Biochemical Characterization of the GTPase-activating protein GAP1IP4BP

Dissertation

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1 Introduction

Cells possess multiple mechanisms to regulate the intercellular communication as well as the intracellular signal transduction. Through intercellular communication a single cell influences the behavior of other cells in a specific manner. There are various forms of communication between cells either through chemical messengers, junctions or via cell surface proteins. The intracellular signal transduction ensures that an incoming signal is passed on from the receptor to downstream proteins, which themselves interact with other proteins as the next partner in the signaling cascade. In this specific manner, further signaling proteins are recruited to act in the signaling chain.

Among the most important components of the intracellular signal transduction are the receptors, protein kinases, protein phosphatases, adaptor proteins, and regulatory guanine nucleotide binding proteins. The latter will be discussed in further details.

1.1 Guanine nucleotide binding proteins

1.1.1 G proteins

Guanine nucleotide-binding (G) proteins are found in all bacterial, plant and animal systems. They are involved in a variety of cellular processes including protein synthesis, vesicular trafficking within cells, signal transduction pathways leading to cell growth and differentiation, receptor mediated endocytosis (Bourne et al., 1990). G proteins are involved in the regulation of all these processes rather than providing energy for mechano-chemical work or chemical synthesis.

The G proteins superfamily, with estimated 200 different proteins per eukaryotic cell, includes members of the Ras-related proteins, the heterotrimeric G-proteins with the $\alpha$, $\beta$, $\gamma$ subunits, the factors involved in protein synthesis and other less abundant families.

A relatively new evolutionary classification, based on sequence and structural signatures, divides the G proteins superclass into two large classes. The first class, designated TRAFAC (after translation factors) includes proteins involved in translation (initiation, elongation and release factors), signal transduction (the Ras like superfamily, including the heterotrimeric G-proteins subfamily), cell motility and intracellular transport. The second class, designated SIMIBI consists of signal recognition particle (SRP), the assemblage of MinD-like ATPases, which are involved in protein localization, chromosome partitioning, and membrane transport, and a group of metabolic enzymes with kinase or related
phosphate transferase activity. These two classes together contain over 20 distinct families that are further subdivided into 57 subfamilies on the basis of conserved sequence motifs, shared structural features and domain architecture (Leipe et al., 2002).

One of the best known families within the SIMIBI class is the **Signal-Recognition-Associated G protein family**. The signal recognition particles (SRPs) are ribonucleoproteins that catalyze the co-translational targeting of secretory and membrane proteins to the prokaryotic plasma membrane or endoplasmic reticulum (ER) in mammalian cells (Egea et al., 2005). SRP binds to the signal sequences of nascent polypeptide chains as they emerge from the ribosome. The resulting targeting complex consisting of the ribosome, the nascent chain with its signal sequence and the SRP, interacts with the SRP receptor (SR) in the ER membrane. This interaction releases SRP from the ribosome/nascent polypeptide chain complex. Concomitantly, the ribosome becomes associated with the translocation channel through which the protein chain is fed across the lipid bilayer (Walter and Johnson, 1994). Intriguingly, SRP and SR interact with each other in a GTP dependent manner and stimulate each others GTP-hydrolysis activity. The complex dissociates upon GTP hydrolysis. Thus, SRP and SR function as ‘initiation factors’ for protein translocation (Egea et al., 2005).

The **TRAFAC family** mostly consists of the **Translation Factors family**, the **Myosin-kinesin**, and the **Ras-like superfamily**.

The complex process of translation is regulated at its different stages (initiation, elongation and termination) by a specific set of translation factors. The initiation factor IF2 (eIF5B in eukaryotes), eIF2γ, elongation factors EF-Tu and EF-G are four members of the translation factor superfamily that are widespread in bacteria and eukaryotes. IF2/eIF5B mediates the binding of Met-tRNA$_{i}^{Met}$ to the ribosome and, in eukaryotes, joining of the two ribosomal subunits (Pestova et al., 2001; Allen and Frank, 2007). EF-G is a ubiquitous five domain G protein that catalyzes the translocation of tRNA to the ribosome (Nilsson and Nissen, 2005). EF-Tu is a three domain protein that forms a ternary complex with aminoacyl-tRNA and protects the aminoester bond against hydrolysis until a correct match between codon and anticodon is achieved. The aminoacyl-tRNA complex is released upon GTP hydrolysis to allow incorporation of the amino acid into the nascent protein chain (Ogle and Ramakrishnan, 2005).
Within the TRAFAC class of G proteins, the myosin-kinesin superfamily consists of the eukaryotic cellular motor ATP-binding proteins kinesin and myosin. It has been argued that these proteins have evolved from an ancestral GTP-binding protein at the onset of eukaryotic evolution, and have lost their specificity towards GTP (Leipe et al., 2002). These proteins mediate ATP-dependent movement of chromosomes, vesicles and organelles along tubulin-microtubules in the case of the kinesins, and along actin filaments in the case of myosins (Mallik and Gross, 2004).

Other members of the TRAFAC superfamily of GTP-binding proteins are the dynamin and the GB1 family. The members of the large family of dynamin are multi domain GTP-binding proteins involved in numerous fundamental cellular processes including membrane fission, maintenance of mitochondrial morphology, plant cell plate formation and chloroplast biogenesis. Dynamin itself is the prototype member of the entire family and is essential for receptor mediated endocytosis and recycling of synaptic vesicles (Praefcke and McMahon, 2004). The GB1 family including GBP's (guanylate-binding proteins) is the most abundant class of proteins strongly induced by interferon-γ and weakly by interferon-α/β (Martens and Howard, 2006).

**Heterotrimeric G proteins** (classified as belonging to the Ras-like superfamily) transduce extracellular signals from the cell-surface receptors of the seven-transmembrane-helix class to the intracellular compartment through a GTP-dependent cycle in which the α and βγ subunits regulate effector proteins such as adenylyl-cyclase, PI3Kinase or phospholipase Cβ. At least 20 different genes for α-subunits, 5 for β-subunits and 12 for γ-subunits are known. Although these proteins are highly homologous they differ considerably with respect to their effectors, regulators and receptor specificity.

The Ras-like superfamily comprises, along with the Heterotrimeric G-proteins, the Ras proteins. **Ras proteins** are the founding members of the Ras-like superfamily. Although similar to the Heterotrimeric G protein α subunits in biochemical properties and function, Ras family proteins function as monomeric G proteins, with molecular masses between 20-25 kD, and are classified as small guanine-nucleotide binding proteins (GNBPs) or small G proteins. They predominantly appear in eukaryotic cells. The Ras-superfamily of small G proteins comprises over 150 human members with evolutionary conserved orthologs found
in *Drosophila*, *C. elegans*, *S. cerevisiae*, *S. pombe*, *Dictyostelium discoideum* and plants (Colicelli, 2004). Based on sequence and functional similarities the Ras superfamily is divided into seven major branches: Ras, Rab, Rho, Arf, Ran, Rag and Rad (Table 1).

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Table 1. The small G protein superfamily

Nevertheless, with increasing evaluation of genomes one can distinguish apart from these major branches other families with growing functional and structural diversity. Members of the Ras branch, comprising 33 human members, represent those with greatest amino acid sequence similarity (up to 55%) and functional similarity with Ras and include R-Ras, Ral, Rheb, Rit and Rap proteins. The Rho and Rab family members share ~30% amino acid identity with members of the Ras family.

The Ras subfamily members are involved in signaling networks that control gene expression and regulation of cell proliferation, differentiation, and survival (Wennerberg et al., 2005). The Rho family proteins (Ras homologous) are key regulators of actin organization, they regulate cell cycle progression and gene expression (Etienne-Manneville and Hall, 2002), they have been implicated in the regulation of cell polarity, cell movement, cell shape, and cell-cell and cell-matrix interactions, as well as regulation of endocytosis and exocytosis (Ridley, 2001). Rab proteins (Ras-like proteins in brain) are regulators of intracellular vesicular transport and trafficking of proteins between different organelles of the endocytic and secretory pathways (Zerial and McBride, 2001). The Ran family proteins (the Ras-like nuclear protein) are best known for their function in
nucleocytoplasmatic transport of both RNA and proteins (Weis, 2003); they also regulate mitotic spindle assembly, DNA replication and nuclear envelope assembly (Li et al., 2003). The Arf family proteins (ADP-ribosylation factor) are involved in regulation of vesicular transport, formation of vesicle coats at different steps in the exocytic and endocytic pathways (Nie et al., 2003; Memon, 2004). Rag proteins are defined as regulators of Ran (Nakashima et al., 1999) and Rad proteins are involved in the regulation of voltage-dependent Ca\textsuperscript{2+} channels and cytoskeleton remodeling (Correll et al., 2008).

1.1.2 The molecular switch function

G proteins function as biological switches that serve as critical regulators of cytoplasmatic signaling networks (Vetter and Wittinghofer, 2001). Similar to the heterotrimeric G protein α subunits, the Ras family of proteins function as binary molecular switches where binding to guanine diphosphate (GDP) or guanine triphosphate (GTP) controls whether they are turned “off” or “on”, respectively. These proteins possess high-affinity for binding GDP and GTP, and an intrinsic GTP hydrolysis (GTPase) activity. However, their intrinsic GTP hydrolysis and GDP/GTP exchange activities are too low to account for the rapid GDP/GTP cycling that occurs following extracellular stimulation. Thus, the GDP/GTP cycle is controlled by two main classes of regulatory proteins that accelerate these intrinsic activities: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Figure 1).

Figure 1. The molecular switch of guanine nucleotide binding proteins
GEFs function as positive regulators of Ras proteins by promoting the displacement of GDP leading consequently to the binding of GTP, which is the more prevalent nucleotide in the living cell. GAPs, the negative regulators, accelerate the intrinsic GTPase activity by several orders of magnitude to promote the formation of the inactive GDP-bound form. When bound to GTP these proteins undergo a conformational change that allows them to interact with downstream effectors (Herrmann, 2003).

1.2 The Ras superfamily – Biochemistry and Regulation

1.2.1 Structural elements of GNBP

All small GNBP share a high degree of sequence similarity and a common three-dimensional structural motif, called the GTP-binding (G) domain. This domain enables them to act as molecular switches cycling between the two defined conformational states: GDP- and GTP-bound. This fold is found in all standard GTP-binding proteins, including the heterotrimeric G proteins and other GNBP. The Ras protein, as the prototype of the Ras superfamily, is the best characterized small GNBP. It consists of 189 amino acids with five conserved sequence elements that are necessary for binding guanine nucleotides and for the hydrolysis of the GTP.

The last four amino acids (CVLS) constitute what is called the CAAX box which represents the signal recognition sequence for the enzyme farnesyltransferase which alkylates the cysteine residue with a C15 prenyl group. This modification is the first in a series of posttranslational modifications which are responsible for anchoring Ras into the plasma membrane, a process required for its biological activity (Willumsen et al., 1984; Cox and Der, 1997; Hancock et al., 1990).

The central G domain consists of a six-stranded β-sheet and five α-helices connected by ten loop regions. Five of these loops contain the signature motifs G1-G5 and are responsible for the specificity and high affinity for the nucleotide. Ras, as most other small GNBP, has an extremely high affinity for both GDP and GTP with a $K_D$ in the picomolar range. No other standard nucleotide binds to Ras with a comparable affinity showing the high specificity for guanine nucleotides. The β-phosphate is required for the high affinity binding since guanosine monophosphate (GMP) has a $10^5$-fold reduced affinity in comparison to GDP/GTP. The binding affinity strongly depends on the presence of $\text{Mg}^{2+}$ ions. In their absence the dissociation rate constant ($k_{off}$) increases by several hundredfold
with a concomitant increase in the equilibrium dissociation constant ($K_D$) (John et al., 1990; Bourne et al., 1991; Schmidt et al., 1996; Via et al., 2000). The G motifs are also called according to their function, PM and G motifs which describe their involvement in the binding of either phosphate and $\text{Mg}^{2+}$ ions or the guanine base, respectively (Wittinghofer and Waldmann, 2000).

Figure 2. Structural overview of the Ras protein. Secondary structure elements are illustrated as cylinders ($\alpha$-helices) and arrows ($\beta$-strands). The guanine nucleotide binding site is formed by five peptide loops (G1-G5; yellow boxes), which are highly conserved throughout all G proteins. The bold lines indicate the position of the G proteins signatures: P-loop (orange), switch I (magenta), switch II (green). The isoprenylation site (CAAX box) is shown at the C-terminus (A). Ribbon structures of the C-terminal truncated Ras GDP (B) (Franken et al., 1993, PDB code 4q21) and Ras GppNHp (C) (Pai et al., 1990, PDB code 5p21) are shown with the P-loop in orange, switch I in magenta, switch II in green, the nucleotide as stick and $\text{Mg}^{2+}$ ion as a cyan sphere. To highlight the nucleotide dependent conformational changes, critical residues like Tyr32, Thr35, Gly60 and Gln61 are indicated.

The most important contribution to high-affinity binding of the nucleotide is provided by the G1 or L1 loop with the consensus sequence $^{10}\text{GxxxxGK(ST)}^{17}$. This loop, also known as the phosphate binding loop or P loop is involved in binding of the nucleotide phosphate groups (Saraste et al., 1990). It contains three important residues: codon 12, encoding for Gly12, is the most frequently mutated Ras codon in human tumors (Barbacid, 1987; Bos,
1989); **Lys16** forms a ring like structure that wraps tightly around the \( \beta \)-phosphate of the GDP/GTP and creates a positively charged environment; **Ser17** in Ras coordinates both, the important \( \text{Mg}^{2+} \)-ion (which is essential for high affinity binding of the nucleotide) and the \( \beta \)-phosphate. Its substitution by Ala or Asn severely modifies the interaction between the protein, metal ion, and nucleotide. As a result, the affinity to guanine nucleotides is reduced and in turn, the protein has a relatively high affinity to guanine nucleotide exchange factors that bind and stabilize the nucleotide free form. The GEFs are then sequestered by the mutated Ras proteins. Therefore such a mutation is called dominant negative because it inhibits signaling through GEFs (Farnsworth and Feig, 1991; John et al., 1993; Feig, 1999).

**G2** or L2 is an integral part of the effector binding loop containing the invariant **Thr35**. This residue is a direct ligand for \( \text{Mg}^{2+} \) in the GTP-bound state, it binds the \( \gamma \)-phosphate of the GTP, and it is a key residue that triggers the conformational change after GTP hydrolysis (John et al., 1993), being therefore crucial for the interaction with effector proteins (Spoerner et al., 2001).

**G3** or L4 contains the \(^{57}\text{DxxGQ}^{61}\) motif. The **Asp57** side chain is involved in \( \text{Mg}^{2+} \) binding, whereas **Gly60** coordinates the \( \gamma \)-phosphate by a main chain hydrogen bond and is an important sensor for the conformational change after GTP hydrolysis (Wittinghofer et al., 1993). Most G proteins contain a **Gln** residue (**Q61**-in Ras) crucial for GTP hydrolysis. This residue is replaced by Thr in Rap proteins and by His in elongation factors.

**G4** and **G5** are responsible for the guanine base recognition. The **G4** motif \(^{116}\text{NKxD}^{119}\) interacts tightly with the guanine base. Mutation of the **Asp119** in Ras has been shown to change the nucleotide specificity from guanosine to xanthosine nucleotides (Schmidt et al., 1996; Cool et al., 1999). The **G5** motif \(^{145}\text{SAK}^{147}\) provides **Ser145** that stabilizes Asp119. **Ala146** binds the guanine base and is another determinant for the guanine binding ability of Ras. The network of interactions between Ras and the GTP analogue GppNHp is shown in Figure 3.
1.2.2 The conformational switch

During the GTPase cycle, the transition between the GTP- and the GDP- bound form of Ras leads to conformational changes that dramatically affect its affinity for downstream signaling molecules. Structures in both the inactive (GDP-bound) and the active (GTP-bound) form have been solved and revealed that structural differences are mainly confined to two highly mobile regions, designated as switch I (residues 30-40 in Ras) and switch II (residues 60-76) (See Figure 2 for a structural overview of the Ras protein). In the active state Tyr32 and Thr35 in switch I and Gly60 in switch II form hydrogen bonds with the $\gamma$-phosphate of GTP (Figure 4). GTP hydrolysis triggers drastic rearrangements of the switch regions, resulting in the reorientation of these critical residues away from the active site (Schlichting et al., 1990; Diaz et al., 2000; Milburn et al., 1990).
In the case of Ras, Rap, Rho, Rac and Rab the switch mechanism involves only the switch I and switch II regions (Figure 5). Ran, in contrast, after transition to the active GTP-bound form, undergoes a large conformational change in switch I with unfolding of an extra β strand and a dramatic relocation of the COOH-terminal extension, the so-called C-terminal switch that is required for its function in nuclear transport (Vetter et al., 1999; Chook and Blobel, 1999). An even more dramatic change upon triphosphate binding involves the change in register of two β strands relative to the rest of the sheet in switch I of Arf and the detachment and membrane insertion of the N-terminal helix, the so-called N-terminal switch (Goldberg, 1998). Ga proteins use an extra structural element for the transition which is correspondingly called switch III.

![Figure 5. GTP/GDP conformations of GNBPs.](image)

The switch region is also conserved not only between GNBPs but also within the family of ATP-binding motor proteins, like kinesin and myosin which also have switch regions that sense the presence of the γ-phosphate. Their switch I regions contain a conserved serine and their switch II regions the same invariant DXXG motif, with glycine forming a main chain contact to the γ-phosphate (Vetter and Wittinghofer, 2001).
1.2.3 Ras signaling

Ras is the first human proto-oncogene identified. It was originally identified as the active principle of tumor-inducing sarcomaviruses in rats. The beginning of RAS research dates back over forty years to the discoveries that some retroviruses caused rapid tumor formation in rats (HARVEY, 1964; Kirsten and Mayer, 1967). The viral oncogenes responsible for their oncogenic properties, were named Harvey and Kirsten ras (H-ras and K-ras), for rat sarcoma, and were found to be derived from the rat genome.

Later, activated forms of Ras were found in human tumors and it is now estimated that mutations in Ras gene are associated with 20-25% of human tumors, with the prevalence as high as 90% in pancreatic cancer and 50% in colon cancers (Cox and Der, 2002; Malumbres and Barbacid, 2003).

The activation of the Ras gene from a proto-oncogene to an oncogene is the result of a point mutation at either positions 12, 13 or 61, the consequence of which is to render the protein unable to hydrolyze GTP, even in the presence of GAPs, thus leading to constitutively active Ras. Even in cancers in which Ras is not mutated, Ras activity is often increased as a result of overexpression and/or mutational activation of receptor tyrosine kinases that function upstream of Ras.

Ras is implicated in all aspects of malignant tumorigenesis, including increased cell proliferation, survival, invasion and metastasis, and thus the Ras pathways and Ras itself are an attractive target for anti-cancer drug development.

There are three human Ras genes (H-, K- and N-Ras) which encode four highly related 21 kDa proteins (K-Ras encodes the related 4A and 4B splice variants). H-, K- and N-Ras are widely expressed, K-Ras being found in almost every cell. Knockout studies have shown that H- and N-Ras are not required for normal development in mice, while K-Ras is essential (Johnson et al., 1997; Koera et al., 1997; Esteban et al., 2001).

Ras proteins are synthesized initially as cytosolic proteins that undergo a series of post-translational modifications that are vital to their normal and oncogenic functions. After translocation of biologically active Ras proteins to the plasma membrane, Ras is activated by several upstream signals. Growth factors such as EGF or PDGF bind to the extracellular domain of receptor tyrosine kinases which induces dimerization and phosphorylation of the receptor. This modification activates the receptor and generates binding sites for protein containing SH2 domains, like Grb2 (growth factor receptor binding). Grb2 is complexed with the RasGEF SOS which interacts with Ras and activates it.
Once activated, Ras interacts with several downstream effectors, the best characterized of which being the Raf kinase. Activated Raf in turn activates the protein kinase MEK by phosphorylation and this activates the protein kinase ERK (which are part of the MAP kinase module). Erk, in turn, phosphorylates and activates a class of transcription factors. Ras signaling is terminated by the action of GAPs, which are also recruited at the plasma membrane for an efficient interaction with Ras, and also by ERK phosphorylation of SOS, which is then not longer able to interact with Grb2 (reviewed in Wittinghofer and Waldmann, 2000).

Other effectors of Ras are PI3Ks and RalGEFs. The catalytic subunit of PI3K can be activated via its interaction with Ras-GTP. The activated PI3K converts the plasma membrane lipid phosphatidylinositol 4,5- bisphosphate (PI(4,5)P2) to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3). Signaling proteins with pleckstrin-homology (PH) domains accumulate at sites of PI3K activation by directly binding to PI(3,4,5)P3. Of particular interest are the protein serine-threonine kinases Akt, also called protein kinase B (PKB) and phosphoinositide-dependent kinase 1 (PDK1). Association with PI(3,4,5)P3 at the membrane brings these proteins into proximity and facilitates phosphorylation of Akt by PDK1. This phosphorylation stimulates the catalytic activity of Akt, resulting in the phosphorylation of a host of other proteins that affect cell growth, cell cycle entry, and cell survival (Cantley, 2002).

Last, RalGEFs are a family of guanine nucleotide exchange factors four of which are known to be activated by recruitment to the plasma membrane by Ras-GTP, where they promote the activation of Ral G-proteins to the GTP-bound state (Wolthuis and Bos, 1999; Peterson et al., 1996). Some other, recently identified, effectors of Ras are Phospholipase Cε, Tiam1, Nore1, and Ras associated domain family 1 (RASFF1).

### 1.2.4 The Ras branch of the Ras superfamily

**Ras, Rap, Ral, R-Ras, Rheb, Rit/Rin and others**

The Ras branch of the Ras superfamily can be presently classified in seven subgroups: Ras (H-Ras, K-Ras, with the two spliced variants A or B, and N-Ras), Rap (with the Rap1 A and B proteins and Rap2A, B and C proteins), Ral (A and B), R-Ras (comprising the R-Ras, R-Ras2/TC21 and R-Ras3/M-Ras proteins), Rit/Rin, Rheb, Di-Ras/ARHI proteins. All these proteins share the conserved characteristics of the G domain with regions involved in interactions with either phosphate or Mg2+ ions or the guanine base. With the
exception of Rin and Rit, they all have a C-terminal CAAX box, and are predicted to be farnesylated (the four Ras proteins, Rap2 A and C, R-Ras2/TC-21, Rheb, Di-Ras 1 and 2, ARHI) or geranylgeranylated (Rap1 A and B, Rap2B, Ral A and B, R-Ras and R-Ras3/M-Ras).

Rap Proteins
The Rap group of G proteins is composed of five different members: Rap1A, Rap1B, Rap2A, Rap2B and Rap2C that share 50-60% sequence homology with Ras proteins. Rap1A and Rap1B are 95% homologous and define the Rap1 subgroup. Similarly, the Rap2 subgroup contains Rap2A, Rap2B and Rap2C that share about 90% sequence identity. Rap1 and Rap2 subgroups altogether share close to 60% sequence identity.

Originally Rap1 was identified as a protein that could revert the morphological phenotype of Ras-transformed cells (Kitayama et al., 1989), raising the possibility that Rap1 might either antagonize the activity of Ras by competing for a common target (or regulatory protein) or function independently of Ras and mediate growth-inhibitory signals.

Rap proteins are found in all multicellular eukaryotes, with a single member of each subgroup represented in *C. elegans* and *D. melenogaster*. Only one Rap homologous protein is found in *Dictyostelium* (DdRap) and *S. cerevisiae* (Rsr1) and none in *S.pombe*.

Rap1 is bound to the membrane through a geranylgeranyl group that is added posttranslationally to the processed Rap1 carboxyl terminus. Rap1 is found predominantly at intracellular membranes in the perinuclear region and on endocytic and exocytic vesicles, but also at the plasma membrane. In malignant oral keratinocytes Rap1 also exhibits a prominent nuclear distribution, Rap1-GTP being able to translocate to the nucleus upon growth factors stimulation (Mitra et al., 2003). In this way, besides Ran, Rap1 is the only other small G protein present in the nucleus.

One of the hallmarks of Rap proteins is that glutamine 61 of the $^{57}$DxxGQ$^{61}$ sequence in the switch II region, which is conserved in proteins of the Ras, Rho, Rab and Ran branches of the Ras superfamily, is replaced by a threonine. This residue plays an important role in the catalytic mechanism of the GTPase reaction (Scheffzek et al., 1997). Despite this substitution, Rap proteins retain an intrinsic GTPase activity, with an intrinsic GTP hydrolysis rate of Rap1 10-fold slower than for Ras, which can be reconstituted by exchanging threonine 61 to glutamine (Frech et al., 1990).

Like for all other small G proteins, Rap1 was found to be activated upon stimulation of several transmembrane receptors, including receptor tyrosine kinases, heterotrimeric G-
protein coupled receptors, cytokine receptors and cell-adhesion molecules. Second messengers such as cyclic AMP, Ca²⁺ and diacylglycerol (DAG) are also involved in transducing the extracellular signals to Rap1 (Bos et al., 2001).

Extracellular stimulation induces the conversion of the inactive GDP-bound form into the active GTP-bound form, by stimulating different guanine nucleotide exchange factors (GEFs). **C3G** was the first RapGEF to be identified (Crk SH3-domain-binding guanine-nucleotide releasing factor). It contains a catalytic region responsible for the exchange reaction and several proline-rich sequences that associate with the SH3 domain of Crk adaptor protein. The Crk-C3G complex binds to phosphotyrosine containing proteins, in this way facilitating the phosphorylation of Tyr504 on C3G which is required for its activity. If the formation of this complex is disrupted, Rap activation is blocked (Bos et al., 2001).

**Epac** family proteins are other GEFs for Rap. They have auto-inhibitory domains capable of binding cAMP. In the absence of cAMP, the cAMP binding domains bind to the GEF moiety and repress its catalytic activity; the binding of cAMP releases this inhibition, hence leading to the activation of the GEF (Rehmann et al., 2006).

Another family of GEFs, **CalDAG-GEFs** are activated by Ca²⁺ or diacylglycerol binding. Two closely related RapGEFs are **PDZ-GEFs** or **RA-GEFs**. They both contain a PDZ and a RA domain. RA domain binds active Rap1 which induces the translocation of the GEF to the perinuclear region or to the plasma membrane and the activation of Rap at these locations (de Rooij et al., 1999; Gao et al., 2001).

Rap1 might function in diverse processes, ranging from modulation of growth and differentiation to secretion, integrin-mediated cell adhesion and morphogenesis. Some of these biological activities are mediated by several distinct effector proteins that interact with Rap proteins. Due to the similarity between their effector domains, Ras and Rap1 share a subset of common potential effector proteins.

Similarly to Ras, Rap1 binds to the **Raf kinases** through an interaction with both the N-terminal Ras Binding Domain (RBD) and the adjacent cysteine-rich domain (CRD) of Raf kinases (Nassar et al., 1995; Herrmann et al., 1996). Though both Ras and Rap bind to the Raf1 kinase, Rap1 is not able to activate Raf1 (Shirouzu et al., 1998). In contrast, Rap1 binds to and activates the Raf family member B-Raf. The evidence for this interaction includes the direct binding of Rap1 to, and activation of, B-Raf **in vitro** (Ohtsuka et al., 1996), and the fact that inhibitors of Rap1 such as Rap1GAP and Rap1N17 abolish the activation of the B-Raf-ERK pathway (Vossler et al., 1997; York et al., 1998). Other
effectors of Rap are **RalGEFs**, **AF-6**, and **PLC-ε**. Several putative Rap effectors like **PI3K**, the Ankyrin-repeat containing protein **Krit1**, and the RA domain containing protein **RapL** (Katagiri et al., 2003) have been described. Rap1 positively regulates integrin-mediated cell adhesion. Rap1 activates all integrins that are associated with the actin cytoskeleton, like those of the β1, β2, and β3 family, and controls both their activity and clustering depending on the integrin and the cell type (Reedquist et al., 2000; Bos et al., 2003).

The Rap1 signaling is terminated by the action of different RapGAPs which stimulate the hydrolysis of GTP to GDP-bound forms, bringing the Rap proteins to their inactive form. A detailed description of Rap inactivators will be discussed in the next chapters.

**Ral**

The Ral group consists of two closely related proteins, RalA and RalB that are 82% identical. Both Ral proteins contain an 11 residue N-terminal extension, which binds the downstream effectors, phospholipase D (PLD) or phospholipase C-δ1 (PLC-δ1) in a nucleotide dependent manner (Jiang et al., 1995). At the C-terminal tail there is a short calmodulin binding amphipathic helix and a geranylgeranyl modification at the CAAX motif. The crystal structure of RalA reveals a catalytic domain of 6 stranded β-sheets, 5 α-helices and 10 connecting loops (Fukai et al., 2003). Upon GTP binding the largest conformational changes are in the switches I and II regions. Most effector proteins bind to a single switch or stretch across both. At least four Ral GEFs are known, which in turn are stimulated by Ras. Several proteins show GAP activity towards RalA, but they remain poorly characterized. Calmodulin binds and also activates Ral. Phosphorylation at Ser-194 in the calmodulin binding motif of RalA (which is missing in RalB) by Aurora-A kinase also stimulates RalA activity (Wu et al., 2005).

RalA is a positive regulator of calcium-evoked exocytosis via binding phospholipase D and is involved in G protein coupled receptor signaling by binding phospholipase C-δ1. The binding of Ral to calmodulin links to intracellular trafficking events. Another link is direct binding of activated Ral (Ral-GTP) to the endocytic and exocytic machineries. Ral-GTP binds RalBP1, which connects to receptor-mediated endocytosis via AP-2. Alternatively, Ral-GTP binds the exocyst complex, which controls secretory vesicle trafficking in regulated secretion and filopodia formation (van Dam and Robinson, 2006).
The R-Ras group

This group contains 3 closely related, yet functionally different, proteins: R-Ras, R-Ras2/TC21 and R-Ras3/M-Ras. Their effector domain is conserved relative to Ras, and is flanked at the N-terminal end by an acidic residue, which may play a role in the interaction with certain effectors. All these proteins contain a N-terminal extension relative to Ras to which no function has yet been described. TC21 is farnesylated at its C-terminal extremity. In contrast both R-Ras and M-Ras are geranylgeranylated.

The principal biological role of R-Ras is the induction of integrin activation (Zhang et al., 1996), TC21 was found to be constitutively activated by a single point mutation and to carry a high transforming activity which is dependent on the activation of PI3K (Rosario et al., 2001; Rong et al., 2002), but its physiological function it is still a matter of debate. The most recently described member of this group, M-Ras, exhibits an expression pattern restricted to brain and heart (Kimmelman et al., 1997). In cells of neuronal origin it is a potent activator of the PI3K/Akt pathway through which it may play a role in the survival of neuronal-derived cells (Kimmelman et al., 2000; Kimmelman et al., 2002).

RHEB

The Rheb group consists of Rheb1 and Rheb2 proteins that share 51% sequence identity. The RHEB1 gene is expressed in all adult human tissues with highest levels found in skeletal and cardiac muscle (Gromov et al., 1995). Homologues of the Rheb proteins have been identified in yeasts, Drosophila, Dictyostelium.

There are three key structural features that define the Rheb proteins. First, an arginine residue corresponding to the glycine at the 12th position of Ras is conserved in all the Rheb homologues. Second, they have very similar effector domain sequences. Third, all Rheb homologues terminate in the CAAX motif, where X is usually methionine, alanine, serine, glutamine, or cysteine and is required for farnesylation (Clark et al., 1997; Yang et al., 2000).

Rheb plays critical roles in the regulation of cell growth and cell cycle progression in yeasts, Drosophila and mammalian cells and it is a component of the insulin/TOR signaling. Rheb’s activity is regulated by the tuberous sclerosis complex consisting of the tumor suppressors Tsc1 and Tsc2, which together act as a GTPase-activating protein (GAP), for Rheb. Mutations in either Tsc1 or Tsc2 cause the tuberous sclerosis (TSC) syndrome, an autosomal-dominat genetic disorder with severe pathological consequences. Tsc1 and Tsc2 proteins that carry point mutations from TSC patients have lost their ability
to downregulate the level of Rheb-GTP in cells, suggesting that overactivation of Rheb is a major cause for the tuberous sclerosis syndrome.

In its active, GTP-bound form, Rheb enhances the activity of mTOR (Target of Rapamycin) which is a conserved Ser/Thr Kinase that regulates cell growth and metabolism in response to environmental cues. Excessive cell growth results as a physiological consequence of mTOR dysregulation. Insulin activates the mTOR pathway through PI3K which phosphorylates Tsc2 and inhibits its activity, thereby resulting in an elevated level of Rheb-GTP. This pathway requires the proper post-translational modification (farnesylation) and membrane localization of Rheb (Aspuria and Tamanoi, 2004; Wullschleger et al., 2006).

The RIT/RIN group

This group contains the human proteins Rit and Rin (Lee et al., 1996; Wes et al., 1996). One striking feature of these proteins is that they lack the C-terminal prenylation motifs, but contain in that region a cluster of basic residues through which Rin binds Calmodulin (Lee et al., 1996). The central part of their effector domain is conserved relative to Ras, which is consistent with their reported interaction with a subset of Ras effectors such as Ral-GEFs, RalGDS, but neither Raf kinases nor PI3 kinase.

While Rin is only expressed in adult neurons, Rit is ubiquitously expressed and it is associated with membranes, which requires the presence of the C-terminal basic stretch. Expression of dominant negative Rin inhibits Ras-dependent extension of neurite in response to NGF (Spencer et al., 2002).

Rit interacts and activates RGL3 (a Ral-GEF), that can also be activated by Ras and Rap (Shao and Andres, 2000), suggesting that Rit may act through the Ral-GEF/Ral pathway.

