The Catalytic Mechanism of Rho GTPase-Activating Protein

A Thesis Submitted to the University of London for the Degree of Doctor of Philosophy

By

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Rho guanine nucleotide binding proteins are downregulated by an intrinsic GTPase, which is enhanced by GTPase-activating proteins (GAPs). Rho-GAPs contain a single conserved arginine residue within their catalytic domain, that has been proposed to be involved in the catalytic GTP hydrolysis mechanism. The role of this arginine in RhoGAP has been elucidated by mutagenesis followed by determination of catalytic and equilibrium binding constants. The turnover numbers for wild-type, R282A and R282K RhoGAPs were 5s\(^{-1}\), 0.025s\(^{-1}\) and 0.010s\(^{-1}\), respectively. Thus, the function of this arginine could not be replaced by lysine or alanine. Nevertheless, the R282A mutation had a minimal effect on binding affinity of RhoGAP to Rho.GMPPNP, which confirms the importance of the arginine residue for catalysis as opposed to formation of the protein:protein complex.

Aluminium fluoride enhances binding of the GDP-bound form of small G proteins to their GAPs. These complexes have been interpreted as being analogues of the transition state of the hydrolytic reaction. The catalytic arginine has been shown to have a role in coordinating and stabilising these analogues. In the presence of aluminium fluoride, R282A RhoGAP binds almost as well as wild-type to Rho.GDP, demonstrating that Arg 282 is not required for the interaction. In addition, the affinity of wild-type RhoGAP for the triphosphate form is similar to that for Rho.GDP with aluminium fluoride. These observations suggest that proposed transition state analogue formed in solution does not have the anticipated properties of the true transition state.

Additionally, it has been observed that, in the absence of aluminium, magnesium can support fluoride-mediated complex formation between Rho.GDP and RhoGAP. Quantitative information on the requirements for both aluminium- and magnesium-dependent fluoride-mediated effects has been obtained. This has led to a high resolution crystal structure of Rho.GDP complexed with RhoGAP and magnesium fluoride, with the magnesium adopting an unusual configuration.
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ABBREVIATIONS

The abbreviations used are those described in the Biochemical Journal except for the following:

\[ \text{AlF}_x \] aluminium fluoride (exact species not defined)

CRIB Cdc42/Rac-interactive-binding

DH Dbl homology

DNPA 2,4-dinitrophenyl acetate

DTT dithiothreitol

\( G_\alpha \) α subunit of heterotrimeric G protein

GAP GTPase activating protein

GDI guanine nucleotide dissociation inhibitor

GEF guanine nucleotide exchange factor

GMPPNP guanylyl imidodiphosphate

GST glutathione-S-transferase

IPTG isopropyl β-D-thiogalactopyranoside

ITC isothermal titration calorimetry

JNK c-Jun N-terminal kinase

\( K_d \) equilibrium dissociation constant

\( K_a \) equilibrium association constant

L Broth Luria-Bertani medium

LPA lysophosphatidic acid

mant 2′(3′)-O-(N-methylanilino)(y)

MAPK mitogen-activated protein kinase
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<th>Abbreviation</th>
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<tr>
<td>MME</td>
<td>monomethyl ether</td>
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<tr>
<td>NF1</td>
<td>neurofibromin</td>
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<tr>
<td>NF-κB</td>
<td>nuclear transcription factor- κB</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>RBD</td>
<td>Ras-binding domain</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SAX</td>
<td>strong ion exchange</td>
</tr>
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<td>SH2/3</td>
<td>src homology 2/3</td>
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<tr>
<td>SPA</td>
<td>scintillation proximity assay</td>
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<tr>
<td>SRF</td>
<td>serum-response factor</td>
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<tr>
<td>TEAB</td>
<td>triethylammonium bicarbonate</td>
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<tr>
<td>TNFα</td>
<td>tumour necrosis factor-α</td>
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CHAPTER 1

Introduction
1.1 Guanine Nucleotide Binding Proteins (G Proteins)
Many cell signalling pathways that relay signals from cell-surface receptors to downstream targets are regulated by guanine nucleotide binding proteins. Whether the G proteins are heterotrimeric G proteins associated with receptors at the cell surface, or members of the Ras superfamily of G proteins, the key feature that enables them to function as efficient signal transducers is their ability to switch between distinct conformations (GTP-bound and GDP-bound), each possessing different biochemical activities.

1.1.1 The Ras Superfamily of Small G Proteins
Studies of the development of neoplastic disease led to the identification of viral genes capable of inducing neoplastic transformation in animals. The small gene family became known as ras genes due to their identification as those required for the transforming potential of the Harvey and Kirsten rat sarcoma viruses. In 1982 the first human ras genes were identified, H-ras and K-ras, and this was followed by the discovery of a third human tumour oncogene in neuroblastomas, N-ras. These three members of the ras gene family have been identified as active oncogenes in about 25-30% of human tumours including sarcomas, carcinomas, neuroblastomas, leukaemias and lymphomas. A comparison of the nucleotide sequences of the oncogene and proto-oncogene show that transforming properties are induced by single point mutations. Mutations in naturally occurring ras oncogenes have been localised in codons 12, 13 and 61 (reviewed in Barbacid, 1987).

The ras genes belong to a superfamily of genes encoding guanine nucleotide binding proteins with molecular masses of 20-25kDa. Human H-, K- and N-Ras proteins show
80% amino acid sequence identity. More than 50 Ras-related proteins are known to exist and they are divided into families based on the degree of sequence homology. Members of this superfamily include the Ras, Rho, Rab, Ran and Arf families. Ras proteins are involved in the control of cell growth and differentiation (Vojtek and Der, 1998), Rho family proteins (Section 1.2) have roles in cytoskeletal organisation and regulation of gene transcription (Mackay and Hall, 1998), the Rab family regulate secretory and endocytic pathways (Schimmöller et al., 1998), the Ran family are involved in nuclear import and export of proteins and RNA (Moore, 1998) and the Arf family regulate vesicular trafficking pathways (Moss and Vaughan, 1998).

1.1.1.1 The Molecular Switch

Small G proteins of the Ras superfamily are molecular switches that control signalling pathways for a wide variety of cellular functions. The cyclical regulatory mechanism relies on the ability of these proteins to undergo a guanine nucleotide-dependent conformational change (Figure 1.1). When bound to GDP, Ras superfamily proteins are in their inactive conformation and are unable to initiate downstream signalling events. A slow intrinsic mechanism exists to cause dissociation of GDP, allowing association of GTP, which is present in the cell at a concentration ten times higher than that of GDP. Guanine nucleotide exchange factors (GEFs), activated by extracellular signals at the surface of the membrane, catalyse this exchange of nucleotide. When bound to GTP, Ras superfamily proteins are in their active conformation and interact with effector proteins, thus initiating downstream signals. They have an intrinsic ability to hydrolyse GTP to GDP, although this activity is slow and in vivo is catalysed by GTPase-activating proteins (GAPs). In the case of the Rho and Rab families, the GDP-bound conformation can be stabilised by interaction with GDP dissociation inhibitors (GDIs).
Although the cyclical regulation of all members of the Ras superfamily is similar, the example shown is for Rho family proteins. All Ras-related proteins are regulated by GAPs and GEFs, however, the Rho and Rab families are also regulated by GDIs. Rho proteins exist in an inactive GDP-bound state complexed to a GDI. In response to an incoming signal, Rho-GDI dissociates and a GEF catalyses nucleotide exchange. When bound to GTP, Rho proteins are in their active conformation and can interact with cellular targets, thus initiating downstream signalling events. The hydrolysis of bound-GTP, which is catalysed by GAPs, causes inactivation and the GDP-bound Rho family protein can then re-associate with the GDI.
1.1.1.2 Three-Dimensional Structure and Sequence Motifs

X-ray structure determination of H-Ras has shown it to contain six strands of β sheet (five parallel and one anti-parallel) and five α helices, connected by ten hydrophilic loops (Brünger et al., 1990; Pai et al., 1990) (Figure 1.2). Rho family proteins share a similar fold although they contain a unique 13 amino acid insertion between β-strand 5 (β5) and α-helix 4 (α4) (Hirshberg et al., 1997; Wei et al., 1997) (Figure 1.2). It has been suggested that the insert may mediate association with the membrane or be involved in effector interactions (Wei et al., 1997). More recently, the insert region in Cdc42 has been shown to be essential for the ability of Cdc42 to stimulate cell growth (Wu et al., 1998; Section 1.2.3).

As the sequences of a number of guanine nucleotide binding proteins became available, common sequence motifs were observed. One sequence motif that is found in many nucleotide binding proteins is GXXXXGK(S/T), which connects a β-strand with an α-helix. In H-Ras, this sequence is found in loop L1, known as the ‘phosphate binding loop’ or ‘P-loop’. It connects β1 and α1, and corresponds to residues 10-17 (\textsuperscript{10}GAGGGVGKS\textsuperscript{17}) (Pai et al., 1989). There are a large number of interactions formed between the P-loop and the phosphate groups of GDP or GTP (Figure 1.3). There are only two weak hydrogen bonds between the α-phosphate and the main chain amide of Ser 17 and Ala 18 (Pai et al., 1990). In comparison, the β-phosphate is hydrogen bonded to the main chain amino groups of residues 13-17 and with the side chain of Lys 16 (Pai et al., 1990). The γ-phosphate is also extensively hydrogen bonded to Lys 16, Tyr 32, Thr 35 and Gly 60 (Pai et al., 1990). The guanine base of the nucleotide is bound by interactions with the conserved sequence motifs NKXD (residues 116-119) and SAK
Figure 1.2 – Structures of Rac.GMPPNP and Ras.GMPPNP

A comparison between the structures of Rac1 and H-Ras bound to GMPPNP. Ribbon diagrams of the overall fold of Rac1 (a) and H-Ras (b), with a yellow ball-and-stick drawing of the nucleotide. The α-helices are shown in green, β-strands in red and 3_10 helices in blue.

(From Hirshberg *et al.*, 1997)
Schematic diagram of the interactions between the GTP analogue, GMPPNP, and residues of H-Ras. All hydrogen bonds (dotted lines) up to a length of 3.1Å have been included. The guanine base is shown to be held between the aliphatic part of the Lys 117 side chain and the tip of the benzene ring of Phe 28 which, in turn, is propped up against the side chain of Lys 147. Lys 117 interacts with the main chain carbonyl of Gly 13 and is also involved in a weaker interaction (not shown) with the oxygen on the ribose ring. The amide of the side chain of Asn 116 links the carbonyl group of Val 14 of the P-loop and the hydroxyl of Thr 144 through hydrogen bonds. The amide side chain is also involved in weaker interaction (not shown) with the guanine ring. The magnesium ion is shown coordinated to the β- and γ-phosphates. mc, main chain.

