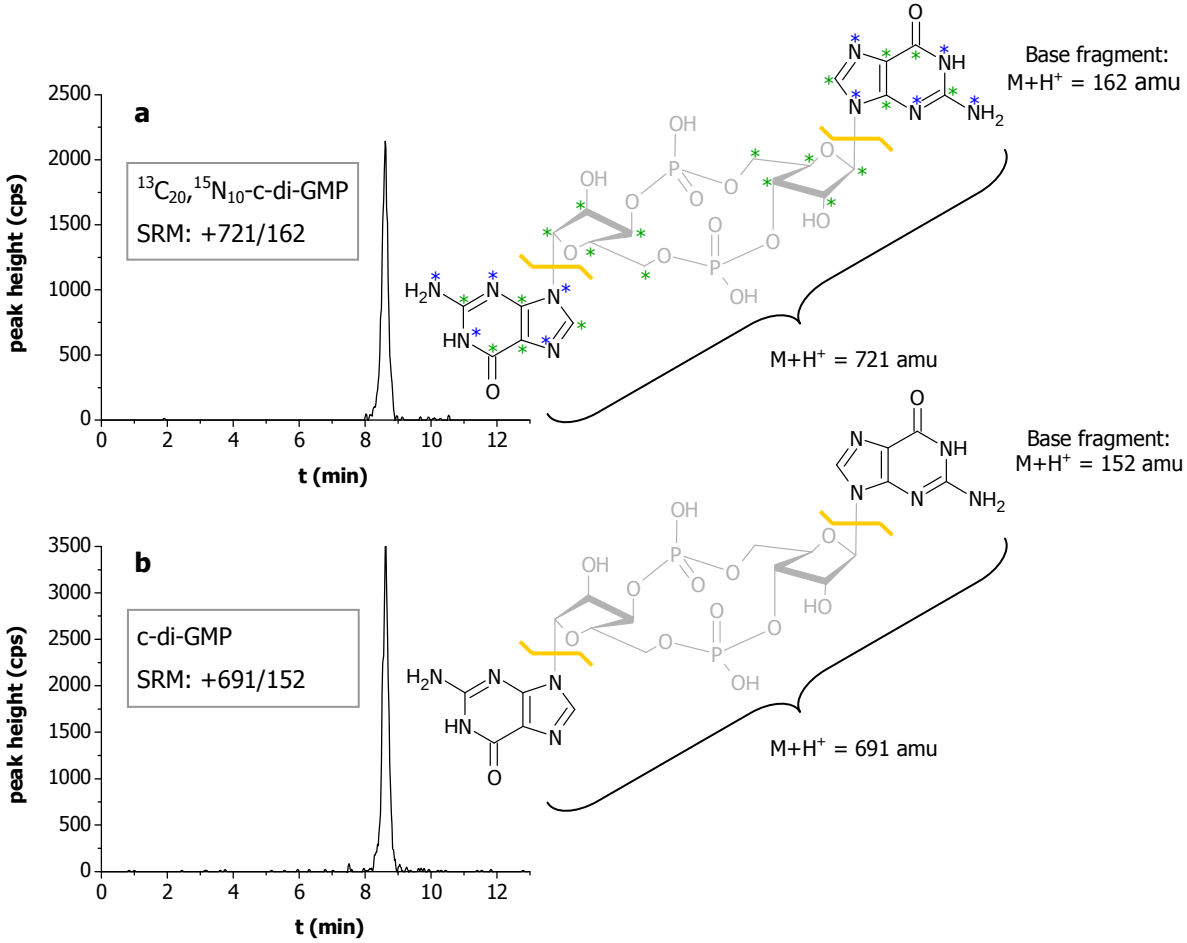


Fig. 5.3 Detection of $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP (**a**) and unlabeled c-di-GMP (**b**) by HPLC-coupled tandem mass spectrometry (HPLC-MS/MS) at concentrations of 200 ng/mL and 128 ng/mL, respectively. Panels **a** and **b** show representative chromatograms of cyclic di-nucleotides dissolved in water with an identical retention time of 8.6 min. Boxes indicate the detected SRM transition. The fragmentation pattern of the respective cyclic di-nucleotides is indicated in the corresponding structures. Atoms with an asterisk are isotope-labeled: ^{13}C (green), ^{15}N (blue).