ARHI

Originally called NOEY 2, ARH1 was identified as a gene normally expressed in ovarian and breast epithelial cells, but not in ovarian and breast cancer cells (Yu et al., 1999). The protein exhibits 60% identity to Ras and Rap proteins. It has a low intrinsic GTPase activity and it is maintained in a constitutively active GTP-bound form in cells (Luo et al., 2003). As compared to Ras, in the P loop of this protein glycine 12 is replaced by alanine, and glutamine 61, residue important for GTP hydrolysis, is replaced with a glycine, in the switch II region. In addition, compared to Ras, ARHI contains a 34 residue N-terminal extension that is essential for the growth inhibitory effect of ARH1 (Luo et al., 2003).
**Di-Ras**

Di-Ras 1 and 2 are specifically expressed in brain (D-Ras1) or heart and brain (Di-Ras2). They have a reduced intrinsic GTPase activity and are predominantly GTP-bound in cells, which might be due to the replacement of Glutamine 61 residue to Serine in the switch II region. The only functional indication about these proteins is that their overexpression in HEK 293 cells leads to the formation of large vacuoles (Kontani et al., 2002).

**1.2.5 The Mechanism of GTP Hydrolysis**

Small G proteins are inefficient enzymes, having a rather slow intrinsic GTPase activity, with GTP hydrolysis reaction rates of $0.028 \text{ min}^{-1}$ for Ras (Schweins et al., 1995), $0.11 \text{ min}^{-1}$ for Rac (Fiegen et al., 2004), and $0.0031 \text{ min}^{-1}$ for Rap (Hart and Marshall, 1990). In the proposed catalytic mechanism of the intrinsic GTP hydrolysis reaction, GTP itself plays a central role and acts as a general base (substrate-assisted catalysis) (Schweins et al., 1995). In all Ras structures, the $\gamma$-phosphate of the GTP is coordinated by interactions with Mg$^{2+}$ ion, five conserved residues (Lys16, Tyr32, Thr35, Gly60, Gln61) and two water molecules (Pai et al., 1990; Scheidig et al., 1999).

According to the substrate-assisted catalysis mechanism, the $\gamma$-phosphate of the GTP abstracts a proton from the catalytic water molecule leading to the formation of a nucleophilic hydroxyl ion (Schweins et al., 1995; Schweins et al., 1997). The hydroxyl ion will subsequently attack the less charged $\gamma$-phosphate and produce a trigonal bipyramidal transition state, which then proceeds to the reaction products $P_i$ and GDP. The role of a second water molecule has been discussed, based on the GTP-bound Ras structure determined at 1.26 Å resolution, proposing a proton-shuffling mechanism between two attacking water molecules and one oxygen of the $\gamma$-phosphate (Scheidig et al., 1999).

The carbonyl group of Gln61 forms a hydrogen bond to the catalytic water while the amide group interacts directly with one of the $\gamma$-phosphate oxygen atoms (Wittinghofer et al., 1993; Schweins and Warshel, 1996; Scheidig et al., 1999). Thereby, the role of the Gln61 is to position the nucleophilic water molecule close to the $\gamma$-phosphate of the GTP and to stabilize the transition state of the reaction (Pai et al., 1990; Wittinghofer and Pai, 1991). Mutations of this glutamine to any other residue dramatically reduce the intrinsic hydrolysis rate and prevent GAP-inactivation, in this way inducing oncogenic transformation by constitutive activation of the small G protein (Krengel et al., 1990; Sprang, 1997b; Ahmadian et al., 1999).
A second amino acid that is important for GTP hydrolysis in Ras is Gly12. Mutations of Gly12 perturb the conformation of Gln61 and its interaction with the catalytic water molecule and consequently the GTP-hydrolysis reaction (Scheffzek et al., 1997; Ahmadian et al., 1999).

The GTPase reaction catalyzed by small G proteins involves two possible reaction pathways ranging from: a) *associative*, where there is no bond cleavage to the leaving group, complete bond formation with the incoming nucleophile and a decrease in bond order of the nonbridging oxygens (Schweins et al., 1995; Graham et al., 2002); and b) *dissociative*, that involves a metaphosphate intermediate characterized by complete bond cleavage on the leaving group, absence of bond formation to the incoming nucleophile and an increase bond order of the non-bridging oxygens. However, the associative or dissociative character of the phosphoryl transfer reaction is controversially discussed (Maegley et al., 1996; Glennon et al., 2000; Allin et al., 2001; Graham et al., 2002; Seewald et al., 2003; Kotting et al., 2006).

![Diagram of dissociative and associative transition state of the phosphoryl transfer reaction](image_url)

*Figure 6. Dissociative and associative transition state of the phosphoryl transfer reaction from (Maegley et al., 1996)*
1.3 GTPase activating proteins (GAPs) - general mechanism

The intrinsic GTP hydrolysis for most G proteins is too slow to control signal transduction processes in a meaningful time frame, which requires complete inactivation within minutes after GTP loading. Efficient hydrolysis is therefore achieved by the interaction with GTPase activating proteins (GAPs), which accelerate the hydrolysis reaction by several orders of magnitude. The GTPase mechanism has attracted considerable attention mostly due to the observation that characteristic G protein mutants are not able to hydrolyze GTP anymore contributing to pathological developments including cancer. In these cases and with Ras in particular, the role of GAPs has been investigated in specific detail since GTPase deficient mutants are not responsive to GAP regulation.

GAPs specific for different families of small G proteins have been described. Accordingly they are termed RasGAPs, RhoGAPs, etc. While the catalytic domains of GAPs for members of the different branches of the Ras superfamily share no sequence homology, the majority of GAPs for sGNBP within each group are related. Many GAPs have a modular architecture to fulfill various other functions in the cell, including signaling (Scheffzek and Ahmadian, 2005).

GAPs are regulated by either protein-protein or protein-lipid interactions, binding of second messengers, and/or post translational modifications. These interactions and modifications induce translocations to the site where the target small G protein is located, release from autoinhibition by a flanking domain and, in some cases, allosteric modification of the catalytic activity.

1.3.1 RasGAPs

Several mammalian Ras specific GAPs of various size and modular architecture have been described to date. In addition to homologs in Drosophila, yeasts, Dictyostelium discoideum and Caenorhabditis elegans, 14 RasGAPs related genes are known in mammalian species (Scheffzek and Ahmadian, 2005).

p120GAP, NF1 (Neurofibromin) and GAP1 are the main, distinct GAPs currently identified for the Ras family of proteins (Bernards, 2003). p120GAP is the prototype of this class of proteins as it was the first one to be isolated (Trahey and McCormick, 1987; Trahey et al., 1988; Vogel et al., 1988). Neurofibromin is the product of neurofibromatosis type 1 gene, which has been found frequently mutated in patients with the disease neurofibromatosis type 1, a common genetic disorder which predisposes to the formation
of tumors and is associated with numerous clinical complications, including learning disabilities. GAP1 was identified as a negative regulator in *Drosophila* eye development (Gaul et al., 1992). GAP1 family includes four mammalian members GAP1\textsuperscript{m}, GAP1\textsuperscript{IP4BP}, CAPRI and RASAL.

Typically, the GAP activity of all these proteins is located within a highly conserved domain of around 330 residues, called the catalytic RasGAP domain. Frequently, the catalytic module is accompanied by other domains like pleckstrin homology (PH), C2 (called conserved domain 2 in PKC), which are involved in signaling or membrane recruitment.

The crystal structures of the catalytic domain of p120GAP and neurofibromin show an elongated helical protein that contains all residues conserved among RasGAPs (Ahmadian et al., 1996; Scheffzek et al., 1996; Scheffzek et al., 1998). The structure of the Ras-RasGAP complex, formed by Ras-GDP and GAP334 (the catalytic domain of p120GAP) in the presence of AlFx (which mimics the $\gamma$-phosphate in its pentavalent transition state of the GAP-G protein reaction complex during GTP-hydrolysis) has shown that GAP binds and stabilizes the switch I and II regions of Ras and supplies an arginine residue (Arg789 in p120GAP or Arg1276 in NF1) into the catalytic machinery of Ras that stabilizes the transition state of the GTP-hydrolysis reaction (Figure 7).

![Figure 7. Structure of the Ras-RasGAP complex in the GDP-AlFx transition state.](image-url)

The catalytic domain of p120GAP (GAP334) is shown in red, Ras in blue, and the bound GDP and AlFx are shown in stick representation (yellow). Image based on Protein Data Bank entry 1WQ1.
There are three prominent regions within the conserved area of the catalytic domain that contain structural fingerprints governing the GAP function: the arginine-finger loop, the invariant phenylalanine-leucine-arginine (FLR) motif and the $\alpha$7/variable loop.

**The finger loop** provides the catalytic arginine residue and is crucial for the stabilization of the transition state of the GAP-catalyzed GTPase reaction.

The oxygen of the backbone carbonyl group of the arginine finger makes a hydrogen bond to the side-chain amide group of the Gln61 in Ras thereby stabilizing its position. Additionally, the guanidinium group of the arginine residue interacts with the $\beta$-phosphate and with AlF$_3$, neutralizing the negative charges that develop during phosphoryl transfer. Gln61 also contacts AlF$_3$ and a water molecule that corresponds to the attacking nucleophile (Figure 8).

Amino acids proximal to the arginine residue which are different for p120GAP and NF1 control the specific orientation of the arginine finger within the active site of Ras. Mutational analyses have shown that deletion of the arginine finger R1276 or its substitution by lysine, glutamine, asparagine and alanine dramatically reduces the GAP-stimulated GTP-hydrolysis of Ras (Ahmadian et al., 1997c; Ahmadian et al., 2003). The R1276P mutation found in patients with type 1 neurofibromatosis has been shown to nearly abolish the GAP-function of NF1 (Klose et al., 1998).

**The invariant FLR region** indirectly contributes to the stimulation of the GTPase reaction by forming a scaffold, which stabilizes the Ras switch regions. The arginine from the FLR motif (R1391 in NF1 and R903 in p120GAP) can be replaced by lysine but not by alanine or methionine, which drastically interfere with the formation of the transition state and consequently with the GAP activity (Mittal et al., 1996; Ahmadian et al., 1997c). Phenylalanine from the FLR motif contributes to the stabilization of the hydrophobic core while the leucine has been demonstrated to be important for the functional interaction between GAP and Ras (Ahmadian et al., 2003).

**The alpha7/variable loop** uses several conserved residues including two lysine residues, which are involved in numerous interactions with the switch I region of Ras, in this way determining the specificity of the Ras-RasGAP interaction (Ahmadian et al., 2003).
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Figure 8. The active site of the RasGAP-Ras-GDP-AlF₃ complex, with Ras shown in yellow and RasGAP in red and orange. Critical elements for catalysis are indicated (from Scheffzek et al., 1997).

The structure of the Ras-RasGAP complex has also provided an explanation for the oncogenicity of the Gly12 mutation, which prevents Ras from being switched off. Gly12 is in close proximity to the finger loop and even its mutation to alanine would sterically interfere with the geometry of the transition state (Scheffzek et al., 1997).

The basic mechanism of the GTPase stimulation relies on two major features: firstly, GAPs stabilize flexible residues in the switch I and II regions of the G protein such as the essential catalytic residue Gln61; secondly, GAPs supply a catalytically critical arginine to the G protein active site that stabilizes the transition state of the GTP hydrolysis reaction.

1.3.2 Rho/Cdc42/Rac-specific GAPs

Since the initial identification of Breakpoint cluster region protein (Bcr) as a RhoGAP over 30 RhoGAPs have been reported in eukaryotes ranging from yeast to human (Moon and Zheng, 2003). The RhoGAP family is defined by the presence of a conserved RhoGAP domain in the primary sequence that consists of about 150 amino acids and shares at least 20% sequence identity with other family members.

Despite the fact that sequences of RhoGAP domains are different from those of other classes of GAPs such as RasGAPs, the tertiary structure as well as the basic GTPase-activating mechanism of the RhoGAP domain appear to be similar to that of RasGAPs.
RhoGAP domain supplies an arginine finger directly into the active site of Rho (Figure 9) to stabilize the transition state of the hydrolysis reaction (Rittinger et al., 1997). In contrast to the Ras-RasGAP system, mutation of the arginine finger in RhoGAPs still results in a significant GTPase stimulation and in the formation of the GDP-AlF₃ bound transition state mimic. It has been suggested that the catalytic arginine is necessary but may not be sufficient for mediating the GAP activity. Of particular importance for the GAP catalysis in Rho/Cdc42 appears to be the conserved Tyr32 in the switch I region which in complex with Cdc42 interacts with the guanidinium group of the arginine finger (Fidyk and Cerione, 2002).

Another example is β2-Chimaerin, a Rac-specific GAP. It contains three conserved domains, an N-terminal SH2, a central C1 and a C-terminal Rac-specific GAP domain, which shows the typical RhoGAP topology (Figure 10). The N-terminal SH2 and C1 domains specifically interact with the GAP domain, thereby blocking its interaction with Rac. Binding of phospholipids to the C1 domain results in the release of the GAP domain which is then able to stimulate the GTPase reaction on Rac (Canagarajah et al., 2004).
Figure 10. Structure of the β2-Chimaerin, a Rac-specific GAP, in its inactive conformation. The RacGAP domain is shown in red, the SH2 domain in magenta and the C1 domain in green. The N-terminus protrudes into the active site of the RacGAP domain, sterically blocking Rac binding. Phospholipids binding to C1 allows the N-terminus to move out of the active site and thereby activating the enzyme. Image based on Protein Data Bank entry 1XA6.

1.3.3 Regulators of G-protein signaling: GαGAPs

Heterotrimeric guanine-nucleotide-binding proteins (subunits α, β, γ) couple the activation of heptahelical receptors at the cell surface to intracellular responses. The α-subunit is responsible for GDP/GTP binding and for the hydrolysis of GTP which takes place much more rapidly than for other G proteins (3 min⁻¹) (Temeles et al., 1985). The structures of the Gα-subunits revealed a conserved protein fold composed of two domains, the GTPase domain and the helical domain (Noel et al., 1993). The higher intrinsic GTPase activity is attributed to the helical domain that forms a lid over the nucleotide binding site burying the bound nucleotide in the core of the protein. A catalytic glutamine contributed by the GTPase domain and a threonine from the helical domain stabilize the attacking water molecule contributing to the mechanism of GTP hydrolysis (Coleman et al., 1994).

Strikingly, the helical domain positions an arginine in cis (from Gα itself) into the active site of the G-domain, in contrast to GAPs for the small G proteins that supply the arginine in trans. Here, the cis-arginine also functions in stabilizing the developing negative charges during the phosphotransfer reaction. Consistent with its importance for catalysis mutations of this arginine abolish the GTPase activity of the Gα-proteins (Coleman et al., 1994).
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Despite the high intrinsic GTPase rates, the processes of G-protein mediated cell responses require rapid downregulation by GAPs specific for the Gα-subunit, which are called RGSs, regulators of G-protein signaling and are highly diverse in structure and specificity. The first three-dimensional structure of an RGS-domain was solved in the GDP-AlF₃-bound transition state complex of RGS4 with its Giα target (Figure 11) (Tesmer et al., 1997). RGS proteins share the conserved RGS domain which folds into a helical module that primarily interacts with and stabilizes the switch regions of the Gα as the major determinant of GAP activity. In contrast to Ras- and RhoGAPs, RGSs do not provide catalytic residues to the active site of the Gα except for one conserved asparagine, which does not contribute significantly to catalysis but rather appears to be important for binding to Gα and stabilization of the catalytic glutamine.

Figure 11. The α-subunit of the heterotrimeric G-proteins in complex with its GAP RGS4. RGS4 is shown in red, Giα in blue, its helical insert domain in light blue and the bound GDP and AlF₃ are shown in stick representation (yellow). Image based on Protein Data Bank entry 1AGR.

1.3.4 GTPase activating proteins of the Rap1GAP family

Several RapGAPs have been found to downregulate Rap specifically, with Rap1GAP being the first identified (Rubinfeld et al., 1991). Its GAP activity resides in a 36 kD fragment encompassing 341 amino acids (residues 74-415) which has been detected in several other proteins like the tumor suppressor Spa1 (Kurachi et al., 1997), the papilloma virus oncoprotein target E6TP1 (Gao et al., 1999), and tuberin which is the product of the tumor suppressor gene Tsc2 (Wienecke et al., 1995) (Figure 12).
RapGAP-stimulated GTP hydrolysis on Rap1 displays a number of features distinct from its close relative Ras and from other families of small G proteins. Biochemical studies and the X-ray structures of Rap1GAP (Daumke et al., 2004) and of the Rap1-Rap1GAP complex (Scrima et al., In press) showed that the GAP-accelerated GTPase reaction of Rap1 proceeds via a mechanism completely different from that of other small G proteins. First of all, in contrast to Gα, Ras, Rho, Rab or Ran, Rap1 does not posses a catalytic glutamine residue at position 61 that is crucial for the GTPase reaction, but it has instead a threonine. As a result the intrinsic GTP hydrolysis rate of Rap1 is 10-fold slower than for Ras but can be reconstituted by replacing the threonine 61 with a glutamine (Frech et al., 1990). Threonine 61 is not required for the intrinsic GTPase reaction and plays no role in catalysis (Maruta et al., 1991).

The crystal structure of the catalytic domain of Rap1GAP (Daumke et al., 2004) revealed a two domain arrangement with the C-terminal part representing the catalytic portion and the N-terminal α/β fold being involved in the stabilization of the RapGAP dimer, but with no critical role for the GAP activity.

Furthermore, unlike most other GAPs, which supply an arginine finger as a crucial element for catalysis, no arginine residues contribute significantly to the RapGAP mediated reaction on Rap1 (Brinkmann et al., 2002). The Rap-Rap1GAP system lacks both the...
intrinsic glutamine from Rap and the Arg-finger in trans. Instead, the GAP stimulated reaction requires a crucial asparagine residue (N290 in Rap1GAP), the so called “Asn thumb” that inserts into the active site and is in close proximity to the $\gamma$-phosphate (Figure 13).

Figure 13. Structure of the Rap-Rap1GAP complex. Rap1GAP molecule is shown in red, Rap1A in blue, and the bound GDP and BeF$_3$ are shown in stick representation (yellow). Image based on PDB coordinates provided by A. Scrima.

It is proposed that the asparagine residue compensates for the missing glutamine in Rap by positioning the attacking water molecule close to the $\gamma$-phosphate. This is supported by the Rap-Rap1GAP structure (Scrima et al., In press) in which the carboxamide of the Asn-thumb occupies the position of the catalytic Gln side chain in other G protein-GAP systems (Figure 14 A). Mutations of this residue result in a drastically reduced activity without any negative effect on complex formation.
Figure 14. Active site of the Rap-Rap1GAP complex. The active site is shown as superimposition with Ras (orange), Ran (cyan), Rho (blue) from the complexes with their cognate GAPs. The catalytic helix of RapGAP with the Asn-thumb is shown in magenta. Asn290 in Rap1GAP occupies the position of the catalytic Gln in Ras (Gln61), Ran (Gln69) and Rho (Gln64). The G12 position in Rap1B is marked with a red sphere (A). Surface representation of Arg286 (magenta) in the catalytic helix of Rap1GAP, G12V (cyan) and RheB R15 (green) modeled into the structure of the Rap-Rap1GAP complex, showing the steric clash of Val12 (cyan) with R286 (magenta), while Arg15 can be accommodated (B) (Scrima et al. In press).

Contrary to the G12 mutants of most other small G proteins which can not be downregulated by their respective GAPs, the G12V mutant of Rap1 is efficiently inactivated by Rap1GAP. In the presence of wt-Rap1GAP the G12V mutant of Rap shows a reduced rate of complex formation and a slower GTPase activity. By modeling Val12 into the Rap1-Rap1GAP complex structure it has been proposed that Val12 will sterically interfere with arginine 286 from the Rap1GAP (Figure 14 B). The positive charge of R286 mediates a fast interaction between Rap1 and Rap1GAP, its mutation results in a five fold slower association reaction, but its effect in catalysis remains marginal (Scrima et al., In press).

1.3.5 Rab/YptGAPs

RabGAPs comprise a family of GTPase activating proteins for the small G proteins Rabs (Ypt in yeasts). Rabs are essential regulators of membrane trafficking being implicated in diverse aspects of vesicular transport including vesicle formation, motility, docking and fusion.

Most GAPs for Rab proteins possess a TBC domain (Tre-2, Bub2 and Cdc16) and are broadly conserved in eukaryotic organisms. Together with the ubiquitously expressed
Rab3GAP and RN-tre, several yeast TBC-domain GAPs, termed Gyps (GAPs for Ypt/Rab proteins) and a mammalian RabGAP (GAPCenA) have been identified (Strom et al., 1993; Du et al., 1998; Cuif et al., 1999; Gao et al., 2003; Miinea et al., 2005; Haas et al., 2005). The catalytic glutamine of Ras and Rho is also conserved in Rab, and the arginine finger is observed in RabGAP, but the mechanism is somewhat different. The crystal structure of Gyp1-Rab33-AlF₃ complex (Figure 15), which approximates the transition state intermediate for GTP hydrolysis, showed that RabGAP provides two catalytic residues in trans, an arginine finger analogous to Ras/Rho family GAPs and a glutamine finger that substitutes for the glutamine in the DxxGQ motif of the G protein. The glutamine from Rab points away from the active site and does not stabilize the transition state as expected, but instead is involved in the binding to GAP (Pan et al., 2006).

Figure 15. Crystal structure of Gyp1 in complex with Rab33-AlF₃. The corresponding GAP (Gyp1) is shown in red, Rab33 is shown in blue, and the bound GDP and AlF₃ are shown in stick representation (yellow). Image based on Protein Data Bank entry 2G77.

1.3.6 ArfGAPs
ADP-ribosylation factors (Arfs) are small G proteins that play a critical role in many vesicular trafficking events in eukaryotic cells, mediated by protein-coated carrier vesicles (Spang, 2002). During the early stages of vesicle formation, GDP-Arf1 is recruited to the site of vesicle emergence. Once at the membrane, GDP-Arf1 undergoes guanine nucleotide exchange mediated by ArfGEFs (Renault et al., 2003). Upon nucleotide exchange, a myristate residue at the amino terminus of Arf1 becomes exposed and stably anchors GTP-
Arf1 in the membrane. Subsequently, coatmer proteins are recruited to this site and a COPI-coated vesicle is formed (COPI-coated vesicles are involved in transport from the Golgi compartment to the ER). Before vesicle fusion with a target membrane the protein coat is shed. Coat removal is thought to be mediated by the hydrolysis of Arf1-GTP stimulated by ArfGAPs. ArfGAPs are also present in the coat protein complex I and when the membrane curvature of the vesicle increases, the activity of ArfGAP is stimulated dramatically, leading to GTP hydrolysis and Arf-GDP dissociation (Bigay et al., 2003).

ArfGAP proteins are classified into two major types, ArfGAP1 and AZAP types, according to the overall domain structure. The former type of ArfGAPs has an ArfGAP domain at the extreme N-terminus and the latter contain an ArfGAP domain between the PH and ankyrin (ANK) repeat domains. The catalytic domain of ArfGAPs family comprises 140 residues including a zinc finger motif (Inoue and Randazzo, 2007).

In the structure of the ArfGAP domain in complex with Arf-GDP (Goldberg, 1999), ArfGAP binds to Arf at a position far away from the active site (Figure 16).

![Figure 16. Overall view of the ArfGAP structure in complex with Arf-GDP. ArfGAP is shown in red, Arf in blue and the bound GDP is shown in stick representation (yellow). ArfGAP binds to Arf at a position far away from the nucleotide binding site. Image based on PDB coordinates provided by J.Goldberg.](image)

An invariant Arg of ArfGAP, which has been proposed as a candidate arginine finger, is at least 15 Å away from the active site and makes extensive contacts with the Zn finger motif. It was proposed that ArfGAP can accelerate GTP hydrolysis by stabilizing the switch region, but it does not provide a catalytic arginine finger. A unique feature of this structure is that the Switch I of Arf1 does not participate in GAP binding. Instead, the vesicle coat
protein, coatamer, binds to the switch region of Arf1GAP complex and dramatically stimulates GTP hydrolysis. Thus it has been suggested that the coatamer rather than GAP may contribute a catalytic residue (Goldberg, 1999).

Nevertheless, the crystal structure of a homologous complex composed of Sar1 (an Arf related protein)-GppNHp bound and the GAP component, Sec23 in yeasts, revealed a conserved arginine protruding into the active site (Bi et al., 2002). Sar1 together with two heterodimeric complexes Sec23/24 and Sec13/31 are components of the COPII-coated vesicles (involved in trafficking from the ER to the Golgi compartment). Sar1 has a histidine in the equivalent position of the catalytic glutamine present in most other G proteins, and a slower intrinsic GTP hydrolysis. This catalytic histidine is used to position the water molecule close to the \( \gamma \)-phosphate. GTP hydrolysis is stimulated by the Sec23 subunit which does not show any sequence homology with other GAPs, but it provides a catalytic arginine into the active site of Sar1 (Bi et al., 2002).

### 1.3.7 RanGAP

Ran is a nuclear Ras-related protein that regulates both transport between the nucleus and cytoplasm during interphase, and formation of the mitotic spindle and/or nuclear envelope in dividing cells (Seewald et al., 2002).

Ran-mediated GTP hydrolysis is thought to be the main driving force behind cargo transport across the nuclear pore complex. The location of Ran-specific exchange factor RCC1 in the nucleus and that of RanGAP in the cytoplasm creates a gradient of Ran-GTP across the nuclear pore complex, so that nuclear Ran is predominantly GTP-bound while cytosolic Ran is GDP-bound (Gorlich and Kutay, 1999). RanGTP is hydrolyzed by the combined action of RanGAP and Ran-binding proteins RBPs (Seewald et al., 2002). All known RanGAPs show a common modular architecture with a leucine-rich repeat (LRR) domain comprising 350 residues, followed by a highly acidic region.

The structure of the yeast RanGAP Rna1p (Hillig et al., 1999) showed that eleven LRR form a crescent shaped molecule. A single LRR forms a \( \beta \) hairpin consisting of a \( \beta \)-strand, a loop and an \( \alpha \)-helix parallel to the \( \beta \)-strand. RanGAP does not have any structure similarity to the purely helical GAPs of Ras and Rho.

The structure of the ternary complex of RanGAP with activated Ran (bound to either GppNHp or GDP-AlF\(_4\)) and the GTPase co-activating RanBP1 (Seewald et al., 2002)
shows that the GTPase mechanism does not involve an arginine finger and that RanBP1 does not participate directly in mediating hydrolysis (Figure 17).

Figure 17. Crystal structure of the ternary complex Ran-RanBP1-RanGAP. RanGAP is shown in red, RanBP1 in magenta, Ran in blue and the bound GppNHp is shown in stick representation (yellow). Image based on Protein Data Bank entry 1K5D.

Candidate residues from the RanGAP like Arg74 and Arg170 are not involved in nucleotide contact, neither in the ground nor in the transition state of the GTPase reaction, and they do not show any appreciable effect on the GTPase reaction (Scheffzek and Ahmadian, 2005). Instead of an arginine, Tyr39 of Ran forms hydrogen bonds with both the $\gamma$-phosphate oxygen and the Gln69 side chain of Ran, but, however it does not contribute significantly to catalysis as it was concluded from mutational analysis.

RanBP1 is a coactivator of RanGAP-mediated hydrolysis (Bischoff et al., 1995). Nevertheless, the intrinsic GTPase reaction is only marginally accelerated by RanBP1. Ran protein has a C-terminal D-E-D-D-L motif. This motif inhibits the interaction of RanGAP with Ran. Therefore, the rate of RanGAP-mediated GTP hydrolysis is accelerated in the absence of the C-terminal D-E-D-D-L motif of Ran. Coactivation of the reaction by
RanBP1 is dependent on the presence of this C-terminal motif of Ran (Gorlich and Kutay, 1999). From the structure it appears that the RanGAP interaction with Ran is indeed inhibited by the C-terminus motif of Ran, and this inhibition is released by RanBP1 (Seewald et al., 2002). The role of RanGAP in promoting GTP hydrolysis appears to be the stabilization of the switch II region and the correct orientation of the catalytic glutamine. Similar to other small G proteins such as Ras, mutation of the catalytic glutamine to alanine or leucine reduces the GAP activity by several orders of magnitude (Bischoff et al., 1994; Klebe et al., 1995).

1.3.8 Signal-recognition particle and its receptor

Cotranslational translocation of proteins across or into membranes requires that the translating ribosome is targeted to the membrane by the signal recognition particle (SRP), an evolutionarily conserved ribonucleoprotein. Newly synthesized proteins destined for secretion or membrane insertion carry a hydrophobic signal sequence at their N terminus. SRP interacts with the signal sequence as soon as it emerges from the ribosomal polypeptide exit tunnel. In eukaryotes, peptide elongation is retarded upon binding of SRP to the ribosome nascent chain complex (RNC) (Halic et al., 2004; Halic et al., 2006a). Subsequently, the SRP–RNC complex is targeted to the endoplasmic reticulum (ER) membrane by the interaction with the SRP receptor (SR). The RNC is then transferred to the protein-conducting channel in the membrane (the translocon) after which, as a result of GTP hydrolysis in SRP and SR, the SRP–SR complex dissociates, leaving the RNC bound to the translocon (Keenan et al., 2001; Koch et al., 2003; Wild et al., 2004; Halic et al., 2006b; Bange et al., 2007).

SRP proteins form a unique family among the small G proteins, having only three members: the signal-sequence-binding protein SRP54/Ffh (fifty-four homolog), the SRP receptor SRα/FtsY (filamentation temperature sensitive) and FlhF, a protein involved in flagellar biosynthesis.

Both SRP and SR contain G domains and are conserved across all kingdoms of life. GTP binding to SRP and SR has been shown to be a prerequisite for formation of the SRP–SR complex. In the SRP–SR complex, each G protein acts as a GTPase-activating protein (GAP) for the other G protein.
In prokaryotes SRP consists of FfH, whereas SR consists of FtsY. The structure of the complex between the two G proteins each bound to a nonhydrolyzable GTP-analog, revealed a highly symmetric heterodimer stabilized by extensive interactions between the two proteins (Focia et al., 2004; Egea et al., 2004). Both proteins contain an unusual domain termed insertion box domain (IBD) between the switch regions and the N-terminal helical domain, which is tightly packed against the G-domain core (Figure 18). In the complex the IBD domain contributes critical residues to the active site. In each chain a catalytic asparagine positions the water molecule close to the $\gamma$-phosphate of the bound nucleotide. Additionally, an arginine and a glutamine are supplied from each molecule into the active site and are responsible for the in trans transition state stabilization or for in cis catalysis involving interactions with the $\beta$, $\gamma$-phosphates.

![Figure 18. Crystal structure of the heterodimer between Signal recognition particle (FfH) and its receptor (FtsY) in the presence of a non-hydrolyzable GTP analog GMPPCP.](image)

The catalytically important interaction that spans the interface occurs between the two bound GTP molecules: the 3'-OH of one GTP is hydrogen bonded to the $\gamma$-phosphate of
the other and vice versa. This reciprocal interaction between the twinned GTPs is severed twice upon hydrolysis, leading to complex dissociation after cargo delivery (Egea et al., 2005).

1.4 The GAP catalyzed GTPase reaction monitored by FTIR Spectroscopy

The molecular reaction mechanism of the GAP-catalyzed GTP hydrolysis reaction can be investigated by time-resolved Fourier transform infrared (trFTIR) difference spectroscopy using caged GTP as photolabile trigger. This approach provides the complete reaction pathway with time resolution of milliseconds at the atomic level. Performing difference spectra between a ground state and an activated state selects the absorbance changes of a few residues involved in the reaction from the background absorbance of the whole protein. A summary of the FTIR studies on the Ras-RasGAP and the Rap-RapGAP reactions will be discussed in the following two subchapters.

1.4.1 Summary of previous FTIR studies on the Ras-RasGAP reaction

The time course of the GTPase reaction of Ras and the protein-protein complex Ras-RasGAP was measured by FTIR spectroscopy and revealed important aspects of the RasGAP catalyzed GTPase reaction of Ras (Cepus et al., 1998; Allin et al., 2001; Allin and Gerwert, 2001; Kotting et al., 2006). In the presence of GAP the reaction is increased from 2 h 50 min to 80 s at 260 K (Figure 19).

![Figure 19](image.png)

Figure 19. The absorbance changes in the infrared spectrum between 1800 and 950 cm$^{-1}$ for the intrinsic (A), and during the GAP catalyzed reaction of Ras (B) are shown as a function of wave...
number and time (logarithmic scale). In the GAP catalyzed reaction of Ras an intermediate, P_i, accumulates as seen at 1114 cm\(^{-1}\). Figure provided by P. Chakrabarti.

The IR absorbance changes for the intrinsic reaction of Ras and the GAP catalyzed reaction are shown in Figure 19 as a function of wave number and time (logarithmic). The absorbance changes of the intrinsic reaction of Ras can be described by a single exponential function as seen at 1143 cm\(^{-1}\) for GTP and 1104 cm\(^{-1}\) for GDP, while the GAP catalyzed reaction is described by three exponential functions (\(k_1, k_2\) and \(k_3\)) as shown in the scheme below.

![Scheme 1. The RasGAP-catalyzed GTPase reaction on Ras](image)

The reaction was initiated by UV laser flashes which release GTP from the precursor caged GTP. The first process with the rate constant \(k_1\) describes the conformational change from the Ras\(_{off}\)-GTP to Ras\(_{on}\)-GTP. The GAP-catalyzed reaction takes place in two steps described by the apparent rate constants \(k_2\) and \(k_3\). \(k_2\) indicates the appearance of an intermediate (Ras-GDP-P_i) and \(k_3\) describes the formation of reaction products (Ras-GDP and P_i).

The GTP hydrolysis reaction is primarily catalyzed by drawing more negative charge towards the non-bridging beta oxygen of GTP and by additional positive charges provided by GAP binding. The charge redistribution towards the \(\beta\)-phosphate of GTP is probably due to the positively charged Lys16 and Mg\(^{2+}\), which are equidistant from the non-bridging \(\beta\) and \(\gamma\)-oxygen (Pai et al., 1990). This leads to a redistribution of negative charge from \(\gamma\) to \(\beta\) phosphate. In addition, in the P-loop backbone N-H groups point towards the non-bridging \(\beta\)-oxygen and might assist in this charge shift (Cepus et al., 1998; Allin and Gerwert, 2001). In the presence of GAP, there is an even larger charge shift towards \(\beta\) oxygen of GTP and it is assumed that this is due to the presence of the additional positive charge of the arginine finger (Allin et al., 2001).