(From Wittinghofer and Pai, 1991)
(residues 145-147) (Figure 1.3), which are identical in the majority of guanine nucleotide binding proteins (Pai et al., 1990; Tong et al., 1991). Asn 116, Asp 119, Ser 145 and Ala 146 form hydrogen bonds with groups on the guanine ring (Pai et al., 1989, Pai et al., 1990), and Asn 116 also hydrogen bonds with Val 14 and Thr 144. Thus, Asn 116 ties together the three elements that are involved in nucleotide binding: the phosphate binding loop, \(^{10}GXXXXGKS\), the \(^{116}NKXD\) and the \(^{145}SAK\) motifs (Pai et al., 1990). Lys 117 assists, linking the phosphate binding loop and the NKXD motif by bonding to the main chain carbonyl of Gly 13 (Pai et al., 1990).

Unlike the guanine base, which has many strong interactions with the protein, the ribose ring is only weakly bound. Asp 30 forms weak hydrogen bonds with the 2'- and 3'-hydroxyl groups and the 2'-hydroxyl is additionally hydrogen bonded to the main chain carbonyl of Val 29 (Pai et al., 1990). The oxygen on the ribose ring is weakly bonded to Lys 117 (Pai et al., 1990).

A single Mg\(^{2+}\) ion is bound to Ras, which is essential for GTP hydrolysis and tight nucleotide binding (Pai et al., 1990). The Mg\(^{2+}\) is octahedrally coordinated, and in the GMPPNP (Pai et al., 1989; Pai et al., 1990), GTP (Schlichting et al., 1990) and GDP-CP (Brünger et al., 1990) bound forms of Ras, Mg\(^{2+}\) is coordinated by the \(\beta\)- and \(\gamma\)-phosphates, the side chains of Ser 17 and Thr 35, and two axial water molecules. In Rac and Rho the equivalent position of Ser 17 corresponds to a threonine residue (Hirshberg et al., 1997; Wei et al., 1997). Asp 33, Asp 57 and the \(\gamma\)-phosphate interact with the two axial water molecules (Pai et al., 1990). However, in the GDP bound form of Ras the interaction between the Mg\(^{2+}\) and the \(\gamma\)-phosphate is lost and has been reported to be replaced by either a direct coordination to Asp 57 (Schlichting et al., 1990) or a water
molecule (Tong et al., 1991). Thr 35 is no longer close enough to form an interaction with the Mg\(^{2+}\) and is likely replaced by a water molecule (Schlichting et al., 1990).

The structural differences between the 'inactive' and 'active' forms of Ras have been determined by comparing the structures of the protein complexed with either GDP or a GTP analogue (Milburn et al., 1990), or using time-resolved crystallographic analysis (Schlichting et al., 1990). The most dramatic differences are localised in two areas: residues 30 to 38, the switch I region or 'effector loop' in loop L2 and the N-terminus of the adjacent \(\beta\)-strand 2, and residues 60 to 76, the switch II region, consisting of part of \(\beta\)-strand 3, loop L4 and part of \(\alpha\)2. In the switch I region a hydrogen bond between Tyr 32 and Tyr 40 in the GDP complex is broken in the GTP complex and the side chain of Tyr 32 swings out, covering a part of the phosphate pocket of GTP. The interactions of Thr 35 with the \(\gamma\)-phosphate and Mg\(^{2+}\) in the GTP-bound form are lost in the GDP-bound form causing the side chain to change orientation and point away from the protein into the solvent. Also, the side chain orientations of residues 36 and 38 are different in the two forms of Ras. Within the switch II region is the DXXG sequence motif (residues 57-60) which is conserved in all GTPases. In the GTP bound form of Ras the invariant aspartate (Asp 57) binds the catalytic Mg\(^{2+}\) through an intervening water molecule, while Gly 60 forms a hydrogen bond with the \(\gamma\)-phosphate of GTP. The hydrogen bond between Gly 60 and the \(\gamma\)-phosphate is lost in the GDP complex leading to conformational changes in loop L4 (Milburn et al., 1990; Schlichting et al., 1990). These changes have been difficult to evaluate since the electron density for this part of the protein is poorly defined. NMR data has shown that these switch regions are dynamically mobile (Kraulis et al., 1994), consistent with the poorly defined electron density seen in the X-ray structures being due to the high flexibility of the region.
Although many of the above structural elements are very similar between Ras-related proteins, the switch I regions in Rac (Hirshberg et al., 1997) and Rho (Wei et al., 1997) differ considerably from that observed in Ras. In the Rac.GMPPNP structure the switch I region is partially disordered, however, in the Rho.GDP structure switch I is well ordered and adopts a different conformation to that seen in the corresponding Ras complex. As a consequence the manner in which switch I contributes to Mg$^{2+}$ coordination differs in these proteins and may reflect differences in the mechanism of GEF-catalysed nucleotide exchange, which is proposed to disrupt the Mg$^{2+}$ binding site (Sprang, 1997).

1.1.1.3 Mutations

Activation of Ras induces proliferation in many cell types. Mutant forms of Ras are found in 25-30% of human tumours. They have an impaired intrinsic GTPase activity and are insensitive to downregulation by GAPs, thus being unable to switch off the transmitted signal leading to uncontrolled cell growth and differentiation (Bollag and McCormick, 1991). Single point mutations that alter the Ras structure so as to prolong the active GTP-bound form include substitutions at amino acids 12 and 61. Mutating these amino acid residues in Rho family proteins (or those analogous to them; 12 and 61 in Rac and Cdc42; 14 and 63 in Rho) results in the same effect, a constitutively active GTP-bound protein unable to efficiently hydrolyse GTP to GDP.

Position 61 in Ras is one of the main sites of oncogenic mutations. Several structures of Ras containing mutations at position 61 have been solved (Krengel et al., 1990; Privé et al., 1992) although in each case no significant changes in the structure compared to the wild-type protein could be detected. It was suggested by Privé et al. (1992) that Gln 61 may have a role in transition state stabilisation, hence the lack of structural changes seen
in the GMPPNP-bound structures (Krengel et al., 1990; Privé et al., 1992), proposed to represent analogues of the ground state of the GTPase reaction. This was confirmed by the publication of the transition state analogue of Ras in complex with the catalytic domain of the Ras-GAP, p120GAP (Scheffzek et al., 1997). Gln 61 was shown to interact with the hydrolytic water molecule involved in GTP hydrolysis, thus, mutation of this residue leads to destabilisation of the transition state and reduced GTP hydrolysis (Scheffzek et al., 1997).

Several structures of oncogenic Val 12 Ras have also been solved (Krengel et al., 1990; Milburn et al., 1990; Schlichting et al., 1990; Privé et al., 1992). The bulk of the Val side chain causes it to push residues 59-61 further from GTP so that Gly 60 can no longer interact with the γ-phosphate and the catalytic configuration of Gln 61 and the catalytic water is disturbed. Mutations at residue 13 can also lead to similar structural distortions that prevent hydrolysis (Krengel et al., 1990).

The introduction of single amino acid substitutions has been widely used to investigate the molecular mechanisms of Ras superfamily proteins. As already discussed, substitutions at positions 12 or 61 in Ras, Rac and Cdc42 (14 or 63 in Rho) severely reduce the GTPase activity of the protein and, hence, have been used as ‘constitutively active’ proteins. Mutation of residue 17 (19 in Rho) to asparagine has been widely used to generate ‘dominant negative’ proteins that can no longer become activated. It is thought that these proteins remain bound to GEFs, thus sequestering the GEFs and inhibiting activation of any normal protein present (Feig and Cooper, 1988).
1.1.1.4 Post-translational Modifications

Ras-related G proteins undergo a variety of post-translational modifications that are essential for membrane association. Most members of the Ras superfamily are modified by the covalent attachment of isoprenoids to cysteine residues located near the C-terminus of the protein (Brown and Goldstein, 1993). The cysteine motifs are structurally diverse and, to a certain extent, can determine the specificity of the modification. Ras proteins and Rho family proteins end with a motif known as the CAAX box, where C is a cysteine, A is any aliphatic residue and X is usually methionine or serine in Ras and leucine in Rho proteins. The CAAX box of Ras proteins undergoes farnesylation (addition of a C-15 isoprenoid), whilst that of Rho family proteins undergoes modification by addition of a geranylgeranyl moiety (C-20 isoprenoid) (Adamson et al., 1992a; Brown and Goldstein, 1993). An exception to this is RhoB, which can be both farnesylated and geranygeranylated (Adamson et al., 1992a). The enzymes that catalyse these reactions are CAAX farnesyltransferase, and CAAX geranylgeranyl transferase. Following these modifications, the three terminal amino acids from Ras and Rho proteins are removed and the remaining cysteine is methylated on its carboxyl group. Many Ras proteins (a notable exception being isoform B of K-Ras) and RhoB, undergo further modification with the cysteine residues upstream of the CAAX box becoming palmitoylated (Hancock et al., 1991; Adamson et al., 1992b). K-(B)Ras and other members of the Ras and Rho families are not palmitoylated but instead have a stretch of polybasic amino acids close to their CAAX box that is essential for correct localisation (Hancock et al., 1991).
1.1.1.5 Guanine Nucleotide Exchange Factors (GEFs)

GEFs exist for each family of Ras-related proteins and most are multidomain proteins that have catalytic domains of 200-300 residues. The structures of representative GEFs of Ran (RCC1) (Renault et al., 1998), ARF (ARNO) (Cherfils et al., 1998), Ras (SOS) (Boriack-Sjodin et al., 1998), and Rho (βPIX) (Aghazadeh et al., 1998) have been solved. The catalytic domains of the different classes of GEFs show no sequence homology and are structurally unrelated (Cherfils and Chardin, 1999). In the case of the Ras-GEF, SOS, the structure was obtained in the presence of Ras and absence of nucleotide (Boriack-Sjodin et al., 1998), providing some clues regarding the mechanism of guanine nucleotide exchange. It appears that SOS inserts an α-helix into Ras, thereby displacing switch I and opening up the nucleotide binding site. Also, the side chains presented by this helix and by the distorted conformation of switch II create an unfavourable chemical environment for the binding of the phosphate groups and the nucleotide associated Mg$^{2+}$. These factors cause the release of GDP (Boriack-Sjodin et al., 1998).

Rho family proteins are activated by a group of GEFs that are related in sequence to the proto-oncogene Dbl. Each member of this group contains a Dbl homology (DH) domain and also a pleckstrin homology (PH) domain that is located immediately C-terminal to the DH domain. PH domains have been implicated as mediators of both protein:lipid and protein:protein interactions and it has been suggested that these domains may regulate membrane translocation (Musacchio et al., 1993; Lemmon et al., 1996). Removal of this PH domain in Dbl does not result in a loss of GEF activity towards Rho and Cdc42 in an in vitro assay, but does result in a loss in the transforming ability of Dbl, illustrating the importance of the PH domain to Dbl function (Zheng et al., 1996). GEFs for Rho family proteins include the proto-oncogenes, Lbc, Ost, Vav and Ect, that were identified on the
basis of their transforming ability, and the proteins Tiam-1, βPIX and FGD1 (reviewed in Whitehead et al., 1997). Recently the structure of βPIX has been published which, together with mutagenesis studies, has revealed the GTPase interaction site to be at the C-terminus of the DH domain (Aghazadeh et al., 1998). It remains to be seen whether the GEF-catalysed mechanism of nucleotide release for Rho family proteins is similar to that seen with Ras.