The rate of c-di-GMP production by YdeH was determined dependent on substrate concentration as indicated in Fig. 5.4. YdeH possessed exceptional enzyme kinetics. DGC activity was characterized by a steep increase at low substrate concentrations followed by a decreasing rate of c-di-GMP formation which then leveled off and remained

constant. Under the applied assay conditions, substrate turnover was higher than 10% for GTP concentrations up to 10 µM. Hence, it is likely for DGC activity to be subject to substrate depletion. Moreover, declining DGC activity determined for GTP concentrations higher than 10 µM is probably ascribed to product inhibition, resulting in a residual rate of c-di-GMP formation of about 0.75 min^{-1}.

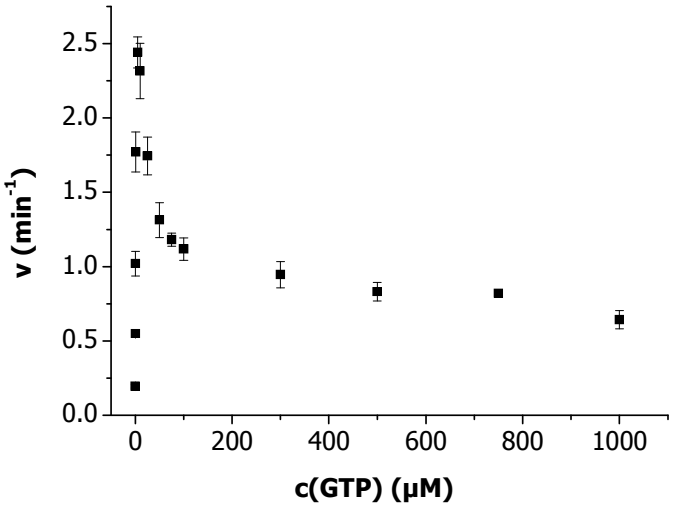


Fig. 5.4 Rate of c-di-GMP formation by YdeH as a function of substrate concentration using HPLC-coupled tandem mass spectrometry (HPLC-MS/MS). YdeH was present at 10 nM. For assay conditions see "Materials and Methods". Due to the unusual enzyme kinetics, data were not analyzed by non-linear regression to calculate K_m and V_{max} values. Error bars are standard deviations of the mean.

The above-described fluorescence assays indicate that MANT-GTP seems to possess a relatively high affinity to YdeH compared to other MANT nucleotides. Therefore, we investigated the effect of MANT-GTP on the DGC activity of YdeH in detail (Fig. 5.5). The activity of YdeH was considerably reduced with increasing concentrations of MANT-GTP. Under the applied assay conditions (see "Materials and Methods") an IC_{50} of 0.5 µM was determined. We additionally performed inhibition assays with a set of MANT- and TNP-nucleotides which were applied at a concentration of 10 µM: MANT-ATP, -CTP, -ITP, -UTP, -XTP, -GTPγS, and TNP-GTP. The activity of YdeH was reduced to a level near the limit of detection only in presence of substituted GTP (MANT-GTPγS and TNP-GTP) whereas no change in DGC activity was observed for all other MANT-substituted nucleotides. MANT-GTPγS and TNP-GTP had IC_{50} values of 0.2 µM and 0.1 µM, respectively (see Fig. 5.5).

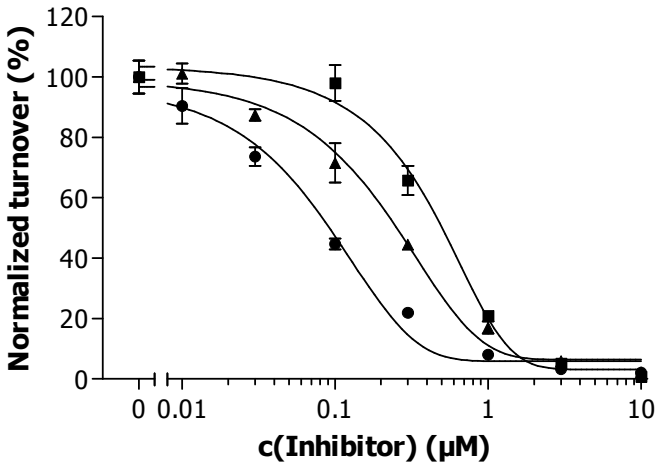


Fig. 5.5 Inhibition experiments of YdeH with MANT-GTP (squares), MANT-GTPγS (triangles), and TNP-GTP (circles) using HPLC-coupled tandem mass spectrometry. DGC activity was normalized with respect to the initial activity in absence of inhibitor. YdeH was present at 10 nM. For assay conditions see "Materials and Methods". Error bars are standard deviations of the mean.