As the first step towards hydrolysis, the attacking water might transfer a proton to GTP. In this way a hydroxyl ion is generated. After the attack of the generated hydroxyl ion on the \(\gamma\)-phosphate and the cleavage of the \(\beta-\gamma\) bond, the GAP catalyzed reaction of Ras proceeds via a H$_2$PO$_4^-$ intermediate that is generated as the initial product. The phosphate is already
cleaved from the GDP, but despite the absence of a covalent bond to the phosphate, it is retained in the binding pocket via hydrogen bonds with the β-phosphate of GDP and electrostatic interactions with the magnesium atom (Kotting et al., 2006).

The rate-limiting step for the GAP-catalyzed reaction is the $P_i$ release from the Ras-GDP-$P_i$-GAP complex into the bulk medium during the last step of the hydrolysis reaction described by the rate constant $k_3$ (Scheme 1). The GTPase reaction is partially reversible since a considerable amount of back reaction to GTP was observed (Allin et al., 2001; Kotting et al., 2006).

### 1.4.2 Summary of previous FTIR studies on the Rap-RapGAP reaction

The RapGAP mediated activation of the GTPase reaction of Rap has been investigated by FTIR and compared to the Ras-RasGAP reaction. As for the Ras-RasGAP system, the Rap1GAP catalyzed reaction involves a three step mechanism described by the following three apparent rate constants (Scheme 2).

\[
\begin{align*}
\text{hv} & \quad \ Rica\text{gted GTP} \quad k_1 \quad \text{Rapoff-GTP} \quad k_2 = 0.6 \text{ s}^{-1} \quad \text{Rapon-GTP} \quad k_3 = 0.07 \text{ s}^{-1} \quad \text{Rap-GDP-P_i} \quad \text{Rap-GDP + P_i}
\end{align*}
\]

**Scheme 2.** The Rap1GAP-catalyzed GTPase reaction on Rap

The three dimensional structure of Rap1GAP (Daumke et al., 2004) and of the Rap-Rap1GAP complex (Scrima et al., In press) together with biochemical experiments showed that the RapGAP catalyzed GTPase reaction is completely different from the RasGAP reaction and employs a catalytic Asn inserted into the active site of Rap. These distinct features of the Rap-RapGAP system raised the question whether RapGAP, without an arginine finger, still shifts charge towards the β-phosphate.

FTIR studies showed that, while the intrinsic hydrolysis reaction of Ras and Rap are very similar, the GAP catalyzed reactions clearly show several distinct features (Chakrabarti et al., 2004). The charge shift toward β-phosphate observed for RasGAP is also observed for RapGAP, but is more pronounced. The downshift of the β-vibration for Ras (from 1219 cm$^{-1}$ to 1140 cm$^{-1}$) is attributed to the catalytic arginine. Because Rap1GAP does not employ an arginine, the similarly large shift from 1215 cm$^{-1}$ to 1130 cm$^{-1}$ seen in the Rap-RapGAP reaction is probably due to a different structural rearrangement. Instead of the positively charged guanidinium group of Arg, in the Rap1GAP catalyzed reaction, H-
bonding of the polar carboxamide group of Asn might induce a charge shift toward $\beta$-phosphate. Moreover, Rap1GAP binding induces a different GTP conformation in the educt state, which is characterized by highly coupled vibrations. The coupled vibrations indicate a different orientation of the three phosphate groups to each other as compared with the GAP-catalyzed hydrolysis of Ras and the intrinsic reaction. This unusual conformation of GTP might contribute to rapid phosphoryl transfer (Chakrabarti et al., 2004). As for the RasGAP catalyzed reaction, a GDP-P$_i$ intermediate is observed in which the cleavage of $\gamma$-phosphate has already taken place, but P$_i$ is at shorter distance from GDP as compared with Ras-RasGAP. The GDP in the intermediate also shows coupled vibrations (Chakrabarti et al., 2004). As in the case of the Ras-RasGAP system, the GTPase reaction appears to be reversible, but a more pronounced back reaction to GTP is observed (Chakrabarti et al., 2004). The IR absorbance changes during the Rap1GAP catalyzed reaction are shown in Figure 20 as a function of wave number and time (logarithmic). Marker frequencies of GTP-\(\beta\) at 1128 cm$^{-1}$, an intermediate at 1172 cm$^{-1}$, GDP-\(\beta\) at 1104 cm$^{-1}$ and the released P$_i$ at 1075 cm$^{-1}$ are shown for illustration (Allin et al., 2001; Chakrabarti et al., 2004). In the case of the Rap1GAP catalyzed reaction the time evolution of the spectra is completely different from the RasGAP catalyzed reaction. The typical GTP-\(\gamma\) band at 1143 cm$^{-1}$, which has also been observed for intrinsic reaction of Rap, is missing in the case of RapGAP catalyzed reaction. In addition, an intermediate like vibration (P$_{int}$) is observed at 1172 cm$^{-1}$ for Rap-RapGAP system instead of 1114 cm$^{-1}$ as seen for Ras-RasGAP system. This indicates that the GAP catalyzed reactions on Ras and Rap are different.

Figure 20. The absorbance changes in infrared between 1800 and 950 cm$^{-1}$ for the GAP catalyzed reaction of Rap are shown as a function of wave number and time (logarithmic scale). P$_{int}$: intermediate. Figure provided by P. Chakrabarti.
1.5 Promiscuous GAPs

The human genome predicts ~170 proteins that are structurally related to GAPs for the Ras superfamily members. Among the approximately 80 proteins that have been analyzed experimentally, all but a few possessed GAP catalytic activity, arguing that most proteins in this group are likely to be functional. The fact that up to 0.5% of human genes may encode functional GAPs suggest an important and widespread role for these GTPase regulators. Interestingly, 70 putative GAPs are predicted to be specific for Rho proteins, whereas another 30 genes predict putative GAPs for members of the Ras branch (Bernards and Settleman, 2005).

Typically GAPs act rather specific for a certain family of small G proteins, being unrelated in sequence or structure. A surprising finding came with the discovery of several GAPs (Bud2, SynGAP and some members of the GAP1 family) which seem to stimulate the GTPase activity towards both Ras and Rap. These proteins contain a catalytic RasGAP domain shearing all residues required for the stimulation of the GTPase reaction on Ras, but they do not have any detectable sequence homology with any known RapGAP. Despite their close homology to RasGAPs, these proteins are able to stimulate the GTPase reaction of Rap. Considering the distinct mechanism by which Ras and Rap GAPs enhance the GTPase activity of their respective G proteins, the discovery of such proteins raises the question on how this dual activity towards both Ras and Rap is achieved.

1.5.1 SynGAP

Synaptic GTPase activating protein (SynGAP) is a brain specific GAP which occurs at high concentrations in the postsynaptic region of the excitatory synapses and plays an important role in synaptic plasticity by regulating signal transduction via the MAP kinase cascade. It was shown that SynGAP is involved in neuronal development (Vazquez et al., 2004), apoptosis (Knuesel et al., 2005), glutamate receptor trafficking (Zhu et al., 2002) and in the induction of hippocampal long-term-potentiation (LTP) (Komiyama et al., 2002). Based on sequence homology, SynGAP was classified as a RasGAP (Kim et al., 1998). It contains a RasGAP domain preceded by a C2 (Ca\(^{2+}\) dependent phospholipids-binding domain) and a pleckstrin homology (PH) domain. Despite its sequence homology with RasGAPs, SynGAP shows dual specificity towards both G proteins Ras and Rap. Moreover, it has been shown that SynGAP activates the GTPase reaction of Rap1 and Rap2 much more potently than that of Ras (two-fold maximum stimulation of the GTPase
reaction on Ras compared to ten-fold stimulation of the Rap GTPase reaction) (Krapivinsky et al., 2004).

Recent structural and biochemical studies (Pena et al., unpublished) showed that the C2 domain of SynGAP is essential for its RapGAP activity. While the GAP domain of SynGAP does not show any RapGAP activity, in the presence of C2 domain there is a dramatic increase in the RapGAP activity. Surprisingly, the catalytic mechanism towards Rap also seems to involve an arginine finger as mutations of the candidate Arg470 in SynGAP completely abolished both RasGAP and RapGAP activities. In support of this observation came the finding that RapG12V, whose GTPase activity can be efficiently accelerated by Rap1GAP, is insensitive to SynGAP, resembling in this way RasG12V which can not be downregulated by RasGAPs. Moreover, an asparagine residue close to the catalytic site might play a role in catalysis as its mutation decreases the RapGAP activity about 20-fold (Pena et al., unpublished). However, the actual mechanism of this reaction awaits structural information of the complex.

### 1.5.2 Bud2

Bud2 is another example of a GTPase activating protein with a RasGAP domain that stimulates GTP hydrolysis on the yeast homolog of Rap1, Bud1. Bud1 (Rsr1) is the most likely orthologue of Rap1 as it lacks a glutamine at position 61, and Rap1 can partially complement the BUD1 defect (McCabe et al., 1992). Bud1 recruits polarity-establishment factors including the small G protein Cdc42, Cdc24 (a GEF for Cdc42) and the scaffold protein Bem1 to assemble the actin cytoskeleton for bud formation (Park et al., 1999). Bud2 is also involved in this process being required for bud site selection. Chromosomal deletion of BUD2 causes a random budding pattern but no obvious growth defects. Overexpression of Bud2 also causes a random budding pattern in wild type cells (Park et al., 1993).

Bud2 has a central GAP domain with 20% sequence identity with the RasGAP domain of NF1. Bud2 protein is localized at the presumptive bud site, where it stimulates conversion of Bud1-GTP to Bud1-GDP, ultimately leading to activation of Cdc42 involved in the recruitment of the actin cytoskeleton to the bud site (Park et al., 1997). This GAP activity of Bud2 is important for proper bud-site selection as both Bud2 null mutations and overexpression of Bud2 cause a random budding pattern (Park et al., 1993).
Bud2 has two important roles in bud-site selection, a structural role to identify the presumptive bud site and a regulatory role as a GAP for Bud1, thus allowing activation of bud-site assembly at the proper bud site.

1.5.3 GAP1 like proteins

The initial member of this family was the *Drosophila* protein GAP1, first characterized as a part of the sevenless signal-transduction cascade (Gaul et al., 1992).

In mammalian cells, the GAP1 family comprises four proteins: GAP1m, GAP1IP4BP, CAPRI (Ca\textsuperscript{2+}-promoted Ras inactivator) and RASAL (Ras-GTPase activating-like protein) (Figure 21).

![Figure 21. Schematical view of the GAP1 family. C2A and C2B (Ca\textsuperscript{2+}-dependent/independent phospholipids binding); GAP (GTPase activating protein domain); PH (pleckstrin homology domain-mediates protein-protein or protein-lipids interactions); Btk (Bruton’s tyrosine kinase domain).](image)

Each protein has a basic, conserved domain architecture, although subtle variations in the function of each domain have pronounced effects on the regulation of each protein. GAP1 family members contain a centrally located Ras-GAP domain. Surrounding the Ras-GAP domain at the N-terminal end are tandem C2 domain repeats (with homology to the high affinity Ca\textsuperscript{2+}-dependent phospholipids-binding C2 domains from synaptotagmin III and protein kinase C \textbeta II). C2 domains are known to function as calcium dependent phospholipid-binding motifs that allow membrane association and/or activation of their host proteins following an elevation of intracellular free calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}).

Immediately C-terminal to the Ras-GAP domain is a PH (pleckstrin homology domain) involved in binding phosphoinositides from the cell membranes with varying degrees of specificity and affinity. The PH domain is followed by a Bruton’s tyrosine kinase (Btk)
motif, a protein module that is typically found in members of the Tec family of protein kinases (Bolen, 1995).

1.5.3.1 CAPRI and RASAL

CAPRI and RASAL are prototypical Ca\(^{2+}\)-triggered RasGAPs (Lockyer et al., 2001; Walker et al., 2004). C2 domains of both CAPRI and RASAL contain the conserved C2 motif that is required for calcium-dependent phospholipids binding. This explains the Ca\(^{2+}\) mobilization induced membrane translocation of CAPRI and RASAL.

In unstimulated cells, CAPRI and RASAL are cytosolic and inactive RasGAPs. Upon agonist-evoked increase in the concentration of intracellular free Ca\(^{2+}\) both proteins undergo a rapid translocation to the plasma membrane mediated by their C2 domains. This plasma membrane association leads to an elevation of their RasGAP activities (Lockyer et al., 2001; Walker et al., 2004).

Changes in [Ca\(^{2+}\)]\(_i\), induced by electrical or chemical stimulation, are known to occur in an oscillatory manner, where the strength of the stimulus is encoded within the frequency of oscillations (Berridge et al., 2000; Berridge et al., 2003; Yarwood et al., 2006). In addition to such oscillations, the amplitude of calcium signals can also be used to transfer information. It has been established that RASAL senses calcium oscillations, while CAPRI is an amplitude sensor (Lockyer et al., 2001; Liu et al., 2005). RASAL undergoes an oscillatory association with the plasma membrane and hence an oscillatory series of activations, in synchrony with the underlying calcium oscillations (Walker et al., 2004). In contrast, CAPRI does not respond to the frequency of calcium oscillations, rather it senses the amplitude of the initial calcium elevation and undergoes a rapid but sustained translocation, and hence activation, to the plasma membrane (Lockyer et al., 2001; Liu et al., 2005).

Surprisingly however, given that these proteins contain no detectable sequence homology with any known RapGAP, both CAPRI and RASAL are also capable of enhancing the GTPase activity of Rap. In vivo, when localized in the cytosol RASAL has a basal RapGAP activity. This basal RapGAP activity is dependent on the RasGAP domain. Q507 in RASAL corresponds to the highly conserved Q1426 in NF1 and Q938 in p120GAP. The structure of GAP334, the catalytic domain of p120GAP, in complex with Ras, shows that Q938 participates in the proper positioning of N942 which directly contacts Ras. Mutations of this conserved glutamine might affect the N942 function in contacting Ras and result in
a reduced GAP activity (Ahmadian et al., 2003). Expression of RASAL (Q507N) mutant lacks the RasGAP activity even after Ca\(^{2+}\)-induced plasma membrane translocation (Walker et al., 2004). Furthermore, expression of RASAL (Q507N) has no effect on Rap1-GTP levels pulled down from cells (Kupzig et al., 2006), suggesting that the RapGAP activity depends on the integrity of the RasGAP domain.

In contrast, the RapGAP activity is independent of the Ca\(^{2+}\)/phospholipid-binding C2 domains, as RASAL (D202A), a C2 domain mutant unable to undergo Ca\(^{2+}\)-induced membrane association, leads to the same decrease in the amount of Rap1-GTP recovered from cells as wild type RASAL (Kupzig et al., 2006).

However, whereas the RasGAP activity of RASAL is dependent on its Ca\(^{2+}\)-induced plasma membrane translocation (Walker et al., 2004), the RapGAP activity appears to be irrespective of such a membrane association.

CAPRI can also function as a dual Ras and RapGAP, but in contrast to RASAL, the RapGAP activity is only detectable upon the Ca\(^{2+}\)-induced plasma membrane association (Kupzig et al., 2005).

1.5.3.2 GAP1m

GAP1m was originally isolated out of rat brain (Maekawa et al., 1993) and subsequently cloned from blood and endothelial cells (Lockyer et al., 1999b; Kobayashi et al., 1996). GAP1m is ubiquitously expressed in human tissues, particularly abundant in brain, spleen, peripheral blood leucocytes, placenta, pancreas and with a lower expression in skeletal muscles (Lockyer et al., 1999a).

In contrast to CAPRI and RASAL, the C2 domains of GAP1 lack residues required for Ca\(^{2+}\) coordination, therefore they do not appear to bind phospholipids in a Ca\(^{2+}\) dependent manner, and in vivo GAP1m is not regulated by an increase in [Ca\(^{2+}\)].

In vivo, GAP1m is a cytosolic protein with predominant perinuclear localization. Upon activation of cell-surface receptors that couple with class I PI3K (phosphoinositide3-kinase), and hence give rise to an elevation in plasma membrane PtdIns(3,4,5)P\(_3\) (PIP\(_3\)) levels, GAP1m undergoes a rapid \(t/2 \approx 30\) s translocation to the cytosolic face of the plasma membrane (Lockyer et al., 1999b). This recruitment results from the ability of the GAP1m PH domain to specifically bind PIP\(_3\).

GAP1m has some basal RasGAP activity when cytosolic. However, upon PIP\(_3\)-induced plasma membrane recruitment, this activity is significantly enhanced (Yarwood et al.,
Moreover, it has been described that GAP1m also interacts through its PH domain with the heterotrimeric G protein subunit Gα12, and its RasGAP activity was stimulated by this interaction (Jiang et al., 1998). In serum starved cells stimulated with epidermal growth factor (EGF), an agonist that induces the plasma membrane association of GAP1m, no detectable decrease in the level of Rap1-GTP was observed after co-transfection with plasmids encoding GAP1m (Kupzig et al., 2006). In contrast with the other members of the GAP1 family, GAP1m is the only member of this family of proteins for which no detectable RapGAP activity was observed in vitro and in vivo (Kupzig et al., 2006; Bottomley et al., 1998).

1.5.3.3 GAP1IP4BP

GAP1IP4BP (IP4BP: Inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄)-binding protein) also called R-RasGAP (Yamamoto et al., 1995) or GAP III (Baba et al., 1995), has been originally isolated from pig platelets (Cullen et al., 1995a; Cullen et al., 1995b) and later on cloned from a human circulating blood cDNA library (Cullen et al., 1995c).

The protein consists of 834 amino acid residues, with a molecular mass of 96 kDa. At the amino acids level GAP1IP4BP and the rat brain GAP1m are approximately 72% identical, with the major difference being an alanine-rich 26-amino-acid N-terminal extension in GAP1m which is absent from GAP1IP4BP. GAP1IP4BP appears to be ubiquitously expressed in human tissues, being particularly abundant in brain, skeletal muscle, spleen and peripheral blood leucocytes (Lockyer et al., 1999a; McNulty et al., 2001).

As previously mentioned GAP1IP4BP has a domain architecture typical for members of the GAP1 family (Figure 21). The N-terminal C2A and C2B domains of GAP1IP4BP do not contain the conserved C2 motif that is known to be required for calcium-dependent phospholipid binding. Its C2 domains do not appear to bind phospholipids in a calcium-dependent manner. Following the C2 domains there is a central, highly conserved RasGAP domain. This domain is 60% identical with the corresponding domain in GAP1m and has sequence homology with other RasGAP domains but none with RapGAPs. It contains the RasGAP consensus sequence, F-L-R and a number of invariant residues that are crucial for the RasGAP activity, including a conserved arginine at position 371, which corresponds to Arg1276 and Arg789 in NF1 and p120GAP, respectively.
GAP1\textsuperscript{IP4BP} was first purified based on its intrinsic ability to bind the second messenger inositol 1,3,4,5 tetrakisphosphate (IP\textsubscript{4}) with high affinity (\(K_D\) of 11.3 nM) (Bottomley et al., 1998) and specificity, being therefore considered a putative IP\textsubscript{4} receptor. The functional IP\textsubscript{4} binding site was located within the C-terminal PH/Btk domain. This domain shows binding activity towards inositol 1,3,4,5 tetrakisphosphate (IP\textsubscript{4}), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\textsubscript{2}) and phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P\textsubscript{3}) (Cozier et al., 2000a; Cozier et al., 2000b; Cozier et al., 2003). These phosphoinositides specificities are translated into distinct subcellular distribution compared to GAP1m.

GAP1\textsuperscript{IP4BP} is constitutively localized at the plasma membrane via binding to PI(4,5)P\textsubscript{2}, whereas GAP1m is mainly localized in the cytosol and translocated to the plasma membrane in a PI(3,4,5)P\textsubscript{3}-dependent manner (Cozier et al., 2000b; Lockyer et al., 1997). Expression of GAP1\textsuperscript{IP4BP} mutants has shown that deletion of either C2A or C2B domains has no effect on the plasma membrane localization, whereas site direct mutagenesis of the PH/Btk domain (K599Q; K600Q; R601Q) has a dual effect of completely abolishing IP\textsubscript{4} binding resulting in a complete loss of plasma membrane association. These results suggested that the binding site for IP\textsubscript{4} overlaps with that of PIP\textsubscript{2} within the PH/Btk motif (Lockyer et al., 1997).

More than 100 different PH-domain containing proteins have been identified, many of which are involved in intracellular signaling. Several tree-dimensional structures of PH domains from different proteins have so far been determined. Each of these structures contains clusters of lysine and arginine residues that form a positively-charged surface, which interacts with the negatively charged phosphate groups of their respective phosphoinositide ligands. Based on the crystal structure of the Btk (Burton tyrosine kinase) PH domain in complex with IP\textsubscript{4} (Baraldi et al., 1999) a model of the PH domain of GAP1\textsuperscript{IP4BP} in complex with IP\textsubscript{4} has been generated and verified by site directed mutagenesis (Cozier et al., 2000a; Cozier et al., 2003). Two mutants (K591T and R604C) show a drop in affinity for PIP\textsubscript{2} resulting in a loss of constitutive association with the plasma membrane. However, these cytosolically localized mutants retained a high affinity for PIP\textsubscript{3} manifested \textit{in vivo} through their ability to undergo a PIP\textsubscript{3}-dependent association with the plasma membrane following growth factors stimulation (Cozier et al., 2003).

GAP1\textsuperscript{IP4BP} contains neither transmembrane-spanning domains nor post-translational membrane-targeting sequences (Cullen, 1998).

GAP1\textsuperscript{IP4BP} functions \textit{in vitro} as a dual RasGAP and RapGAP (Cullen et al., 1995c; Bottomley et al., 1998). Despite the fact that GAP1\textsuperscript{IP4BP} contains no sequence homology
with any known RapGAP, GAP1\textsuperscript{IP4BP} is also able of enhancing the GTPase activity of Rap. GAP1\textsuperscript{IP4BP} stimulates the GAP activity on H-Ras with about five fold lower potency than p120GAP, and it is equally effective against Rap1A, but shows no GAP-stimulated activity against Rac or Rab3A (Cullen et al., 1995c). Moreover it has been reported that GAP1\textsuperscript{IP4BP} has higher GAP activity towards R-Ras than towards H-Ras (Yamamoto et al., 1995). Very little is known about how GAP1\textsuperscript{IP4BP} is regulated. One model argues that it is an inactive RasGAP that only becomes active when it binds the water-soluble IP\textsubscript{4} (Cullen et al., 1995c). In the resting cell GAP1\textsuperscript{IP4BP} is located at the plasma membrane via the ability of its PH/Btk domain to bind PIP\textsubscript{2}. In this state GAP1\textsuperscript{IP4BP} is unable to function as a RasGAP (Cozier et al., 2000b). It has been argued that the binding of IP\textsubscript{4}, that is generated in cells following receptor mediated activation of PI-PLC, to the PH domain serves to remove the inhibitory influence of PIP\textsubscript{2}, thereby allowing activation of the RasGAP activity (Cozier et al., 2000b).

Recently, transgenic mice that express mutant GAP1\textsuperscript{IP4BP} with knocked down activity were generated (Iwashita et al., 2007). The GAP1\textsuperscript{IP4BP} mutant mice lacked 88 amino acids, including the arginine finger loop responsible for both catalytic activity and substrate specificity. While heterozygotes are normal, homozygous mice die at E12.5–13.5 of massive subcutaneous and intraparenchymal bleeding, probably due to underdeveloped adherens junctions between capillary endothelial cells. These results show essential roles of GAP1\textsuperscript{IP4BP} in development and differentiation, its expression being needed for embryonic development of blood vessel barriers, whereas its down-regulation facilitates NGF-induced neurite formation of PC12 cells (Iwashita et al., 2007).

1.6 Inositol-1,3,4,5-tetrakisphosphate

For many different cell types agonist exposure often causes the activation of phosphoinositide-specific phospholipase C (PLC) which leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\textsubscript{3}). While diacylglycerol remains in the plasma membrane where it helps to activate protein kinase C enzymes, IP\textsubscript{3} is released into the cytosol where it serves to activate IP\textsubscript{3} receptors and to allow Ca\textsuperscript{2+} to be released from intracellular stores which reside in the endoplasmic reticulum. IP\textsubscript{3} is phosphorylated in the presence of ATP to IP\textsubscript{4} in a reaction catalyzed by a Ca\textsuperscript{2+}-regulated Ins(1,4,5)P\textsubscript{3} 3-kinase.
Introduction

It is known that IP$_4$ is hydrolyzed by the same 5-phosphatase that hydrolyzes IP$_3$, but the enzyme has a 10-fold higher affinity and 100-fold lower $V_{\text{max}}$ for IP$_4$ than IP$_3$ (Connolly et al., 1987). So, IP$_4$ can protect IP$_3$ against hydrolysis and therefore increase its effectiveness (Hermosura et al., 2000).

IP$_4$ is classically presented as a modulator of calcium mobilization in various cells. It has been linked to a potential role in the regulation of intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{i}$) following cellular stimulation with agonists that activate phosphoinositide-specific phospholipase C. For example, in endothelial cells there is direct evidence (Luckhoff and Clapham, 1992) and in neurons direct and indirect evidence (Tsubokawa et al., 1996; Szinyei et al., 1999) that IP$_4$ can activate Ca$^{2+}$ channels in the plasma membrane. In neurons the consequence is the enhancement of LTP (long-term potentiation). In HeLa cells electroporation of IP$_4$ causes a transient increase in the frequency of calcium oscillations in response to histamine (Zhu et al., 1996). In platelets application of IP$_4$ on purified plasma membranes induces a calcium flux distinguishable from that induced by IP$_3$ (El Daher et al., 2000).

IP$_4$ can also interact with IP$_3$ receptors on the endoplasmic reticulum membrane, but only at high concentrations, and with effects that seem contradictory. In some cell types it inhibits the action of IP$_3$ (Hermosura et al., 2000; Bird and Putney, Jr., 1996) whereas in others it mimics it by mobilizing Ca$^{2+}$ (Gawler et al., 1991). There are three isoforms of the IP$_3$ receptors in mammals and it is possible that they respond differently to IP$_4$. The most elusive effect of IP$_4$ is to synergize with IP$_3$ to mobilize Ca$^{2+}$ and to activate stored-operated Ca$^{2+}$ entry (Irvine and Schell, 2001).

Multiple IP$_4$ binding sites have been found in different tissues and several IP$_4$-binding proteins have been identified. Among them, GAP1IP$_4$BP has the potential to integrate the inositol phosphate pathway and the small G proteins pathways, both of which participate in the activation or regulation of calcium entry.

Currently, the only direct evidence for a role of GAP1IP$_4$BP in Ca$^{2+}$ homeostasis has emerged from L-1210 cells (Loomis-Husselbee et al., 1996). When these cells are permeabilized, the addition of exogenous GAP1IP$_4$BP enhances the ability of IP$_4$ to potentiate IP$_3$-stimulated Ca$^{2+}$ release. This observation clearly suggests that in these cells under these particular conditions the binding of IP$_4$ to GAP1IP$_4$BP does appear to play a role in the regulation of IP$_3$-stimulated Ca$^{2+}$ release (Walker et al., 2002).

Moreover, it has been shown that disruption of the gene encoding the ubiquitously expressed IP$_3$-kinase isoform B (Itpkb) in mice is associated with a notably decreased
concentration of IP4 in stimulated thymocytes and caused a severe T cell deficiency due to major alterations in thymocyte responsiveness and selection (Pouillon et al., 2003). Recently it was shown that upon its generation by IP3 3-kinase, IP4 binds to the PH domain of the enzyme Itk (belonging to the tec homology protein tyrosine kinases family) and induces its association with the plasma membrane PIP3 (Huang et al., 2007). In this form Itk is able to phosphorylate and activate PLC-γ1 which subsequently hydrolyzes PIP2 and produces the second messengers IP3 and diacylglycerol. It turns out that by activating protein kinase C, diacylglycerol drives T cell development. When T cells from mutant mice lacking IP3 3-kinase are stimulated, PIP3-dependent recruitment of Itk to the plasma membrane does not occur because it requires obligatory help from IP4 (Irvine, 2007). This defines IP4 as an essential messenger during T cell selection and differentiation in thymus. GAP1IP4BP might represent a good candidate responsible for the effects of Ins(1,3,4,5)P4 in thymocytes, as it is highly expressed in peripheral blood leukocytes, and is active on Ras and Rap molecules, which are important during thymocytes differentiation.
1.7 Objectives of this work

The GTPase activating protein, GAP1 IP4BP, is a member of the GAP1 family of Ras GTPase-activating proteins (RasGAPs). Composed of a central Ras GAP domain, surrounded by amino-terminal C2 domains and a carboxy-terminal PH/Btk domain, GAP1 IP4BP has high sequence homology with other RasGAPs, but no sequence homology to any known RapGAPs. Despite its sequence homology to RasGAPs, GAP1 IP4BP has previously been shown to possess besides the predicted RasGAP activity, an unexpected GAP activity on the Ras-related protein Rap.

The mechanism by which Ras and RapGAPs enhance the GTPase activity of their respective GTPases is distinct. RasGAP works by inserting an arginine residue, the arginine finger, into the nucleotide-binding pocket of Ras, which, on one hand, fixes the conformation of the glutamine in the switch region of Ras that in turn positions a water molecule ready for the nucleophilic attack, and, on the other hand, neutralizes the negative charges that develop at the γ-phosphate of the GTP during phosphoryl transfer.

The stimulation of GTP hydrolysis in Rap1 involves a substantially different mechanism. Rap1 does not possess a glutamine in switch II that is crucial for GTP-hydrolysis in Ras and Rap1GAP does not provide a catalytic arginine, but it supplies an asparagine residue, the Asn thumb, which is inserted into the active site and takes over the role of glutamine 61 in Ras.

Considering these two different mechanisms, the discovery of GAP1 IP4BP raises the question on how such a dual activity towards both Ras and Rap is achieved. Therefore, in this study, I attempted to explore the dual GAP mechanism of GAP1 IP4BP by biochemical and structural analyses.

The main goal of this study was to determine the biochemical specificity of GAP1 IP4BP towards both small G proteins Ras and Rap, to identify the domains and the catalytic residues that are responsible for both GAP activities towards Ras and Rap and to characterize the GAP stimulated GTP hydrolysis reaction. Based on the biochemical data, the catalytic centre should be identified and further characterized by mutational and biochemical analyses. Consequently, it was attempted to determine the $k_{cat}$ and $K_M$ of both GAP1 IP4BP mediated reactions by saturating the GAP1 IP4BP with increasing concentrations of the small G proteins and treating the data with the Michaelis-Menten equation. Furthermore, to obtain important information about the mechanism of the phosphoryl
transfer reaction, the GAP1IP4BP-stimulated GTPase activity towards both small G-proteins Ras and Rap was explored by Time-Resolved Fourier-transform infrared spectroscopy (FTIR).
2 Materials and Methods

2.1 Materials

2.1.1 Chemicals
All chemicals were purchased from Aldrich (Steinheim), GE Healthcare (Freiburg), Riedel-de Haen (Seelze), Merck (Darmstadt), Invitrogen (Karlsruhe), Qiagen (Hilden), Roth (Karlsruhe), Serva (Heidelberg), Sigma (Deisenhofen), Jena Bioscience (Jena), Roche Diagnostics (Mannheim), Gerbu (Gaiberg), Bio-Rad (München). The radioactive [γ-\(^{32}\)P] GTP was ordered from GE Healthcare (Freiburg) with a specific activity of >5000 Ci/mmol at the reference day. Deionized and sterile water (Milipore) was used for all buffers and solutions. pH values were adjusted at room temperature.

2.1.2 Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAase-I</td>
<td>Roche Diagnostics (Mannheim)</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>New England Biolabs (Beverly, MA)</td>
</tr>
<tr>
<td>Restriction enzymes</td>
<td>New England Biolabs (Beverly, MA)</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>New England Biolabs (Beverly, MA)</td>
</tr>
<tr>
<td>Trypsin, α-chymotrypsin</td>
<td>Sigma (Deisenhofen)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Serva (Heidelberg)</td>
</tr>
<tr>
<td>Protease Inhibitor Cocktail tablets</td>
<td>Roche Diagnostics (Mannheim)</td>
</tr>
</tbody>
</table>

2.1.3 Kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAprep Spin Miniprep Kit</td>
<td>Qiagen (Hilden)</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>Qiagen (Hilden)</td>
</tr>
<tr>
<td>BigDye Terminator Sequencing Kit</td>
<td>Perkin-Elmer (Überlingen)</td>
</tr>
<tr>
<td>ECL plus Western Blotting Detection system</td>
<td>GE Healthcare (Freiburg)</td>
</tr>
</tbody>
</table>

2.1.4 Standards

<table>
<thead>
<tr>
<th>Standard</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ-DNA standard</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>1Kb Ladder for Agarose gels</td>
<td>Gibco (Eggenstein)</td>
</tr>
<tr>
<td>Wide Range, SDS7 protein marker</td>
<td>Sigma (Deisenhofen)</td>
</tr>
</tbody>
</table>
Materials and Methods

2.1.5 Chromatography Media

DEAE-Sepharose fast Flow  GE Healthcare (Freiburg)
Glutathion Sepharose 4B  GE Healthcare (Freiburg)
Superdex 75 and 200  GE Healthcare (Freiburg)
Ni-NTA-Agarose Superflow  Qiagen (Hilden)

2.1.6 Microorganisms

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK600K</td>
<td>supE, hsdM+, hsdR-, kanR (Hoffmann-Berling, Heidelberg)</td>
</tr>
<tr>
<td>TG1</td>
<td>K12, supE, hsdΔ5, thi, Δ(lac-proAB), F’mtraD36, proAB+, lacIq, lacZΔM15</td>
</tr>
<tr>
<td></td>
<td>(Promega)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>B, F-, hsdSB (rB-, mB-), gal, dcm, ompT, λ(DE3) (Novagen)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F–ompT hsdSB(rB– mB–) gal dcm (DE3) pRARE2 (CmR)</td>
</tr>
<tr>
<td>BL21(DE3)-RIL</td>
<td>(E. coli B) F –ompT hsdS (rB– mB–) dcm + Ter gal λ(DE3) endA Hte (argU ileY leuW Cam³)</td>
</tr>
<tr>
<td>Rosetta(DE3)</td>
<td>pRARE containing the tRNA genes argU, argW, ileX, glyT, leuW, proL, metT, thrT, tyrU, and thr (Novagen)</td>
</tr>
<tr>
<td>DH10Bac</td>
<td>F mcrA Δ(mrr-hsdRMS-mcrBC) ΔlacZHΔM15 ΔlacX74 recA1 endA1 araD139</td>
</tr>
<tr>
<td></td>
<td>Δ(ara, leu)7697 galU galK λ- rpsL nupG1bMON14272/pMON7124 (Invitrogen)</td>
</tr>
</tbody>
</table>

Table 2. Genotypes of used bacterial strains.