1.1.1.6 Guanine Nucleotide Dissociation Inhibitors (GDIs)

GDIs exist for the Rho and Rab families of small G proteins, although there is no significant homology between RhoGDIs and RabGDIs. Interaction between GDIs and Rho or Rab family proteins requires post-translational modification of the small G protein. Properties reported for these proteins include the formation of stable complexes with the GDP-forms of their respective small G protein partners (Sasaki et al., 1990; Ueda et al., 1990) and presentation of the G proteins to membranes (Abo et al., 1994; Ullrich et al., 1994). Only a small number of GDIs exist and the best characterised GDI for Rho family proteins is RhoGDI (Ueda et al., 1990). RhoGDI is ubiquitously expressed and interacts with Rho, Rac and Cdc42. Structural studies of RhoGDI (Keep et al., 1997) have shown it to consist of an ordered domain that is responsible for binding to the isoprenyl group of the post-translationally modified small G protein, and a flexible N-terminal region that is essential for the Rac-binding function of RhoGDI. It has been suggested that the N-terminal domain may interact with the P-loop of the small G protein, preventing dissociation of the nucleotide (Keep et al., 1997). RhoGDIs appear to play a crucial role in the translocation of Rho family proteins from the cytosol to the membrane. In resting neutrophils Rac is tightly bound to RhoGDI in the cytosol, and
activation of Rac is associated with the release of GDI and translocation to the membrane (Abo et al., 1994).

1.1.1.7 GTPase-Activating Proteins (GAPs)

During microinjection studies to determine the role of guanine nucleotides in Ras function, Trahey and McCormick (1987) noticed that Ras was predominantly GDP-bound due to a ‘dramatic stimulation’ of Ras-associated GTPase activity. The cytosolic protein responsible for this increased activity, now known as p120GAP, was shown to stimulate GTP hydrolysis by normal Ras in vitro, but had no effect on oncogenic Ras mutants (Trahey and McCormick, 1987). Since the discovery of this first GAP, many more proteins with GAP activity towards members of the Ras, Rho, Rab, Ran and Arf families of small G proteins have been described. Since a member of the Rho-GAP family was the subject of investigations described in this thesis, members of this family will be described in more detail below (Section 1.3). Although the members of each family of GAPs show high sequence homology, GAPs from different families only have limited or no homology (Ahmed et al., 1994; Scheffzek et al., 1998; Sermon et al., 1998; Ahmadian et al., 1998). The first structural determination of a GAP was of the catalytic domain of p120GAP (Scheffzek et al., 1996). This was closely followed by the structure of the catalytic domain of RhoGAP (Barrett et al., 1997). A comparison of these two structures has shown that, although there is little sequence homology, there are some structural similarities, and it has been suggested that they may have evolved from a common ancestor (Bax, 1998; Rittinger et al., 1998). The mechanism of GAP-activated GTP hydrolysis is discussed in Section 1.5.
1.1.2 Heterotrimeric G Proteins

Many transmembrane signalling processes caused by extracellular hormones and neurotransmitters are mediated by receptors that interact with heterotrimeric G proteins. Heterotrimeric G proteins are made up of one α, one β and one γ subunit. At least 20 distinct α, 6 β and 12 γ subunits have been identified. The β and γ subunits form a functional dimer (Gpγ) that is tightly associated and only dissociated by denaturation (Hamm, 1998). The α subunit contains two domains, a domain involved in binding and hydrolysing GTP (the G domain) that is structurally closely related to the Ras superfamily of G proteins (Kjeldgaard et al., 1996), and a helical domain that buries the GTP in the core of the protein (Noel et al., 1993; Coleman et al., 1994). Both the α and the γ subunit contain sites for lipid modification that allow attachment to the membrane. The G protein is in an inactive conformation when the α, β and γ subunits exist as a trimer with GDP bound to Gα. When an activated receptor interacts with Gαβγ, it induces nucleotide exchange so that GDP is released from the Gα subunit, and GTP then binds. The conformational change associated with GTP binding causes Gα.GTP to dissociate from Gpγ, allowing both to interact with distinct effector molecules. Gα acts as a GTPase and hydrolysates the bound GTP to GDP, thereby causing inactivation and re-association with Gpγ (Hamm, 1998).

Heterotrimeric G proteins are potently activated by aluminium fluoride, as first described by Sternweis and Gilman (1982). Initially, aluminium fluoride was thought to be tetrahedrally coordinated and bound to GDP, thus acting as an analogue of the γ phosphate (Bigay et al., 1985; Bigay et al., 1987). However, the X-ray structures of G10.GDP.AlF4− and G111.GDP.AlF4− revealed that the bound AlF4− was octahedrally coordinated, with the fluorines present in a planar configuration (Sondek et al., 1994;
Coleman et al., 1994). By analogy to the transition state of the phosphoryl transfer reaction, in which the phosphate adopts a trigonal bipyramidal arrangement, this configuration led to the proposal that these G\textsubscript{\alpha} complexes were in fact transition state analogues (Sondek et al., 1994; Coleman et al., 1994). These structures provided information on the mechanism of GTP hydrolysis by heterotrimeric G proteins (Section 1.4).

As well as GAPs for small G proteins, GAPs for heterotrimeric G\textsubscript{\alpha} subunits also exist and are known as RGS proteins (Regulators of G Protein Signalling) (Dohlman and Thorner, 1997; Berman and Gilman, 1998). There at least 16 family members and they are able to increase the basal rate of GTP hydrolysis by 50-fold. RGS proteins are unrelated in primary sequence to GAPs for members of the Ras superfamily of small G proteins (Dohlman and Thorner, 1997).

1.2 Rho Family Proteins

Rho family proteins have around 30% amino acid sequence identity to Ras proteins and 50% identity to each other. At least fourteen genes encoding mammalian Rho family members have been identified, and many homologous genes have been isolated from other species. The mammalian Rho family consists of RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, Cdc42, RhoD, RhoE, RhoG, RhoH, TC10, Rnd1 and Rnd2 (Aspenström, 1999). Rac1, RhoA and Cdc42 are the best characterised. Throughout this thesis Rac1 is referred to as Rac and RhoA as Rho. Rho family proteins are involved in a wide variety of cellular activities including organisation of the cytoskeleton, actin reorganisation in cell adhesion, cell cycle progression, endocytosis, and the mediation of gene transcription.
(reviewed in Mackay and Hall, 1998). Rac also has a role in the activation of an NADPH oxidase in phagocytic cells (Abo et al., 1991).

1.2.1 Rho Family Proteins and the Cytoskeleton

The first indications of the biological function of Rho proteins came from the use of the C3 exoenzyme produced by Clostridium botulinum, which ADP-ribosylates Rho family proteins (Sekine et al., 1989; Didsbury et al., 1989). ADP-ribosylation of Rho occurs on asparagine 41 (Sekine et al., 1989) and has the specific effect of preventing downstream signals (Paterson et al., 1990). The introduction of C3 transferase into a variety of cell types produced morphological changes, causing cells to lose their actin stress fibres and round up (Rubin et al., 1988; Chardin et al., 1989; Paterson et al., 1990). Microinjection of recombinant Rho or plasmids expressing rho cDNAs into a variety of cells had the opposite effect of inducing rapid changes in actin organisation leading to formation of stress fibres (Paterson et al., 1990). This provided evidence suggesting a role for Rho proteins in the regulation of cytoskeletal organisation.

Using constitutively activated forms of Rho, Rac and Cdc42, and utilising the inhibitory properties of C3 transferase as tools, the involvement of these small G proteins in regulation of the organisation of the actin cytoskeleton has been elucidated. In serum-starved Swiss 3T3 fibroblasts Rho rapidly stimulated formation of actin stress fibres and focal adhesions (Ridley and Hall, 1992). Addition of the extracellular factors, lysophosphatidic acid (LPA) and bombesin, produced a similar response, which led to the conclusion that Rho acts as a molecular switch to control a signal transduction pathway that links membrane receptors to the cytoskeleton (Ridley and Hall, 1992). Rac was the next family member to be studied and it was found to stimulate membrane ruffling and
lamellipodia in rat fibroblasts and serum starved Swiss 3T3 cells (Ridley et al., 1992). These effects were also induced by platelet-derived growth factor (PDGF) and insulin (Ridley et al., 1992). In addition, a later effect involved formation of actin stress fibres and focal adhesions. C3 transferase eliminated formation of stress fibres but not membrane ruffles indicating that Rho acts downstream of Rac (Ridley et al., 1992). Later, it was shown that Cdc42 induced filopodium formation in fibroblasts and macrophages and this effect was also induced by bradykinin (Kozma et al., 1995; Allen et al., 1997). Microinjection of Cdc42 also stimulated the formation of lamellipodia, a factor found to be attributable to the downstream activation of Rac by Cdc42 (Kozma et al., 1995; Allen et al., 1997).

1.2.2 Rho Family Proteins and Gene Transcription

It has been observed that Rho, Rac and Cdc42 mediate signalling pathways upstream of transcriptional activation. Expression of constitutively activated forms of Rac and Cdc42 in a range of cell types results in stimulation of c-Jun N-terminal kinase (JNK) and p38 kinase, both mitogen-activated protein kinases (MAPKs) (Coso et al., 1995; Minden et al., 1995). MAPKs are serine/threonine kinases that are activated upon stimulation of a variety of cell surface receptors, and their function is to convert extracellular stimuli to intracellular signals controlling the expression of genes essential for many cellular processes, including cell growth and differentiation. It has also been suggested that this Rac/Cdc42-mediated activation may not occur in all cell types, as Rho and Cdc42, but not Rac, have been shown to activate JNK in human kidney cells (Teramoto et al., 1996b). Rho, Rac and Cdc42 have also been shown to activate serum-response factor (SRF)-dependent transcription (Hill et al., 1995). In this pathway, the effects of Rho are mediated by LPA and inhibited by C3 transferase, whilst Rac and Cdc42 appear to
activate the pathway independently of Rho. Therefore, it appears that Rho regulates the activation by a different mechanism to Rac and Cdc42, with the two pathways converging at some point to result in activation of SRF-regulated genes (Hill et al., 1995). Rho, Rac and Cdc42 can also activate the nuclear transcription factor, κB (NF-κB) (Sulciner et al., 1996; Perona et al., 1997). Activation of NF-κB is stimulated by a variety of agents, including viruses, tumour necrosis factor-α (TNFα) and UV light. Activation of NF-κB by TNFα is dependent on Cdc42 and Rho, but not on Rac, whereas activation by UV light is not dependent on these Rho family proteins (Perona et al., 1997). Rac has been shown to regulate reactive oxygen species (ROS) in fibroblasts (Sundaresan et al., 1996) and it has been suggested that the generation of ROS by Rac may lead to activation of NF-κB, as it is known that NF-κB stimuli can also induce ROS production (Sulciner et al., 1996).