5.5 Discussion

Microorganisms preponderantly exist in biofilm forming communities which account for the high persistence in chronic infections and may cause severe problems due to the adherence to implanted medical devices[5]. The development of efficient therapeutic strategies against the formation of biofilms is difficult[24]. In this study, we assessed the inhibitory potential of MANT and TNP nucleotides on the model DGC YdeH as a basis for intervening in c-di-GMP metabolism of biofilm forming bacteria.

Our study demonstrates that MANT nucleotides (MANT-ATP, -CTP, -GTP, -ITP, -UTP, and -XTP) undergo distinct fluorescent changes in both FRET and direct fluorescence experiments after interaction with YdeH (Fig. 5.1). All fluorescence experiments were performed with an excess of YdeH compared to the fluorescent ligand in order to obtain quantitative ligand binding and sufficient fluorescence signals. Notably, an increase in fluorescence was detected for all MANT nucleotides, not only for the MANT-substituted DGC substrate GTP. Both, the reduced FRET signals and decreased intrinsic MANT fluorescence after addition of GTP to the YdeH-MANT nucleotide complexes point to the displacement of MANT nucleotides from YdeH and binding of the original substrate GTP. However, Figs. 5.1c and i indicate that displacement of MANT-GTP is clearly less pronounced compared to all other MANT nucleotides. This points at a high affinity of the GTP-group to YdeH. Competition experiments of MANT-ATP and

MANT-GTP with NTPs (ATP, CTP, GTP, ITP, and UTP) as exemplarily shown in Fig. 5.2 support this finding: The direct fluorescence of MANT-ATP bound to YdeH is strongly reduced after successive addition of increasing concentrations of all NTPs examined, whereas in contrast, MANT-GTP fluorescence is decreased to a lesser extent in the same experiment. GTP is the most potent nucleotide in the MANT-GTP competition kinetics which can be ascribed to its function as natural substrate of DGCs. However, given the detected interaction between YdeH and MANT-NTPs other than MANT-GTP, one can speculate that YdeH optionally accepts other nucleoside 5'-triphosphates as substrate. In an *in vitro* assay with the highly active enzyme WspR the synthesis of c-di-dGMP and c-di-IMP from dGTP and ITP, respectively, was proposed, indicating a rather low substrate specificity of DGCs[25]. These data are in accordance with our findings.

The obvious binding capability of MANT-NTPs by YdeH offers the possibility of developing DGC inhibitors. Therefore, the mechanisms of inhibitor binding have to be elucidated in detail. The inhibitory characteristics of MANT-ATP and MANT-GTP and the underlying molecular binding mechanisms have been studied intensively for mammalian membrane ACs (mACs)[15,22]. From these studies we know that an increase in MANT fluorescence and a blue-shift of the emission maximum goes along with the interaction of the MANT fluorophore with nonpolar amino acid residues in a hydrophobic binding pocket. The homology between mAC and DGC catalytic domain may mislead to the assumption that the nucleotide binding mode was the same which, in contrast, is substantially different as shown for the crystal structure of the DGC PleD from *Caulobacter crescentus* solved in complex with the product c-di-GMP[20]. The authors proposed that the positions of the guanine, ribose and α-phosphoryl moieties in case of GTP binding are the same as in the complex structure with c-di-GMP.

In order to evaluate the nucleotide binding mode of YdeH we performed an amino acid alignment of PleD with YdeH (Fig. 5.6). Residues involved in GTP binding and the catalytic mechanism of c-di-GMP synthesis are conserved among the two DGCs. Hence, it is likely that the nucleotide binding mode of YdeH is comparable to PleD. We suggest that the MANT group of MANT-NTPs is transferred into a so far unspecified hydrophobic binding pocket as can be deduced from the fluorescence experiments. The exact binding mechanism of MANT nucleotides by YdeH remains to be elucidated experimentally.

```
PleD   292 DQLTGLHNRRYMTGQLDSLVKRATLGGDPVSALLIDIDFFKK 333
YdeH   132 DVLTGLPGRRVLDESFDHQLRNAEPLN--LYLMLLDIDRFKL 171

PleD   334 INDTFGHDIGDEVLREFALRLASNVRAIDLPCRYGGEEFVVI 375
YdeH   172 VNDTYGHLIGDVVLRTLATYLASWTRDYETVYRYGGEEFIII 213
```

Fig. 5.6 Amino acid alignment of the DGCs PleD from *Caulobacter crescentus* and YdeH from *Escherichia coli*. Residues necessary for GTP binding and catalytic activity in PleD (according to Ref. 20) are shaded gray and represent conserved amino acids also found in YdeH.