2.1.7 Media

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani (LB)</td>
<td>10 g/l Bactotryptone, 10 g/l NaCl, 5 mM NaOH,</td>
</tr>
<tr>
<td></td>
<td>5 g/l yeast extract</td>
</tr>
<tr>
<td>Terrific Broth (TB)</td>
<td>12 g/l BactoTryptone, 24 g/l Bacto-yeast-extract,</td>
</tr>
<tr>
<td></td>
<td>4 g/l glycerol, 17 mM KH2PO4, 72 mM K2HPO4</td>
</tr>
<tr>
<td>Standard I (ST I)</td>
<td>25 g/l standard-I powder (Merck, Darmstadt)</td>
</tr>
<tr>
<td>LB Plate Medium</td>
<td>10 g/l bacto tryptone; 5 g/l yeast extract; 10 g/l NaCl;</td>
</tr>
<tr>
<td></td>
<td>15 g/l bacto agar</td>
</tr>
<tr>
<td>Grace’s Insect Cells Medium</td>
<td>3,330 mg/L lactalbumin hydrolyzate;</td>
</tr>
<tr>
<td></td>
<td>3,330 mg/L yeastolate; and L-glutamine</td>
</tr>
</tbody>
</table>
**Materials and Methods**

**Cellfectin Reagent** 1:1.5 (M/M) liposome formulation of the cationic lipid N, NI, NII, NIII Tetramethyl-N, NI, NII, NIII-tetrapalmitylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water.

**2.1.8 Antibiotics**

All antibiotics used were purchased from Gerbu (Gaiberg) and were prepared as follows:
Ampicillin was prepared as stock solution of 50 mg/ml in water.
Kanamycin was prepared as a stock solution of 50 mg/ml in water.
Chloramphenicol was prepared as stock solution of 25 mg/ml in isopropanol.

**2.1.9 Vectors**

**pProEX-HT vectors** (Invitrogen, Karlsruhe)
The pProEX-HT vectors contain the IPTG-inducible lac promoter and are Ampicillin resistant. They contain N-terminal hexa-histidine residues as fusion partner. During affinity purification, these hexa-histidine residues bind to Nickel-NTA-Agarose resin. As the pProEX-HT vectors contain a TEV protease cleavage site after the N-terminal hexa-histidine affinity tag, the protein of interest can be cleaved off from the fusion protein.

**pGEX vectors** (GE Healthcare, Freiburg)
The pGEX vectors contain the IPTG-inducible lac promoter and are Ampicillin resistant. They contain the N-terminal Glutathion-S-Tranferase (GST) fusion protein from Schistosoma Japonicum. During affinity purification the GST part of the fusion protein binds to a Glutathion (GSH)-Sepharose column. pGEX-6P-2 vector encodes the recognition sequence for site-specific cleavage by PreScission Protease, between the GST domain and the multiple cloning site. pGEX-4T-1 contains a thrombin recognition site and therefore the protein of interest can be cleaved off from the fusion protein.

**pET20 2xHis** (provided by Dorothee Kühlmann- MPI Dortmund) contains the IPTG-inducible lac promoter. It is Ampicillin resistant. It contains N-terminal hexa-histidine and C-terminal hexa-histidine residues that bind to the Ni-NTA–Agarose column.
### 2.1.10 General buffers

**HPLC running buffer**
- 100 mM Potassium phosphate
- 10 mM tert.-Butylammoniumbromide (TBA-Bromide)
- 7.5% Acetonitrile

**Acrylamide/Bis-acrylamide stock solution**
- 30% acrylamide
- 0.8% bis-acrylamide
  
  \((N,N^\prime\text{-methylenbis-acrylamide})\)

**2.5x separating gel buffer**
- 1.875 M Tris-Cl
- 0.25% SDS
- pH 8.9

**5x stacking gel buffer**
- 0.3 M Tris-phosphate
- 0.5% SDS
- pH 6.7

**1x electrophoresis running buffer**
- 25 mM Tris / HCl
- 190 mM Glycine
- 0.1% SDS

**5x SDS sample buffer**
- 0.225 M Tris / HCl, pH 6.8
- 50% glycerol
- 5% SDS
- 0.05% bromophenol blue
- 0.25 M dithiothreitol (DTT)

**Coomassie Staining solution**
- 0.05% (w/v) Coomassie Brilliant Blue R-250
- 40% (v/v) ethanol
- 10% (v/v) glacial acetic acid
- 50% (v/v) water

**Destaining solution for SDS gels**
- 40% (v/v) ethanol
- 10% (v/v) glacial acetic acid
- 50% (v/v) water

**TAE Buffer**
- 40 mM Tris-acetate
- 1mM EDTA

**6xDNA sample buffer**
- 0.25 % (w/v) bromphenolblue
0.25 % (w/v) xylencyanol
30 % (v/v) Glycerol in TAE-buffer

**1x T4 DNA ligase buffer**
50 mM Tris / HCl pH 7.5
10 mM MgCl2
1 mM ATP
10 mM Dithiothreitol
25 μg/ml BSA

**Ponceau S staining solution**
0.5% (w/v) Ponceau S
1% (v/v) glacial acetic acid

**Tank-blotting transfer buffer**
25 mM Tris base
150 mM Glycine
20% (v/v) methanol

**Semi-dry transfer buffer**
25 mM Tris base
150 mM glycine
10% (v/v) methanol

**PBS**
50 mM potassium phosphate (0.5 M K₂HPO₄; 0.5 M KH₂PO₄)
150 mM NaCl
pH 7.2 without adjustment

**Tris buffered saline (TBS)**
10 mM Tris base
150 mM NaCl
pH 7.5

### 2.1.11 Crystallization screens

- Crystal Fast Screen I: Hampton Research (Laguna Hills, USA)
- Crystal Fast Screen II: Hampton Research (Laguna Hills, USA)
- Index Screen HT: Hampton Research (Laguna Hills, USA)
- PEG/Ion-Screen: Hampton Research (Laguna Hills, USA)
- Salt RX HT: Hampton Research, (Laguna Hills, USA)
- Grid Screen: Hampton Research, (Laguna Hills, USA)
- PO₄ Screen: Hampton Research, (Laguna Hills, USA)
- (NH₄)₂SO₄ Suite: Qiagen (Hilden)
- Anions Suite: Qiagen (Hilden)
2.2 Molecular biology methods

2.2.1 Isolation of plasmid DNA
The isolation of plasmid DNA was performed using QIAprep Spin Miniprep Kit from Qiagen (Hilden) according to the manufacturer’s protocol. The procedure uses for bacterial lysis the modified alkaline lysis method (Birnboim and Doly, 1979). The bacterial lysate is neutralized and adjusted to high-salt binding conditions. Denatured proteins and precipitated cellular components are removed by centrifugation. The cleared lysates are then applied to the QIAprep module where plasmid DNA adsorbs to a silica gel membrane in high salt buffer. Impurities are washed away and pure DNA is eluted in low-salt buffer or water.

2.2.2 Agarose gel electrophoresis
DNA was evaluated by agarose gel electrophoresis where the negatively charged DNA fragments migrate through polymerized agarose (1.0%) towards the anode, by applying electric current (less than 5 volts per centimeter of the gel) in a buffer that establishes a pH and provides ions for conductivity (TAE). DNA was stained with Ethidium bromide (0.5 μg/ml), a fluorescent dye which intercalates between nucleotide bases, and the migration of the DNA molecules was recognized using bromophenol blue as tracking dye.

2.2.3 Polymerase chain reaction (PCR)
The selective amplification of specific DNA fragments from vectors for cloning was performed by PCR using Pfu polymerase, a thermostable DNA polymerase and two
specific primers. The principle of this method is as follows: two synthetic, single stranded oligonucleotides are synthesized. One is complementary in sequence to the 5’ end of one strand of DNA to be amplified, the other complementary to the 3’ end of the other strand. The DNA from the vector is then denaturated and the oligonucleotides anneal to their target sequences. Thermostable DNA polymerase and deoxynucleotides are added and the enzyme extends the two primers. This reaction generates double stranded DNA over the region of interest on both of the strands of the DNA. In the first cycle of the PCR reaction, two double-stranded copies of the starting fragment are produced. The DNA is subjected to further rounds of amplification. Each cycle doubles the number of copies, until a large quantity of the target sequence is produced. Fragments are then digested and purified using QIAquick Gel Extraction Kit (Hilden) according to manufacturer’s protocol.

2.2.4 Hydrolysis of DNA with restriction endonucleases
Restriction endonucleases are enzymes that hydrolyze DNA at specific sites by recognition of short (4-8 base pairs) target sequences, usually palindromic. The DNA to be cloned into a vector (the insert DNA) and the vector DNA to be cloned into, were independently restricted with the respective enzymes to make the compatible termini with 5’ phosphate residues and 3’ hydroxyl moieties. 5-10 U of enzyme were used per μg of DNA, 1x adequate buffer for the enzyme, 1x BSA was used when suggested for the respective enzyme, in a final volume of 20 μl/μg of DNA and incubated for the indicated time and temperature according to the manufacturer’s protocol. For the identification of positive clones after transformation and the control of ligation products, restriction enzymes were used and the result analyzed by agarose gel electrophoresis. All enzymes used were purchased from New England Biolabs (Beverly, MA) and were used according to manufacturer’s protocol.

2.2.5 Purification of DNA fragments
The specific size of the restricted DNA to be cloned was cut and purified from the agarose gel after adequate separation. For the isolation of separated DNA from the agarose gels QIAquick Gel Extraction Kit from Qiagen (Hilden) was used according to manufacturer protocol. The principle of this method consists in the disintegration of the agarose gel slices and the subsequent DNA isolation using a silica gel membrane under the correct salt concentrations and pH conditions.
2.2.6 Ligation of DNA fragments
Vector and insert DNA were quantified in agarose gels using digested λ-marker (New England Biolabs, Beverly, MA) as a reference. 10 ng of vector was ligated with a 5-10 fold molar excess of insert overnight at 16°C using T4 DNA ligase (Roche, Mannheim) according to the manufacturer’s protocol.

2.2.7 Determination of DNA concentration
The concentration of DNA was measured using a spectrophotometer, at 260 nm. The purity of the DNA solution was estimated using the ratio of OD readings at 260 nm and 280 nm, where pure preparations of DNA have an OD$_{260}$/OD$_{280}$ ratio of 1.8. The concentration was calculated according to the following equation $c = A_{260} \times 50 \mu g/ml \times$ dilution factor.

2.2.8 Preparation of competent cells
Competent cells were prepared according to (Chung et al., 1989). 200 ml LB medium was inoculated with 2 ml preculture and grown at 37°C until an OD$_{600}$ of 0.4. Bacteria were incubated for 20 min on ice, pelleted for 5 min at 1200 x g (4 °C), resuspended in 20 ml ice-cold sterile TSS buffer (85% LB medium without NaOH, 10% PEG 8000, 5% DMSO, 50 mM MgCl$_2$, pH 6.5), flash frozen and stored at -80°C.

2.2.9 Transformation of bacterial cells with circular DNA
Transformation is the process by which a host organism can take up DNA from its environment. Some bacteria, but not E.coli can do this naturally and are therefore considered to have a genetic competence. The heat shock method was used for transformation according to standard procedures (Sambrook and Russel, 1989). In general, transformation is a relatively inefficient process, only a small percentage of DNA-treated cells take up the circular DNA.
Competent bacteria (150 µl) were thawed on ice, the ligation mix was added to the competent bacteria and incubated on ice for 30 min, heat-shock was given at 42°C for 60 sec, 1 ml of LB medium was added and the cells were incubated at 37°C in a rotator for an hour. The bacteria were pelleted by centrifuging at 8000 rpm for 1 min, resuspended in 50 µl medium and plated on LB agar plates with the corresponding antibiotics (1 g/l).
2.2.10 **Storage of bacterial cells**

A 2 ml culture was grown overnight at 37°C. The pelleted cells were resuspended in 1 ml 30% glycerol in LB medium and the bacterial stocks were flash frozen and stored at -80°C.

2.2.11 **DNA sequencing**

DNA sequencing was performed according to (Sanger et al., 1977) using the Bid Dye terminator kit (Applied Biosystems, Langen). A sequencing reaction contained 1 µg plasmid DNA Qiaprep solution, 4 µl terminator mix, 3.5 pmol sequencing primer in a final volume of 20 µl. The sequencing reaction was carried out as follows: 1x (96°C; 2 min); 25x (96°C, 30 sec; 48°C, 20 sec; 60°C, 4 min); 1x 60°C, 10 min; hold temperature at 4°C. The annealing temperature was calculated using the equation $2x (\text{AT}) + 4x (\text{GC})$. Following the temperature cycling the DNA was precipitated by adding 2 µl 3 M sodium acetate pH 4.8 and 60 µl 100% ethanol followed by centrifugation at 13000 rpm for 30 min at 4°C. The pellet was washed once with 400 µl 70% ethanol, followed by centrifugation 10 min at 10000 rpm, 4°C. After centrifugation the supernatant was removed and the pellet was dried at room temperature for 30 min. Analysis of the sequencing products was done in house using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Langen).
### 2.2.12 Constructs

<table>
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</table>

Table 3. Overview of the constructs used in the present study.
2.3 Biochemical Methods

2.3.1 Sequence alignment
Sequences were aligned using the ClustalW algorithm (Thompson et al., 1994) and manually refined using the program Genedoc (Nicholas et al., 1997).

2.3.2 SDS polyacrylamide gel electrophoresis
Separation of proteins of different molecular mass was performed according to (Laemmli, 1970) using denaturing, discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Discontinuous one-dimensional gel electrophoresis was performed under denaturing conditions in the presence of 0.1% SDS (Laemmli method). The discontinuous gel system comprises a separating gel overlaid by a stacking gel. The concentration of acrylamide used for the separating gel depends on the size of the proteins to be analyzed. The protein samples were dissolved in 1x SDS-PAGE sample buffer. β-mercaptoethanol was used as reducing agent (added fresh), to disrupt the disulphide bonds. The samples were boiled at 95°C for 5 minutes prior to loading. The gels were run at 20 mA for 2 hours in a running buffer in mini-Protean II gel chambers (Biorad) or big gel chambers. Visualization of protein bands was carried out by incubating the gels with Coomassie staining solution followed by destaining.

2.3.3 Total protein staining
Ponceau S total protein staining solution was used for rapid protein detection in solution. This method can only detect high protein concentrations (>1µg). 2 µl protein solution were added onto a nitrocellulose membrane, incubated for 2-4 minutes in Ponceau S and rinsed several times with water to visualize the proteins.

2.3.4 Determination of protein concentration
Protein concentration was determined according to (Bradford, 1976) using the Biorad protein assay (Biorad). The solution was calibrated using bovine serum albumin. This assay can detect protein concentrations as low as 0.05-0.5 µg/ml.
Protein concentration was also determined by ultraviolet-visible spectrophotometry at 280 nm with the calculated molar absorption coefficient based on the number of tyrosine, tryptophan and cysteine residues (Gill and von Hippel, 1989).
2.3.5 Western blot

In the western blot analysis, proteins are transferred to a membrane from a polyacrylamide gel after separation according to size by SDS-PAGE. This allows confirmation of the size of the expressed 6xHis-tagged or GST-tagged protein by comparison with molecular weight markers and detection of any degradation products that might be present. Following electrophoresis, proteins in polyacrylamide gel were transferred to a PVDF membrane (Hybond-P, GE Healthcare) in a buffer-tank-blotting apparatus or by semi-dry electroblotting. After transfer of proteins the remaining protein free sites on the membrane were blocked with blocking buffer (3% BSA in TBS) to prevent high background due to the unspecific binding of the primary or secondary antibody. Once transferred to the membrane the proteins can be probed with epitope-specific antibodies or conjugates. The membrane was incubated in the primary antibody solution (anti-His or anti-GST antibody) (1:1000 or 1:2000 dilution of antibody stock solution in blocking buffer) at room temperature for 1 h. After incubation, the membrane was washed 3 times with TBST (0.05% Tween in TBS), and subsequently incubated in the secondary antibody solution (horseradish peroxidase conjugated anti-mouse IgG, 1:3000 dilution in 5% nonfat dried milk in TBS). After 1 hour incubation at room temperature, the membrane was washed three times with TBST. Chemiluminescent detection reaction was performed using the ECL plus Western Blotting Detection system (GE Healthcare) followed by exposure to X-ray film.

2.3.6 Partial digestion

GAP1IP4BP was partially digested to find a smaller, compact folded fragment. The reaction (100 µl) containing 100 µg GAP1IP4BP was started by adding the indicated amount of proteases and incubated at room temperature.

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<td>Carboxypeptidase</td>
<td>4 µg / ml</td>
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<tr>
<td>Elastase</td>
<td>8 µg / ml</td>
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</table>

Table 4. Proteases used for partial digestion.
After 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 60 min, 120 min, 240 min and 1000 min, 10 μl aliquots were transferred from the reaction mixture in SDS sample buffer and immediately flash frozen. Aliquots were analyzed by SDS-PAGE.

2.3.7 Expression of GAP1<sup>IP4BP</sup> in insect cells

The Bac-to-Bac Baculovirus expression system

Recombinant baculoviruses are used to express heterologous genes in cultured insect cells. Several GAP1<sup>IP4BP</sup> constructs (Table 3) were cloned into a pFastBac vector under the control of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) promoter for high-level expression in insect cells. Figure 22 depicts the generation of recombinant baculovirus and the expression of a gene of interest using the Bac-to-Bac Baculovirus Expression System.

In the pFastBac vector the expression cassette is flanked by the left and right arms of Tn7 transposon. The DH10Bac *E. coli* strain is used as the host for the pFastBac vector. DH10Bac cells contain a baculovirus shuttle vector (bacmid) with a mini-att<sub>Tn7</sub> target site. Once the pFastBac expression plasmid is transformed into DH10Bac cells, transposition occurs between the mini-Tn7 element on the pFastBac vector and the mini-att<sub>Tn7</sub> target site on the bacmid to generate a recombinant bacmid. The bacmid present in DH10Bac *E. coli* contains a segment of DNA encoding the LacZα peptide. Insertions of the mini-Tn7 into the mini-att<sub>Tn7</sub> attachment site on the bacmid disrupt the expression of the LacZα peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid.

Recombinant plasmids containing different constructs of GAP1<sup>IP4BP</sup> were transformed in DH10Bac cells. The cells were subsequently incubated for 48 hours at 37°C. After incubation white colonies were selected for analysis. White colonies were restreaked on fresh LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Bluo-Gal, and 40 μg/ml IPTG and incubated overnight at 37°C.

From a single colony confirmed to have a white phenotype on restreaked plates containing Bluo-gal and IPTG, a liquid culture containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, and 10 μg/ml tetracycline was inoculated and the recombinant bacmid DNA was subsequently isolated and analyzed to verify successful transposition to the bacmid.
Materials and Methods

Figure 22. Generation of recombinant baculoviruses and gene expression using the Bac-to-Bac expression system.

Transfecting insect cells
Sf21 cells were transfected using a cationic lipid such as Cellfectin as transfection reagent. In a 6-well culture plate, 9 x 10⁵ Sf21 cells per well were seeded in 2 ml growth medium containing antibiotics (e.g. 2 ml of Sf-900 II SFM containing 50 units/ml penicillin and 50 μg/ml streptomycin final concentration). In order to allow cells to attach the cultures were incubated at 27°C for at least 1 hour. For each transfection sample 1 µg of purified bacmid DNA was diluted in 100 μl of unsupplemented Grace’s Medium. 6 µl Cellfectin Reagent were diluted in 100 unsupplemented Grace’s Medium. The diluted bacmid DNA and Cellfectin Reagent were combined, mixed gently and incubated at room temperature for 15 to 45 minutes. The Sf21 cells were once washed with 2 ml of unsupplemented Grace’s Medium. 0.8 ml unsupplemented Grace’s Medium was added to the DNA-Cellfectin mixture, gently mixed and subsequently added to each well containing cells. The cells were incubated in a 27°C incubator for 5 h. Subsequently, the DNA-Cellfectin complexes were removed and 2 ml of complete growth media (e.g. Sf-900 II SFM containing...
antibiotics) were added and the cells were then incubated in a 27°C humidified incubator until signs of viral infection were visible (e.g. 72 h).

**Insect cells culture**

*Spodoptera frugiperda* Sf9 or Sf21 insect cells are commonly used for baculovirus expression. Suspension cultures of Sf9 or Sf21 cells were cultured at 27°C in shaker flasks under constant stirring. The culture volume should not exceed 20% of the flask volume in order to ensure proper aeration. The cells were counted and subcultured every 2 days in order to maintain a cell density of 1-2 x 10^6 cells/ml. Sf9 cells were cultured in Grace’s Insect Cells Media supplemented with 10% FBS while Sf21 cells were cultured in TC10 media supplemented with 5% FBS.

**Isolating the viral stock**

Budded virus should be released into medium 72 hours after transfection. Cells were visually inspected daily for signs of infection (increased cell diameter, increased size of the cell nuclei, detachment of cells from the culture plate). Once the transfected cells demonstrated signs of late stage infection the medium containing virus from each well was collected, centrifuged and the clarified supernatant which represents the first viral stock (P1) was kept at 4°C.

**Performing a viral plaque assay**

In order to determine the titer of the viral stock a viral plaque assay was performed. Sf21 cells were harvested and a 12 ml cell suspension at 6 x 10^6 cells/ml in complete growth medium was prepared. 2 ml of cell suspension were aliquoted into each well of two 6-well plates. Cells were allowed to settle to the bottom of the plate and incubated, covered, at room temperature for 1 hour. Serial dilutions (1:10^6, 1:10^7, 1:10^8) of the clarified baculoviral stock in medium were prepared. To do this, 0.5 ml of the baculoviral stock were sequentially diluted in 4.5 ml medium. The medium from the 6-well plates containing Sf21 cells was removed and immediately replaced with 1 ml of the appropriate virus dilution. The cells with virus were incubated for 1 hour at room temperature. Sequentially starting from the highest dilution to the lowest dilution, the medium containing virus was removed from the wells and replace with 2 ml of plaquing medium. The cells were incubated in a 27°C humidified incubator for 7-10 days until plaques were visible and ready to count. To facilitate counting, the plaques were stained with the dye
Neutral Red. The number of plaques present in each dilution was counted and the titer of the viral stock - plaque forming units (pfu/ml) - was calculated.

**Amplifying the viral stock**

Sf21 cells were plated in a 6-well-plate at 2 x 10^6 cells/well and incubated at room temperature for 1 hour to allow attachment. To amplify the viral stock cells were infected at MOI (multiplicity of infection, representing the number of virus particles per cell) ranging from 0.05 to 0.1. The appropriate amount of P1 viral stock was added to each well and cells were incubated for 48 hours in a 27°C humidified incubator. 48 hours post-infection, 2 ml of medium containing virus from each well were collected and centrifuged at 500 x g for 5 minutes to remove cells and large debris. The supernatant representing the P2 viral stock was stored at 4°C, protected from light. The titer of the initial P1 viral stock obtained from transfecting Sf21 cells generally ranged from 1 x 10^6 to 1 x 10^7 plaque forming units (pfu)/ml. Amplification allowed production of a P2 viral stock with a titer ranging from 1 x 10^7 to 1 x 10^8 pfu/ml.

**Expression of recombinant proteins in Sf21 cells**

Once a baculoviral stock with a suitable titer (e.g. 1 x 10^8 pfu/ml) was generated, it was used to infect insect cells and to assay for expression of the recombinant proteins. Usually 2.5 x 10^8 cells were cultured in fresh growth media and subsequently infected with the baculoviral stock at different MOI. The cells were incubated at 27°C and harvested at different times post-infection (24, 48 or 72 hours). The cells were opened by sonication. The efficiency of lysis and the expression of recombinant proteins were verified by western-blot analysis.

2.3.8 **Expression of GAP1<sup>IP4BP</sup> in E. coli**

**Test expression**

A number of various conditions and their effects on expression and solubility of proteins were analyzed in a series of test expressions. Vectors containing the desired insert were transformed in E. coli BL21(DE3), BL21(DE3) Codon Plus-RIL and BL21(DE3) Rosetta. A 50 ml bacterial culture in TB medium was grown in a 37°C shaker at 160 rpm to an OD<sub>600</sub> of ~ 0.5. The proteins were expressed as fusion proteins upon overnight induction with 50 µM IPTG (Isopropyl-β-D-thiogalactoside) at 18°C. For the expression tests, 1 ml
of the overnight culture was pelleted, resuspended in water and the total protein fraction was analyzed using SDS-PAGE. Additionally, the soluble protein fraction was tested. For this purpose, 1 ml of each overnight bacterial culture was pelleted and resuspended in 1 ml lysis buffer. Cells were lysed by sonification (50% Duty cycle, 50% Output control) on ice using a Sonifier 450 (Branson Ultrasonics, Danbury, USA). After 30 minutes centrifugation at 4°C, 25000 x g, fractions of 10 µl aliquots of the supernatant and 5 µl of the pellet previously resuspended in water were analyzed together by SDS-PAGE.

In order to attain maximum solubility, the proteins were also expressed under varying temperature conditions (ranging from 16°C to 25°C), different IPTG concentrations (from 5 µM to 200 µM) and varying the time of induction from 4h to overnight.

**Parameters used for protein overexpression**

Based on the results of the expression tests the following parameters (described in Table 5) were used for proteins overexpression.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>E.coli strain</th>
<th>Medium</th>
<th>Antibiotics</th>
<th>Induction at OD₆₀₀</th>
<th>[IPTG] / µM</th>
<th>T/°C</th>
<th>Expression time / h</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX-4T-3 Igase</td>
<td>BL21(DE3) TB</td>
<td>Amp, Cam</td>
<td>0,2</td>
<td>50</td>
<td>18</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>GAP₁IP₄BPᵢ ₂₉₁₋₅₆₉</td>
<td>Rosetta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEX-4T-1</td>
<td>BL21(DE3) TB</td>
<td>Amp, Cam</td>
<td>0,8</td>
<td>50</td>
<td>18</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>GAP₁IP₄BPᵢ ₂₉₁₋₅₆₉</td>
<td>Rosetta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPROEX-HTc</td>
<td>BL21(DE3) TB</td>
<td>Amp, Cam</td>
<td>0,8</td>
<td>25</td>
<td>17</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>GAP₁IP₄BPᵢ ₂₉₁₋₅₆₉</td>
<td>Rosetta</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pPROEX-HTb</td>
<td>BL21(DE3) TB</td>
<td>Amp, Cam</td>
<td>0,8</td>
<td>25</td>
<td>17</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

*Table 5. Expression constructs and expression conditions.*

**2.3.9 Glutathione-Sepharose Affinity Chromatography**

The GST fusion proteins were purified using a Glutathione-Sepharose-Superflow affinity column (GE Healthcare, Freiburg).

Protein overexpression was induced as specified in Table 5. After overnight expression bacterial cells were pelleted at 4000 g, 15 min at 4°C and resuspended in lysis buffer (50 mM Hepes (pH 7.5), 5 mM DTE, 50 mM NaCl, 2.5 mM EDTA) supplied with 100 µM PMSF (phenylmethysulfonylfluoride) and Complete EDTA-free Protease Inhibitor
Cocktail tablets (Roche, Mannheim) (two tablets/100 ml buffer) as proteases inhibitors. The cells were opened by sonification (50% Duty cycle, 70-80% Output control) using a Branson Ultrasonic 450 Sonifier (Danbury, USA). The lysate was centrifuged (100000 g for 45 minutes at 4°C) and the resulting supernatant was applied to a 40 ml Glutathione-Sepharose column (column capacity 200-400 mg GST-fusion protein, 10 mg protein/1 ml column resin) pre-equilibrated with 50 mM Hepes (pH 7.5), 5 mM DTE, 50 mM NaCl. The column was then extensively washed (at least 10x column volumes) with the same buffer. The buffer was changed to 50 mM Hepes (pH 7.5), 5 mM DTE, 50 mM NaCl and 5 mM CaCl₂ to improve the cutting conditions for thrombin. The GST was cleaved overnight by the addition of 300-600 units of thrombin (Serva). The cut protein was eluted with 50 mM Hepes (pH 7.5), 5 mM DTE, 50 mM NaCl (1x column volume) and subsequently concentrated using an Amicon Ultra concentrator. The cut GST fragment was eluted with 30 mM Glutathione in 50 mM Hepes (pH 7.5), 5 mM DTE, 50 mM NaCl. The Glutathione-Sepharose column was regenerated with 6M guanidine hydrochloride (2x column volume) and afterwards extensively washed with water.

2.3.10 Ni-NTA Affinity Chromatography

The N-terminal hexa-histidine fusion proteins were purified using a Nickel-NTA-Superflow Agarose column (Qiagen, Hilden). In case of His-tagged fusion proteins, the bacterial cells were cultivated by fermentation in a 15 or 30 L fermenter. The protein overexpression was induced with 25 μM Isopropyl-β-D-thiogalactoside (IPTG) at 16-18°C at an OD₆₀₀ of 0.8 and cells were incubated overnight. After overnight expression, bacterial cells were pelleted at 4000 g, 15 min at 4°C, washed once with NaCl 0.9% and froze at -80°C until further purification procedures. The cells were thawed in a cool water bath and resuspended in the lysis buffer containing 25 mM Tris (pH 8.8), 5% Glycerol, 5 mM β-mercaptoethanol. To avoid proteases degradation of the overexpressed proteins, 100 μM PMSF and Complete EDTA-free Protease Inhibitor Cocktail tablets (Roche, Mannheim) were added (2 tablets/100ml buffer) as proteases inhibitors. Subsequently, DNase I was added 20 mg/ml (stock 20 g/l in 1 mM MgCl₂). The cells were lysed by sonification with a medium size tip (50% Duty cycle, 70-80% Output control) using a Branson Ultrasonic 450 Sonifier (Danbury, USA). The lysate was centrifuged (100000 g for 45 minutes at 4°C) and the resulting supernatant was used for further purification steps. To ensure the proper lysis of cells, the lysis buffer did not
contain any salt or imidazole. NaCl in a final concentration of 150 mM and Imidazole 10 mM were added to the soluble fractions only prior to applying on the Ni-NTA column. The cell supernatant was applied to a 50 ml Ni-NTA column, pre-equilibrated with 200 ml (4 x column volumes) Buffer A (25 mM Tris (pH 8.8), 5% Glycerol, 150 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol). The column was excessively washed with Buffer B (Buffer A + 100 mM KCl, 1 mM MgCl$_2$, 0.25 mM ATP). His-fusion proteins were eluted with an Imidazole gradient from 10 mM to 500 mM in Buffer A. The fractions containing the fusion protein were analyzed by SDS-PAGE. The fusion protein was concentrated using an Amicon Ultra-30 concentrator and immediately dialyzed or applied to a HiLoad Superdex 200 gel filtration column pre-equilibrated with 25 mM Tris (pH 8.8), 150 mM NaCl, 5 mM DTE in order to exchange the buffer and remove the excess of Imidazole. Alternatively the His tag can be cleaved directly on the Ni-NTA column or in solution using TEV protease (10 U/ml, provided by Carolin Koerner).

### 2.3.11 Size Exclusion Chromatography

Size exclusion chromatography (or gel filtration) separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. The medium is a porous matrix equilibrated with buffer that fills the pores and is referred as the stationary phase. Buffer (mobile phase) and sample move continuously through the column. Molecules that are larger than the pores of the matrix are unable to diffuse into the pores and pass through the column. Smaller molecules diffuse into the pores and are delayed in their passage down the column. Large molecules leave the column first followed by smaller molecules in order of their size. The entire separation process takes place as one total column volume of buffer passes through the gel filtration medium. Depending on the size of the pores the gel filtration columns exist in different sizes. The choice for one specific column depends on the molecular weight of the protein to be separated.

<table>
<thead>
<tr>
<th>Column material</th>
<th>Molecular mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiLoad Superdex 30 Prep Grade</td>
<td>&lt; 10 kDa</td>
</tr>
<tr>
<td>HiLoad Superdex 75 Prep Grade</td>
<td>3-70 kDa</td>
</tr>
<tr>
<td>HiLoad Superdex 200 Prep Grade</td>
<td>10-600 kDa</td>
</tr>
</tbody>
</table>

Table 6. Gel filtration columns and molecular mass of proteins that can be separated.
Materials and Methods

Molecules that do not enter the matrix are eluted in the exclusion volume as they pass directly through the column at the same speed as the flow of the buffer. The exclusion volume is equivalent to approximately 30% of the total volume of a specific column. Molecules with partial access to the pores of the matrix elute from the column in order of decreasing size. To achieve maximal resolution, the volume of the sample to be applied should not exceed 1-2% of the total volume of the column. The amount of protein that can be applied on the column and the flow rate depend on the specific size of each column.

<table>
<thead>
<tr>
<th>Column size</th>
<th>Column volume (ml)</th>
<th>Exclusion Volume (ml)</th>
<th>Amount of protein (mg protein)</th>
<th>Flow Rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiLoad 16/60</td>
<td>120</td>
<td>40</td>
<td>5-30</td>
<td>1</td>
</tr>
<tr>
<td>HiLoad 26/60</td>
<td>320</td>
<td>116</td>
<td>30-100</td>
<td>2</td>
</tr>
<tr>
<td>HiLoad 36/60</td>
<td>610</td>
<td>200</td>
<td>100-200</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 7. Chromatographic properties of used gel-filtration columns

After GSH-affinity chromatography or Ni-NTA-affinity chromatography the proteins were subjected to size exclusion chromatography in order to exchange the buffer and as a further purification procedure. Superdex 75 (in 50 mM Hepes (pH 7.5), 5 mM DTE, 50 mM NaCl) was used for GAP1IP4BP291-569 and Superdex 200 (in 25 mM Tris (pH 8.8), 5 mM DTE, NaCl 150 mM) for GAP1IP4BP FL/WT and GAP1IP4BP291-834, size of the column depending on the amount on protein. After gel filtration proteins were analyzed by SDS-PAGE. Fractions containing the protein of interest were collected, concentrated by ultra-filtration in an Amicon Ultra concentrator and aliquots were subsequently flash frozen and stored at -80°C.