1.2.3 Rho Family Proteins and Cell Growth Control

A potential role for Rho family proteins in cell growth control was initially suggested by the rapidly expanding number of oncogenes that encode exchange factors for Rho family members (Whitehead et al., 1997). More recently, Rho, Rac and Cdc42 have been shown to stimulate progression through the G1 stage of the cell cycle and subsequent DNA synthesis, whereas the dominant negative forms of these proteins block stimulation of DNA synthesis in response to serum (Olson et al., 1995).

Ridley et al. (1992) showed that oncogenic Ras could activate Rac, and further investigation revealed that Rac and Rho are essential for Ras-induced transformation (Khosravi-Far et al., 1995; Prendergast et al., 1995; Qiu et al., 1995). One well-characterised signal transduction pathway involving Ras is the activation of the
serine/threonine kinase, Raf, which in turn activates a MAPK cascade leading to activation of transcription factors and cellular proliferation. Unregulated stimulation of this pathway by oncogenic Ras has a role in cellular transformation (reviewed in Vojtek and Der, 1998). Dominant negative mutants of Rac and Rho were shown to inhibit Ras transforming activity, but not the transforming activity of activated Raf. Also, Rac and Rho caused a synergistic enhancement of the transforming activity seen with activated Raf. These data are consistent with the idea that a number of pathways contribute to Ras-induced transformation, and both the Raf/MAPK and Rac/Rho pathways are required for full transformation effects (Prendergast *et al.*, 1995; Qiu *et al.*, 1995). More recently, a role for Cdc42 in cell transformation has been established, although it is not clear by what mechanism this transformation takes place (Lin *et al.*, 1997). However, the insert region present in Rho family proteins that is non-essential for many known signalling pathways initiated by Cdc42 has been shown to be essential for cell growth and the transforming ability of Cdc42 (Wu *et al.*, 1998).

It is not yet clear whether stimulation of G1 progression and cell transformation are due to effects on the actin cytoskeleton or on gene transcription, although they correlate well with the ability of the small G proteins to induce cytoskeletal effects (Joneson *et al.*, 1996; Lamarche *et al.*, 1996).

### 1.2.4 Effectors for Rho Family Proteins

In order to understand the mechanisms by which Rho family proteins control these diverse cellular processes, it is important to identify proteins that associate with them. The yeast two-hybrid system and biochemical affinity purification methods have been used extensively to identify a large number of interacting proteins for Rho, Rac and
Cdc42 (Table 1.1). Information is still lacking, however, on the specific role of each effector in cellular signalling pathways. The number of effector proteins is likely to be a consequence of the numerous biological processes controlled by these small G proteins.

It should be noted that some effectors have specific tissue and cell type distribution and not all are present in any particular cell. Rac and Cdc42 each have a number of effector proteins, some of which are common to both. The first Rac target identified was the serine/threonine kinase PAK (p21-activated kinase), that also interacts with Cdc42 (Manser et al., 1994). There are at least three isoforms of PAK in mammalian cells and they have been ascribed a role in activation of JNK and actin regulation (Sells and Chernoff, 1997). Activation of JNK has also been shown to occur via the Cdc42- and Rac-interacting mixed lineage kinases (MLK2 and MLK3) (Van Aelst and D’Souza-Schorey, 1997; Nagata et al., 1998). However, mutant Rac and Cdc42 proteins that still stimulate JNK, cannot bind either PAK or MLK. A recently discovered Rac target, known as POSH, may act as a bridge between Rac and PAK or MLK in the activation of JNK, as the mutant Rac protein that is able to stimulate JNK but does not bind PAK or MLK, can bind to POSH (Tapon et al., 1998). POR1, another Rac binding protein, has been implicated in Rac-induced lamellipodia formation but its precise role is unknown. Truncated forms of POR1 abolish Rac-mediated membrane ruffling suggesting that POR1 is a downstream target of Rac in the membrane ruffling pathway (Van Aelst et al., 1996). One Cdc42-specific effector that has been reported to be involved in rearrangement of actin is WASP (Symons et al., 1996), and a related protein, N-WASP has been shown to be essential for filopodia formation (Miki et al., 1998). Human mutations in WASP cause severe defects in some cell functions, for example, they cause
Table 1.1 – Rho Family Target Proteins

This table shows some of the proteins that bind to either Rho, Rac or Cdc42, which may represent downstream effector molecules for these small G proteins. Where it has been identified, the effector function is indicated.

+, ability to bind to the corresponding small G protein; -, inability to bind to the corresponding small G protein

ACK, activated Cdc42 kinase; PAK, p21-activated kinase; MRCK, myotonic dystrophy kinase-related Cdc42-binding kinase; MLK, mixed lineage kinase; MEKK, MAP kinase kinase kinase; pp70S6k, 70kDa ribosomal S6 kinase; PI3K, phosphatidylinositol 3-kinase; CIP4, Cdc42-interacting protein 4; MSE, marrow stromal/endothelial cell protein; PORI, partner of Rac; POSH, plenty of SH3 domains; p67PHOX, p67 subunit of NADPH oxidase; WASP, Wiskott-Aldrich syndrome protein; PRK/PKN, protein kinase N; ROK, rho-kinase; ROCK, Rho-associated coiled-coil kinase; MBS, myosin binding subunit of the myosin light-chain phosphatase; PIP5K, phosphatidylinositol 4-phosphate 5-kinase, PIP2; phosphatidylinositol 4,5-bisphosphate.
<table>
<thead>
<tr>
<th>Effector</th>
<th>Function</th>
<th>Rho</th>
<th>Rac</th>
<th>Cdc42</th>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>Ishizaki et al., 1996; Nakagawa et al., 1996; Ishizaki et al., 1997</td>
</tr>
<tr>
<td>Kinectin</td>
<td>Kinesin binding</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>MBS</td>
<td>MLC phosphorylation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Kimura et al., 1996</td>
</tr>
<tr>
<td>p140mDia</td>
<td>Actin organisation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Watanabe et al., 1997</td>
</tr>
<tr>
<td>Rhophilin</td>
<td>Unknown</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Watanabe et al., 1996</td>
</tr>
<tr>
<td>Rhotekin</td>
<td>Unknown</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Reid et al., 1996</td>
</tr>
<tr>
<td>PIP5K</td>
<td>Regulates formation of PIP2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Tolias et al., 1995</td>
</tr>
</tbody>
</table>
an immunodeficiency syndrome resulting from distorted microvilli on T lymphocytes and lack of chemotactic properties of macrophages and monocytes (Aspenström, 1999). One further effector protein for both Cdc42 and Rac is IQGAP, which appears to be needed for the formation of Cdc42- and Rac-induced cell-cell contacts (Kuroda et al., 1998).

Targets for Rho include a family of Rho kinases that have been shown to induce stress-fibre formation and assembly of focal adhesions (Leung et al., 1996). Substrates that have been identified for these kinases are the myosin binding subunit of the myosin phosphatase (MBC) and myosin light chain (MLC), which both regulate the activity of myosin filament assembly and actin bundling, leading to stress fibre formation (Van Aelst and D’Souza-Schorey, 1997). Rho has also been shown to regulate the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2), a lipid that has been shown to bind to and regulate a number of actin-binding proteins (Toker, 1998).

Recently, the NMR structures of Cdc42 bound to either the GTPase-binding domain of WASP (Abdul-Manan et al., 1999) or the tyrosine kinase ACK (Mott et al., 1999) have been solved. Both WASP and ACK are Cdc42-specific effectors, although they contain a region common to both Cdc42- and Rac-binding proteins, known as the CRIB (Cdc42/Rac-interactive-binding) motif. Both structures show that this motif is involved in extending the β-sheet of Cdc42 by an additional strand, causing interactions with the effector loop of Cdc42. This interaction is similar to that seen in the structures of Rap1a (a Ras family protein) bound to the Ras-binding domain (RBD) from Raf-1 (Nassar et al., 1995) and Ras complexed with the RBD from RalGDS (Huang et al., 1998). Both Raf-1 and RalGDS interact by forming an intermolecular β-sheet with the G protein. However, the interactions outside the CRIB motif seen in the Cdc42/ACK structure are unlike those
seen in the Ras complexes and involve extensive interactions with residues within the C-terminal α-helix and the switch II region of the protein (Mott et al., 1999). Although WASP also interacts with these regions of Cdc42, the interactions are different (Abdul-Manan et al., 1999) and these differences have been predicted to determine the specificity of these effectors (Abdul-Manan et al., 1999; Mott et al., 1999).

1.3 Rho GTPase-Activating Proteins (Rho-GAPs)

There is a large family of sequence related proteins that possess activity towards Rho family proteins. The first GAP activity discovered for Rho family proteins (Garrett et al., 1989) was found to be due to the protein, RhoGAP (also known as p50rhoGAP or Cdc42GAP). RhoGAP was first identified as a 29-kDa protein present in a crude cytoplasmic extract of human spleen and was shown to interact with and increase the GTPase activity of Rho (Garrett et al., 1989). RhoGAP was also found to be present in cytoplasmic extracts of a variety of mammalian cells and Xenopus oocytes (Garrett et al., 1989). Other groups described almost identically sized proteins which were either isolated from bovine adrenal glands with GAP activity towards Rho (Morii et al., 1991) or from human platelets with activity towards Cdc42 (Hart et al., 1991). Isolation of a human RhoGAP cDNA revealed that the short protein originally observed was the carboxyl terminal proteolytic fragment of the full length 50kDa protein (Barfod et al., 1993; Lancaster et al., 1994). These two groups almost simultaneously cloned this GAP protein, one group naming it Cdc42GAP (Barfod et al., 1993) and the other naming it RhoGAP (Lancaster et al., 1994). RhoGAP was found to have activity towards Rho, Rac and Cdc42 (Barfod et al., 1993; Lancaster et al., 1994).
The catalytic domain of RhoGAP is situated towards the carboxy terminus of the protein and extends over approximately 140 amino acids (Barfod et al., 1993; Lancaster et al., 1994). Sequence analyses have identified several other proteins containing a Rho-GAP domain (Table 1.2) with the domains showing between 20 and 40% amino acid identity (Lamarche and Hall, 1994). The substrate specificity of these GAP proteins towards Rho, Rac and Cdc42 is varied (Table 1.2). In the case of the regulatory subunit of phosphatidylinositol 3-kinase, p85, the presence of a Rho-GAP domain does not confer GAP activity towards Rho, Rac or Cdc42, although p85 can bind to Rac and Cdc42 (Zheng et al., 1994).