The enzymology of DGCs representing putative inhibitor targets needs to be elucidated in detail in order to develop new antimicrobial therapeutics. The conversion of GTP to c-di-GMP by YdeH dependent on substrate concentration was monitored with a modified HPLC-MS/MS method. The enzymatically synthesized internal standard (IS) $^{13}C_{20}$,$^{15}N_{10}$-c-di-GMP serves as ideal IS due to its identical molecular structure and retention time and further improves the described method. Hence, a reliable quantitation of c-di-GMP synthesis and DGC activity is possible. So far, the catalytic activity of YdeH has only been rudimentarily investigated[19] with the objective of identifying YdeH as DGC. In the respective activity assay, YdeH was present at a very high concentration of 2 µM. We performed assays with 10 nM YdeH (Fig. 5.4) and observed exceptional enzyme kinetics. The course of the kinetics at low substrate concentrations (≤ 10 µM GTP) is hard to evaluate since it is likely for DGC activity to be affected by substrate depletion (GTP conversion > 10%). The following decline in DGC activity for substrate concentrations higher than 10 µM is probably due to product inhibition[19], a general feature known for DGCs. However, YdeH has a relatively high residual DGC activity which is the reason for its application in the mass-spectrometric activity assay. In order to find out whether YdeH follows a Michaelis-Menten kinetic we tried to shorten the reaction time of the assay in order to avoid too high substrate conversions also for low substrate concentrations. Unfortunately, incubation times of only a few seconds would have had to be chosen which is experimentally difficult to accomplish. In addition, mass-spectrometric signals would have been too small for accurate assessment.

Facing the unusual enzyme kinetics of YdeH, we performed *in vitro* inhibition assays under fixed conditions. To our knowledge, effective inhibitors of DGC activity have not been identified so far. Some recently described compounds are able to repress biofilm formation supposedly in an indirect fashion rather than binding directly to DGCs[8,9]. We clearly identified direct inhibitors of YdeH activity in mass-spectrometric inhibition experiments. The higher affinity of MANT-GTPγS compared to MANT-GTP may

be due to stronger interactions of the bulky sulfur with surrounding amino acid residues in the binding pocket of YdeH. The TNP group is quite rigid and relatively polar. It can be speculated that it is located in a large and rather polar compartment in the binding pocket of YdeH and, thus, is responsible for the higher affinity of TNP-GTP compared to MANT-substituted guanine nucleotides. The affinities of MANT/TNP-nucleotides to DGCs seem to be lower compared to those of other cyclase families (adenylyl or guanylyl cyclases). However, absolute affinities cannot be determined due to the exceptional enzyme kinetics of YdeH for which reason we only state relative affinities for MANT/TNP nucleotides. It is likely that the lower affinities can be attributed to a different binding mechanism. This is consistent with a previous report[20]: The authors state that the nucleotide binding mode of DGCs is substantially different compared to mACs. Regardless of the relatively low affinity, it has become obvious that affinities of guanine nucleotides are significantly higher in comparison to all other examined purine and pyrimidine nucleotides. Hence, we suggest that GTP serves as an auspicious core structure for the development of potent DGC inhibitors. However, sufficient cell membrane permeability often represents a great challenge with regard to the establishment of efficient therapeutic strategies. Hence, the development of lipophilic pronucleotide inhibitors can offer new perspectives in the treatment of persistent biofilm-related infections.

Most of the examined nucleotide derivatives (except for guanine nucleotide-based derivatives) have a very low affinity to YdeH, and an inhibitory effect could not be shown in mass-spectrometric activity assays. In contrast, the described fluorescent experiments are very suitable for detecting binding events even in case of low affinity inhibitors. Additionally, competition assays with NTPs can provide a fast estimation of affinities. To our knowledge, this is the first fluorescence-based analysis of a DGC and offers a very elegant possibility to circumvent the problems arising from the unusual enzyme kinetics. As a first approximation, the identification of newly developed inhibitors *via* binding to DGCs can be accomplished straightforwardly by fluorescence spectroscopy in a high-throughput manner given the fact that YdeH can be purified in large quantities.

In conclusion, our present study provides insights into the interaction of MANT nucleotides with the DGC YdeH. Binding events were monitored *via* FRET-based and direct fluorescence experiments and the influence of potential DGC inhibitors was analyzed by sensitive HPLC-coupled tandem mass spectrometry. We identified direct DGC inhibitors based on GTP derivatives. Hence, our results provide a promising starting

point for the development of effective DGC inhibitors with the objective of inhibiting the formation of highly persistent biofilms *via* the influence of intracellular c-di-GMP metabolism.