2.3.12 Analytical gel filtration

Analytical gel filtration can be used as a method to analyze protein-protein interactions as well as oligomerization. For analytical separations sample volumes between 0.5-1% of the total volume of the column can be used. The analytical gel filtration was performed using a Superdex 200 10/30 (column volume 24 ml; exclusion volume 8.5 ml; flow rate 0.5 ml/min). 1 mg protein in 50-200 μl buffer was applied on the column.
Materials and Methods

2.3.13 Rap1GAP purification

Rap1GAP75-415 was purified as GST-fusion according to Brinkmann et al. (2002) with a subsequent gel filtration. The first buffers contained ATP, potassium and magnesium ions to remove bound GroEL. Bacterial cells were broken in PBS, 5 mM MgCl₂, 5 mM DTE, 1 mM ATP, 100 μM PMSF. The cell supernatant was applied to a GST-column equilibrated with PBS, 5 mM MgCl₂, 5 mM DTE, 1 mM ATP and excessively washed with at least 500 ml of the same buffer. The buffer was exchanged to PBS, 5 mM DTE or 50 mM Hepes (pH 7.5), 100 NaCl, 2 mM CaCl₂, 5 mM DTE. The GST-tag was cleaved overnight by the addition of 300 units thrombin (Serva). The cut protein was eluted with PBS, 5 mM DTE or 50 mM Hepes (pH 7.5), 100 NaCl, 2 mM CaCl₂, 5 mM DTE. The protein was concentrated (30 mg/ml) using an Amicon Ultra-10 concentrator and further purified in a Sephadex 200 gel filtration column using 20 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM DTE as running buffer. From the gel filtration column, Rap1GAP eluted in two peaks, the first one containing bound chaperone and eluting in the exclusion volume, the second eluting as protein with an apparent molecular mass of 100 kDa and containing pure Rap1GAP. The protein from the second peak was pooled, concentrated and twice washed with 20 mM Hepes (pH 7.5), 5 mM DTE in an Amicon-Ultra concentrator to remove NaCl. The protein was finally concentrated and flash frozen.

2.3.14 Purification of H-Ras C’₁-166 and Rap1B C’₁-166

C-terminally truncated versions of H-Ras (H-Ras C’₁-166) and Rap1B (Rap1B C’₁-166) were purified according to (Tucker et al., 1986). Bacteria were broken in 32 mM Tris (pH 7.6), 100 μM PMSF, 2 mM EDTA.

The cell supernatant was applied on a Q-Sepharose column (anion exchanger) equilibrated with 32 mM Tris (pH 7.6), 5 mM MgCl₂, and 10 mM DTE. The column was excessively washed with the same buffer and the bound proteins were eluted using a salt gradient (0-300 mM NaCl) in 32 mM Tris (pH 7.6), 5 mM MgCl₂, and 10 mM DTE. Fractions containing H-Ras or Rap1B were identified using SDS-PAGE and pooled. Proteins were precipitated by slowly adding solid ammonium sulfate (3 M final concentration). Precipitated proteins were pelleted by centrifugation for 60 min at 16000 g and resuspended in 32 mM Tris (pH 7.6), 5 mM MgCl₂, 0.4 M NaCl, 200 μM GDP and 10 mM DTE. H-Ras or Rap1B were further purified on a Sephadex 75 gel filtration column equilibrated with 32 mM Tris (pH 7.6), 5 mM MgCl₂, 0.4 M NaCl, 200 μM GDP and 10
Materials and Methods

mM DTE. Fractions containing the protein of interest were pooled and concentrated using an Amicon concentrator (10 kD cutoff) and flash frozen. The point mutants Ras-Q61T and Rap1B-T61Q were prepared following the same purification protocol as previously described.

2.3.15 Nucleotide exchange

Small guanine nucleotide-binding proteins are isolated in their inactive, GDP-bound form. Nucleotide exchange was performed according to (Tucker et al., 1986). This method is based on the fact that the binding of guanine nucleotides to the small GNBPs is strongly dependent on Mg\(^{2+}\) ions. Addition of EDTA which binds Mg\(^{2+}\) ions leads to an increase in the dissociation rate (K\(_D\)) of the nucleotide by many hundred folds. As a result, the nucleotide can then be exchanged by an excess of freshly added nucleotide (John et al., 1988).

200 \(\mu\)M Rap1B in 50 mM Hepes (pH 7.6), 100 mM NaCl were incubated with 15 mM EDTA, 150 mM ammonium sulfate and 10 mM nucleotide (stock 100 mM nucleotide in 1 M Hepes, pH 7.6) for 60 min at room temperature or overnight at 4°C. The exchange reaction was stopped by adding 30 mM MgCl\(_2\). Non-bound nucleotide was removed by washing the protein several times in an Amicon concentrator (10 kDa cutoff) at 4°C. To confirm successful nucleotide exchange, the nucleotide concentration was determined (see 2.3.16) and compared to the protein concentration. The protein was flash frozen and stored at -80 °C.

2.3.16 Nucleotide detection using Reversed-phase HPLC

Reverse-phase HPLC was used to monitor the kinetics of the hydrolysis of GTP to GDP stimulated by GAP1\(^{IP4BP}\) \(_{F/WT}\) and different GAP1\(^{IP4BP}\) constructs.

This method was performed according to (Lenzen et al., 1995). The principle of reversed-phase HPLC is based on the interaction between a polar, mobile phase, with a nonpolar, stationary phase. The sample was applied on a HPLC system Gold 166 (Bechman, Palo Alto, USA) and separated via a reversed-phase Octadecyl-SiO\(_2\) (ODS) Hypersil C18 column (Biscoff, Leonberg). The running buffer contained 10 mM tetrabutylammoniumbromide, 100 mM potassium phosphate (pH 6.5) and 7.5% acetonitrile.
Tetrabutylammoniumbromide (TBAB) binds to the negatively charged phosphate groups of the nucleotide and increases its hydrophobicity. The degree of hydrophobicity depends on the number of charged phosphates and therefore the number of bound TBAB molecules. Increased hydrophobicity leads to an increase in the retention time on the column. The elution of the bound complex is the result of the competition between the complex and a nonpolar organic solvent for the same binding sites. Therefore, the higher the hydrophobicity of the nucleotide-TBAB-complex, the longer its retention time on the column. The denaturing proteins resulting from the interaction with the running buffer were absorbed and separated over a nucleosil-100-C18 precolumn. Nucleotide peaks were detected by measuring adsorption at 254 nm and quantified by integration. The concentration and nature of the nucleotide was determined via the comparison of retention times and the area under the peak using standard nucleotide solutions as a reference.

Different constructs of GAP1IP4BP were tested for the GAP activity towards H-Ras or Rap1B using reversed-phase chromatography. Different concentrations of GAP1IP4BP were incubated at 25°C with H-Ras-GTP or Rap1B-GTP (100 µM) in 50 mM Hepes (pH 7.5), 50 mM NaCl, 5 mM MgCl₂ and 5 mM DTE. After 0, 5, 10, 15, 20 and 30 min aliquots were removed and analyzed by HPLC. As a control, aliquots of H-Ras-GTP and Rap1B-GTP (100 µM) without any addition of GAP were analyzed and preliminary hydrolysis rates could be estimated.

2.3.17 Radioactive charcoal assay

The kinetic characterization of the hydrolysis reaction stimulated by GAP1IP4BP was performed using the radioactive charcoal assay as described by (Leupold et al., 1983). Reaction samples containing \([γ-^{32}P]GTP\)-loaded Ras or Rap together with the GTP-ase activating protein (GAP1IP4BP) were incubated at 25°C. As a result of the hydrolysis reaction, free phosphate \((P_i)\) is produced. At different time points aliquots from the reaction mixture were removed and added to a charcoal solution which contains 20 mM phosphoric acid and 5% activated carbon. The charcoal solution immediately denatures and binds proteins as well as nucleotides of the reaction mixture, but it will not bind the free phosphate (resulted from the GAP mediated hydrolysis and from the intrinsic hydrolysis) as the charcoal solution is already saturated with phosphate. Upon removal of the activated carbon together with the bound proteins and nucleotides by centrifugation, the supernatant containing the radioactive free phosphate can be quantified by scintillation counting. To
determine only the initial rates of the reaction, the concentration of the enzyme (the GTPase activating protein) was chosen so that at 25°C after 8 minutes incubation not more than 30% of the substrate (Ras or Rap) was hydrolyzed. H-Ras and Rap1B were preloaded with GTP. The GTP was then partially exchanged with [γ-32P]GTP by incubating 1.5 mM H-Ras-GTP or Rap1B-GTP with 20 µCi [γ-32P]GTP in the presence of 12 mM EDTA for 30 minutes on ice. The nucleotide exchange reaction was stopped by adding 25 mM MgCl2.

The initial rates of the GAP-stimulated hydrolysis reaction were determined by using a fixed concentration of the enzyme (the GTPase activating protein) and increasing concentrations of the substrate (in this case, Ras/Rap-[γ-32P]GTP) at 25°C in standard buffer (50 mM Hepes (pH 7.6), 5 mM MgCl2, 50 mM NaCl and 3 mM DTE). At different time points 10 µl of the reaction mixture were removed, added to the 400 µl charcoal solution and vortexed. The end point of the GAP-mediated GTP hydrolysis (when all GTP has been hydrolyzed to GDP) was determined through the addition of highly concentrated GAP protein and incubation of the reaction for 20 minutes at 25°C. Upon centrifugation, 200 µl supernatant containing the free Pi were added to 3 ml scintillation cocktail and subjected to scintillation counting. Initial rates were evaluated by linear regression fitting, and K_M and k_cat were determined by fitting the initial rates to the Michaelis-Menten equation using the program Grafit5 (Erythacus software, Horley UK).

2.4 Biophysical Methods

Fluorescence spectroscopy
Fluorescence is one type of emission of light from an excited electronic state. A molecule absorbs light, which results in the excitation of the molecule from its normal, or ground state to a higher energy, or excited electronic state. Once in this state, the excited molecule can then rapidly lose some of its excited vibrational energy that is reemitted into the environment either as heat radiation or fluorescence. Upon emission of this energy the molecule returns to the ground vibrational state.

The intensity of fluorescence depends on the environment of the fluorescing molecule, the fluorophore. In a polar environment a large amount of energy is transferred to the surrounding solvent. This effect is known as fluorescence quenching. In a hydrophobic
environment the fluorescence quenching is strongly reduced, resulting in an increase in the fluorescence intensity.

The fluorescence spectroscopy takes advantage of the effect of the environment on the fluorescence intensity. The fluorophore is attached to the nucleotide (GDP or GTP). When the nucleotide binds to a protein (e.g. small G protein) the fluorescence quenching is reduced since the fluorophore is now in a hydrophobic environment, and so the fluorescence intensity is increased. If the nucleotide would be free in solution and unbound to the small G protein the fluorescence intensity would be low due to fluorescence quenching.

Protein-protein interactions can be monitored using environmentally sensitive fluorophores attached to positions where they experience environmental changes upon binding of an interaction partner (Kraemer et al., 2002). The presence of a fluorescence label, which can either be intrinsic (e.g., tryptophan residue) or it can be introduced into one of the components should not grossly disturb the biochemical properties of the proteins involved. The use of fluorescence also requires that the reporter group is sensitive enough to changes in the local environment to produce a detectable fluorescence change at reasonable protein concentrations.

Widely applicable is the introduction of fluorescently labeled methyl-anthraniloyl (mant) guanine nucleotides into GTP-binding proteins. In this study mant guanine nucleotides such as mGDP and the nonhydrolyzable GTP analog GppNHp (guanosine (5’-β, γ-imidotriphosphate) were used.

In direct fluorescence experiments, the change in fluorescence is due to a change in the environment of the fluorophore, while in fluorescence polarization the change in fluorescence is due to a change in the molecular mass.

Fluorescence polarization is a technique specially applied to study molecular interactions. This method is based on the fact that fluorescent molecules in solution when excited with plane-polarized light emit light back into a fixed plane (i.e. the light remains polarized) if the molecules remain stationary during the excitation of the fluorophore. If the molecule rotates out of this plane during the excited state, light is emitted in a different plane from the excitation light. Small molecules rotate quickly during the excited state and the emitted light is depolarized relative to the excitation plane. Therefore, upon emission, small molecules have low polarization values. Large molecules, caused by binding of a second molecule, rotate little during the excited state and the emitted light remains highly polarized, having therefore high polarization values.
In this study, fluorescence measurements were performed in a FluoroMax II spectrofluorimeter (S.A. Instruments, USA), at 20°C, in Tris 25 mM, (pH 8.8), NaCl 150 mM, DTE 3 mM and 5% Glycerol. Titration experiments were performed at fixed wave lengths (excitation 350 nm, emission 480 nm). A total of 2 µM mant nucleotide (GDP or GppNHz)-bound to Ras or Rap was titrated with increasing amounts of GAP1IP4BP. The increase in polarization upon addition of GAP1IP4BP was integrated over at least 15 min, corresponding to at least 30 measured values. The fluorescence change was fitted to Equation (1) yielding the KD of the interaction:

\[
\frac{F_{\text{min}} - (E + L + K) - \sqrt{(E + L + K)^2 - 4 \times E \times L \times (F_{\text{max}} - F_{\text{min}})}}{2 \times E} \quad \text{(Equation 1)}
\]

where \(F_{\text{min}}\), minimal fluorescence; \(F_{\text{max}}\), maximal fluorescence; \(E\), concentration of fluorophore, \(L\), concentration of ligand (GAP protein); \(K\), \(K_D\). Data analysis, fitting, and plotting were done with the Grafit 5.0 program (Erithracus software, Horley, UK).

Aluminium fluoride and beryllium fluoride solutions were freshly prepared from NaF (1 M) and AlCl3 or BeCl3 (0.1 mM) stock solutions, respectively.

### 2.5 Crystallization methods

The first step in determining the X-ray structure of a protein is obtaining crystals of sufficient size and quality. Crystal formation involves two separated phases: screening of crystallization conditions to obtain the first crystal and optimization of these conditions in order to improve the size and the quality of the crystal. For all crystallization trials GAP1IP4BP was used either freshly prepared or thawed after freezing and diluted to different concentrations in buffer containing 25 mM Tris (pH 8.8), 150 mM NaCl, 3 mM DTE.

A classical procedure for inducing proteins to separate from solution and produce a solid phase is to gradually increase the level of saturation by the addition of a precipitating reagent which can be a salt such as ammonium sulphate, an organic solvent such as ethanol or methylpentanediol (MPD), or a highly soluble synthetic polymer such as polyethylene glycol (PEG).

A widely used crystallization method is the vapor diffusion technique. Here a droplet containing the protein to crystallize diluted in the appropriate buffer and the crystallizing
reagent are equilibrated against a reservoir. During this equilibration process volatile species diffuse until the vapor pressure in the droplet equals the vapor pressure in the reservoir. As a result the concentration of the sample in the droplet increases leading eventually to the formation of a crystal.

Initial crystallization trials were performed using the sitting drop technique were a small droplet of the protein sample mixed with the crystallization reagent was placed on a platform in vapor equilibration with the crystallization reagent.

Crystallization was performed using the in-house high-throughput facility, a nano drop crystallization robot (Mosquito, TTPLabtech, UK) programmed to set up 100 nl-scale sitting drop crystallization experiments in 96-well plates (Linbro, Flow Laboratories Inc, USA) (containing 70 μl reservoir solutions that were manually pipeted). In each well 100 nl protein solution were pipeted together with 100 nl reservoir solution. An overview of the crystallization experiments is provided in Table 8.

The initial conditions that yielded crystalline forms using the nano drop robot were subsequently scaled up to microliter range. For the refinement screening the effect of precipitant, additives and pH was tested. Here the hanging drop vapor diffusion method was used. In this method a droplet containing the protein to crystallize diluted in the appropriate buffer and the crystallizing reagent (with or without additives) were mixed on a siliconized glass cover slide inverted over a reservoir. 1 ml of reservoir solution pre-equilibrated at the desired temperature was placed in 24-well cell culture linbro plates (Linbro, Flow Laboratoires Inc, USA). The hanging drop consisted of 1-2 μl protein solution and 1-2 μl reservoir solution. All crystallization trials were performed at 4°C and 20°C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conditions Screened</th>
<th>Source</th>
<th>T °C</th>
<th>Protein concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP1IP4BP</td>
<td>672</td>
<td>Hampton Research</td>
<td>20</td>
<td>6 mg/ml</td>
</tr>
<tr>
<td></td>
<td>1056</td>
<td>NeXtal Qiagen</td>
<td>4, 20</td>
<td>5, 8, 10, 15 mg/ml</td>
</tr>
<tr>
<td></td>
<td>291-834</td>
<td>Hampton Research</td>
<td>20</td>
<td>10 mg/ml</td>
</tr>
<tr>
<td>GAP1IP4BP</td>
<td>672</td>
<td>Hampton Research</td>
<td>20</td>
<td>5, 10, 15 mg/ml</td>
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<tr>
<td></td>
<td>1056</td>
<td>NeXtal Qiagen</td>
<td>4, 20</td>
<td>5, 10, 15 mg/ml</td>
</tr>
</tbody>
</table>

Table 8. Overview of the crystallization experiments.

GAP1IP4BP<sub>FL/WT</sub> yielded tiny needles appearing after 2 days at 20°C using Crystal Fast Screen from Hampton (Hampton Research, Laguna Hills, USA) condition 2 containing
30% PEG 4000, 0.1 M Na Citrate (pH 5.6), 0.2 M Ammonium Acetate. Crystallization trials were also performed using GAP1^{IP4BP}_{Fl/WT} together with Ras-GDP or Rap-GDP in the presence of AlF₃. For this purpose equal amounts of both proteins (GAP1^{IP4BP}_{Fl/WT} and Ras/Rap-GDP) were mixed along with 1 mM AlF₃ in buffer containing 25 mM Tris (pH 8.8), 150 mM NaCl and 3mM DTE. GAP1^{IP4BP}_{291-834} yielded crystals appearing after 2 days at 20°C using Crystal Fast Screen from Hampton (Hampton Research, Laguna Hills, USA) condition B12 containing 20% iso-Propanol, 0.1 M Na Acetate (pH 4.6), 0.2 M CaCl₂. However, these crystals did not diffract x-rays. A second condition resulting in spherulites was found using EasyXtal PACT Suite from NeXtal Qiagen condition D11 containing 0.2 M CaCl₂, 0.1 M Tris (pH 8), 20% PEG 6000. Screening different PEG 6000 concentrations, different CaCl₂ concentrations, different pHs and 192 different additives did not significantly improve the size and quality of these crystals.

2.6 Time resolved infrared spectroscopy

2.6.1 Basic principles of infrared spectroscopy

Information on protein-protein interactions can be obtained by comparing the absorbance spectra of individual proteins with the absorbance spectra of a protein-protein complex. Time-resolved Fourier-transform infrared (FTIR) difference spectroscopy can reveal exact molecular details of a protein-protein interaction over time (Gerwert, 1999). By analysis of difference spectra, one can select the absorbance bands of the protein groups involved in the protein-protein interactions against the background absorbance of the whole sample. The absorbance changes can be monitored with nanoseconds time resolution and followed for time periods ranging over nine orders of magnitude. This technique is suitable for many proteins, including membrane proteins with sizes up to 100000 daltons. It can also be used to examine the interactions of proteins with small ligands (Kotting and Gerwert, 2005b; Remy and Gerwert, 2003). Like any molecule, a protein can be visualized as a system of mass points (the atoms) connected by springs (the bonds). Such system can undergo distinct vibrations. An electromagnetic field can induce a vibration if the energy of the field coincides with the energy of the vibrational mode and if the dipole moment of the molecule changes during this vibration. These interactions result in absorption of distinct energies. Some functional groups of atoms always absorb at approximately the same energy. The stretching mode of the C=O in the peptide backbone (the amide I band)
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absorbs around 1650 cm\(^{-1}\) in an \(\alpha\)-helix. A change in environment will lead to a shift of this absorption. The same C=O stretching mode in a \(\beta\)-sheet will absorb at around 1630 cm\(^{-1}\).

Even a relatively small protein of 20 kDa (like Ras) contains about \(10^4\) vibrational modes. Thus the absorption spectrum alone does not give information on individual bands of the protein, but only on the global features of the protein. The spectrum is dominated by the amide I (C=O stretch) and amide II (NH bend coupled with C-N stretch) bands where every amino acid contributes. From these backbone absorptions information on the secondary structure can be obtained. The components of the amide I band (\(\alpha\)-helix, \(\beta\)-sheet, and turn/coil) can be fitted by separate curves, because different hydrogen bonding leads to shifts of the band. Water absorptions (O-H bend) are found in the same region as the amide I, and therefore this experiment should be performed with samples dissolved in D\(_2\)O, which absorbs at around 1250 cm\(^{-1}\) (Kotting and Gerwert, 2005b).

For an FTIR difference spectrum of a reaction A\(\rightarrow\)B, one calculates the absorbance spectrum of B minus the absorbance spectrum of A. Thus, the vibrations from groups that are not changed during the reaction annihilate each other, and only the changes during the reaction are seen. With such a subtraction process, individual absorptions can then be resolved (Kotting and Gerwert, 2005a).

### 2.6.2 Instrumentation

A typical setup for a time-resolved FTIR experiment consists of a light source, an interferometer, sample chamber, and a detector. The light source is a globar (SiC heated at 1500K). Infrared light from the globar passes an aperture (0.25-12 mm) before entering a Michelson interferometer. Subsequently the light passes the sample chamber, which is equipped with a thermostatic transmission cell in which the sample is placed. Finally, the infrared light reaches a liquid-nitrogen-cooled MCT (mercury-cadmium-telluride) detector. For reaction triggering, the sample in the cell can additionally be irradiated by a laser.

### 2.6.3 Sample cells

The most common cell is a simple transmission cell with IR-transparent windows (CaF\(_2\)). Due to the high absorptivity of water in the mid-infrared spectral region, meaningful spectra of hydrated proteins are obtained only by transmission measurements through very thin (2-10 \(\mu\)m) films. This involves placing a drop of a protein suspension or solution onto
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an IR-transparent window and then concentrating it under a nitrogen stream or under vacuum. A typical measurement requires about 100-200 µg of protein. The concentration of the protein in the film is 6-10 mM. The sample chamber is closed by a second IR window, which is separated from the first by a Mylar spacer (Kotting and Gerwert, 2005a).

2.6.4 Trigger techniques

The number of functional groups involved in the protein-protein interaction is small compared with the total number of groups in each protein. Thus the absorbance changes arising from an interaction are several orders of magnitude smaller than the background absorbance of the proteins. To avoid changing the background, the sample must be activated without removing it from the sample chamber. The FTIR difference technique requires a sharp initiation (triggering) of the protein reaction. This can be achieved by a laser flash, activating protein or setting free a caged compound, or it can be fast mixing of two different proteins.

In case of the GTPase reaction the triggering reaction is obtain by photolysis of caged GTP. Caged GTP is protected by the 1-(2-nitrophenyl) ethyl moiety that is frequently used to protect phosphate, nucleotides and nucleotide analogues. Application of UV flashes leads to photolysis followed by formation of GTP and the by-product 2-nitrosoacetophenone. A spectrum of caged GTP is measured prior to photolysis, as reference, and after photolysis. Further spectra are recorded and the absorbance difference spectra \( \Delta A \) are calculated. Only those vibrational modes that have undergone reaction-induced absorbance changes give rise to bands in the difference spectra. Negative bands in the difference spectra are due to the caged GTP, whereas positive bands are due to the photolysis products (Kotting and Gerwert, 2005a).

2.6.5 The rapid scan mode

All spectra recorded were collected in the rapid scan mode. The principle of rapid scan FTIR mode of time-resolved spectroscopy is as follows: after taking a reference spectrum of the protein in its ground state, the protein is activated (e.g. by a laser flash) and interferograms are continuously recorded in much shorter time than the half lives of the reactions (Gerwert et al., 1990).
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Figure 23. Photolysis of caged [1-(2-nitrophenyl) ethyl] GTP (a). Time course of a rapid scan FTIR experiment investigating the interaction of two proteins (colored in red and blue) (b). Time course of the data acquisition (c). First, reference spectra (R) are taken. The laser flash initiates the reaction. During the reaction, via intermediate C toward product D, interferograms are continuously recorded. After kinetic analysis, e.g., by global fit, amplitude spectra are obtained, showing the bands of the groups involved in the interaction (d) (Kotting and Gerwert, 2005a).

In the ground state (A) the interaction is hindered by a cage. A laser flash removes the cage and activates the protein. The activated protein (B) leads to the formation of an intermediate (C), and to a final protein-protein complex (D). During this reaction interferograms are recorded continuously. The first 4 reference (R) interferograms represent the ground state (A) while the following are taken during the reaction (B to C to D). Thus, the first interferogram will mainly represent the spectrum of B and the following interferograms the ratio of C and subsequently D and will increase according to the kinetics of the observed reaction. In a kinetic analysis amplitude spectra for the individual conversions can be calculated (Kotting and Gerwert, 2005a).

2.6.6 Experimental setups
The experimental procedures were performed as previously described for the Ras-RasGAP and Rap-RapGAP systems (Allin and Gerwert, 2001; Chakrabarti et al., 2004).

Sample composition: Protein solutions were concentrated to a final concentration of 6-10 mM on 20x2 mm CaF₂ or BaF₂ windows under nitrogen stream along with the selected
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buffer system. Typical buffer composition is: 200 mM Buffer (HEPES for pH 7.6, MES for pH 6.0), 20 mM DTE/DTT, 20 mM MgCl₂. GAP catalyzed reactions were performed at 260, 270 or 280 K, along with 12% ethylene glycol to avoid freezing.

**Instruments**: IFS 66v/s FTIR spectrometer (Bruker Optics, Karlsruhe, Germany) with KBr beamsplitter, MCT detector KMPV11 – 1 – J1 (Kolmar, Newburyport, MA, USA), Excimer Laser LPX 240i (Lambda Physik, Göttingen, Germany) with XeCl (308 nm), CaF₂ / BaF₂ windows (Korth GmbH, Kiel, Germany), Mylar film (DuPont, Circleville, OH, USA).

Briefly, 1 µl of the sample solution was placed between two CaF₂ windows. The windows were fixed by a metal cuvette in the thermostated sample holder of the spectrometer. The sample solutions for the assessment of the photolysis reaction contained 10 mM Ras (or Rap) caged nucleotide complex, 20 mM MgCl₂, 20 mM DTE/DTT, 200 mM MES (pH 6.0), and 12 vol % ethylene glycol. The sample solutions for the assessment of the GTPase reaction contained 10 mM Ras (or Rap) caged GTP complex, 0.1 mM GAP1IP4BP, 20 mM MgCl₂, 20 mM DTE/DTT, 200 mM MES (pH 6.0) or HEPES (pH 7.0), and 12 vol % ethylene glycol. Dithiothreitol was used to scavenge the reactive photolysis byproduct 2-nitrosoacetophenone.

The FTIR measurements were performed on modified IFS 66v and IFS 66v/S spectrometers (Bruker, Karlsruhe, Germany) equipped with a mercury cadmium telluride (MCT) detector using the fast scan technique. The OPUS software (Bruker) was used for data acquisition. The spectra were measured between 2000 and 950 cm⁻¹ and analyzed by a global fit algorithm with one exponential term.

The global fit analysis provides amplitude spectra \( a_i(\mathbf{S}) \) and the rate constants \( k_i \). The photolysis of caged GTP was performed at 308 nm using an LPX 240 XeCl excimer laser (Lambda Physics, Göttingen, Germany). The laser energy was between 90 and 120 mJ per flash with a pulse duration of ~20 ns. The conversion of caged GTP was checked by HPLC analysis with 50 mM potassium phosphate (pH 6.5), 5 mM tetrabutylammonium bromide, and 20% acetonitrile. For the investigation of the photochemical release of GTP, 30 flashes were applied to achieve 70-80% conversion of caged GTP to GTP in 90 ms.

The photolysis measurements with the Ras or Rap caged GTP complex were performed at 260 K to inhibit the hydrolysis reaction.

For the investigation of the GTPase reaction, 60 flashes were applied to achieve complete conversion of caged GTP to GTP. The measurements of the GTPase reaction were
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performed at 260, 270 or 280 K. The hydrolysis difference spectra were calculated from the 400 averaged scans between 1 and 8 s after photolysis, representing the Ras or Rap-GTP state, and from the 800 averaged scans recorded between 48 and 62 s after photolysis, representing the Ras or Rap-GDP state.
3 Results

3.1 Overexpression and purification of GAP1IP4BP

In order to characterize the mechanism of the GAP-mediated GTP hydrolysis reaction of GAP1IP4BP, to define its biochemical specificity towards both small GTP-binding proteins Ras and Rap, and to identify the domains and the catalytic residues that are responsible for such a dual activity several GAP1IP4BP constructs comprising different domains of the molecule were designed based on secondary structure predictions. To achieve optimal protein expression the designed GAP1IP4BP constructs were overexpressed using an insect cells expression system and subsequently an *E. coli* expression system.

3.1.1 Overexpression and purification of GAP1IP4BP using the baculovirus system in insect cells

Initially GAP1IP4BP was expressed in *Spodoptera frugiperda* (SF21) insect cells using the baculovirus system for heterologous gene expression. This system has several advantages as it permits folding, post-translational modification and oligomerization of proteins in manners that are often identical to those that occur in mammalian cells.

Several constructs of GAP1IP4BP were used for testing the expression efficiency and solubility. Initially, a recombinant virus containing the expression cassette encoding for GAP1IP4BP was generated. The virus was amplified in SF21 cells and the expression of the desired proteins was analyzed by western-blotting (chapter 2.3.5). All constructs tested yielded to only a low amount of overexpressed protein (Table 9).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector</th>
<th>tag</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP1IP4BP&lt;sub&gt;FL/WT&lt;/sub&gt;</td>
<td>pFastBac A</td>
<td>N-6xHis</td>
<td>Low expression level, low binding to Ni-NTA</td>
</tr>
<tr>
<td>GAP1IP4BP&lt;sub&gt;1-717&lt;/sub&gt;</td>
<td>pFastBac A</td>
<td>N-6xHis</td>
<td>Low expression level</td>
</tr>
<tr>
<td>GAP1IP4BP&lt;sub&gt;1-569&lt;/sub&gt;</td>
<td>pFastBac A</td>
<td>N-6xHis</td>
<td>Low expression level</td>
</tr>
<tr>
<td>GAP1IP4BP&lt;sub&gt;291-834&lt;/sub&gt;</td>
<td>pFastBac A</td>
<td>N-6xHis</td>
<td>Low expression level</td>
</tr>
<tr>
<td>GAP1IP4BP&lt;sub&gt;274-569&lt;/sub&gt;</td>
<td>pFastBac A</td>
<td>N-6xHis</td>
<td>Low expression level</td>
</tr>
</tbody>
</table>

Table 9. Constructs of GAP1IP4BP recombinantly expressed in SF21 cells using the baculovirus system
However, several attempts to obtain pure and soluble protein were made using recombinant GAP1\textsuperscript{IP4BP\textsubscript{Fl/WT}} expressed as N-terminal 6xHis-fusion protein. For this purpose, 1 l SF21 cells expressing GAP1\textsuperscript{IP4BP\textsubscript{Fl/WT}} were harvested after 3 days cultivation, lysed by sonication and the soluble fraction was subsequently applied on a Ni-NTA column. GAP1\textsuperscript{IP4BP\textsubscript{Fl/WT}} was purified according to standard purification procedures for His-fusion proteins as described under Materials and Methods (chapter 2.3.10). Since this approach led to only a small amount of protein no further size exclusion chromatography could be applied.

Despite the advantages that the baculovirus expression system offers it did not prove to be efficient for GAP1\textsuperscript{IP4BP} expression and therefore no other attempts to overcome these expression problems were carried out.

3.1.2 Cloning, overexpression and purification of GAP1\textsuperscript{IP4BP} from E. coli

As the first attempts to express and purify a stable GAP1\textsuperscript{IP4BP} fragment in insect cells turned out to be unsuccessful, the expression of GAP1\textsuperscript{IP4BP} was subsequently carried out using a bacterial expression system. Different GAP1\textsuperscript{IP4BP} constructs comprising different domains of the molecule were designed based on secondary structure predictions. The constructs were cloned into different expression vectors and expressed as either GST- or His- fusion proteins. The expression of the desired proteins was analyzed by SDS-PAGE or western-blotting using anti-GST or anti-His specific antibodies as described under Material and Methods. An overview of the GAP1\textsuperscript{IP4BP} constructs used in the present study is shown in Table 10.
### Table 10. Overview of the constructs used in the present study. Domains architecture of GAP1IP4BP. C2A and C2B (Ca$^{2+}$-dependent/independent phospholipids binding); GAP (GTPase activating protein domain); N-6xHis (6x Histidine tag); N-GST (N-terminal GST tag).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector</th>
<th>tag</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP1IP4BP 274-569</td>
<td>pGEX-6P-2</td>
<td>N-GST</td>
<td>Expressed, protein soluble</td>
</tr>
<tr>
<td>GAP1IP4BP 291-834</td>
<td>pGEX-6P-2</td>
<td>N-GST</td>
<td>Expressed, protein degraded upon purification</td>
</tr>
<tr>
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<td>N-GST</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 291-834</td>
<td>pPROEX b</td>
<td>N-6xHis</td>
<td>Expressed, protein soluble, purified in high amounts</td>
</tr>
<tr>
<td>GAP1IP4BP 274-717</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein partly soluble, purified in low amounts</td>
</tr>
<tr>
<td>GAP1IP4BP 1-717</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein soluble, low binding to Ni-NTA column</td>
</tr>
<tr>
<td>GAP1IP4BP 1-569</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 1-802</td>
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<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
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<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 1-750</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 100-750</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 139-834</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 139-802</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 139-770</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 139-750</td>
<td>pPROEX c</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 100-617</td>
<td>pPROEX b</td>
<td>N-6xHis</td>
<td>Expressed, protein partly soluble, purified in low amounts</td>
</tr>
<tr>
<td>GAP1IP4BP 141-617</td>
<td>pPROEX b</td>
<td>N-6xHis</td>
<td>Expressed, protein insoluble</td>
</tr>
<tr>
<td>GAP1IP4BP 158-617</td>
<td>pPROEX b</td>
<td>N-6xHis</td>
<td>Expressed, protein partly soluble, purified in low amounts</td>
</tr>
<tr>
<td>GAP1IP4BP 244-617</td>
<td>pPROEX b</td>
<td>N-6xHis</td>
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<tr>
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<td>N-10xHis</td>
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<td>GAP1IP4BP 291-569</td>
<td>pGEX-4T-3 Igase</td>
<td>N-GST</td>
<td><strong>Expressed, protein soluble, purified in high amounts</strong></td>
</tr>
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<td>pGEX-4T-3 Igase</td>
<td>N-GST</td>
<td>Not tested</td>
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<td>N-GST</td>
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</tr>
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<td>N-GST</td>
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<tr>
<td>GAP1IP4BP 1-712</td>
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<td>N-GST</td>
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</tr>
<tr>
<td>GAP1IP4BP 118-619</td>
<td>pGEX-4T-1</td>
<td>N-GST</td>
<td>Expressed, protein partly soluble, purified in low amounts</td>
</tr>
<tr>
<td>GAP1IP4BP 118-834</td>
<td>pGEX-4T-1</td>
<td>N-GST</td>
<td>Expressed, protein partly soluble, purified in low amounts</td>
</tr>
<tr>
<td>GAP1IP4BP FLR317A</td>
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<td>N-GST</td>
<td>Expressed, protein partly soluble, purified in low amounts</td>
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<td>H-Ras C1-166 Q61T</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rap1B C1-166 T61Q</td>
<td></td>
<td></td>
<td>Expressed, protein soluble, purified in high amounts</td>
</tr>
</tbody>
</table>
Results

PH (pleckstrin homology domain); Btk (Bruton’s tyrosine kinase domain). The indicated GAP1\textsuperscript{IP4BP} constructs were recombinantly produced in \textit{E. coli}.