As well as a GAP domain there are several other domains that are common to some members of the Rho-GAP family (Figure 1.4). The presence of these additional domains suggests that these proteins are multifunctional. Many of the Rho-GAP proteins contain proline-rich sequences that are known to interact with Src homology 3 (SH3) domains that were first identified as conserved sequences in Src-related non-receptor tyrosine kinases. SH3 domains have since been identified in a variety of signalling molecules, although their function is still not clear. RhoGAP has been shown to interact with the SH3 domains of c-Src and p85 (Barfod et al., 1993), whereas 3BP-1 interacts with the SH3 domain of Abl-tyrosine kinase (Cicchetti et al., 1995). However, it is not known whether these interactions occur in vivo. Bcr and Abr contain domains that have sequence homology to the Dbl family of GEFs, and these proteins have both GAP and GEF activity towards Rho family proteins (Chuang et al., 1995). DdRacGAP in D. dictostelium also contains a Dbl domain although no GEF activity has so far been reported (Ludbrook et al., 1997). Additionally, Bcr has phosphotransferase activity due
Table 1.2 – GTPase-Activating Proteins for Rho Family Proteins

This table lists a number of proteins containing a domain homologous to the catalytic domain of RhoGAP. Their specificity towards Rho, Rac or Cdc42 is also indicated.

**Key**

+ ability to stimulate the intrinsic GTPase activity of the corresponding species specific protein

- inability to stimulate the intrinsic GTPase activity of the corresponding species specific protein

ND not determined
<table>
<thead>
<tr>
<th>Protein containing Rho-GAP Domain</th>
<th>Organism</th>
<th>Rho</th>
<th>Rac</th>
<th>Cdc42</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>RhoGAP</td>
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<td>+</td>
<td>+</td>
<td>Barford et al., 1993; Lancaster et al., 1994</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>Diekmann et al., 1991</td>
</tr>
<tr>
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<td>+</td>
<td>Lamarche-Vane and Hall, 1998</td>
</tr>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>Leung et al., 1993</td>
</tr>
<tr>
<td>n-chimaerin</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>Diekmann et al., 1991</td>
</tr>
<tr>
<td>GRAF</td>
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<td>ND</td>
<td>+</td>
<td>Hildebrand et al., 1996</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>Touré et al., 1998</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>Reinhard et al., 1995</td>
</tr>
<tr>
<td>Myr7</td>
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<td>+</td>
<td>-</td>
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<td>Oligophrenin-1</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>p85α</td>
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<td>-</td>
<td>-</td>
<td>Otsu et al., 1991</td>
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<tr>
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<td>Mammalian</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Settleman et al., 1992b</td>
</tr>
<tr>
<td>3BP-1</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Bem3</td>
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<td>+</td>
<td>ND</td>
<td>+</td>
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</tr>
<tr>
<td>CeGAP</td>
<td>C.elegans</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Chen et al., 1994</td>
</tr>
<tr>
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<td>D.dictostelium</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>Ludbrook et al., 1997</td>
</tr>
</tbody>
</table>
Figure 1.4 – Domain Structure of Proteins Containing a Rho-GAP Domain

A schematic drawing of the domain structure of proteins containing a Rho-GAP domain. The length of the protein sequence and the domains are drawn roughly to scale with their Rho-GAP domains shown directly in line with one another. Homologous domains are indicated by their colour and identified at the bottom of the figure.
to the presence of a serine/threonine kinase domain (Maru and Witte, 1991). Cysteine rich sequences are found in a number of Rho-GAP proteins and in \( n \)-chimaerin this sequence has been shown to be involved in lipid binding (Ahmed et al., 1993). Myr5 (Reinhard et al., 1995) and myr7 (Chieregatti et al., 1998) are class 9 unconventional myosins that have characteristic motor and tail domains thought to be involved in the directed movement of myosin along actin filaments. However, they also contain sequence elements not previously seen in myosins: an N-terminal extension, an insertion in the tail domain, and a Rho-GAP domain. It has been suggested that myr5 and myr7 regulate the activity of Rho in neurones (Chieregatti et al., 1998). The ZPH domain present in PARG1 is not present in any of the other Rho-GAPs identified so far, and although it has identity to the gene product of the \textit{C.elegans} gene \textit{ZK669}, its function is unknown (Saras et al., 1997). p190 was first identified as a tyrosine-phosphorylated RasGAP-associated protein, although in addition to the GTPase, or GTP binding domain, responsible for this interaction, p190 was also shown to have a GAP domain with activity towards Rho family proteins (Settleman et al., 1992a). This has implicated p190 as a coupling protein between Ras and Rho family proteins, although the biological importance of this is not yet understood.

In addition to accelerating the intrinsic GTPase activity of small G proteins, it has been proposed that GAPs may also mediate downstream signals. It has been reported that \( n \)-chimaerin has GAP activity towards Rac and Cdc42, and microinjection of its GAP domain into fibroblasts prevents both Rac- and Cdc42-induced cytoskeletal changes (Kozma et al., 1996).
It is not clear why there is a need for such a large, diverse group of proteins containing a Rho-GAP domain. Apart from the tissue-specific chimaerins, most of these proteins appear to be ubiquitously expressed, therefore, in order to ensure that the activity of the Rho family protein is not always switched off, there needs to be a high degree of regulation within the cell. One form of regulation is the substrate specificity of the GAPs and it is likely, from the multifunctional characteristic of the Rho-GAP domain-containing proteins, that another results from protein-protein interactions that regulate their subcellular site of action (Lamarche and Hall, 1994).

1.3.1 Three-dimensional Structure of RhoGAP

The structure of the catalytic domain of RhoGAP alone (Barrett et al., 1997; Figure 1.5), and in complex with either Cdc42.GMPPNP (Rittinger et al., 1997a), Rho.GDP and AlF4⁻ (Rittinger et al., 1997b) or Cdc42.GDP and AlF3 (Nassar et al., 1998) shows that the molecule is extensively α-helical. There are nine α-helices with the core of the structure being formed by helices A, B, E, and F, which form a four-helix bundle. Helices A and B are separated by a 38-residue segment containing a short α-helix, A1, and extending from the core structure at the opposite side to A1 is a hairpin made up of helices C and D. The molecule possesses two large faces with a shallow pocket formed between the B and F helices on one of the faces (Barrett et al., 1997). There is an abundance of conserved residues associated with this pocket (Figure 1.6) and this is the primary region of contact between RhoGAP and Rho family proteins (Rittinger et al., 1997a). The Rho family protein contacts RhoGAP through its switch I and II regions, and the P-loop. The crystal structure of the RhoGAP-homology domain of p85α has also been solved and, similarly, shows an extensively helical molecule with a four-helix bundle in the core of the structure (Musacchio et al., 1996).
Figure 1.5 – Structure of RhoGAP

Ribbon representation of the catalytic domain of RhoGAP. The helices are labelled from the N to C terminus as A0 to G. There are nine helical segments and two short helical turns (labelled E’ and G’ in the top figure), all of which are shown with the starting and ending residues numbered. Two short regions of $3_{10}$ helix between helices A1 and B, and helices C and D, are shown in green.

Top figure - the molecule is viewed from the side looking into the B-F face

Bottom figure - the molecule is viewed from the top showing the four helical bundle formed between helices A, B, E and F.

(From Barrett et al., 1997)
Figure 1.6 - Sequence Alignment of Six Members of the Rho-GAP Family

Sequence alignment of six proteins containing a Rho-GAP domain. Residues identical in all six family members are shown in red, with residues identical in at least four of the proteins shown in blue. The helical segments are shown in yellow. The numbering relates to the residues present in the fragment of RhoGAP used in these studies, such that the first residue of the fragment corresponds to residue 198 in full-length RhoGAP.

(From Barrett et al., 1997)
Although there is little sequence homology between Rho-GAPs and Ras-GAPs, their G-protein binding sites are similar in terms of the binding surface primarily involving two adjacent α-helices (Gamblin and Smerdon, 1998).

1.3.2 Sequence Comparisons of Rho-GAP Proteins

Analysis of sequence conservation amongst proteins containing a Rho-GAP domain shows that there are ten identical residues (shown in red in Figure 1.6) and 42 that are semi-conserved (shown in blue) (Barrett et al., 1997). These two categories of residues are shown with the same colour coding in the space-filling model (Figure 1.7; Barrett et al., 1997). As previously mentioned, there is an abundance of conserved residues located within the B and F helices, now known to be the primary region of contact between RhoGAP and Rho family proteins (Rittinger et al., 1997a). Of the totally conserved and semi-conserved residues situated within the Rho-GAP domain of RhoGAP, Arg 282 (Arg 85 when numbered from the first residue of the crystallized catalytic fragment) was proposed to be involved in binding to Rho family proteins and Asn 391 (Asn 194 in fragment) was proposed to be involved in the catalytic function of RhoGAP (Barrett et al., 1997). Arg 282 is situated in the A-A1 loop and Asn 194 is in helix F (Barrett et al., 1997). From deletion studies and through comparison to the conserved arginine required for maximum functioning of Ras-GAPs, the arginine residue had also previously been implicated as an important residue required for GAP activity (Ahmed et al., 1994).

1.4 Mechanism of GTP Hydrolysis by G Proteins

The lifetime of the ‘activated’ GTP-bound form of G proteins is limited due to their intrinsic ability to hydrolyse GTP to GDP. The turnover rates differ between families of G proteins, with Gα having a $k_{cat}$ of ~2-4 min$^{-1}$ (Bourne et al., 1991) compared to
Figure 1.7 – **Space-filling Model of the Rho-GAP Domain of RhoGAP**

Space-filling model of the Rho-GAP domain of RhoGAP viewed in the same orientation as in Figure 1.5 (top figure). In accordance with Figure 1.6, identical residues are shown in red and highly conserved residues in blue. Arg 85, Lys 122 and Asn 194 (corresponding to Arg 282, Lys 315 and Asp 391 of the full-length protein) are labelled.

(From Barrett et al., 1997)
<0.03 min\(^{-1}\) for Ras and Rho family proteins (Eccleston et al., 1993; Sermon et al., 1998; Zhang and Zheng, 1998; Graham et al., 1999a). Feuerstein et al. (1989) demonstrated that GTP hydrolysis by Ras involved an inversion of the configuration of the \(\gamma\)-phosphate, leading them to suggest that the mechanism is a single step, in-line transfer of the terminal phosphate from the GTP to water. Since this mechanism involves the formation of a pentacoordinate transition state, where the nucleophile and the leaving group occupy the apical positions of the trigonal bipyramid, the nucleophilic water must attack from the opposite side of the leaving group, the \(\beta\)-phosphate. The X-ray structure of Ras (Pai et al., 1990) identified the presence of this water molecule. The location of the water between the \(\gamma\)-phosphate and the side chain of Gln 61, together with the knowledge that mutations of Gln 61 decrease the GTPase rate, led to the suggestion that this residue plays an important role in catalysis by acting as a general base for the nucleophilic water molecule (Pai et al., 1990). The role of the general base would be to activate the water for nucleophilic attack by abstracting a proton. The corresponding glutamine residue in Rac and Cdc42 (Gln 61), Rho (Gln 63) and \(G_{i\alpha}\) (Gln 204), at the amino-terminus of switch II, is essential for the efficient catalytic function of these G proteins. Mutations of this residue in both \(G_{\alpha}\) and Ras-related proteins, largely abolish hydrolytic activity and thus stabilise the GTP-bound state (Der et al., 1986; Coleman et al., 1994). Gln 61 mutations in Ras are a common cause of cellular transformation (Barbacid, 1987) and mutations of the corresponding glutamine in \(G_{s\alpha}\) and \(G_{i\alpha}\) are associated with human endocrine tumours (Landis et al., 1989; Lyons et al., 1990). However, Privé et al. (1992) noted that mutations at position 61 in Ras do not alter the position of the attacking water molecule and suggested that Gln was too weak a base to abstract a proton. Schweins et al. (1995) went on to show that catalysis is substrate-assisted, with the catalytic base being the \(\gamma\)-phosphate. Also, substitution of the bound nucleotide-associated Mg\(^{2+}\) with
Mn$^{2+}$ was shown to increase the hydrolysis rate, most likely by increasing the basicity of the $\gamma$-phosphate (Schweins et al., 1997).