5.6 References

1 Costerton JW, Lewandowski Z (1995) Microbial biofilms. *Annu Rev Microbiol* **49:** 711-745

2 Branda SS, Vik A, Friedman L, Kolter R (2005) Biofilms: the matrix revisited. *Trends Microbiol* **13:** 20-26

3 Whittaker CJ, Klier CM, Kolenbrander PE (1996) Mechanisms of adhesion by oral bacteria. *Annu Rev Microbiol* **50:** 513-552

4 Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413:** 860-864

5 Donlan RM (2001) Biofilm formation: a clinically relevant microbiological process. *Clin Infect Dis* **33:** 1387-1392

6 Mah TFC, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9:** 34-39

7 Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358:** 135-138

8 Ueda A, Attila C, Whiteley M, Wood TK (2009) Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist. *Microb Biotechnol* **2:** 62-74

9 Antoniani D, Bocci P, Maciag A, Raffaelli N, Landini P (2010) Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. *Appl Microbiol Biotechnol* **85:** 1095-1104

10 Jenal U, Malone J (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* **40:** 385-407

11 Paul R, Weiser S, Amiot NC, Chan C, Schirmer T, Giese B, Jenal U (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev* **18:** 715-727

[12] Schmidt AJ, Ryjenkov DA, Gomelsky M (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol* **187:** 4774-4781

[13] Galperin MY (2005) A census of membrane-bound and intracellular signal transduction proteins in bacteria: Bacterial IQ, extroverts and introverts. *BMC Microbiology* **5:** 35-53

[14] Taha HM, Schmidt J, Göttle M, Suryanarayana S, Shen Y, Tang WJ, Gille A, Geduhn J, König B, Dove S, Seifert R (2009) Molecular analysis of the interaction of anthrax adenylyl cyclase toxin, edema factor, with 2'(3')-*O*-(*N*-(methyl)anthraniloyl)-substituted purine and pyrimidine nucleotides. *Mol Pharmacol* **75:** 693-703

[15] Mou TC, Gille A, Suryanarayana S, Richter M, Seifert R, Sprang SR (2006) Broad specificity of mammalian adenylyl cyclase for interaction with 2',3'-substituted purine- and pyrimidine nucleotide inhibitors. *Mol Pharmacol* **70:** 878-886

[16] Suryanarayana S, Göttle M, Hübner M, Gille A, Mou TC, Sprang SR, Richter M, Seifert R (2009) Differential inhibition of various adenylyl cyclase isoforms and soluble guanylyl cyclase by 2',3'-*O*-(2,4,6-trinitrophenyl)-substituted nucleoside 5'-triphosphates. *J Pharmacol Exp Ther* **330:** 687-695

[17] Pei J, Grishin NV (2001) GGDEF domain is homologous to adenylyl cyclase. *Proteins: Struct Funct Bioinform* **42:** 210-216

[18] Jonas K, Edwards AN, Simm R, Romeo T, Römling U, Melefors Ö (2008) The RNA binding protein CsrA controls cyclic di-GMP metabolism by directly regulating the expression of GGDEF proteins. *Mol Microbiol* **70:** 236-257

[19] Boehm A, Steiner S, Zaehringer F, Casanova A, Hamburger F, Ritz D, Keck W, Ackermann M, Schirmer T, Jenal U (2009) Second messenger signalling governs *Escherichia coli* biofilm induction upon ribosomal stress. *Mol Microbiol* **72:** 1500-1516

[20] Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T (2004) Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci USA* **101:** 17084-17089

[21] Spangler C, Böhm A, Jenal U, Seifert R, Kaever V (2010) A liquid chromatography-coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine monophosphate. *J Microbiol Meth* **81:** 226-231

[22] Mou TC, Gille A, Fancy DA, Seifert R, Sprang SR (2005) Structural basis for the inhibition of mammalian membrane adenylyl cyclase by 2′(3′)-*O*-(*N*-methylanthraniloyl)-guanosine 5′-triphosphate. *J Biol Chem* **280:** 7253-7261

[23] Hiratsuka T (1983) New ribose-modified fluorescent analogs of adenine and guanine nucleotides available as substrates for various enzymes. *Biochim Biophys Acta* **742:** 496-508

[24] Jabra-Rizk MA, Meiller TF, James CE, Shirtliff ME (2006) Effect of farnesol on *Staphylococcus aureus* biofilm formation and antimicrobial susceptibility. *Antimicrob Agents Chemother* **50:** 1463-1469

[25] Lory S, Merighi M, Hyodo M (2009) Multiple activities of c-di-GMP in *Pseudomonas aeruginosa*. *Nucleic Acids Symposium Series* **No. 53:** 51-52

6. Summary

6.1 Summary in English

Background

Guanosine 5′-triphosphate (GTP) serves a central substrate of a diverse set of proteins and enzymes which are involved in specific signaling pathways in both eukaryotes and prokaryotes. These pathways are responsible for a variety of different cellular functions and the involved proteins and enzymes can play a key role in the development of dangerous diseases.