3.1.2.1 Purification of GAP1\textsuperscript{IP4BP}{\textsubscript{291-569}} (the minimal catalytic RasGAP domain)

As previously mentioned, GAP1\textsuperscript{IP4BP} contains a central highly conserved GAP domain sharing sequence homology with other RasGAPs but none with RapGAPs. Based on sequence homology with the GAP domain constructs of GAP334 and NF1-333 from p120\textsuperscript{GAP} and Neurofibromin, respectively, the GAP domain of GAP1\textsuperscript{IP4BP} was defined as comprising residues 291 to 569. To characterize its Ras and RapGAP mediated reactions, the minimal RasGAP catalytic domain of GAP1\textsuperscript{IP4BP} (GAP1\textsuperscript{IP4BP}{\textsubscript{291-569}}) was cloned into pGEX-4T-3 Igase vector, expressed in \textit{E. coli} as GST-fusion protein and purified via affinity chromatography. The expression and purification conditions were previously established by C. L. Polte.

Initially GAP1\textsuperscript{IP4BP}{\textsubscript{291-569}} was purified over a glutathione sepharose 4-B column. The GST tag was cleaved on the column with thrombin and the protein was subsequently subjected to size exclusion chromatography. GAP1\textsuperscript{IP4BP}{\textsubscript{291-569}}, with a molecular weight of 30 kDa, eluted as a monomer from the gel filtration column (Figure 24). In a preliminary GAP activity test, GAP1\textsuperscript{IP4BP}{\textsubscript{291-569}} showed considerable RasGAP activity but no RapGAP activity could be detected under standard conditions using reverse phase HPLC (chapter 2.3.16).

![Figure 24. Purified GAP1\textsuperscript{IP4BP}{\textsubscript{291-569}}. GAP1\textsuperscript{IP4BP}{\textsubscript{291-569}} was expressed in bacteria as GST fusion protein and purified via affinity chromatography followed by size exclusion chromatography. 1) Marker; 2) Purified protein as eluted from the gel filtration column.](image-url)
3.1.2.2 Purification of GAP1\textsuperscript{IP4BP}_{Fl/WT}

**GST-GAP1\textsuperscript{IP4BP}**

GAP1\textsuperscript{IP4BP}_{Fl/WT} comprises 834 amino acids with a molecular mass of 96 kDa. Initially, the purification procedures were performed using GST fusion GAP1\textsuperscript{IP4BP}_{Fl/WT}. GST full-length wild-type GAP1\textsuperscript{IP4BP} purified according to standard purification procedures for GST-fusion proteins (chapter 2.3.9) led to only a small amount of impure protein (data not shown). After applying the soluble extract over a Glutathione Sepharose 4-B column, the column was extensively washed with buffer containing high salt concentration, ATP, KCl and MgCl\textsubscript{2} to remove bound chaperones and degradation products. For stability reasons, full-length wild type GAP1\textsuperscript{IP4BP} was purified as GST fusion protein (GST-GAP1\textsuperscript{IP4BP}), while the mutant full-length GST-GAP1\textsuperscript{IP4BP} R371A was cleaved from GST. After elution from the GSH column, the proteins were concentrated and snap frozen in liquid nitrogen and stored at -80°C. Since very low amount of protein could be obtained after the first purification step no further size exclusion chromatography could be performed. GST-tagged full-length protein could not be fully purified and contained additional lower molecular mass bands, most probably due to the presence of chaperones or due to proteolytic degradation.

However, in a preliminary GAP assay preformed using reverse phase HPLC, the GST-fusion GAP1\textsuperscript{IP4BP}_{Fl/WT} displayed dual specific Ras and RapGAP activity as it has previously been shown (Cullen et al., 1995c).

**6xHis-tagged GAP1\textsuperscript{IP4BP}**

Subsequently, recombinant GAP1\textsuperscript{IP4BP}_{Fl/WT} was expressed as N-terminal 6xHis-fusion protein. Two *E. coli* cell strains were used as hosts for the expression of 6xHis-tagged GAP1\textsuperscript{IP4BP}_{Fl/WT}: BL21(DE3) Rosetta and BL21(DE3) Codon Plus-RIL. In order to identify the best protein-overexpressing bacterial strain, similar expression and purification procedures for both expression strains were carried out. Both bacterial strains led to the expression of GAP1\textsuperscript{IP4BP}_{Fl/WT}, however BL21(DE3) Codon Plus-RIL yielded a higher amount of purified protein.

To obtain a larger amount of pure, soluble protein, transfected bacterial cells were cultivated in a 30 l fermentor, the protein expression was induced with 25 µM Isopropyl-β-D-thiogalactoside (IPTG) at 16-18°C at an OD\textsubscript{600} of 0.8, and the cells were incubated
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overnight. The low IPTG concentration was necessary, as induction with higher concentrations of IPTG led to a higher fraction of insoluble protein. Cells were harvested, washed once in 0.9% NaCl and frozen at -80°C until further purified. The cells were lysed by sonification while constantly kept on ice. To ensure the proper lysis of the cells, the lysis buffer did not contain any salt or imidazole. NaCl in a final concentration of 100 mM and 10 mM Imidazole were added to the soluble fractions only prior to applying the supernatant on the Ni-NTA column. The recombinant protein was purified by Ni-NTA affinity chromatography (Figure 25) with an extensive washing step in buffer containing ATP, KCl and MgCl₂, followed by elution of the bound His-fusion protein with an imidazole gradient from 10 to 500 mM as described in Materials and Methods (chapter 2.3.10).

Figure 25. FPLC purification of GAP1\textsuperscript{IP4BP\_F\_WT} on Ni-NTA superflow. 6xHis-tagged GAP1\textsuperscript{IP4BP\_F\_WT} (~96 kDa) was expressed in \textit{E. coli} and purified via affinity chromatography. Coomassie-stained SDS gels. T: total cell lyzate; S: soluble fraction; FT: flow-through; W: washing fraction; 20-56: fractions eluted with the imidazole gradient.

In order to separate aggregated proteins and to remove the high concentration of imidazole from the protein sample, the eluted protein was further purified by size exclusion chromatography. The rapid removal of imidazole from the protein solution was required as high imidazole concentration affected protein stability and solubility. 6xHis-tagged GAP1\textsuperscript{IP4BP\_F\_WT} was applied on a gel-filtration column S200 16/60 or 26/60 depending on the amount of protein obtained in the first purification step. GAP1\textsuperscript{IP4BP\_F\_WT} eluted as a monomer with an apparent molecular mass of 100 kDa. The elution profile, illustrated in
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Figure 26 showed that the protein eluted in one peak with a shoulder on the right side. To ensure homogeneity of the protein sample, only fractions corresponding to the central part of the peak were collected, concentrated by ultrafiltration, flash frozen in liquid nitrogen and stored at -80°C. The protein yield was ~15 mg/10 l culture. The protein could be concentrated up to 90 mg/ml without any precipitation.

![Figure 26. Purification of GAP1IP4BP<sub>Fl/WT</sub>. SDS-PAGE Coomassie stained gel showing GAP1IP4BP<sub>Fl/WT</sub> fractions as eluted from the gel filtration column (A); Chromatogram representing the elution profile of GAP1IP4BP<sub>Fl/WT</sub> on a HighLoad 26/60 Superdex S200 Prep Grade column (B). Western-blot analysis with an anti-His antibody (C).](image)

3.1.2.3 Degradation and partial digest

GAP1IP4BP<sub>Fl/WT</sub> was more than 90% pure after size exclusion chromatography as shown by SDS-PAGE analysis (Figure 26). However, a western-blot analysis performed with an anti-His antibody showed that GAP1IP4BP<sub>Fl/WT</sub> contained several smaller degradation bands that could not be removed after purification. This implies that GAP1IP4BP<sub>Fl/WT</sub> contains flexible loops which are accessible for bacterial proteases. In order to obtain a stable GAP1IP4BP fragment, several proteases were tested in a partial digestion experiment as described in Materials and Methods (chapter 2.3.6). Chymotrypsin, aminopeptidase and carboxypeptidase did not lead to any specific degradation bands. Trypsin and elastase cleaved the full-length GAP1IP4BP fragment into several small peptide fragments (Figure 27). Due to their close molecular mass the isolation of these peptides fragments from the SDS gel proved to be difficult, and no further analyses for mass and sequence determination could be performed.
Figure 27. Partial digestion of GAP1\textsuperscript{IP4BP\textsubscript{Fl/WT}} with trypsin (A), elastase (B), chymotrypsin (C), aminopeptidase (D), carboxypeptidase (E). The digestion reaction was started with the addition of the specific protease at point 0. After the indicated time points aliquots were removed and analyzed by SDS-PAGE.

3.1.2.4 6xHis-tagged GAP1\textsuperscript{IP4BP\textsubscript{291-834}}

In order to obtain a stable fragment for crystallization and for further activity tests, several constructs of GAP1\textsuperscript{IP4BP} were tested for optimal expression and stability (Table 10). Among them, GAP1\textsuperscript{IP4BP\textsubscript{291-834}} turned out to be a soluble and stable fragment that could be purified in considerable amounts.

The protein was expressed in BL21(DE3) Rosetta cells. Transfected cells were cultivated by fermentation using the same parameters as for GAP1\textsuperscript{IP4BP\textsubscript{Fl/WT}}. The protein was purified via Ni-NTA column following the protocol previously described for full-length wild-type GAP1\textsuperscript{IP4BP}. 6His-tagged GAP1\textsuperscript{IP4BP\textsubscript{291-834}} was subsequently further analyzed by size exclusion chromatography. As shown in the elution profile illustrated in Figure 28 GAP1\textsuperscript{IP4BP\textsubscript{291-834}} eluted from the gel filtration column in two adjacent peaks that were analyzed by SDS-PAGE. Fractions corresponding to each peak were collected independently and subsequently subjected to analytical gel filtration. The first, smaller, peak contains a mixture of tetramer (with a molecular mass of ~230 kDa) and monomer, while the second, larger peak corresponds to a monomer with the expected molecular mass (~66 kDa).
3.2 The hydrolysis mechanism of GAP1\textsuperscript{IP4BP}

In order to characterize the enzymatic activity of GAP1\textsuperscript{IP4BP} and to explore the dual GAP mechanism towards both small GTP-binding proteins Ras and Rap, the GAP1\textsuperscript{IP4BP} constructs GAP1\textsuperscript{IP4BP}\textsubscript{291-569}, GAP1\textsuperscript{IP4BP}\textsubscript{291-834}, and GAP1\textsuperscript{IP4BP}\textsubscript{F1/WT} were tested for their GAP activity and their specificity towards Ras and Rap. A detailed kinetic analysis was performed using a radioactive charcoal assay as described in Materials and Methods (chapter 2.3.17). Here, the GAP activity was monitored by following the production of free phosphate (P\textsubscript{i}) as a result of the intrinsic and GAP stimulated GTP hydrolysis reaction. On the basis of this experiment Michaelis-Menten parameters of the GAP1\textsuperscript{IP4BP}-mediated reaction were obtained.

3.2.1 Characterization of GAP1\textsuperscript{IP4BP}\textsubscript{291-569}

The isolated GAP domain of GAP1\textsuperscript{IP4BP}, residues 291-569, was initially tested for its Ras and Rap GAP activity. Michaelis-Menten kinetics of the GAP1\textsuperscript{IP4BP}\textsubscript{291-569}-mediated reactions were determined using the radioactive charcoal assay by incubating increasing concentrations of the small GTP-binding proteins H-Ras and Rap1B (10-800 µM) with constant concentration of GAP1\textsuperscript{IP4BP}\textsubscript{291-569} (100 nM GAP1\textsuperscript{IP4BP}\textsubscript{291-569} for H-Ras and 20 µM GAP1\textsuperscript{IP4BP}\textsubscript{291-569} for Rap1B).
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GAP1^{IP4BP}_{291-569} showed a pronounced RasGAP activity, which in contrast to those of other RasGAPs could not be saturated under the conditions used and therefore the $k_{\text{cat}}$ of the reaction could not be determined. At the highest concentration of Ras-GTP tested (800 $\mu$M), the $k_{\text{obs}}$ of the reaction reached a value of 8 s\(^{-1}\) (Figure 29), which is in the range observed for other RasGAPs (Ahmadian et al., 1997a).

![Diagram](image)

**Figure 29.** Schematic representation of the GAP1^{IP4BP}_{H/WT} and GAP1^{IP4BP}_{291-569} domains architecture (A); Michaelis-Menten kinetics of the GAP1^{IP4BP}_{291-569}–mediated GTP hydrolysis of H-Ras and Rap1B (B, C). 100 nM GAP1^{IP4BP}_{291-569} were incubated in standard buffer at 25°C with increasing concentrations of H-Ras [$\gamma$-32P]GTP or Rap1B [$\gamma$-32P]GTP. The GTPase activity was monitored by measuring Pi release, and data were evaluated as described in chapter 2.3.17. Data are plotted as rates (B) or rate constants (C).

Under the same conditions as for the Ras reaction, no stimulation of the Rap GTPase reaction was observed (Figure 29). However, by increasing the concentration of the GAP domain to 20 $\mu$M, RapGAP activity was detected (Figure 30), and reached a $k_{\text{obs}}$ of only
Results

0.02 s\(^{-1}\) at the highest Rap-GTP concentration tested, indicating that the apparent second order rate constant \(k_{\text{cat}}/K_M\) is at least 2 orders of magnitude lower for the Rap versus the Ras GTPase activation.

Figure 30. Michaelis-Menten kinetics of the GAP1\(^{IP4BP}\)\(_{291-569}\)-mediated GTP hydrolysis of Rap1. 20 µM GAP1\(^{IP4BP}\)\(_{291-569}\) were used to assay GTP hydrolysis with increasing concentrations of Rap1B \([\gamma^{32}\text{P}]\)GTP. The GTPase activity was monitored by measuring P\(_i\) release, and data were evaluated as described in chapter 2.3.17. Data are plotted as rates (A) or rate constants (B).

In summary, the GAP1\(^{IP4BP}\) fragment comprising residues 291 to 569 showed a pronounced RasGAP activity, while no significant RapGAP activity could be detected. Therefore it was assumed that other regions of the molecule such as the N-terminal C2 domains and/or the C-terminal region including the PH/Btk domain are required for an efficient RapGAP activity.

3.2.2 Characterization of GAP1\(^{IP4BP}\)\(_{FL/WT}\)

Since efficient RapGAP activity has been previously reported (Cullen et al., 1995c) and the RasGAP activity is apparently similar to that observed for other RasGAPs, it became obvious that GAP1\(^{IP4BP}\) requires additional domains for an efficient RapGAP activity. When full-length GST-GAP1\(^{IP4BP}\) was used an efficient Ras and RapGAP activity could be observed (Figure 31).
Since the His-tagged GAP1\textsuperscript{IP4BP} proved not to be suitable for the radioactive charcoal assay as the reaction did not reach saturation under the condition used, GST-GAP1\textsuperscript{IP4BP}, enriched via GSH-Sepharose, was used. The kinetic characterization of the hydrolysis reaction was performed using the radioactive charcoal assay.

As previously mentioned, GST-tagged GAP1\textsuperscript{IP4BP\_F/WT} could not be fully purified and contained additional lower molecular mass bands, probably due to the presence of chaperones or derived from proteolytic degradation. Consequently, the concentration of GAP1\textsuperscript{IP4BP} could only be estimated and therefore not all Michaelis-Menten parameters (k\textsubscript{cat} and k\textsubscript{M}) could be determined. The initial rates for both Ras and Rap GAP1\textsuperscript{IP4BP\_stimulated reactions were determined using a constant concentration of GAP1\textsuperscript{IP4BP\_F/WT} of approximately 5 nM and increasing concentrations of GTP-loaded H-Ras or Rap1B. In the case of GAP1\textsuperscript{IP4BP\_F/WT} both reactions displayed a typical saturatable Michaelis-Menten behavior.

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**Figure 31.** Domain architecture of GAP1\textsuperscript{IP4BP\_F/WT} (A); Michaelis–Menten kinetics of the GAP1\textsuperscript{IP4BP\_F/WT}-stimulated reaction on H-Ras (B) and Rap1B (C). Initial rates were obtained by using a constant concentration of 5 nM GAP1\textsuperscript{IP4BP\_F/WT} and increasing concentrations of the GTP-bound H-Ras and Rap1B.
GTP-bound Rap1B. Reactions rates $v$ [µmoles/sec] were plotted against different H-Ras (B) and Rap1B concentrations (in µM) (C).

With 5 nM GAP1IP4BPFl/WT similar maximal rates of 0.24 µmoles/sec for Ras and only a three fold lower rate of 0.08 µmoles/sec for Rap could be observed. Although, these rates could not be directly converted to $k_{cat}$ values due to the degradation products present in the preparation of full-length protein, $k_{cat}$ was estimated to be 48 sec$^{-1}$ for Ras and 16 sec$^{-1}$ for Rap indicating a very efficient catalysis for both GTPase reactions (Figure 32). Furthermore, full-length protein had a five-fold lower $K_M$ for Rap (42 µM) versus Ras (213 µM).

$$k_{cat} = 48 \text{ sec}^{-1}$$
$$K_M = 213 \mu\text{M}$$

$$k_{cat} = 16 \text{ sec}^{-1}$$
$$K_M = 42 \mu\text{M}$$

Figure 32. Michaelis–Menten kinetics of the GAP1IP4BPFl/WT-stimulated reaction. Estimated $k_{obs}$[sec$^{-1}$] were plotted against different H-Ras and Rap1B concentrations.

These results indicate that the additional domains not only affect the catalytic efficiency of the RapGAP reaction but also dramatically enhance the affinity towards Rap-GTP. Thus, whilst the isolated RasGAP domain of GAP1IP4BP is, like other RasGAPs, sufficient to enhance the GTPase activity of Ras, other regions of the molecule such as the N-terminal C2 domains and/or the C-terminal region including the PH/Btk domains, are required for the RapGAP activity.

### 3.2.3 Characterization of GAP1IP4BP$_{291-834}$

To extend the characterization of the Ras and Rap GAP activity of GAP1IP4BP and to identify the structural elements beyond the RasGAP domain that might be responsible for
an efficient RapGAP activity, the enzymatic activity of the fragment comprising the GAP and the PH/Btk domain (GAP\textsuperscript{IP4BP\291-834}) was further analyzed. The kinetic characterization of the hydrolysis reaction stimulated by GAP\textsuperscript{IP4BP\291-834} was performed using the radioactive charcoal assay. In the presence of 200 nM GAP\textsuperscript{IP4BP\291-834} and increasing concentrations of Ras-GTP, the initial rates of the hydrolysis reaction were determined at different Ras-GTP concentrations. Similarly to the GAP domain construct, GAP\textsuperscript{IP4BP\291-834} could not be saturated under the conditions used (Figure 33). With 200 nM GAP\textsuperscript{IP4BP\291-834}, efficient RasGAP activity was detected. At 800 µM Ras-GTP the $k_{\text{obs}}$ of the reaction reached a value of 2.6 sec$^{-1}$ which is in a similar range to that of other GAP stimulated reactions.

![A](image1.png)  

![B](image2.png)  

![C](image3.png)

Figure 33. Schematic representation of the GAP\textsuperscript{IP4BP\291-834} domains architecture (A); Michaelis-Menten kinetics of the GAP\textsuperscript{IP4BP\291-834}–mediated GTP hydrolysis of H-Ras (B, C). 200 nM GAP\textsuperscript{IP4BP\291-834} were incubated in standard buffer at 25°C with increasing concentrations of H-Ras [γ-\textsuperscript{32P}]GTP. The GTPase activity was monitored by measuring P\textsubscript{i} release, and data were evaluated as described in Materials and Methods. Data are plotted as rates (B) or rate constants (C).
As shown in Figure 34, GAP1IP4BP$_{291-834}$ showed no significant RapGAP activity. In the presence of 10 µM GAP1IP4BP$_{291-834}$ and with 800 µM Rap-GTP the $k_{obs}$ of the reaction reached a value of only 0.048 sec$^{-1}$.

![Figure 34. Michaelis-Menten kinetics of the GAP1IP4BP$_{291-834}$-mediated GTP hydrolysis of Rap1B. 10 µM GAP1IP4BP$_{291-834}$ were used to assay GTP hydrolysis with increasing concentrations of Rap-1B [$\gamma$-32P]GTP. The GTPase activity was monitored by measuring P$_i$ release. Data are plotted as rates (A) or rate constants (B).](image)

The $k_{obs}$ values for Rap and Ras were estimated to be in the same range with those obtained for the GAP1IP4BP$_{291-569}$-mediated reaction indicating that the PH/Btk domains do not have any influence on the affinity for Ras and Rap. It might be speculated that N-terminal regions adjacent to the GAP domain such as C2 domains might be responsible for the higher affinity towards Ras and Rap. However a construct of GAP1IP4BP comprising the GAP domain and the N-terminal C2 domains could not be purified due to contamination and excessive degradation and therefore no conclusive information about the Ras or Rap GAP activity could be obtained. Nevertheless, the RapGAP activity of GAP1IP4BP$_{158-617}$ was tested in a preliminary GAP assay but showed no stimulation of the GTPase reaction of Rap (data not shown).
3.3 Probing the dual GAP mechanism by active site analysis

3.3.1 The arginine finger mutation and its effect on the dual activity

Previous studies on the RasGAP reaction have shown that the crucial catalytic arginine cannot be replaced by any other amino acid without drastically reducing the GAP activity. In the case of other RasGAPs (p120GAP and NF1) the mutation of the catalytic arginine to alanine reduces the RasGAP activity more than 1000-fold (Ahmadian et al., 1997c; Ahmadian et al., 2003).

To identify the arginine residue responsible for the RasGAP activity of GAP1IP4BP several sequences corresponding to RasGAP domains from human GAP1IP4BP, human GAP1m, human neurofibromin and human p120GAP were compared (Figure 35).

![Sequence alignment of the catalytic domain of several human RasGAPs. The aligned sequences correspond to human GAP1IP4BP, human GAP1m, human neurofibromin and human p120GAP. Completely conserved residues are showed in red, highly conserved residues (80%) in blue, and less conserved residues (60%) in grey.](image)

A conserved arginine residue in position 371 corresponding to Arg789 in p120GAP and to Arg1276 in neurofibromin was identified. To test its involvement in catalysis this residue was mutated to alanine, the corresponding construct was cloned in pGEX-4T1, and the mutant protein was purified according to standard procedures for GST-fusion proteins (chapter 2.3.9).
The GAP activity of the mutant protein was analyzed using the radioactive charcoal assay, under standard conditions, using a constant concentration of GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLR371A}} of approximately 5 nM with increasing concentrations of GTP-loaded H-Ras and GTP-loaded Rap1B. The initial rates of the hydrolysis reaction were determined and plotted against different H-Ras (Figure 36 A) and Rap1B concentrations (Figure 36 B).

![Graph A](image)

![Graph B](image)

**Figure 36.** Probing the GTPase mechanism by arginine finger mutation. Analysis of GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLR371A}} and comparison to GAP\textsubscript{IP\textsubscript{4}BP} wild-type full-length. 5 nM full-length enriched GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLWT}} and 5 nM GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLR371A}} were incubated in standard buffer at 25°C with increasing concentrations of radioactively labelled $[\gamma^{32}]$GTP H-Ras (A) and $[\gamma^{32}]$GTP Rap1B (B). The GTPase activity was monitored by measuring Pi release. Data are plotted as reactions rates $v$ [µmoles/sec].

As expected, the arginine finger mutant of GAP\textsubscript{IP\textsubscript{4}BP}, GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLR371A}}, severely affected the RasGAP activity. Using a concentration of 5 nM GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLR371A}}, it was estimated that the arginine finger mutant is at least 20-fold less active than the wild-type at a concentration of 100 µM Ras-GTP.

In contrast to RasGAPs, the stimulation of the GTPase reaction in the Rap-RapGAP system does not involve an Arg finger (Brinkmann et al., 2002). Surprisingly, when the GTPase reaction on Rap was analyzed using GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLR371A}}, a large drop in the RapGAP activity could be observed. It was estimated that GAP\textsubscript{IP\textsubscript{4}BP\textsubscript{FLR371A}} is at least 30-fold less active than the wild-type GAP\textsubscript{IP\textsubscript{4}BP} at a concentration of 100 µM Rap-GTP. This result argues that GAP\textsubscript{IP\textsubscript{4}BP} activity towards Rap involves a different mechanism as
the Rap1GAP reaction. It could also be concluded that the basic machinery for both RasGAP and RapGAP activations seems to reside within the GAP domain involving the arginine 371.

Consistent with these in vitro data, it was shown that in cells expressing GAP1\textsuperscript{IP4BP} there is a significant decrease in the levels of Ras and Rap-GTP. This decrease in recoverable Ras and Rap-GTP was not observed in those cells expressing the arginine finger mutant GAP1\textsuperscript{IP4BP}_{FL}R371Q (Kupzig et al., 2006) indicating that also in vivo, the Ras and RapGAP activities of GAP1\textsuperscript{IP4BP} are dependent on the presence of the arginine finger.

### 3.3.2 The Glycine 12 mutation and its effect on the GAP1\textsuperscript{IP4BP}-stimulated reaction of Rap

The glycine 12 residue of Ras appears most frequently mutated in human tumors (Barbacid, 1987; Bos, 1989). This residue is critical for the oncogenic activation of Ras. Any mutation of Gly12 (except to proline) activates the oncogenic potential of Ras (Seeburg et al., 1984). From the structure of p120GAP in complex with Ras (Scheffzek et al., 1997) it appears that mutation of Gly12 even to alanine will produce steric clashes between the arginine finger and the critical glutamine 61. Gly12 mutants of Ras bind to GAP with an affinity similar to that of wild-type without subsequent GTP hydrolysis (Gideon et al., 1992).

In contrast to Ras where no down-regulation of the G12V mutant by RasGAP could be observed, a significant Rap1GAP-catalyzed GTPase reaction was found for the RapG12V mutant (Brinkmann et al., 2002). However, when the GAP activity of GAP1\textsuperscript{IP4BP} towards RapG12V was analyzed (Figure 37), a 40-fold reduced GAP-catalyzed GTPase activity could be observed at a concentration of 50 µM RapG12V-GTP, indicating that the G12 mutation interferes with the GAP1\textsuperscript{IP4BP} catalyzed GTPase reaction.

This result led to the assumption that GAP1\textsuperscript{IP4BP} employs a completely different reaction mechanism for the stimulation of the GTPase reaction of Rap compared to Rap1GAP.
Mutants of Ras and Rap in the catalyzed reaction of GAP1IP4BP

Another key residue of Ras, whose mutation results in a complete loss of the GTPase activity, particularly in the presence of RasGAPs, is glutamine 61. Apart from glycine 12, glutamine 61 has been found to be another biochemical reason for the oncogenicity of Ras as its mutation prevents Ras for being switched off (Der et al., 1986; Barbacid, 1987; Krengel et al., 1990; Ahmadian et al., 1999). Mutations of Gln61 dramatically reduce the intrinsic hydrolysis rate and prevent the GAP-stimulated reaction.

GAP1IP4BP is able to stimulate the hydrolysis reaction on both small GTP-binding proteins Ras and Rap with similar efficiency. We further analyzed the role of glutamine 61 in Ras and threonine 61 in Rap in the GAP1IP4BP-stimulated reaction.

Glutamine 61 of Ras was replaced by threonine (the corresponding residue in Rap) and the GAP1IP4BP-stimulated GTP-hydrolysis reaction was analyzed. Mutation of glutamine 61 in Ras reduced the RasGAP-stimulated reaction for both GAP1IP4BPFl/WT (Figure 38 A) and GAP1IP4BP291-569 (Figure 39), respectively, arguing that Glu61 plays a critical role in the hydrolysis mechanism. Using 5 nM GAP1IP4BPFl/WT it was estimated that the GTPase reaction is at least 25-fold reduced in the presence of 100 µM RasQ61T-GTP than in the presence of wt-Ras. Nevertheless, this result was surprising, considering that GAP1IP4BP stimulates the GTPase reaction on Rap presumably independently of a catalytic glutamine.

We wondered therefore about the effect of the canonical mutation threonine 61 in Rap to glutamine, on the GAP1IP4BP-mediated reaction.
The activity of wild-type GAP\textsubscript{1IP4BP} and of the GAP domain construct (GAP\textsubscript{1IP4BP}\textsubscript{291-569}) against the Rap-T61Q mutant was analyzed using the radioactive charcoal assay. In case of the GAP\textsubscript{1IP4BP}\textsubscript{FL/WT}, catalysis was more efficient for the Rap mutant (RapT61Q) than for wild-type Rap but the affinity was reduced as the reaction could no longer be saturated with 800 µM Rap T61Q-GTP (Figure 38. B).

In case of the GAP domain construct, GAP\textsubscript{1IP4BP}\textsubscript{291-569}, the inefficient hydrolysis on Rap seems to be at least partially due to the absence of a glutamine residue (Figure 39 A and B). Using the Rap-T61Q mutant, the k\textsubscript{obs} of the reaction reached a value of 0.5 sec\textsuperscript{-1} at 400 µM protein concentration, which is only approximately 10-fold lower than for Ras.

Figure 38. GAP\textsubscript{1IP4BP}\textsubscript{FL/WT}-stimulated activity towards RasQ61T (A) and RapT61Q (B) and comparison to Rap1 and Ras wild-type. Initial rates were obtained by using a constant concentration of 5 nM GAP\textsubscript{1IP4BP}\textsubscript{FL/WT} and increasing concentrations of radioactively labelled [\(\gamma\)\textsuperscript{32P}]GTP H-Ras, [\(\gamma\)\textsuperscript{32P}]GTP RasQ61T (A) and [\(\gamma\)\textsuperscript{32P}]GTP Rap1B, [\(\gamma\)\textsuperscript{32P}]GTP RapT61Q (B). Reaction rates v [\(\mu\)moles/sec] were plotted against different Ras/Rap concentrations.
Figure 39. GAP1^{IP4BP}_{291-569}-stimulated reaction towards RasQ61T and RapT61Q and comparison to Rap1 wild-type. 100 nM GAP1^{IP4BP}_{291-569} was incubated in standard buffer at 25°C with increasing concentrations of radioactively labeled $[^{32}\text{P}]$GTP Rap1B, $[^{32}\text{P}]$GTP RasQ61T, $[^{32}\text{P}]$GTP RapT61Q. Data are plotted as reaction rates $v$ [µmoles/sec] (A) and rate constants $k_{obs}$ [sec$^{-1}$] (B).

3.4 Monitoring the interaction of GAP1^{IP4BP} with Ras and Rap by fluorescence approaches

Fluorescently labeled guanine nucleotides can serve as reporter groups to investigate the interaction of GTP-binding proteins with a variety of regulatory and signaling proteins, such as GAPs, GEFs and downstream effectors.

Binding of Ras and Rap to GAP1^{IP4BP} was followed either by measuring direct fluorescence or using a fluorescence polarization assay as described in Materials and Methods (chapter 2.4). For this purpose, fluorescently labeled, non-cleavable GTP analogues (e.g. mGppNHp) or mGDP in the presence of AlFx can be used. AlFx along with GDP (or ADP) mimics the transition state of the phosphoryl transfer reaction in many enzymatic systems such as myosin and G$\alpha$ subunits of the heterotrimeric G proteins (Chabre, 1990). Aluminium fluoride binds to Ras-like proteins only in the presence of their respective GAPs demonstrating that the active site of Ras-like proteins needs to be complemented by residues from GAP (Ahmadian et al., 1997b; Mittal et al., 1996).
The interaction of GAP1\textsuperscript{IP4BP} with Ras was followed in a fluorescence polarization assay. To determine the binding affinity between Ras and GAP1\textsuperscript{IP4BP}, an equilibrium titration was performed by using a constant concentration of Ras bound to mant-nucleotides (either mGDP or mGppNHp) and increasing concentrations of GAP1\textsuperscript{IP4BP\_FL/WT} protein (Figure 40). Addition of GAP1\textsuperscript{IP4BP\_FL/WT} (96 kDa) to Ras (20 kDa) led to an increase in polarization due to the formation of a higher molecular mass complex. The maximal signal change was 20\% for Ras-mGDP and 12\% for Ras-mGppNHp. As a control Ras-mGDP and Ras-mGppNHp, respectively, were titrated with increasing concentrations of BSA and in this case, no increase in polarization was observed. In order to avoid the dilution of the fluorophore with the addition of GAP1\textsuperscript{IP4BP\_FL/WT}, Ras-mGDP (or Ras-mGppNHp) was always premixed with the GAP protein to a final concentration of 2 \mu M.