As mentioned (section 1.1.2), $G_\alpha$ proteins, in the GDP-bound form, bind AlF$_4^-$ into the $\gamma$-phosphate binding pocket and thereby stimulate the actions of $G_\alpha$ proteins in their GTP-bound state. In contrast, Ras and Ras-related proteins are not able to bind aluminium fluoride in the absence of any interacting protein (Kahn, 1991). The structures of $G_\alpha$ proteins in the presence of GDP and AlF$_4^-$ have shown that AlF$_4^-$ mimics the transition state of the reaction by forming an octahedral arrangement, where the AlF$_4^-$ coordinates with the fluorines in a planar configuration, and the $\beta$-phosphate oxygen and attacking water molecule are the axial ligands (Coleman et al., 1994; Sondek et al., 1994). By analogy to the transition state of the phosphoryl transfer reaction, in which the $\gamma$-phosphate adopts a trigonal bipyramidal arrangement, this configuration led to the proposal that the complex was an analogue of the transition state (Coleman et al., 1994; Sondek et al., 1994). Comparisons of the $G_{a11}.\text{GTP}_\gamma\text{S}$ and $G_{a11}.\text{GDP.AlF}_4^-$ structures (Coleman et al., 1994), revealed that two residues, Gln 204 and Arg 178 are reoriented and contact the nucleotide only in the transition state analogue. Arg 178 forms hydrogen bonds with two of the fluoride atoms and interacts with the $\alpha$- and $\beta$- phosphate oxygens of GDP, and Gln 204 interacts with a fluoride atom and the axial water molecule, demonstrating that both residues have a role to play in stabilising the transition state for GTP hydrolysis (Coleman et al., 1994). Mutation or cholera toxin-induced ADP-ribosylation of this arginine residue, which is conserved in $G_\alpha$ proteins, impairs GTP hydrolysis and such mutations in $G_{sa}$ and $G_{ta}$ are associated with human tumours (Landis et al., 1989; Lyons et al., 1990).
Although structural studies have shown that $G_\alpha$ and Ras share conserved sequence elements of guanine nucleotide binding proteins around the active site, the invariant arginine found in the active site of $G_\alpha$ proteins is not found in Ras proteins. The absence of this catalytic arginine residue to assist in transition state stabilisation in Ras proteins may be the cause of the approximate 100-fold lower intrinsic GTP hydrolysis rate compared to $G_\alpha$.

1.5 Mechanism of GAP Catalysed GTPase Activity of Ras Superfamily Proteins

The intrinsic GTP hydrolysis rate of Ras family proteins is increased by the interaction with GAPs. The mechanism by which GAPs accelerate the GTPase reaction has been a matter of considerable debate. The issue has been whether Ras superfamily proteins themselves are efficient GTPase devices and GAPs accelerate a rate determining conformational change into a catalytically competent conformation (Neal et al., 1990; Moore et al., 1993), or whether GAPs participate directly in the chemical cleavage step of the reaction by contributing catalytic residues (Mittal et al., 1996; Scheffzek et al., 1996). At the onset of the studies described in this thesis, evidence was gathering to support the latter mechanism.

Although Ras superfamily proteins do not contain conserved arginine residues, the catalytic domain of GAPs for Ras and Rho proteins do contain conserved arginine residues (Scheffzek et al., 1996; Barrett et al., 1997). In the case of Ras-GAPs there are two invariant arginine residues (Arg 789 and Arg 903 in p120GAP; Arg 1276 and Arg 1391 in neurofibromin (NF1)), and in Rho-GAPs there is a single conserved arginine residue (Arg 282 in RhoGAP). By analogy to the sequence of $G_\alpha$ and the mutation of the conserved arginine causing a decrease in the GTP hydrolysis rate (Landis et al., 1989), it
was suggested that a conserved arginine in p120GAP may have such a role (McCormick, 1989). Indeed, mutation of a conserved arginine in p120GAP and NF1 was shown to reduce the activity of these GAPs (Skinner et al., 1991; Brownbridge et al., 1993; Gutmann et al., 1993). The structure of p120GAP (Scheffzek et al., 1996) shows that both invariant arginines are close to one another and it was suggested that Arg 789 was involved in the catalytic function of RasGAP, whilst the other, Arg 903, was involved in stabilising the orientation of this catalytic residue.

Although Ras family proteins do not bind aluminium fluoride in the absence of other interacting proteins (Kahn, 1991), as do Gα subunits (Coleman et al., 1994; Sondek et al., 1994), Ras.GDP was shown to bind aluminium fluoride in the presence of stoichiometric amounts of Ras-GAPs (Mittal et al., 1996). This implied that the catalytic centre of Ras was incomplete unless GAP is present (Mittal et al., 1996). In support of this aluminium fluoride complex between Ras and RasGAP being a transition state complex, as in the case of Gα.GDP.AlF4⁻ (Coleman et al. 1994; Sondek et al., 1994), it was shown that oncogenic mutants of Ras do not form the complex, nor does the RasGAP, NF1, with one of the invariant arginines mutated (Mittal et al., 1996).

1.6 Aims and Scope of this Project

The overall aim of the project was to characterise the mechanism by which RhoGAP stimulates the GTPase activity of Rho.

At the onset of this project there was little published work describing the interaction of RhoGAP and Rho family proteins. Using the Rho-GAP, n-chimaerin, Ahmed et al. (1994) had shown that deletion of the conserved arginine together with the two amino
acids on either side resulted in a mutant defective in GAP activity. Also, mutagenesis studies had been undertaken to examine the role of conserved arginine residues in Ras-GAPs, identifying that mutations of these residues reduced the GAP-catalysed hydrolysis of Ras.GTP (Skinner et al., 1991; Brownbridge et al., 1993; Gutmann et al., 1993). Based on these pieces of evidence it was thought that the conserved arginine (Arg 282) in RhoGAP was likely to be a residue important for its catalytic function.

Shortly after the project had started the structure of the catalytic domain of RhoGAP was published (Barrett et al., 1997). It was proposed that the conserved arginine 282 (residue 85 of Rho-GAP domain) residue was involved in binding to Rho family proteins, whereas asparagine 391 (residue 194 of Rho-GAP domain) was involved in GAP activity due to its absence in p85, a protein lacking GAP activity but containing sequence homology to Rho-GAPs (Barrett et al., 1997).

In order to investigate whether Arg 282 has a role in the binding interaction between RhoGAP and Rho or a role in the catalytic activity of RhoGAP, mutational studies were undertaken. The arginine residue was mutated to alanine and lysine and the interaction of wild-type and mutant RhoGAPs with Rho was extensively studied. Using single turnover techniques, the catalytic rate constants (k_{cat}) for wild-type and mutant RhoGAPs catalysing Rho-GTPase activity were measured. Both the native GTP nucleotide and the fluorescent nucleotide analogue (mantGTP) bound to Rho were used. Similar single turnover experiments have previously been used to characterise the interaction between Ras and wild-type and mutant Ras-GAPs (Eccleston et al., 1993; Ahmadian et al., 1997a; Ahmadian et al., 1997c; Sermon et al., 1998). The binding affinities of wild-type and
mutant RhoGAPs for Rho were measured using the scintillation proximity assay (SPA), isothermal titration calorimetry (ITC) and fluorescence anisotropy.

In addition, biochemical studies utilising SPA, ITC and fluorescence anisotropy techniques were carried out to examine the role of Arg 282 in the formation of the aluminium fluoride-mediated transition state analogue complexes formed between Rho.GDP and RhoGAP. As well as the formation of high affinity aluminium fluoride-mediated complexes between Rho.GDP and RhoGAP, it has been observed that, in the absence of aluminium, magnesium can support fluoride-mediated complex formation. The requirements for both aluminium- and magnesium-dependent fluoride-mediated effects has been quantified. To complement these studies, the structural determination of a complex between Rho.GDP and RhoGAP in the presence of fluoride and magnesium, but absence of aluminium was performed in collaboration with the Protein Structure division at N.I.M.R.
CHAPTER 2

Materials and Methods
2.1 Plasmids

DNA encoding either the C-terminal fragment of RhoGAP (residues 198-439), RhoA or Q63L RhoA had been cloned into pGEX2T plasmids (Pharmacia Biotech) downstream of the glutathione S-transferase tag (Garrett et al., 1989; Lancaster et al., 1994; Self and Hall, 1995a). These pGEX2T-RhoGAP, pGEX2T-RhoA and pGEX2T-Q63L RhoA plasmids were generous gifts from Prof. Alan Hall (University College London, London). Protein derived from either the pGEX2T-RhoA or the pGEX2T-Q63L RhoA plasmids contained a F25N amino acid substitution to improve stability (Paterson et al., 1990; Self and Hall, 1995a).

2.2 Site-Directed Mutagenesis

The conserved arginine 282 residue (numbered from the first residue of the full length RhoGAP (Lancaster et al., 1994)) of GST-RhoGAP was mutated to alanine and lysine using the Stratagene QuikChange™ mutagenesis kit. The oligonucleotides that were used for mutagenesis were purchased from Life Technologies and are shown below, with the mutagenic nucleotides in bold and underlined.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
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<td>5'-GGGCATCTTCCGCAGGTCGGCCAACACCC-3'</td>
</tr>
<tr>
<td>3'-CCCGTAGAAGCCTCCAGCCTGCTGGG-5'</td>
</tr>
<tr>
<td>R282K RhoGAP</td>
</tr>
<tr>
<td>5'-GGGCATCTTTCAAGAGTCCGGCCAACACCC-3'</td>
</tr>
<tr>
<td>3'-CCCGTAGAAGTTCTCCAGCCTGCTGGG-5'</td>
</tr>
</tbody>
</table>

Mutagenesis was confirmed by DNA sequencing performed by the Core Sequencing group (Glaxo Wellcome), and electrospray mass spectrometry of purified protein performed by Steve Graham (Glaxo Wellcome).
2.3 Transformation of *Escherichia coli*

The plasmids containing GST-RhoGAP (wild-type, R282A or R282K) or GST-Rho (wild-type or Q63L) were transformed into competent *Escherichia coli* cells, strains DH5α (Life Technologies) and BL21 (Novagen), respectively. Typically, 1μl of 0.2-0.3μg.ml⁻¹ DNA was added to 10μl of competent *E. coli* cells on ice. After 20min on ice, cells were heat shocked at 42°C for 45 seconds and placed back on ice. 100μl of L Broth (Sambrook *et al.*, 1989) was added and cells were incubated at 37°C, with shaking at 225rpm, for 30min. Cells were plated out onto a pre-warmed agar plate containing 100μg.ml⁻¹ ampicillin and incubated overnight at 37°C. Plates were stored at 4°C as necessary.