Fluorescence Assay for Ras Proteins

Mutated Ras oncogenes are involved in the progress of cancer. A luminescence-based microwell plate assay for real-time monitoring of the GTPase activity of wildtype and mutated Ras proteins was established. The emission intensity of the applied lanthanide complex quickly responds to changes in analyte concentration. The activity of various Ras proteins and the influence of a regulator as well as of a downstream effector was examined. The presented assay can serve as a cheap, easy, and robust platform for high-throughput screening of regulators affecting the GTPase activity of Ras proteins.

HPLC-MS/MS Assay for Di-Guanylate Cyclases

The formation of bacterial biofilms is the major cause of many chronic infections. Cyclic 3′:5′-di-guanosine monophosphate (c-di-GMP) is a ubiquitous second messenger in bacteria and is involved in the regulation of biofilm formation. A highly specific and sensitive HPLC-MS/MS method was developed and applied to the determination of the *in vitro* activity of the di-guanylate cyclase (DGC) PleD* and the *in vivo* quantitation of c-di-GMP in bacterial liquid cultures at different stages of growth. In *Escherichia coli*, intracellular c-di-GMP concentrations change in a growth dependent manner. Thus, the established quantitation method can serve as a valuable tool to gain more detailed insights into the complex c-di-GMP regulatory system.

Most chronic infections such as in the airways of patients with cystic fibrosis result from the formation of robust bacterial biofilms which are resistant to antimicrobial treatment due to the complex biofilm architecture. DGCs synthesize c-di-GMP which, at high concentrations, induces biofilm formation. Hence, DGCs represent pharmacological

targets for potential inhibitors of c-di-GMP synthesis. The affinities of 2'(3')-*O*-(*N*-methylanthraniloyl) (MANT)- and 2',3'-*O*-(2,4,6-trinitrophenyl) (TNP)-substituted nucleotides to the model DGC YdeH from *Escherichia coli* were examined in fluorescence-based competition assays. Relative affinites were determined using the established HPLC-MS/MS method. The presented fluorescence-based assay can be used to initially screen potential DGC inhibitors even with low affinities.

Conclusions

In conclusion, this work describes the development and application of two novel analytical methods for the analysis of GTP-converting proteins and enzymes and demonstrates the advantages and disadvantages of each method. However, the choice of the appropriate method for a specific scientific question can essentially contribute to the elucidation of the complex signaling networks based on the conversion of GTP.

6.2 Summary in German

Hintergrund

Guanosine-5'-triphosphat (GTP) stellt ein wichtiges Substrat einer Gruppe verschiedenster Proteine und Enzyme dar, die sowohl in Eukaryoten als auch in Prokaryoten mit spezifischen Signaltransduktionswegen in Verbindung stehen. Diese Signalwege sind für eine Vielzahl verschiedener zellulärer Funktionen verantwortlich und die beteiligten Proteine und Enzyme können eine zentrale Rolle bei der Entstehung gefährlicher Krankheiten spielen.

Fluoreszenzassay für Ras-Proteine

Mutierte Ras-Onkogene sind an der Entwicklung von Krebs beteiligt. Es wurde ein Lumineszenz-basierter Mikrotiterplattenassay zur Echtzeit-Bestimmung der GTPase-Aktivität von Wildtyp- und mutierten Ras-Proteinen aufgebaut. Die Emissionsintensität des eingesetzten Lanthanoidkomplexes spricht schnell auf Änderungen der Analytkonzentration an. Die Aktivität verschiedener Ras-Proteine und der Einfluss eines Regulators sowie eines nachgeschalteten Effektors wurden untersucht. Der dargestellte Assay kann als preiswerte, einfache und robuste Plattform für das Hochdurchsatzscreening von Regulatoren, die die GTPase-Aktivität beeinflussen, dienen.