The increase in polarization for GAP1\textsuperscript{IP4BP} was fitted by a quadratic binding equation as described in chapter 2.4. The \( K_D \) value for Ras-mGDP-GAP1\textsuperscript{IP4BP\_FL/WT} complex in the presence of AlF\textsubscript{3} was 62 \mu M. Similarly, for Ras-mGppNHp and GAP1\textsuperscript{IP4BP\_FL/WT} the measured \( K_D \) had a value of 60 \mu M which was 3.5-fold lower than what has been expected from the Michaelis-Menten kinetics (\( K_M \) of 213 \mu M).

![Figure 40. Binding measurements of the GAP1\textsuperscript{IP4BP\_FL/WT—Ras interaction.} A total of 2 \mu M Ras-mGDP in the presence of AlF\textsubscript{3} (A) or 2 \mu M Ras-mGppNHp (B) were titrated with increasing concentrations of GAP1\textsuperscript{IP4BP\_FL/WT} and the increase in polarization was plotted against the GAP1\textsuperscript{IP4BP\_FL/WT} concentrations. Data were fitted to a quadratic equation to determine the equilibrium dissociation constants.](image-url)
An increase in polarization could be also observed in the presence of GAP1\textsuperscript{IP4BP\_291-834} under similar conditions, but in this case the reaction could not be saturated suggesting a much weaker affinity binding between this construct and Rap or Ras, respectively, and subsequently higher $K_D$ values for both Ras and Rap (data not shown).

Beryllium fluoride (as a ground state analogue) was also used together with Ras-mGDP. Titration of Ras-mGDP with increasing concentrations of GAP1\textsuperscript{IP4BP\_FL/WT} in the presence of BeF\textsubscript{3} did not lead to any increase in polarization (data not shown).

Titration of Rap-mGDP in the presence of AlF\textsubscript{x} or Rap-mGppNHp with increasing amounts of GAP1\textsuperscript{IP4BP\_FL/WT} did not change the polarization suggesting that fluorescence polarization might not be suitable for measuring the interaction between Rap and GAP1\textsuperscript{IP4BP} or that the affinity between the two proteins is actually much weaker.

Therefore a direct fluorescence experiment was subsequently employed to study the interaction between GAP1\textsuperscript{IP4BP\_FL/WT} and Rap. In this case, to determine the dissociation constant of the complex, Rap-mGDP was titrated with increasing concentrations of GAP1\textsuperscript{IP4BP\_FL/WT} in the presence of AlF\textsubscript{x}. Upon binding of Rap-mGDP to GAP1\textsuperscript{IP4BP\_FL/WT} in the presence of AlF\textsubscript{x} the titration curve shows a decrease in fluorescence as illustrated in Figure 41 with the change in fluorescence signal of 15%. To validate this experiment and to prove that the fluorescence change is not just an artefact but it is due to complex formation between Rap and GAP1\textsuperscript{IP4BP} several control experiments were performed. Either buffer only or GST were used instead of GAP1\textsuperscript{IP4BP} leading to only a small fluorescence change following titration. Rap-mGDP was also titrated with GAP1\textsuperscript{IP4BP\_FL/WT} in the absence of AlF\textsubscript{x} when no complex formation should be observed. As expected, the fluorescence signal changed very less compared to the change of 15% observed in the presence of AlF\textsubscript{x}.

The affinity of GAP1\textsuperscript{IP4BP\_FL/WT} for Rap-mGDP ($K_D$=42 μM) was higher than for Ras ($K_D$=60 μM) and agreed well with the $K_M$ value (42 μM) determined under multi-turnover conditions using the radioactive charcoal assay (see chapter 3.2.2).
Results

Figure 41. Decrease in fluorescence signal on titration of GAP1\textsuperscript{IP4BP\_FL/WT} into a solution of Rap-mGDP. 2 µM Rap-mGDP in the presence of AlF\textsubscript{3} was titrated with increasing concentrations of GAP1\textsuperscript{IP4BP\_FL/WT} and the decrease in fluorescence was plotted against different concentrations of GAP1\textsuperscript{IP4BP\_FL/WT}.

3.5 Crystallization trials

GAP1\textsuperscript{IP4BP\_FL/WT} and GAP1\textsuperscript{IP4BP\_291-834} were used for crystallization trials using both sitting drop and hanging drop vapor diffusion techniques. All crystallization trials were performed at 4°C and 20°C. GAP1\textsuperscript{IP4BP} was used either freshly prepared or thawed after freezing and diluted in appropriate buffer to different concentrations ranging from 5-15 mg/ml. Crystallization of GAP1\textsuperscript{IP4BP\_FL/WT} proved to be unsuccessful as from a total of 1728 conditions tested at different protein concentrations and different temperatures, only one condition yielded tiny needles using PEG 4000 as precipitant (see chapter 2.5). These needles could not be refined in size by adjusting the crystallization conditions (Figure 42 A).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conditions Screened</th>
<th>Source</th>
<th>T°C</th>
<th>Protein concentration</th>
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<td>NeXtal Qiagen</td>
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<td>5, 10, 15 mg/ml</td>
<td>2</td>
<td>not refinable</td>
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Table 11. High-throughput crystallization experiments with GAP1\textsuperscript{IP4BP}
Co-crystallization of GAP1\textsubscript{FP4BP}\textsubscript{FL/WT} with Ras-GDP (or Rap-GDP) in the presence of AlF\textsubscript{x} was also attempted. However, this approach yielded no crystals in all tested conditions. For GAP1\textsubscript{FP4BP}\textsubscript{291-834}, crystals were found using sodium acetate as precipitant (Figure 42 B) (see chapter 2.5 for details). These crystals were tested for diffraction, but they did not diffract x-rays. Another condition yielding spherulites (microcrystals) was found using PEG 6000 as precipitant (Figure 42 C, D). However, screening different PEG 6000 concentrations, different CaCl\textsubscript{2} concentrations, different pHs and 192 different additives did not significantly improve the size and quality of these spherulites.

Several factors are known to prevent crystallization of proteins. Among them, of particular importance are the purity of the macromolecule, the aggregation state and the stability. Impurities in the macromolecule or in the crystallization solution can often make crystallization less likely. Conformational flexibility can also affect crystallization, due to entropy. Furthermore, molecules that tend to self-assemble into regular helices are often difficult to assemble into crystals. The difficulties to obtain crystals of sufficient size and quality led us to assume that GAP1\textsubscript{FP4BP} might contain flexible regions which might limit the crystallization of this protein.
3.6 The GAP1\textsuperscript{IP4BP}-catalyzed GTPase reaction monitored by FTIR Spectroscopy

Time-resolved Fourier transform infrared (trFTIR) difference spectroscopy has been successfully applied to monitor the molecular reaction mechanisms of proteins at atomic level in real time (Gerwert, 1999; Kotting and Gerwert, 2005b). In this chapter the GAP1\textsuperscript{IP4BP} mediated activation of the GTPase reaction on Ras and Rap has been investigated by FTIR spectroscopy and compared to the RasGAP and the RapGAP catalyzed reactions. For a summary of previous FTIR studies on the Ras-RasGAP and the Rap-RapGAP reactions see chapter 1.4. For a detailed description of the FTIR method see chapter 2.6.

3.6.1 The GAP1\textsuperscript{IP4BP} catalyzed reaction

FTIR spectroscopy was used to investigate the GAP1\textsuperscript{IP4BP}-catalyzed GTPase reaction towards the small GTP-binding proteins Ras and Rap. In order to avoid hydrolysis of GTP during the preparation of the sample, the G proteins were loaded with nonhydrolyzable caged GTP containing a photo labile protecting group. The GTPase reaction was initiated by UV laser flashes that remove the protecting group, releasing GTP from caged GTP. The IR absorbance changes of the hydrolysis reaction were monitored between 1800 cm\textsuperscript{-1} and 950 cm\textsuperscript{-1} simultaneously, with 10 ms time-resolution in the rapid scan mode. The time resolved absorbance changes were analyzed by a multi-exponential global fit analysis which reveals the amplitude spectra and the corresponding apparent rate constants. Alternatively, spectra corresponding to the pre-hydrolysis and post-hydrolysis states were recorded. A direct subtraction led to the difference spectra that are nearly identical to the amplitude spectra. In the amplitude or difference spectra, as a convention, negative bands represent the vibrations of the disappearing molecular groups or interactions whereas positive bands correspond to vibrations of the products.

The bands appearing in the amplitude or difference spectra were assigned to specific residues for interpretation using different site-specific \textsuperscript{18}O labeled GTP. Isotopic labeling shifts the IR bands of the labeled group to lower frequencies. To visualize the frequencies shifts, double difference spectra were used. The double difference spectra were calculated by subtracting the difference spectrum of the unlabelled from the spectrum of the labeled GTP. In principle, in the double difference spectra only the vibrations of the labeled group should appear as difference bands.
3.6.1.1 The RasGAP reaction of the GAP1\textsuperscript{IP4BP} monitored by FTIR

As GAP, the minimal RasGAP catalytic domain of GAP1\textsuperscript{IP4BP} (GAP1\textsuperscript{IP4BP}\textsubscript{291-569}) was initially used. The sample solutions were prepared as described in Materials and Methods. To achieve single-turnover conditions, a 10\% excess of GAP1\textsuperscript{IP4BP}\textsubscript{291-569} over the Ras-caged nucleotide was used.

The GTPase reaction of the GAP1\textsuperscript{IP4BP}\textsubscript{291-569}-Ras-caged GTP complex was initiated by UV laser flashes, 12 flashes for pHP (\(P^3\)-\textit{para}-hydroxyphenacyl)-caged GTP and 40 flashes for NPE (\(P^3\)-1-(2-nitrophenyl) ethyl)-caged GTP with 500 Hz repetition rate. After cleavage of the photolabile protecting group, GTP hydrolysis takes place and the Ras-GDP complex and P\(_i\) are formed.

While a single apparent rate constant can describe the intrinsic hydrolysis reaction of Ras (Figure 19, chapter 1.4.1) the GAP1\textsuperscript{IP4BP}\textsubscript{291-569}-catalyzed reaction was described by at least three exponential functions which implies a three step mechanism. By comparison with the NF1 catalyzed GTPase reaction of Ras, the three steps could be assigned to the following processes: in the first step, the photolysis of the GAP1\textsuperscript{IP4BP}\textsubscript{291-569}-Ras-caged GTP complex to a GAP1 IP4BP\textsubscript{291-569}-Ras-GTP ground state complex occurred. After photolysis the GTPase reaction took place in two steps with apparent rate constants of 0.024 s\(^{-1}\) and 0.006 s\(^{-1}\), respectively (at 260 K) as shown in the scheme below:

\[
\text{hv} \quad \text{Ras-caged GTP} \quad k_1 \quad k_2 = 0.024 \text{ s}^{-1} \quad k_3 = 0.006 \text{ s}^{-1} \quad \text{Ras-GDP + P}_i
\]

\textbf{Scheme 3. The GAP1\textsuperscript{IP4BP}\textsubscript{291-569}-catalyzed GTPase reaction on Ras.}

The apparent rate constants \(k_2\) and \(k_3\) describe the appearance of an intermediate and the decay to GDP and P\(_i\) by the slowest rate, respectively. The spectral signatures of these two steps are discussed in the following sections.

The photolysis and hydrolysis amplitude spectra between GAP1\textsuperscript{IP4BP}\textsubscript{291-569} and NF1-catalyzed reactions on Ras are compared in Figure 43. For this purpose the catalytic domain of neurofibromin, NF1-333 was used.

In case of the photolysis reaction both spectra exhibit the same difference bands (GTP-\(\alpha\) at 1259 cm\(^{-1}\), GTP-\(\beta\) at 1218 cm\(^{-1}\), GTP-\(\gamma\) at 1140 cm\(^{-1}\)).
Figure 43. Comparison of the photolysis and hydrolysis amplitude spectra of GAP1\textsuperscript{IP4BP\textsubscript{291-569}} and NF1-catalyzed reactions on Ras. Photolysis amplitude spectrum of the GAP1\textsuperscript{IP4BP\textsubscript{291-569}} and NF1-catalyzed reactions on Ras at 270 K (A). Hydrolysis amplitude spectrum of the GAP1\textsuperscript{IP4BP\textsubscript{291-569}} and NF1-catalyzed reactions on Ras at 270 K (B). The amplitude spectrum of GAP1\textsuperscript{IP4BP\textsubscript{291-569}} catalyzed reaction is shown in red and the amplitude spectrum of NF1 catalyzed reaction is shown in green.

The hydrolysis spectrum of GAP1\textsuperscript{IP4BP\textsubscript{291-569}}-catalyzed reaction agrees well with the hydrolysis difference spectrum of the NF1-catalyzed reaction. This agreement shows that the protein–bound GTP structure and the charge distribution as probed by FTIR are very similar for both NF1 and GAP1\textsuperscript{IP4BP\textsubscript{291-569}}-catalyzed reactions. Marker frequencies of GDP-\(\alpha\) at 1230 cm\(^{-1}\), GDP-\(\beta\) at 1096 cm\(^{-1}\) and the released P\(_i\) at 1081 cm\(^{-1}\) and 990 cm\(^{-1}\) are highlighted for illustration.

**The Ras\(_{on}\)-GTP to Ras-GDP-P\(_i\) transition (the appearance of the intermediate)**

The transition from the GTP-bound form to the intermediate is represented by the amplitude spectrum of \(k_2\) shown in Figure 44. For the assignment of the phosphate vibrations in the \(k_2\) amplitude spectra of the GAP1\textsuperscript{IP4BP\textsubscript{291-569}} catalyzed reaction different site-specific \(^{18}\)O-labeled caged GTP were used. The hydrolysis difference spectra of the unlabeled GTP and labeled GTP are compared. The former positive GTP product bands in the photolysis difference spectra now become negative educt bands in the hydrolysis difference spectra.
Results

Figure 44. Spectral signatures of the intermediate. The amplitude spectra of $k_2$ which describes the transition from the Ras$_{on}$-GTP to Ras-GDP-P$_i$, are shown (black lines). Negative bands belong to the GTP state and positive bands correspond to the intermediate. In addition, the amplitude spectra with differentially labeled GTP are shown in colored lines corresponding to [$\beta$, $\gamma^{18}$O/$\gamma^{18}$O$_3$], H$_2^{18}$O, [$\alpha$, $\beta^{18}$O]/$\beta^{18}$O$_2$] (A). Differences between the amplitude spectra of labeled and unlabeled GTP are presented to visualize the frequency shifts. Difference bands are illustrated by shaded areas. The original frequency is given in black and the shifted position in color (B). The Ras-caged GTP complex was prepared using either NPE-caged GTP in case of the [$\beta$, $\gamma^{18}$O/$\gamma^{18}$O$_3$] labeling or pHP-caged GTP for all other measurements.

The four fold $\gamma^{18}$-O label shifts the band at 1140 cm$^{-1}$ to 1122 cm$^{-1}$. This is seen in the double difference spectrum as difference band at 1143/1112 cm$^{-1}$. Thus the band at 1140 cm$^{-1}$ can be assigned to the $\gamma$-GTP vibration. Similarly to the Ras-RasGAP reaction in the GAP1$^{IP4BP}_{291-569}$ catalyzed reaction on Ras the GTP-$\gamma$ band at 1140 cm$^{-1}$ is also observed at early time points, such as in the photolysis spectra and appears as negative band in the $k_2$ amplitude spectrum.

As in the case of the Ras-RasGAP reaction (Allin et al., 2001), two intermediate bands are identified. They appear at 1205 cm$^{-1}$ and 1109 cm$^{-1}$ for the Ras-GAP1$^{IP4BP}_{291-569}$ system. Due to $\gamma^{18}$O labeling, the first band of the intermediate at 1205 cm$^{-1}$ is shifted to 1172 cm$^{-1}$, which is seen in the double difference spectra as a difference band at 1204/1177 cm$^{-1}$. The second band of the intermediate shifts from 1109 cm$^{-1}$ to 1091 cm$^{-1}$ due to $\gamma^{18}$-O labeling. This is seen in the double difference spectra as difference band at 1107/1092 cm$^{-1}$. 
These shifts indicate that the intermediate bands at 1205 and 1109 are either GTP-\(\gamma\) or cleaved P\(_i\). In order to determine whether the attack of water on the \(\gamma\)-phosphate group has already taken place in the intermediate the same reaction was performed in H\(_2\)\(^{18}\)O with unlabeled GTP. The water molecule attacks the \(\gamma\)-phosphate in a nucleophilic manner and introduces an \(^{18}\)O label into the released P\(_i\). The incorporated \(^{18}\)O should shift the P\(_i\) bands selectively. In this case a similar shift of the intermediate bands as seen for the \(\gamma\)-label will appear only if the cleavage of the \(\beta\)-\(\gamma\) bond has already taken place.

In the case of GAP1\(_{291-569}\), H\(_2\)\(^{18}\)O measurements, shifted bands are not observed probably because these shifts are not above the noise level (Figure 44 B). Therefore we can not conclude that the cleavage of the \(\beta\)-\(\gamma\) bond has already taken place in the intermediate. The \(\gamma\)-\(^{18}\)O labeling affects not only the \(\gamma\) vibrations but also several other phosphate bands due to coupling. Upon \(\gamma\)-\(^{18}\)O labeling, the GTP-\(\alpha\) band at 1259 cm\(^{-1}\) is also affected as seen from the downshift in the double difference spectra at 1265/1238 cm\(^{-1}\). The effect of the \(\gamma\)-\(^{18}\)O label on the GTP-\(\alpha\) band at 1265/1238 cm\(^{-1}\) is attributed to the coupled \(\alpha\)-\(\gamma\) vibrations in GTP.

The \(\beta\)-\(^{18}\)O label shifts the \(\beta\)-GTP band at 1218 cm\(^{-1}\) to 1184 cm\(^{-1}\). This is observed in the double difference spectra as difference band at 1217/1185 cm\(^{-1}\). Thus the band at 1218 cm\(^{-1}\) can be attributed to \(\beta\)-GTP vibration.

Interestingly, the \(\beta\)-\(^{18}\)O label also shifts the second band of the intermediate from 1109 cm\(^{-1}\) to 1105 cm\(^{-1}\), which is seen in the double difference spectra as a difference band at 1112/1101 cm\(^{-1}\). As found for the NF1 catalyzed reaction of Ras (Allin et al., 2001), this effect of the \(\beta\)-\(^{18}\)O label on the intermediate band can be attributed to coupled vibrations. Since we could not conclude that chemical cleavage due to the breakage of the \(\beta\)-\(\gamma\) bond of GTP has already taken place one explanation for such a coupled vibrations might be that vibrations of the \(\gamma\)-phosphate are still covalently bound to the GDP counterpart.

The Ras-GDP-P\(_i\) to Ras-GDP + P\(_i\) transition (product formation and P\(_i\) release)

Figure 45 shows the amplitude spectrum for \(k_3\), which describes product formation and P\(_i\) release. The former positive bands of the intermediate (1205 cm\(^{-1}\) and 1109 cm\(^{-1}\)) appear as negative bands as it disappears with \(k_3\) while the product peaks of the GDP (\(\alpha\)-GDP at 1236 cm\(^{-1}\) and \(\beta\)-GDP at 1138 cm\(^{-1}\)) and P\(_i\) appear as positive bands.
Figure 45. Spectral signatures of product release. The amplitude spectra of $k_3$, which describes the product (Ras-GDP) formation and Pi release are shown (black lines). Negative bands now belong to the intermediate and positive bands correspond to the GDP state or free Pi. In addition, the amplitude spectra of the labeled GTP are shown in colored lines (A). Differences between the amplitude spectra of labeled and unlabeled GTP are presented (B).

The four fold $\gamma$–GTP label shifts the intermediate band from 1209 cm$^{-1}$ to 1179 cm$^{-1}$. This is seen in the double difference spectra at 1213/1176 cm$^{-1}$. The second band of the intermediate at 1115 cm$^{-1}$ is also shifted with $\gamma$-labeling to 1110 cm$^{-1}$ and appears as a double difference band at 1116/1096 cm$^{-1}$ in the double difference spectrum. These data confirm the assignments of the intermediate bands obtained from the analysis of $k_2$.

The GDP band is shifted from 1138 cm$^{-1}$ to 1128 cm$^{-1}$, which appears as a double difference band at 1138/1126 cm$^{-1}$. In addition, due to four-fold $\gamma$ labeling the bands at 1081 cm$^{-1}$ and 990 cm$^{-1}$ are shifted to 1054 cm$^{-1}$ and 973 cm$^{-1}$, respectively. They are identified in the double difference spectrum as difference bands at 1081/1054 cm$^{-1}$ and 991/969 cm$^{-1}$. Such shifts are also seen for the intrinsic reaction of Ras (Allin and Gerwert, 2001) and Rap (Chakrabarti et al., 2004) and they represent released phosphate.

H$_2^{18}$O labeling experiments showed a decrease in the intensity of the first band of the intermediate as seen in the amplitude spectrum at 1209 cm$^{-1}$. The second band of the intermediate (at 1115 cm$^{-1}$) appears slightly shifted in the amplitude spectrum. This shift is better seen in the double difference spectrum at 1114/1095 cm$^{-1}$. To further understand this
effect of H$_2$O$^{18}$O labeling on the intermediate bands observed in $k_3$, assignments of the $\beta$, $\gamma$-bridging vibration are required. The phosphate bands are also shifted due to H$_2$O$^{18}$O labeling from 1081 cm$^{-1}$ to 1059 cm$^{-1}$ and 990 cm$^{-1}$ to 962 cm$^{-1}$. These effects are seen as double difference bands at 1074/1059 cm$^{-1}$ and 993/977 cm$^{-1}$ and represent either bond breakage with $k_2$ or significant back reaction in $k_3$.

The $\beta$-18O labeling shifts the GDP bands from 1138 to 1117 cm$^{-1}$ and from 1096 to 1080 cm$^{-1}$. These shifts are identified in the double difference spectrum as difference bands at 1137/1116 cm$^{-1}$ and 1102/1075 cm$^{-1}$, respectively. These band positions are in good agreement with the band assignment of GDP-$\beta$ as described for the intrinsic reaction of Ras (Allin and Gerwert, 2001), Rap (Chakrabarti et al., 2004) and for the GAP catalyzed reaction of Ras (Allin et al., 2001). The $\beta$-18O labeling shifts the intermediate bands from 1209 cm$^{-1}$ to 1184 cm$^{-1}$ and from 1115 cm$^{-1}$ to 1106 cm$^{-1}$. The first shift is identified in the double difference spectrum at 1217/1183 cm$^{-1}$. The second band of the intermediate is not visible in the double difference spectrum due to overlapping frequencies with the $\beta$-GDP vibrations.

### 3.6.1.2 Time course of the Ras-GTPase reaction catalyzed by GAP1$^{IP4BP}$

Based on the band assignments, the kinetics of the phosphate groups and one selected protein group are shown in Figure 46 by following the absorbance changes of the respective groups against time. The absorbance changes relax to zero because the final GDP state is used as reference. The marker GTP$_{on}$ bands, GTP$_{on}$-$\gamma$ at 1144 cm$^{-1}$ and GTP$_{on}$-$\alpha$ at 1261 cm$^{-1}$ appear with $k_1$ and disappear with $k_2$. $k_1$ describes the transition from GTP$_{off}$ to GTP$_{on}$. GTP disappears almost completely with $k_2$ which might reflect the cleavage of the $\gamma$-phosphate. Simultaneously, the intermediate indicated at 1205 cm$^{-1}$ appears with the second rate $k_2$ and disappears with the third rate $k_3$. P$_i$ is released with $k_3$ from the Ras-GAP1$^{IP4BP}$$_{291-569}$ complex as monitored at 1080 cm$^{-1}$. $k_3$ represents the rate-limiting step of the reaction. The GDP vibration of the final Ras-GDP state appears at 1095 cm$^{-1}$.

Above 1350 cm$^{-1}$ where different protein side groups and the protein backbone absorb, a band at 1531 cm$^{-1}$ is observed. This band appears with $k_2$ and disappears with $k_3$. For a clear assignment of this band specific non-invasive mutants or site direct isotopic labels of Ras or GAP1$^{IP4BP}$ have to be used.
Figure 46. Time-dependent amplitude changes at different individual IR frequencies corresponding to different bond vibrations. Shown are the data (colored points), the fitted curves (solid curve), and the contributions from the apparent rate constants ($k_2$) of the multi-exponential fit (dotted colored curves). The time axis is logarithmic. $k_1$ represents the transition from the Ras-GTP$_{off}$ to Ras-GTP$_{on}$ state and the appearance of released $\gamma$-phosphate as indicated at 1144 cm$^{-1}$. $k_2$ shows the cleavage of $\gamma$-phosphate seen again at 1144 cm$^{-1}$ and the appearance of protein bound P$_i$ at 1205 cm$^{-1}$. $k_3$ shows the decay of the protein bound P$_i$ at 1205 cm$^{-1}$ and its release from the protein-protein complex into the bulk medium at 1080 cm$^{-1}$. The release of the protein bound P$_i$ to the bulk medium at 1080 cm$^{-1}$ described by $k_3$ is the rate-limiting step of the reaction.

As previously shown, in the case of Ras (Kotting et al., 2006) and Rap (Chakrabarti et al., 2004) the GAP-catalyzed hydrolysis reactions are described by at least two apparent rate constants indicating that both reactions involve the formation and decay of an intermediate. Similarly, in the case of the GAP1$^{IP4BP_{291-569}}$ catalyzed reaction on Ras after photolysis the GTPase reaction takes place in two steps involving the formation of an intermediate. Intermediate like vibrations appear at 1205 and 1107 cm$^{-1}$ (Figure 47 A).

The GAP catalyzed reaction on Ras was also monitored in the presence of full-length wild-type GAP1$^{IP4BP}$. Compared to GAP1$^{IP4BP_{291-569}}$, in the case of GAP1$^{IP4BP_F/WT}$ no intermediate like vibrations could be resolved during the course of the reaction. At 1144 cm$^{-1}$ the $\gamma$-phosphate group appears with photolysis and disappears with hydrolysis.
reflecting the cleavage of the $\gamma$ phosphate. Simultaneously, the $P_1$ vibration at 1080 cm$^{-1}$ rises (Figure 47 B).

![Figure 47](image)

**Figure 47.** Typical absorbance changes of the GAP1$^{\text{IP4BP}_{291-569}}$ and GAP1$^{\text{IP4BP}_{FL-WT}}$ catalyzed reactions. Time-dependent amplitude changes at different individual IR frequencies corresponding to different bond vibrations in the case of GAP1$^{\text{IP4BP}_{291-569}}$ (A) and GAP1$^{\text{IP4BP}_{FL-WT}}$ (B) catalyzed reactions. Shown are the decays of the GTP-$\gamma$ band indicated at 1144 cm$^{-1}$ and the release of the protein bound $P_1$ to the bulk medium at 1080 cm$^{-1}$. In the case of the GAP1$^{\text{IP4BP}_{291-569}}$ stimulated reaction intermediate like vibrations appear at 1205 cm$^{-1}$ (A). For the GAP1$^{\text{IP4BP}_{FL-WT}}$ catalyzed reaction no intermediate could be resolved during the course of the reaction (B). The GAP1$^{\text{IP4BP}_{291-569}}$ catalyzed reaction on Ras was measured at 270 K and the GAP1$^{\text{IP4BP}_{FL-WT}}$ catalyzed reaction was performed at 260 K.

### 3.6.1.3 The dual activity of GAP1$^{\text{IP4BP}}$ monitored by FTIR

The GAP1$^{\text{IP4BP}}$ catalyzed GTPase reaction on Rap was also monitored by FTIR. For this purpose GAP1$^{\text{IP4BP}_{291-569}}$ and GAP1$^{\text{IP4BP}_{FL-WT}}$ were used. For the Rap GTPase stimulated reaction of GAP1$^{\text{IP4BP}_{291-569}}$ and GAP1$^{\text{IP4BP}_{FL-WT}}$ no intermediate state could be resolved at 280 K or 260 K, respectively. Therefore no detailed comparison with RasGAP and RapGAP catalyzed reactions could be made. As an illustrative example (Figure 48), the disappearance of the GTP-$\alpha$ band at 1261 cm$^{-1}$ has been chosen as marker band to monitor the disappearance of GTP since the usual marker band for GTP, GTP-$\gamma$ at 1143 cm$^{-1}$, is masked by the appearing intermediate band in the GAP1$^{\text{IP4BP}_{291-569}}$ catalyzed reaction on Ras.
The GAP1\textsuperscript{IP4BP}-catalyzed reaction on Rap takes place after photolysis in only one step described by the rate constant \( k_2 \).

For the GAP1\textsuperscript{IP4BP}\textsubscript{291-569} catalyzed reaction on Ras the reaction takes place after photolysis in two steps described by the apparent rate constants \( k_2 = 0.024 \text{ sec}^{-1} \) and \( k_3 = 0.006 \text{ cm}^{-1} \), where \( k_2 \) describes the appearance of an intermediate and \( k_3 \) the appearance of reaction products Ras-GDP and P\(_i\) (Figure 48 A, blue points and blue curve). The marker GTP band at 1261 cm\(^{-1}\) primarily disappears with the second rate \( k_2 \), but \( k_3 \) prolongs the decay. In contrast, the GAP1\textsuperscript{IP4BP}\textsubscript{291-569} catalyzed reaction on Rap can be described by a single rate constant after photolysis (\( k_2 = 0.004 \text{ sec}^{-1} \)) (Figure 48 A, red curve), indicating the formation of the GDP-bound Rap and the phosphate release as a result of the hydrolysis reaction.

For the GAP1\textsuperscript{IP4BP}\textsubscript{FL/WT} catalyzed reaction on Ras and on Rap, after photolysis the reactions take place in only one step with similar apparent rate constants (\( k_2 \)) of 0.029 sec\(^{-1}\) and 0.028 sec\(^{-1}\) for Ras and Rap, respectively (Figure 48 B). These rates describe the hydrolysis of the GTP and the appearance of the products Ras/Rap-GDP and P\(_i\).

**Figure 48.** The GAP1\textsuperscript{IP4BP} catalyzed GTPase reactions of Ras and Rap monitored by FTIR. Time dependent absorbance changes of marker band of the GTP hydrolysis (GTP-\(\alpha\)) during GAP1\textsuperscript{IP4BP}\textsubscript{291-569} (A) and GAP1\textsuperscript{IP4BP}\textsubscript{FL/WT} (B) catalyzed reactions of Ras (blue) and Rap (red) are shown. The GAP1\textsuperscript{IP4BP}\textsubscript{291-569} catalyzed GTPase reaction of Ras was measured at 260 K, while the GAP1\textsuperscript{IP4BP}\textsubscript{291-569} catalyzed GTPase reaction of Rap was performed at 280 K. The GAP1\textsuperscript{IP4BP}\textsubscript{FL/WT} catalyzed reactions of Ras and Rap were performed at 260 K.
Results

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<th>GAP1_{IP4BP}^{Fl/WT}</th>
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<td>k_2 = 0.024 s^{-1}</td>
<td>k_2 = 0.028 s^{-1}</td>
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<td>Ras_{on}-GTP → Ras-GDP-P_i → Ras-GDP + P_i</td>
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<tr>
<td>k_3 = 0.006 s^{-1}</td>
<td>k_3 = 0.029 s^{-1}</td>
</tr>
<tr>
<td>Rap_{on}-GTP → Rap-GDP + P_i</td>
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Table 12. Summary of the calculated rate constants for the GAP1_{IP4BP}^{291-569} and GAP1_{IP4BP}^{Fl/WT}-stimulated reactions on Ras and Rap.

The FTIR measurements showed that the hydrolysis of Ras-GTP in the presence of the GAP domain alone (GAP1_{IP4BP}^{291-569}) is almost as fast as with GAP1_{IP4BP}^{Fl/WT}, as seen from the kinetics of the GTP band at 1261 cm^{-1} (Figure 48 A and B, blue curve). Furthermore it could be shown that the acceleration of the GTPase reaction on Rap is much faster (about 8-fold) with GAP1_{IP4BP}^{Fl/WT} compared with GAP1_{IP4BP}^{291-569} (Table 12).

The major difference between wild-type GAP1_{IP4BP} and the GAP domain is that during the Ras GTPase reaction the accumulation of the intermediate could only be resolved in the case of the GAP domain as seen from the single frequency kinetics at 1205 cm^{-1} (Figure 47 A, green curve).
4 Discussion

GAP1\textsuperscript{IP4BP} is a member of the GAP1 family of Ras GTPase-activating proteins (RasGAPs) that includes GAP1m, CAPRI, and RASAL (Yarwood et al., 2006). The domain architecture of these proteins consists of a central RasGAP domain, surrounded by amino-terminal C2 domains and a carboxyl-terminal pleckstrin homology/Bruton’s tyrosine kinase domain (Cullen, 1998). Consistent with the presence of the RasGAP domain all of these proteins have been shown to function as RasGAPs (Cullen et al., 1995c; Lockyer et al., 1999b; Lockyer et al., 2001; Walker et al., 2004).

Even if no sequence homology with other RapGAPs has been detected, GAP1\textsuperscript{IP4BP} has previously been shown to possess besides the predicted RasGAP activity, an unexpected GAP activity on the Ras-related protein Rap (Cullen et al., 1995c). Therefore, GAP1\textsuperscript{IP4BP} has dual Ras and Rap GAP activity.

The investigation of the mechanism by which GAP1\textsuperscript{IP4BP} functions as both Ras and Rap GAP was the main aim of this study. Therefore, the biochemical activity and specificity of GAP1\textsuperscript{IP4BP} towards the small G proteins Ras and Rap, the domains and the residues participating in the reaction and the mechanism of the GAP1\textsuperscript{IP4BP}-mediated hydrolysis towards Ras and Rap were examined.

A series of constructs comprising different domains of GAP1\textsuperscript{IP4BP} were cloned, expressed using an \textit{E. coli} or a baculovirus expression system. The expression efficiency was analyzed in a series of test expressions and the soluble proteins were further purified. The GTPase activity towards Ras and Rap was investigated by kinetic and mutational analyses together with time-resolved FTIR spectroscopy.