A single colony from the plate was used to inoculate 10ml of L Broth containing 200μg.ml⁻¹ ampicillin and cells were grown at 37°C, with shaking at 225rpm, until the A₆₀₀ reached 0.4-0.6. A 1ml aliquot of the culture was mixed with 0.5ml 80% glycerol and frozen at –80°C. These glycerol stocks were then used for inoculation in all protein preparations.

2.4 Expression of GST-fusion Proteins

A 200ml starter culture inoculated with *E.coli*, containing either a pGEX2T-RhoGAP plasmid (wild-type, R282A or R282K) or the pGEX2T-Q63L RhoA plasmid, was grown overnight in L Broth containing 200μg.ml⁻¹ ampicillin at 37°C with shaking at 225rpm. This was used to inoculate 4 litres (8 x 500ml in 2 litre flasks) of prewarmed L Broth containing 200μg.ml⁻¹ ampicillin. Cells were grown at 37°C, with shaking at 225rpm, until the A₆₀₀ reached 0.8. Cultures were then induced for 4hr at 37°C with 1mM isopropyl β-D-thiogalactopyranoside (IPTG). Samples of culture were removed prior to
induction and 4hrs post-induction, and run on a SDS-gel (section 2.6.3) to check expression levels of recombinant protein. A SDS gel showing typical expression levels is shown in Figure 2.1. Centrifugation of the culture was carried out at 5,000g, 4°C for 10min to harvest the cells. The cell pellet was weighed and frozen at −80°C until use. Approximately 20g of cells were obtained using this procedure.

A large scale 40 litre culture of *E.coli* containing the pGEX2T-RhoA plasmid was grown in a fermenter at N.I.M.R by Maggie Goggin. This required a 1 litre starter culture inoculated from a glycerol stock, grown overnight in a 2 litre flask, at 37°C, in L Broth containing 100µg.ml⁻¹ ampicillin. After inoculation with starter culture, cells were grown in NZCYM broth (Sambrook *et al.*, 1989) at 37°C to an OD₆₀₀ of 0.5-0.6 and induced for 3 hours with 0.1mM IPTG. The cells were harvested by centrifugation and the 190g pellet stored at −80°C.

### 2.5 Purification of GST-fusion Proteins

Each step of the following purification procedure was carried out at 4°C unless specified. To purify recombinant RhoGAP (wild-type, R282A or R282K), the entire cell pellet from the 4 litre culture was first thawed and the cells were resuspended in an equal volume of 10mM Tris/HCl pH 7.5, 50mM NaCl, 0.1mM EDTA, 1mM DTT (buffer A) containing 1mM phenylmethylsulphonyl fluoride (PMSF). Cells were then lysed by sonication, on ice, using a Heat Systems Ultrasonics W-385 Ultra sonicator with a 0.5 inch flat tip probe, set at 50% cycle, 5s cycle time, output control setting 7, and using 3 x 30s pulses. Following each sonication, a 10µl aliquot of the cell suspension was removed. This aliquot was spun for 1min at 10,000g and 1µl of the supernatant was assayed for
Figure 2.1 – SDS-PAGE of Samples Obtained at Various Points of the Protein
Expression and Purification Procedure

Samples were taken at various points during a typical protein expression and purification
procedure of RhoGAP, and analysed on a NOVEX 10% NuPAGE gel (section 2.6.3). Lanes 1 and 14 contain Promega mid-range molecular markers (97.4, 66, 55, 42.7, 40, 31, 21.5, 14.4 kDa) and lanes 2 and 15 contain Sigma Dalton Mark VII-L molecular markers (66, 45, 36, 29, 24, 20, 14.2 kDa). Lane 3 contains a pre-induction sample and lane 4 contains a post-induction sample. The expression of GST-RhoGAP following induction can be seen as a 54kDa band on the SDS gel. Lane 5 shows a sample taken following sonication of the cell pellet, lane 6 shows a sample of the supernatant that was loaded onto the glutathione agarose column and lane 7 shows the eluate from the column. Lanes 8-11 contain thrombin cleaved RhoGAP and lanes 12-13 show two fractions collected during elution with 5mM glutathione.
protein concentration using the Bio-Rad assay (section 2.6.1). The concentration of protein in the supernatant reached a maximum of approximately 50mg.ml\(^{-1}\) after 3 x 30s sonications, at which point cell lysis was considered to be complete.

Cell debris and insoluble materials were removed by centrifugation at 125,000g for 60min. The resulting supernatant was loaded onto a 20ml glutathione agarose column (Sigma Product G4510) equilibrated in buffer A, at a flow rate of 0.5ml.min\(^{-1}\) using a Pharmacia P-1 Pump. The eluent was monitored at 280nm using an LKB 2238 Uvicord SII. Buffer A was then applied to the column at the same flow rate until the A\(_{280}\) had returned to baseline. At this point a 5ml p-aminobenzamidine agarose column (Sigma Product A8332) and a 1ml anti-thrombin III agarose column (Sigma Product A8293) were set up in series with the glutathione agarose column and equilibrated with buffer A supplemented with 2mM CaCl\(_2\). Once equilibrated, the flow rate was reduced to 0.04ml.min\(^{-1}\), and 500 Sigma units of thrombin (Sigma Product T6759) were added to 50ml of buffer A containing CaCl\(_2\). This buffer was applied to the columns overnight and 2.5ml fractions were collected using an LKB 2211 Superrac fraction collector. Buffer A was then used at a flow rate of 0.5ml.min\(^{-1}\) to elute all the cleaved protein from the columns (as visualised by observing the A\(_{280}\)). The glutathione agarose column was washed with buffer A containing 5mM glutathione, adjusted to pH 8.0 with Tris base, to elute the GST portion of the cleaved protein and any uncleaved GST-fusion protein. The protein concentrations of fractions corresponding to peaks visualised by observing the A\(_{280}\), were determined using the Bio-Rad assay (section 2.6.1). The fractions containing cleaved protein were then identified using SDS-PAGE (section 2.6.3). These fractions were pooled and the protein concentration was determined using the Bio-Rad assay (section 2.6.1). If the protein concentration was less than 1mg.ml\(^{-1}\), the pooled fractions
were concentrated in an ultrafiltration cell (Amicon) using a 25mm diameter PM10 ultrafiltration membrane (Amicon) before proceeding to the next purification step. Then, to every 1ml of protein solution (>1mg.ml⁻¹), 0.5g ammonium sulphate was added, on ice, to give 78% saturation, and stirred gently for 20min. Centrifugation at 68,000g for 8min produced a pellet of precipitated protein, which was dissolved in a minimum volume of buffer A. All proteins were then dialysed overnight against 20mM Tris/HCl pH 7.5, 2mM MgCl₂ and 1mM DTT, before being snap frozen in liquid nitrogen and stored at -80°C. When uncleaved GST-fusion proteins were required the thrombin cleavage step was omitted and the GST-fusion protein was eluted from the column with 5mM glutathione, pH8 in buffer A.

Rho proteins (wild-type and Q63L) were also purified using the above method, however, the purification buffer used was 10mM Tris/HCl pH 7.5, 50mM NaCl, 1mM MgCl₂, 1mM DTT and 20μM GDP, rather than buffer A described previously. As for RhoGAP purification, the entire cell pellet was used for Q63L Rho purification. However, for wild-type Rho a 35g portion of the cell pellet obtained from the 40 litre culture was used. Due to the larger amount of cells used for Rho, 2 x 20ml glutathione agarose columns, set up in series, were used for the affinity purification, and 750 Sigma units of thrombin were used for the cleavage step.

RhoGAP proteins were shown to be homogenous by SDS-polyacrylamide gel electrophoresis (section 2.6.3; Figure 2.1). However, wild-type Rho ran with a mobility corresponding to a higher molecular weight than expected and smearing of the band was observed, both of which observations have previously been documented (Self and Hall, 1995a). Although Q63L Rho ran at the anticipated molecular weight the smearing seen
with wild-type Rho was also observed with this mutant. Electrospray mass spectrometry, carried out by Steve Graham (Glaxo Wellcome), showed that all proteins were within +/- 2 mass units of their anticipated molecular weights, which are: Rho 21845Da; Q63L Rho 21830Da; wild-type RhoGAP 27828Da; R282A RhoGAP 27742Da; R282K RhoGAP 27800Da. Typical yields were 5mg of purified protein per litre of culture for wild-type and mutant RhoGAPs and 10mg per litre for Rho.

Gladstone Thompson and Peter Chalk (Glaxo Wellcome), donated purified Q61L Rac and GST-PAK (residues 75-132). These proteins were purified using methods similar to those described above.

2.6 Protein Concentration and Purity Determination

2.6.1 Bio-Rad Protein Assay

A commercially available Coomassie brilliant blue G-250 reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories) was used to determine protein concentrations based on the Bradford dye-binding procedure (Bradford, 1976). The Bio-Rad Protein Assay is based on the colour change of the dye in response to various concentrations of protein. In order to obtain a standard curve, 0-25µg of a bovine serum albumin (BSA) standard from Bio-Rad was added to 0.8ml of water, followed by the addition of 0.2ml Bio-Rad reagent. After 5min at room temperature, the absorbance at 595nm was determined and a standard curve of absorbance versus amount (µg) was plotted. The sample of unknown protein concentration was treated in the same way as for the BSA standard and, assuming the same colour yields as for BSA, the absorbance readings were converted to an equivalent protein concentration using the BSA standard curve.
The Bio-Rad Protein Assay was used during protein purification procedures to estimate protein concentrations.