HPLC-MS/MS-Assay für Di-Guanylatzyklasen

Die Bildung bakterieller Biofilme ist die Hauptursache vieler chronischer Infektionen. Zyklisches 3′:5′-Di-guanosinmonophosphat (c-di-GMP) ist ein universeller *second messenger* in Bakterien und ist an der Regulation der Biofilmbildung beteiligt. Es wurde eine hochspezifische und sensitive HPLC-MS/MS-Methode entwickelt und für die Bestimmung der *in vitro*-Aktivität der Di-guanylatzyklase (DGC) PleD* und die *in vivo* Quantifizierung von c-di-GMP in Bakterien-Flüssigkulturen bei verschiedenen Wachstumsphasen angewandt. Bei *Escherichia coli* ändern sich die intrazellulären c-di-GMP-Konzentrationen in Abhängigkeit vom Wachstum. Auf diese Weise kann die entwickelte Quantifizierung als wertvolle Methode eingesetzt werden, um detailliertere Einblicke in das komplexe System der c-di-GMP-Regulation zu erhalten.

Die meisten chronischen Infektionen, wie bei Mukoviszidosepatienten, beruhen auf der Bildung robuster bakterieller Biofilme, die aufgrund der komplexen Biofilmarchitektur resistent gegen konventionelle Antibiotika sind. DGCs bilden c-di-GMP, das bei höheren Konzentrationen die Biofilmbildung induziert. Folglich stellen DGCs hochinteressante pharmakologische Zielstrukturen für potentielle Inhibitoren der c-di-GMP-Synthese dar. Die Affinitäten von 2′(3′)-*O*-(*N*-Methylanthraniloyl) (MANT)- und 2′,3′-*O*-(2,4,6-Trinitrophenyl) (TNP)-substituierten Nukleotiden zur Modell-DGC YdeH aus *Escherichia coli* wurden in fluoreszenz-basierten Kompetitionsassays untersucht. Relative Affinitäten wurden mit Hilfe der etablierten HPLC-MS/MS-Methode bestimmt. Der dargestellte Fluoreszenz-basierte Assay kann zum Screening von DGC-Inhibitoren benutzt werden, auch wenn diese nur relativ niedrige Affinitäten aufweisen.

Schlussfolgerungen

Abschließend kann man festhalten, dass in dieser Arbeit mehrere analytische Methoden zur Analyse GTP-umsetzender Proteine und Enzyme eingeführt wurden und dass jede Methode sowohl Vor- als auch Nachteile mit sich bringt. Wenn jedoch eine angemessene Methode für eine spezielle wissenschafltliche Fragestellung ausgewählt wird, kann man zur Aufklärung der komplexen Netzwerke der Signalweiterleitung, die auf dem Umsatz von GTP basieren, entscheidend beitragen.

7. Curriculum Vitae

Personal Information

Name:	Christian Spangler
Date of birth:	29th May, 1981
Place of birth:	Regensburg

Education

02/2009 – present	Continuation PhD thesis at the Institute of Pharmacology, Hannover Medical School Supervisor: Prof. Dr. Roland Seifert
11/2006 – 01/2009	PhD thesis at the Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg Supervisor: Prof. Dr. Otto S. Wolfbeis
10/2001 – 10/2006	Diploma in Chemistry, University of Regensburg Diploma thesis at the Institute of Analytical Chemistry, Chemo- and Biosensors (Supervisor: Prof. Dr. Otto S. Wolfbeis): "Development of Fluorescent Probes for the Detection of Phosphorylated Amino Acids and Proteins"
09/2003 – 03/2004	Erasmus Student, University of Aberdeen, Scotland, UK Student exchange
10/2000 – 08/2001	Civilian Service, Bezirkskrankenhaus Parsberg
06/2000	Graduation from High School, Gymnasium Parsberg

Additional Qualification

Advanced Training "Bioinformatics", Braunschweig 2010

Advanced Courses in Pharmacology

"Methoden zur Analyse G-Protein-gekoppelter Rezeptoren", Mainz 2010
"Moderne Massenspektrometrie in Pharmakologie und Toxikologie", Mainz 2010
"Tiermodelle in der Pharmakologischen Forschung", Mainz 2009
"Arzneimittelinteraktionen, -metabolismus und -transport", Mainz 2009

Advanced Course "Basics in Laboratory Animal Science and Perioperative Management", Hannover 2010

Advanced Course "Gentechnische Sicherheit für Projektleiter und Beauftragte für biologische Sicherheit nach § 15 GenTSV", Hannover 2009

Proof of Competence "Amtlicher Sachkundenachweis im Umgang mit Gefahrstoffen gemäß § 5 ChemVerbotsV"

8. Abstracts and Publications

Parts of this thesis or related studies were published or presented as posters or short lectures.