GAP1\textsuperscript{IP4BP}\textsubscript{FL/WT} and two other constructs GAP1\textsuperscript{IP4BP}\textsubscript{279-569} and GAP1\textsuperscript{IP4BP}\textsubscript{291-834} yielded soluble proteins and could be purified as biochemically active GTPase-activating proteins. The activation of the GTPase reaction stimulated by these proteins was further analyzed using the charcoal assay in order to obtain the Michaelis-Menten parameters of their respective GAP catalyzed reactions.

The detailed kinetic analysis has demonstrated that full-length, recombinant GAP1\textsuperscript{IP4BP} possesses a robust Ras and Rap GAP activity, with estimated $k_{\text{cat}}$s of 16 and 48 sec$^{-1}$ and $K_M$s of 42 and 213 M$^{-1}$ for Rap and Ras, respectively. The isolated GAP domain of GAP1\textsuperscript{IP4BP} (GAP1\textsuperscript{IP4BP}\textsubscript{291-569}) showed a pronounced RasGAP activity, but under similar conditions no stimulation of the RapGAP activity could be detected. Moreover, addition of
the C-terminal PH/Btk domain did not confer an efficient RapGAP activity. These data indicate that other regions of GAP1IP4BP might be required to support the catalytic activity towards Rap.

4.1 The RasGAP activity of GAP1IP4BP

Sequence analysis of GAP1IP4BP showed that the central GAP domain shares sequence homology with other known RasGAPs such as p120GAP, neurofibromin and GAP1m (Figure 35). Overall GAP1IP4BP has 62% sequence homology with GAP1m which led to the classification of GAP1IP4BP as a member of the GAP1 family of RasGAPs (Cullen et al., 1995c).

The kinetic studies performed in this work have demonstrated that full-length, recombinant GAP1IP4BP acts indeed as an efficient RasGAP. For the GAP1IP4BP stimulated reaction on Ras an estimated $k_{cat}$ value of 48 sec$^{-1}$ and a $K_M$ of 231 µM were obtained. For other known specific GAPs like p120GAP (Ahmadian et al., 1997c), neurofibromin (Wiesmuller and Wittinghofer, 1992), Rap1GAP (Brinkmann et al., 2002), RhoGAP (Graham et al., 1999), RanGAP (Klebe et al., 1995) and RabGAP (Albert et al., 1999) the $k_{cat}$ values vary between 5-20 sec$^{-1}$. The Ras-specific GAPs, NF1 and p120GAP, have $K_M$ values of 0.23 and 5 µM, respectively and the $k_{cat}$ of the GTPase reaction on Ras is 5.4 sec$^{-1}$ for NF1 and 8.0 sec$^{-1}$ for p120GAP (Ahmadian et al., 1997a).

Moreover, it was shown in this study that compared to the intrinsic hydrolysis rate of H-Ras (0.01 min$^{-1}$) GAP1IP4BP$^{FL/WT}$ accelerated the GTP hydrolysis reaction on Ras approximately $10^4$-fold.

The catalytic domains of neurofibromin and p120GAP, known as RasGAPs domains, comprise around 300 residues and are sufficient for the stimulation of the GTPase activity towards Ras. Accordingly, the isolated RasGAP domain of GAP1IP4BP (GAP1IP4BP$^{291-569}$) retains an efficient RasGAP activity. In the presence of 100 nM GAP1IP4BP$^{291-569}$ and 800 µM RasGTP, the $k_{obs}$ of the reaction reached a value of 8 sec$^{-1}$, which is in the same order as those of p120GAP (8 sec$^{-1}$) and neurofibromin (5.4 sec$^{-1}$). Furthermore, by time resolved FTIR spectroscopy it was proved that the acceleration of the GTPase reaction on Ras in the presence of the GAP domain alone is as fast as with full-length GAP1IP4BP (0.024 sec$^{-1}$ for the GAP domain, 0.028 sec$^{-1}$ for GAP1IP4BP$^{FL/WT}$).

Based on the sequence homology of the GAP domain of GAP1IP4BP with other RasGAPs and on the tertiary structure predictions of the GAP domain fold it is expected that the
RasGAP domain of GAP1\textsuperscript{IP4BP} will share the same protein fold as p120GAP and neurofibromin (Ahmadian et al., 1996; Scheffzek et al., 1996; Scheffzek et al., 1998). When a model structure of the GAP domain of GAP1\textsuperscript{IP4BP} in complex with Ras was generated (Figure 49) it predicted a helical, elongated molecule with a similar fold as the GAP domain of p120GAP. From the model it can be speculated that the GAP domain of GAP1\textsuperscript{IP4BP} might bind and stabilize the same switch I and switch II regions of Ras as other RasGAPs.

![Figure 49. The model structure of the GAP domain of GAP1\textsuperscript{IP4BP} in complex with Ras.](image)

Furthermore, sequence alignment of the GAP domain of GAP1\textsuperscript{IP4BP} with other RasGAP domains (Figure 35) suggest that the conserved arginine residue (R371) of GAP1\textsuperscript{IP4BP} might be involved in the catalysis mechanism as for p120GAP and neurofibromin. In the model structure of the GAP domain in complex with Ras (Figure 49) the arginine residue in position 371 appears oriented towards the active site of Ras. It approaches the glutamine 61 of Ras from close proximity and might be responsible for stabilizing its orientation allowing the positioning of the attacking water molecule. According to the predicted model
it could be shown that a site-directed mutant targeting the conserved catalytic arginine, GAP1\textsuperscript{IP4BP}\textsubscript{FL/R371A}, was unable to stimulate the GTPase activity towards Ras. This data is entirely consistent with GAP1\textsuperscript{IP4BP} functioning as a RasGAP and furthermore validates the arginine finger-based catalysis mechanism of GAP1\textsuperscript{IP4BP} towards Ras.

### 4.2 The RapGAP activity of GAP1\textsuperscript{IP4BP}

In this study, detailed kinetic analysis has demonstrated that full-length GAP1\textsuperscript{IP4BP} possesses, besides the predicted RasGAP activity, a robust RapGAP activity with estimated $k_{\text{cat}}$ of 16 sec$^{-1}$ and $K_M$ of 42 µM, corresponding to values observed for Rap1GAP, a Rap specific GAP, which has a $k_{\text{cat}}$ of 5.6 sec$^{-1}$ and $K_M$ of 56 µM (Brinkmann et al., 2002).

RapGAP activity was only displayed by GAP1\textsuperscript{IP4BP}\textsuperscript{FL/WT} (chapter 3.2.2). The isolated GAP domain of GAP1\textsuperscript{IP4BP} (GAP1\textsuperscript{IP4BP}\textsubscript{291-569}) was not sufficient to enhance the GTP hydrolysis activity on Rap. Using 100 nM GAP1\textsuperscript{IP4BP}\textsubscript{291-569} no stimulation of the Rap GTPase reaction could be observed, while with 20 µM GAP1\textsuperscript{IP4BP}\textsubscript{291-569} an inefficient RapGAP activity could be detected and reached a $k_{\text{obs}}$ value of only 0.02 sec$^{-1}$ (chapter 3.2.1). Furthermore, it could be shown that additional C-terminal domains are still not sufficient to stimulate the GTPase reaction towards Rap as the construct comprising the GAP domain and the PH/BtK domain (GAP1\textsuperscript{IP4BP}\textsubscript{291-834}) showed no significant RapGAP activity. In the presence of 10 µM GAP1\textsuperscript{IP4BP}\textsubscript{291-834} and 800 µM Rap-GTP the $k_{\text{obs}}$ of the reaction reached a value of only 0.048 sec$^{-1}$ (chapter 3.2.3).

These data clearly indicate that other regions of GAP1\textsuperscript{IP4BP} are necessary to stimulate the GTPase reaction on Rap and that an efficient RapGAP activity requires structural elements beyond the RasGAP domain, one possibility being that both N- and C-terminal regions that surround the central GAP domain might be required to support an efficient catalytic activity towards Rap.

The time resolved FTIR spectroscopy data presented in this study support this hypothesis as even at high protein concentrations, the GAP domain construct was still not able to elicit an efficient RapGAP activity. This data can, therefore suggest that the inefficient RapGAP activity of the GAP domain is not attributed solely to a weak binding to Rap but is most probably due to a less effective catalysis.
4.3 The dual specificity and activity of GAP1\textsuperscript{IP4BP}

Members of the different branches of the Ras superfamily are regulated by different sets of GAPs which are unrelated by sequence and specific for a single small G protein (Scheffzek and Ahmadian, 2005). However, the majority of GAPs for the sGNBP within each group are related. Compared to other known GTPase-activating proteins that are specific for only a single small G protein, full-length GAP1\textsuperscript{IP4BP} has similar activity towards the individual G proteins Ras and Rap, but unlike the Ras-specific GAPs, neurofibromin and p120GAP, and the Rap-specific GAP, Rap1GAP, GAP1\textsuperscript{IP4BP} retains both of these activities within one molecular entity. This protein has therefore dual Ras and Rap GAP activity. For the dual Ras and RapGAP activity of GAP1\textsuperscript{IP4BP}, regions outside the RasGAP domain are required as the isolated GAP domain (GAP1\textsuperscript{IP4BP}\textsubscript{291-569}) retains a pronounced RasGAP activity but has no observable activity towards Rap. Additional C-terminal regions do not participate in the GAP1\textsuperscript{IP4BP} mediated RapGTP hydrolysis as the construct GAP1\textsuperscript{IP4BP}\textsubscript{291-834} comprising the central GAP domain and the C-terminal PH/Btk motifs is not able to elicit an efficient RapGAP activity.

Furthermore, full-length protein had a five-fold lower \(K_M\) for Rap (42 \(\mu\)M) versus Ras (213 \(\mu\)M), which indicates that the additional domain(s) not only affect the catalytic efficiency of the RapGAP reaction but also dramatically enhance the affinity towards Rap-GTP.

Typically, the basic mechanism of the GAP-mediated GTPase reaction for most Ras-like proteins relies on two main residues. A glutamine located on the sGNBP participates in catalysis, while the so-called arginine finger is usually supplied by the GAP into the GTPase active site stabilizing the catalytic machinery. Mutation of the glutamine in Ras, Rho and Ran constitutively activates the G protein and almost completely abolishes the hydrolysis reaction (Bos et al., 2007). However, the involvement of an arginine finger varies and can not be universally applied to all GAP systems. While the stimulation of the GTPase reaction on Ras and Rab is dependent on the presence of the arginine finger (Ahmadian et al., 1997c; Albert et al., 1999), RanGAP does not employ an arginine residue (Seewald et al., 2002), and the involvement of an arginine finger in the ArfGAP catalyzed reaction is still under debate (Goldberg, 1999).

In contrast to most other small G proteins, Rap1 does not possess a catalytic glutamine but a threonine residue at the equivalent position. This substitution reduces the intrinsic GTP hydrolysis rate (Frech et al., 1990). The threonine residue is also not required for the GAP
Discussion

stimulated GTP hydrolysis on Rap1. Furthermore, Rap1GAP does not act via an arginine finger but provides instead an asparagine for catalysis (Daumke et al., 2004).

In this study, the GAP1IP4BP stimulated GTPase reaction towards Ras and Rap was analyzed using the arginine finger mutant (GAP1IP4BR371A). The mutation not only eliminated the RasGAP activity, but also severely affected the RapGAP activity (Figure 36). The importance of an arginine residue in the GAP mediated GTP hydrolysis of Rap is surprising as no arginine residue was described to be involved in the Rap1GAP-stimulated hydrolysis mechanism (Daumke et al., 2004). This result indicates that the basic machinery for GAP1IP4BP activation seems to reside within the GAP domain and that the mechanism for RapGAP activity of this protein seems to be different from both the RasGAP and Rap1GAP mechanisms. Furthermore, several site-directed mutants, targeting residues within the predicted Ras-binding region of the GAP domain have shown that Rap associates with GAP1IP4BP through the conserved Ras binding site (Kupzig et al., 2006). These results argue that GAP1IP4BP can not function simultaneously as a Ras and a Rap GAP and will only stimulate the GTPase activity of either Ras or Rap.

It is known that glutamine 61 of Ras plays a crucial role in catalysis and its mutation prevents the RasGAP-mediated reaction (Ahmadian et al., 1999; Der et al., 1986; Krengel et al., 1990; Sprang, 1997a), while Rap1GAP stimulates the GTPase reaction on Rap independently of a catalytic glutamine (Daumke et al., 2004; Brinkmann et al., 2002). When a threonine was introduced in Ras at position 61 instead of glutamine, the mutation drastically affected the RasGAP-mediated reaction of GAP1IP4FL/WT and GAP1IP4291-569 argued that glutamine 61 plays a critical role in the hydrolysis mechanism (Figures 38 and 39). The canonical mutation threonine 61 to glutamine in Rap reduced the affinity for the full-length GAP1IP4BP but the catalysis was more efficient for the Rap mutant then for wt-Rap (Figures 38). The activity of the GAP domain construct, GAP1IP4BP291-569, towards the mutant RapT61Q (Figure 39) was more efficient than for wt-Rap which shows that the inefficient hydrolysis stimulated by the GAP domain alone towards Rap-wt is at least partially due to the absence of a glutamine residue.

Another amino acid that is important for GTP hydrolysis is glycine 12 in the P-loop. In most cases, mutations at the third position of the P-loop can not be down-regulated by GAPs since any other side chain at this position will interfere with the geometry of the catalytic glutamine and arginine finger in the transition state (Scheffzek et al., 1997; Rittinger et al., 1997). However, Rap1GAP can down-regulate Rap1G12V (Brinkmann et al., 2002). Another example is the small G protein RheB which has an arginine residue
instead of a glycine in position 12 but can still be inactivated by the Rap1GAP homolog, Tuberin (Tee et al., 2003). As shown from the structure of the Rap-RapGAP complex (Scrima et al., In press) the catalytic asparagine in Rap1GAP adopts a different conformation than the arginine finger in the Ras-RasGAP system and takes over the role of cis-glutamine of Ras, Rho or Ran.

In contrast to Rap1GAP which is able to efficiently down-regulate RapG12V, GAP1IP4BP shows a 40-fold reduced GAP activity at a concentration of 50 µM RapG12V (Figure 37). The inefficiency of GAP1IP4BP to down-regulate RapG12V suggests once more that GAP1IP4BP employs a different reaction mechanism for the stimulation of the GTPase reaction on Rap as Rap1GAP.

A common mechanism for the GAP-stimulated GTP hydrolysis involves three major components that are essential in all sGNBP-GAP systems described. First of all, the GTPase reaction, like any other biological phosphoryl transfer reaction, is strictly dependent on the presence of Mg\(^{2+}\) ions which act as cofactors in both the GDP- and GTP-bound states and counterbalance the negative charge of the nucleotide (Sprang, 1997a). The second element that appears conserved in all systems described is the phosphate binding loop or P-loop which has the most important contribution to high-affinity binding of the nucleotide and plays also a role in catalysis (Saraste et al., 1990). Its totally conserved lysine (K16 in Ras) creates a positively charged environment for the negatively charged phosphate groups of the nucleotide. The serine/threonine residue following the lysine coordinates the Mg\(^{2+}\) ion. The third components are the GTPase-activating proteins which act in two ways to cause rate acceleration. GAPs stabilize the switch II region primarily to position a catalytic glutamine side chain and its associated nucleophilic water molecule ready to attack the $\gamma$-phosphate (Scheffzek and Ahmadian, 2005). The catalytic glutamine residue is present in small G proteins and in the $\alpha$-subunit of the heterotrimeric G proteins, but is absent in Rap1 and Sar1. In the case of Rap1, an asparagine provided from the GAP positions the water molecule (Daumke et al., 2004). In Sar1, a small G protein required for vesicle formation from the ER, a histidine residue replaces the glutamine and is responsible for positioning the water molecule (Bi et al., 2002). In case of the signal recognition particle and its receptor, aspartate residues position the water molecules close to the $\gamma$-phosphate of the bound nucleotide (Egea et al., 2004; Focia et al., 2004). Furthermore, GAPs for Ras, Rho and Rab supply an arginine residue into the active
site whose positive charge neutralizes the developing negative charges during the hydrolysis reaction. However, Rap1 and Ran are not assisted in the stimulation of their respective GTP-hydrolysis by a catalytic arginine (Daumke et al., 2004; Seewald et al., 2002).

Compared to the fact that positioning a water molecule in the proximity of the nucleotide is a principle likely to apply for all G proteins, the presence of an arginine finger in the GAP-assisted GTP hydrolysis can not be regarded as universally applicable.

The biochemical data presented in this study evidently indicates that GAP1IP4BP stimulates the GTPase reaction equally well on both small G proteins Ras and Rap and the stimulation of the GTPase reaction of Rap is clearly different from the Rap1GAP-catalyzed reaction. However, several aspects concerning the RapGAP activity of GAP1IP4BP are not yet understood and await further structural and biochemical analyses. GAP1IP4BP does not contain any sequence homology with any other known RapGAP and does not contain a typical RapGAP domain. While both Ras and RapGAP activities of GAP1IP4BP seem to be dependent on the presence of the arginine finger, it is not yet understood whether GAP1IP4BP employs a catalytic asparagine to stimulate the GTPase reaction of Rap, as no conserved asparagines could be detected close to the catalytic site.

However, it is clear that whilst full-length GAP1IP4BP is able to stimulate the hydrolysis reaction on both G proteins with same efficiency a construct comprising the central GAP domain shows only activity towards Ras. Furthermore, a C-terminal extended construct encompassing apart from the GAP domain the PH/Btk motifs was also not able to elicit a significant activity towards Rap.

It might be speculated that other domains are involved in stimulating an efficient Ras and RapGAP activity. Indeed for other GAP molecules there is evidence that additional domains are required to specifically regulate the interaction with the G proteins and to support the catalytic activity. In the case of p120 RasGAP, the N-terminal PH domain was suggested to bind to the C-terminal catalytic domain and inhibit its GAP activity (Drugan et al., 2000). For the Rho/Rac/CDC42GAP oligophrenin-1 it has been shown that the N-terminal part of the molecule inhibits its RhoGAP activity in fibroblasts (Fauchereau et al., 2003). It has been proposed that the N- and C-terminal of p50 RhoGAP are involved in an intramolecular interaction that restricts the accessibility of the GAP domain (Moskwa et al., 2005). Therefore, suggesting an intramolecular interaction involved in the regulation of a GAP protein is not unprecedented. The extra domains of GAP1IP4BP might mediate an
intramolecular interaction and serve to regulate the binding and the activity towards Ras and Rap.

It has been shown that the RapGAP activity of SynGAP, a brain specific GAP, requires its C2 domain as a key determinant of its catalytic activity (Pena et al., unpublished). However, in a preliminary GAP activity assay, a construct of GAP1IP4BP comprising the GAP domain and the N-terminal C2 domains was not sufficient to stimulate the GTPase reaction of Rap (data not shown).

C2 domains are modular signaling domains that can induce membrane-protein or protein-protein interactions, after binding of several Ca\(^{2+}\) ions (Nalefski and Falke, 1996). Ca\(^{2+}\) binding is mediated by a series of conserved aspartate residues that line pockets on one end of each of the C2A and C2B domains (Sutton et al., 1995; Fernandez et al., 2001). Upon Ca\(^{2+}\) influx the C2A and C2B domains penetrate the plasma membrane, resulting in the local induction of membrane curvature (Hui et al., 2006; Herrick et al., 2006). For synaptotagmin-1, the multiple C2 domain module promotes membrane fusion by Ca\(^{2+}\)-dependent induction of membrane curvature, explaining how synaptotagmin-1 couples Ca\(^{2+}\) entry into a synapse to vesicle fusion (Martens et al., 2007). The promotion of membrane fusion by local induction of membrane curvature stress by C2 domain-containing proteins may be a widespread phenomenon (Martens et al., 2007).

Furthermore, Ca\(^{2+}\) induces the translocation of CAPRI and RASAL to the plasma membrane. However, both proteins decode Ca\(^{2+}\) differently: RASAL follows Ca\(^{2+}\) fluctuations linearly by repetitively oscillating between membrane and cytosol, whereas CAPRI remains associated with the membrane after a pulse of Ca\(^{2+}\). While cytosolic, CAPRI is an inactive RasGAP. Ca\(^{2+}\)-triggered recruitment of CAPRI to the plasma membrane seems to switch on its RasGAP activity, such that, once associated with the membrane, CAPRI reduces the Ras-GTP levels and inhibits subsequent activation of the ERK/MAPK pathway (Lockyer et al., 2001).

There are C2 domains that do not bind Ca\(^{2+}\) but constitutively bind to a membrane, others that might be involved in Ca\(^{2+}\) independent protein-protein interactions, and some that might bind inositol phosphates.

It has been shown that p120 RasGAP can be recruited to the plasma membrane after an elevation in [Ca\(^{2+}\)], translocation that occurs through its C2 domain (Gawler et al., 1995; Chow et al., 1999). However, p120 RasGAP C2 domain contains none of the aspartate residues that are involved in interacting with the Ca\(^{2+}\) ions, being proposed that p120
RasGAP might be regulated indirectly by Ca$^{2+}$ ions through its association with the Ca$^{2+}$ sensor, annexin VI (Davis et al., 1996; Chow et al., 1999). Similarly, the C2A and C2B domains of GAP1<sup>IP4BP</sup> do not contain the conserved C2 motif that is involved in the interaction with Ca$^{2+}$ ions.

However, GAP1<sup>IP4BP</sup> has been proposed to be regulated by InsP<sub>4</sub>, a putative second messenger that is generated directly from InsP<sub>3</sub> by a Ca$^{2+}$-regulated Ins(1,4,5)P<sub>3</sub> 3-kinase. Therefore, GAP1<sup>IP4BP</sup> might function as an InsP<sub>4</sub> regulated RasGAP that determines the level of PLC-dependent Ras activation after cellular stimulation by agonists that generate InsP<sub>3</sub> (Cullen, 1998). It is presently unknown whether Ca$^{2+}$ signals might affect the Ras and RapGAP activity of GAP1<sup>IP4BP</sup> and further studies should be devoted to validate the potential regulation of GAP1<sup>IP4BP</sup> by Ca$^{2+}$ ions.

Another mechanism involved in the regulation of GAPs is based on protein-lipid interactions. The catalytic activity of several GAPs is reportedly inhibited or stimulated by various phospholipids in vitro, indicating a likely physiological role for lipids in regulating small G proteins. The RasGAP activity of p120 RasGAP and NF1 is inhibited by various acidic phospholipids and fatty acids (Serth et al., 1991; Tsai et al., 1989). The catalytic function of the RacGAP, α1-chimaerin, is inhibited by some phospholipids and stimulated by others (Ahmed et al., 1995). Binding of DAG to the C1 domain of β2-chimaerin results in the translocation of the protein to the plasma membrane and in a direct enhancement of its GAP activity (Caloca et al., 2003). In the crystal structure of full-length β2-chimaerin the DAG-binding site is partially covered by the N terminus of the protein, which seems to extend into the binding site for Rac in the GAP domain (Canagarajah et al., 2004). The binding of DAG is proposed to release this block of the active site. Several GAPs for the Arf G proteins depend on phosphoinositides and on liposomes for GAP activity (Miura et al., 2002; Kam et al., 2000; Nie et al., 2002). Phospholipids strongly influence the GAP activity of the p190 RhoGAPs (p190A and p190B), which regulate both G proteins Rho and Rac (Ligeti et al., 2004).

As previously mentioned, GAP1<sup>IP4BP</sup> has the ability to bind phosphoinositides like IP<sub>4</sub>, PIP<sub>2</sub> and PIP<sub>3</sub> (Cozier et al., 2000b). Binding of IP<sub>4</sub> occurs through the same site within the PH domain that binds PIP<sub>2</sub>. It is therefore argued that binding of IP<sub>4</sub> to the PH domain serves to remove the inhibitory influence of PIP<sub>2</sub>, thereby allowing activation of the RasGAP activity. The subtleties of this regulation are not fully understood and whether
specific changes in the selectivity for phosphoinositides regulate the RapGAP activity of this protein remains to be established. However, it is clear that small differences in the ability of the PH domain to associate with distinct phosphoinositides has a pronounced effect on the localization and regulation of GAP1\textsuperscript{IP4BP}.

At present it can not be defined what additional factors, such as phosphorylation, association with particular phospholipids or other protein-protein interactions, modulate the preference of GAP1\textsuperscript{IP4BP} for either Ras or RapGAP activity. However, it is tempting to speculate that one or more of these modulation mechanisms may differentially regulate the activity of GAP1\textsuperscript{IP4BP} and will have an important role on the physiological function of this protein.

An obvious question is why do cells require such dual activity? Rap1 which was originally identified as an antagonist of Ras signaling is currently shown to function independently in a number of signaling pathways. The characterization of proteins that display dual Ras and RapGAP activity is important as it would suggest a relationship between the two Ras and Rap signaling pathways.
5 Summary

The main aim of this study was the biochemical characterization of the GTPase activating protein GAP1\textsuperscript{IP4BP} and the elucidation of the mechanism by which GAP1\textsuperscript{IP4BP} functions as both Ras and Rap GAP.

As a member of the GAP1 family of Ras GTPase-activating proteins (RasGAPs), GAP1\textsuperscript{IP4BP} has high sequence homology to other RasGAPs, but no sequence homology to any known RapGAP. Despite its sequence homology to RasGAPs, GAP1\textsuperscript{IP4BP} possesses besides the predicted RasGAP activity, an unexpected GAP activity on the Ras-related protein Rap.

The mechanism by which Ras and RapGAPs enhance the GTPase activity of their respective G proteins is distinct. In the case of the Ras-RasGAP system, RasGAP inserts a catalytic arginine into the active site of Ras and stabilizes the position of the critical glutamine 61 of Ras which in turn positions the water molecule for optimal nucleophilic attack on the $\gamma$-phosphate of the GTP. In addition, the catalytic arginine neutralizes the negative charges that develop during phosphoryl transfer. In contrast, Rap does not possess a glutamine residue that is essential for the GAP mediated GTP hydrolysis in nearly all other small G proteins. Furthermore, RapGAPs are not related to other GAPs and do not employ a catalytic arginine residue as a crucial element for catalysis. Instead, the reaction involves an asparagine residue that inserts into the active site of Rap and it is proposed to compensate for the missing catalytic glutamine.

In this work, the biochemical activity and specificity of GAP1\textsuperscript{IP4BP} towards the small G proteins Ras and Rap, its domains and residues participating in the reaction and the mechanism of the GAP1\textsuperscript{IP4BP}-mediated hydrolysis towards Ras and Rap were analyzed. A series of constructs comprising different domains of GAP1\textsuperscript{IP4BP} were cloned, expressed using an \textit{E. coli} or a baculovirus expression system and the soluble proteins were further purified. The GTPase activity towards Ras and Rap was investigated by kinetic and mutational analyses together with time-resolved FTIR spectroscopy. GAP1\textsuperscript{IP4BP}\textsubscript{FL/WT} and two other constructs GAP1\textsuperscript{IP4BP}\textsubscript{279-569} and GAP1\textsuperscript{IP4BP}\textsubscript{291-834} yielded soluble proteins and could be purified as biochemically active GTPase-activating proteins. The activation of the GTPase reaction stimulated by these proteins was analyzed using the charcoal assay. It could be shown that full-length, recombinant GAP1\textsuperscript{IP4BP} possesses a robust Ras and Rap GAP activity, with estimated $k_{\text{cats}}$ of 16 and 48 sec$^{-1}$ and $K_M$ of 42 and 213 M$^{-1}$ for Rap and Ras, respectively. The $k_{\text{cats}}$ values of the GAP1\textsuperscript{IP4BP}-stimulated
reaction on Ras and Rap are similar to those of p120GAP, neurofibromin, Rap1GAP, RhoGAP, RanGAP and RabGAP which all have $k_{cat}$ values between 5-20 sec$^{-1}$.

The isolated GAP domain of GAP1$_{IP4BP}$ (GAP1$_{IP4BP}^{291-569}$) showed a pronounced RasGAP activity, but under similar conditions no stimulation of the RapGAP activity could be detected. Time-resolved FTIR spectroscopy proved that the acceleration of the GTPase reaction on Ras in the presence of the GAP domain alone is as fast as with full-length GAP1$_{IP4BP}$ (0.024 sec$^{-1}$ for the GAP domain, 0.028 sec$^{-1}$ for GAP1$_{IP4BP}^{FL/WT}$). Furthermore, it was shown that the acceleration of the GTPase on Rap in the presence of the wild-type protein is at least one order of magnitude faster compared with the GAP domain alone.

A site-directed mutant targeting the conserved catalytic arginine, GAP1$_{IP4BP}^{FL,R371A}$, was unable to stimulate the GTPase activity towards Ras, this data being entirely consistent with GAP1$_{IP4BP}$ functioning as a RasGAP and, furthermore, validating the arginine finger-based catalysis mechanism of GAP1$_{IP4BP}$ towards Ras. Using a concentration of 5 nM GAP1$_{IP4BP}^{FL,R371A}$, it could be estimated that the arginine finger mutant is at least 20-fold less active on Ras-GTP than the wild-type. Interestingly, a large drop of at least 30-fold in the RapGAP activity could also be observed when the GTPase reaction on Rap was analyzed using GAP1$_{IP4BP}^{FL,R371A}$. This result suggests that GAP1$_{IP4BP}$ activity towards Rap involves a different mechanism than the Rap1GAP reaction and that the basic machinery for both RasGAP and RapGAP activations seems to reside within the GAP domain involving arginine 371.

RapGAP activity was only displayed by GAP1$_{IP4BP}^{FL/WT}$. The isolated GAP domain of GAP1$_{IP4BP}$ (GAP1$_{IP4BP}^{291-569}$) was not sufficient to enhance the GTP hydrolysis activity on Rap. It could be shown that additional C-terminal domains are still not sufficient to stimulate an efficient GTPase reaction towards Rap. It is thus evident that other regions of GAP1$_{IP4BP}$ are necessary and that an efficient RapGAP activity requires structural elements beyond the RasGAP domain. One possibility is that both N- and C-terminal regions that surround the central GAP domain are required to support an efficient catalytic activity towards Rap. The time-resolved FTIR spectroscopy data presented in this study support this hypothesis as even at high protein concentrations, the GAP domain construct was still not able to elicit an efficient RapGAP activity. This data suggest that the inefficient RapGAP activity of the GAP domain is not attributed solely to a weak binding to Rap but is most probably due to a less effective catalysis.

A glutamine to threonine mutation in Ras at position 61 drastically affects the RasGAP-mediated reaction of GAP1$_{IP4BP}^{FL/WT}$ and GAP1$_{IP4BP}^{291-569}$, arguing that glutamine 61 plays
a critical role in the hydrolysis mechanism. The GTPase reaction was at least 25-fold reduced in the presence of RasQ61T-GTP than in the presence of wt-Ras. Nevertheless, this result is surprising, considering that GAP1IP4BP stimulates the GTPase reaction on Rap presumably independently of a catalytic glutamine. The canonical mutation threonine 61 to glutamine in Rap reduced the affinity for the full-length GAP1IP4BP but the catalysis was more efficient. Using Rap-T61Q mutant in the presence of the GAP domain, the k_{obs} of the reaction reached a value of 0.5 sec^{-1} at 400 µM Rap-T61Q-GTP, which is only approximately 10-fold lower than for Ras. The more efficient activity of the GAP domain construct, GAP1IP4BP_{291-569}, towards the mutant RapT61Q shows that the inefficient hydrolysis stimulated by the GAP domain alone towards Rap-wt is at least partially due to the absence of a glutamine residue. When the GAP activity of GAP1IP4BP towards RapG12V was analyzed, a 40-fold reduced GAP-catalyzed GTPase activity could be observed at a concentration of 50 µM RapG12V-GTP, indicating that the G12V mutation interferes with the GAP1IP4BP catalyzed GTPase reaction. The inefficiency of GAP1IP4BP to down-regulate RapG12V suggests once more that GAP1IP4BP employs a different reaction mechanism for the stimulation of the GTPase reaction on Rap as Rap1GAP.

The apparent affinities of GAP1IP4BP_{Fl/WT} for active Ras and Rap determined by fluorescence approaches were 60 µM and 42 µM for Ras and Rap respectively, indicating that GAP1IP4BP_{Fl/WT} binds active Rap with a somewhat higher affinity than Ras. Furthermore, full-length protein had a five-fold lower Km for Rap (42 µM) versus Ras (213 µM), which indicates that the additional domain(s) not only affect the catalytic efficiency of the RapGAP reaction but also dramatically enhance the affinity towards Rap-GTP.

In conclusion, biochemical data presented in this study evidently indicate that GAP1IP4BP stimulates the GTPase reaction equally well on both small G-proteins Ras and Rap. Furthermore, it could be shown that the stimulation of the GTPase reaction of Rap is clearly different from the Rap1GAP-catalyzed reaction. Additional regions of GAP1IP4BP seem to be required for an efficient dual Ras and RapGAP activity and several factors such as phosphorylation, association with particular phospholipids or other protein-protein interactions might serve to regulate the binding and the activity towards Ras and Rap. However, the final understanding of the modulation mechanisms that may differentially regulate the activity of GAP1IP4BP awaits further structural and biochemical analyses.
6 References


Ref Type: Generic


Ref Type: Generic


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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Å</td>
<td>Ångström (0.1 nm)</td>
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<td>DTE</td>
<td>1,4-Dithioerythritol</td>
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<td>GAP</td>
<td>GTPase-activating Protein</td>
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<td>GDP, GTP</td>
<td>GTP Guanosine-5’-diphosphate, Guanosine-5’-triphosphate</td>
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<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>GNBP</td>
<td>Guanine nucleotide binding Protein</td>
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<td>GppNHp</td>
<td>Guanosine-5’-(β,γ-imido)-triphosphate</td>
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<td>Tween-20</td>
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For amino acids, the one and three letter code was used:

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Curriculum vitae

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June 2003 - August 2007: Doctoral studies with Prof. Dr. Alfred Wittinghofer in the Department of Structural Biology at the Max Planck Institute of Molecular Physiology in Dortmund. Topic: Biochemical characterization of the GTPase-activating protein GAP1^IP4BP

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Publications


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