Original Publications

Christian Spangler, Volkhard Kaever, Roland Seifert. Interaction of the di-guanylate cyclase YdeH of *Escherichia coli* with 2',(3')-substituted purine and pyrimidine nucleotides, *submitted*

Christian Spangler, Alex Böhm, Urs Jenal, Roland Seifert, Volkhard Kaever (2010) A liquid chromatography-coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine monophosphate. *J Microbiol Meth* **81:** 226-231

Jacob G. Malone, Tina Jaeger, **Christian Spangler**, Daniel Ritz, Anne Spang, Cécile Arrieumerlou, Volkhard Kaever, Regine Landmann, Urs Jenal (2010) YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. *PLoS Pathog* **6(3):** e1000804

Alex Boehm, Matthias Kaiser, Hui Li, **Christian Spangler**, Christoph A. Kasper, Martin Ackermann, Volkhard Kaever, Victor Sourjik, Volker Roth, Urs Jenal (2010) Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141:** 107-116

Mark-Steven Steiner, Robert J. Meier, **Christian Spangler**, Axel Duerkop, Otto S. Wolfbeis (2009) Determination of biogenic amines by capillary electrophoresis using a chameleon type of fluorescent stain. *Microchim Acta* **167:** 259-266

Christian Spangler Corinna M. Spangler, Michael Spoerner, Michael Schäferling (2009) Kinetic Determination of the GTPase Activity of Ras Proteins by Means of a Luminescent Terbium Complex. *Anal Bioanal Chem* **394:** 989-996

Corinna M. Spangler, **Christian Spangler**, Martin Göttle, Yuequan Shen, Wei-Jen Tang, Roland Seifert, Michael Schäferling (2008) A fluorimetric assay for real-time monitoring of adenylyl cyclase activity based on terbium norfloxacin. *Anal Biochem* **381:** 86-93

Review Articles

Corinna M. Spangler, **Christian Spangler**, Michael Schäferling (2008) Luminescent lanthanide complexes as probes for the determination of enzyme activites. *Ann NY Acad Sci* **1130:** 138-148

Christian Spangler, Michael Schaeferling, Otto S Wolfbeis (2008) Fluorescent probes for microdetermination of inorganic phosphates and biophosphates. *Microchim Acta* **161:** 1-39

Short Lectures

Christian Spangler, Alex Böhm, Urs Jenal, Roland Seifert, Volkhard Kaever (2010) Determination of *in vitro* di-guanylate cyclase activity and *in vivo* levels of cyclic di-guanosine monophosphate by liquid chromatography-coupled tandem mass spectrometry. *51. Jahrestagung der Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie (DGPT)*, Mainz, Germany

Corinna M. Spangler, **Christian Spangler**, Michael Schäferling, Roland Seifert (2008) Fluorimetric assay for adenylyl cyclase activity. *49. Jahrestagung der Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie (DGPT)*, Mainz, Germany

Poster Presentations

Christian Spangler, Corinna M. Spangler, Michael Spoerner, Michael Schäferling (2009) Kinetic determination of the GTPase activity of Ras proteins by means of a phosphate-sensitive luminescent Tb^{3+}-complex. *11th Conference on Methods and Applications of Fluorescence: Spectroscopy, Imaging and Probes*, Budapest, Hungary

Christian Spangler, Roland Seifert, Volkhard Kaever (2009) Quantitation of cyclic dinucleotides by reversed-phase LC-MS/MS. *4th International Conference on cGMP Generators, Effectors and Therapeutic Implications*, Regensburg, Germany

Corinna M. Spangler, **Christian Spangler**, Roland Seifert, Michael Schäferling (2008) A fluorimetric assay for adenylyl cyclase activity facilitating real-time enzyme kinetics and high throughput screening. *Symposium on "Signaltransduktion: Innovative Quelle für die Pharmakologie"*, Hannover, Germany

Christian Spangler, Corinna M. Spangler, Michael Spoerner, Michael Schäferling (2008) Probing the GTPase activity of Ras proteins by means of the luminescent lanthanide complex terbium-norfloxacin. *13th International Symposium on Luminescence Spectrometry*, Bolgona, Italy

Corinna M. Spangler, **Christian Spangler**, Roland Seifert, Michael Schäferling (2007) Luminescent probes for nucleoside phosphates and their application to the determination of adenylyl cyclase activity. *10th Conference on Methods and Applications of Fluorescence: Spectroscopy, Imaging and Probes*, Salzburg, Austria

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet. Weitere Personen waren an der inhaltlich materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Hannover, den_____ _____

Christian Spangler