### 2.6.2 Ultra-violet Spectroscopy

Where precise concentrations were required, the concentration of purified proteins was determined from their absorbance at 280nm and their calculated molar extinction coefficients. All absorbance measurements were carried out on a Perkin Elmer Lambda 7 or 15 UV/Vis Spectrophotometer. Molar extinction coefficients were calculated based on the contributing absorbance at 280nm of tyrosine (1480\( \text{M}^{-1}\text{cm}^{-1} \)) and tryptophan (5540\( \text{M}^{-1}\text{cm}^{-1} \)) residues (Mach et al., 1992) and any guanine nucleotide present (7950\( \text{M}^{-1}\text{cm}^{-1} \)) (Dawson et al., 1994), assuming 1mol of nucleotide bound per mol of protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Extinction coefficient at 280nm (( \text{M}^{-1}\text{cm}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type, R282A or R282K RhoGAP</td>
<td>14420</td>
</tr>
<tr>
<td>GST-RhoGAP</td>
<td>57300</td>
</tr>
<tr>
<td>Wild-type or Q63L RhoA</td>
<td>26430</td>
</tr>
</tbody>
</table>

### 2.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A Hoefer gel apparatus was used with gels measuring 180 x 80 x 0.7mm. The stacking gel consisted of 5% acrylamide, 0.13% methylene bis-acrylamide, 125mM Tris/HCl pH 6.8, 0.1% SDS, 0.01% N, N, N’, N’-tetramethylethylenediamine (TEMED), 1mg.ml\(^{-1}\) ammonium persulphate and the resolving gel contained 19.5% acrylamide, 0.088% methylene bis-acrylamide, 375mM Tris/HCl pH 8.8, 0.1% SDS, 0.005% TEMED and 0.5mg.ml\(^{-1}\) ammonium persulphate. Protein samples were heated to 95°C for 3min in sample buffer containing 100mM Tris/HCl pH 6.8, 1% SDS, 10% glycerol, a trace of bromophenol blue and 1% (v/v) 2-mercaptoethanol. Typically, 10\( \mu l \) of protein sample
(containing 3-5μg protein) was mixed with 10μl sample buffer and the entire sample was loaded onto the gel. Electrophoresis was carried out at 4°C in a running buffer containing 50mM Tris, 384mM glycine, 0.1% SDS (w/v) at a current of 43mA until the bromophenol blue dye front has reached the bottom of the gel (45-60min). NOVEX NuPAGE pre-cast gels (NOVEX, San Diego, California, USA) were also used. Mini gels (NuPAGE Gradient 4-12% or NuPAGE 10% Bis-Tris; 80 x 80 x 1mm) were run in 2-(N-morpholino) ethane sulphonic acid (MES) running buffer in a NOVEX Xcell II Mini-Cell electrophoresis unit according to manufacturers instructions. Typically, 10μl sample containing 2μg of protein was mixed with 2.5μl of 4x NuPAGE sample buffer containing 4% 2-mercaptoethanol before heating at 95°C for 3min. The entire sample was then loaded onto the gel. NuPAGE gels were run at a constant voltage of 200V for approximately 35min.

Following gel electrophoresis, gels were placed in fixing solution comprising 45% (v/v) methanol and 9% (v/v) acetic acid for 10min before being stained for 10min with 0.1% Coomassie blue R250 in fixing solution. Destaining was carried out overnight in 7.5% (v/v) acetic acid, 5% (v/v) methanol. When destaining was complete, gels were soaked in 2% glycerol for 30min before being secured between 2 sheets of cellulose gel drying film (Promega) and allowed to dry at room temperature overnight until ready for storage.

2.7 Synthesis and Purification of Fluorescent Guanine Nucleotide Analogues

Fluorescent 2'(3')-O-(N-methylanthraniloyl) (mant) analogues of GTP (Figure 2.2), GDP and GMPPNP were synthesised according to the method of Hiratsuka (1983), modified by Neal et al. (1990). Nucleotide (0.27mmol) was dissolved in 4ml of water at 37°C.
Figure 2.2 – Structure of MantGTP

The structure of 2'-O-(N-methylanthraniloyl)GTP (mantGTP).
The pH of the solution was adjusted to 9.6 using 1M NaOH. Methylisatoic anhydride (0.4mmol; Molecular Probes) was added and the reaction mixture maintained at pH 9.6 using 1M NaOH whilst being continuously stirred. The reaction was deemed complete when the pH ceased to drop and at this point 1M HCl was added to bring the pH down to 7.6. Unreacted methylisatoic anhydride, its hydrolysis product N-methylanthranilic acid, and nucleotide were separated from the resultant mant nucleotide using an ion exchange DEAE-Cellulose DE52 column in the bicarbonate form (36cm x 3cm). The reaction mixture was diluted to a volume of 20ml with water and was applied to the column at a flow rate of 2ml.min⁻¹. Water (250ml) was applied to the column before a linear gradient of triethylammonium bicarbonate (TEAB) (0M to 0.6M in 3 litres) was used to elute the components of the mixture. The eluent was monitored at 254nm. The elution profile consisted of three peaks that were scanned in a spectrophotometer in order to identify the peak of interest containing the required mant nucleotide. Unreacted methylisatoic anhydride and N-methylanthranilic acid were eluted first, followed by unreacted nucleotide and finally the required mant nucleotide, recognisable due to an absorbance peak at 252nm with a shoulder at 276nm and a smaller peak at 350nm. The mant nucleotides can be identified by calculating the ratio of the absorbance at 252nm to the absorbance at 350nm, which should be 3.96 (Hiratsuka, 1983). The fractions containing mant nucleotide were pooled and concentrated by rotary evaporation. Residual TEAB was removed by repeated washing with methanol and subsequent evaporation. The concentrated product was taken up in water and stored at -20 °C. Typical yields were 25-35%.

The purity of the preparation in terms of the required nucleotide being bound to the mant fluorophore (e.g. mantGTP vs. mantGDP) was determined using HPLC (section 2.9) and
found to be >96% pure. The concentration of the preparation was determined from the molar extinction coefficient of mant fluorophore at 350nm, which is 5700M⁻¹cm⁻¹ (Hiratsuka, 1983).

2.8 Preparation of Rho.nucleotide Complexes

Purified wild-type Rho contained stoichiometric amounts of bound GDP, whereas Q63L Rho typically contained a mixture of bound GTP and GDP. Nucleotide exchange was required to replace endogenous nucleotide with either [³H]GTP, mantGTP, GMPPNP, mantGMPPNP, [³H]GDP or mantGDP. The purity of all the nucleotide complexes prepared was checked using HPLC (section 2.9).

2.8.1 Exchange with Mant Nucleotides

Mant nucleotide complexes of Rho were prepared using a method similar to that described for Ras by Brownbridge et al. (1993). Typically, a twenty-fold molar excess of mantGTP, mantGDP or mantGMPPNP was added to 1mg of protein and made up to no more than 1ml in 10mM Tris/HCl pH7.5, 100mM ammonium sulphate and 20mM EDTA. This was incubated for 10min at room temperature. The reaction was quenched by addition of MgCl₂ to a final concentration of 40mM and complexes were gel filtered on a PD-10 column (Pharmacia) equilibrated in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT at 4°C. The passage of the complex down the column was visualised by fluorescence using an ultra-violet light source, as separation between complex and free nucleotide could be seen.

The purity of the mant-labelled proteins in terms of the proportions of fluorescent nucleotides (e.g. mantGTP vs. mantGDP) was determined using HPLC with fluorescence
detection (section 2.9). UV absorbance spectrometry was used to check the extent of exchange with mant-guanine nucleotide, that is, to calculate the ratio of mant-nucleotide to protein, which should be a 1:1 ratio if 100% exchange has occurred. First, the absorbance of a sample of mant-nucleotide was scanned over the wavelengths 260 to 400nm. From the known molar extinction coefficient of the mant group at 350nm, which is 5700M\(^{-1}\)cm\(^{-1}\) (Hiratsuka, 1983), and the absorbance reading at 350nm, the concentration of the mant nucleotide was determined. Using this determined concentration and the absorbance reading at 280nm, the molar extinction coefficient for mant nucleotides at 280nm was determined as 9777M\(^{-1}\)cm\(^{-1}\). This was then used, together with the absorbance contributions of tyrosine and tryptophan (section 2.6.2), to calculate a molar extinction coefficient for Rho bound to mant nucleotide. This calculated molar extinction coefficient for Rho.mant-nucleotide at 280nm was 28257M\(^{-1}\)cm\(^{-1}\), compared to 26430M\(^{-1}\)cm\(^{-1}\) for Rho.nucleotide (section 2.6.2). Using these values, the ratio of the absorbance contribution of Rho.mant-nucleotide at 280nm to that at 350nm is 4.96, whereas 50% exchange of mant-nucleotide for nucleotide would give a ratio of 9.6. Thus, this method of comparing the A\(_{280}\) with the A\(_{350}\) allows the purity of the Rho.mant-nucleotide complex to be calculated, in terms of the extent of exchange of mant-nucleotide for unlabelled nucleotide. Typically, the A\(_{280}/A_{350}\) ratio was 5.2, indicating that 95% of the protein was bound to mant-nucleotide.

### 2.8.2 Exchange with GMPPNP

Complexes between Rho and GMPPNP were prepared in a similar manner to that described by John et al. (1990). Rho (0.6mM) was incubated, with gentle agitation, at 37°C in the presence of 4.2mM GMPPNP, 350mM ammonium sulphate and alkaline phosphatase-agarose (75 Sigma Units; Product P0762) in a volume of 450\(\mu\)l. After
45 min, MgCl₂ was added to 40 mM, and the sample applied to a 1 ml Sephadex G-25 superfine centrifuge gel filtration column (Penesky, 1977), equilibrated in 20 mM Tris/HCl pH 7.5, 2 mM MgCl₂, 1 mM DTT. The column was centrifuged at 840 g, 4°C for 2 min to remove unbound nucleotide. The purity of each preparation was determined using HPLC with UV detection (section 2.9).

2.8.3 Exchange with [³H]GTP

Rho[³H]GTP, Q63L Rho[³H]GTP and Q61L Rac[³H]GTP were prepared as described for Q61L Ras[³H]GTP by Skinner et al. (1994). Typically, [³H]GTP (400 μCi; 1 mCi.ml⁻¹; 30.8 Ci.mmol⁻¹; NEN Life Science Products) was dried down under vacuum. This vacuum dried [³H]GTP was then incubated in the presence of Rho (0.4 mM), phosphoenol pyruvate (10 mM), potassium chloride (5.5 mM), ammonium sulphate (280 mM) and pyruvate kinase (14 Sigma units; Product P7768), in a final volume of 180 μl, for 30 min at 37°C. Gel filtration to remove unbound nucleotide was carried out as for GMPPNP complexes.

2.8.4 Exchange with [³H]GDP

[³H]GDP complexes of Rho were made by incubating [8-5’-³H]GDP (26 Ci.mmol⁻¹; 150 μCi; NEN Life Science Products), dried down under vacuum, with protein (0.8 mM), in the presence of 20 mM EDTA, in a final volume of 100 μl, for 30 min at 37°C. After incubation, MgCl₂ was added to 40 mM and unbound nucleotide was removed as described for GMPPNP complexes.
2.9 HPLC Analysis of Fluorescent and Non-fluorescent Guanine Nucleotides

Fluorescent nucleotides were analysed by anion exchange HPLC with fluorescence detection on a Perkin Elmer LS-50B fluorimeter (Ex 365nm; Em 435nm) using a Partisphere SAX 5μm column (Whatman; 4.6 x 110mm) with a mobile phase of 0.5M ammonium phosphate (monobasic) pH4: methanol (4:1 v/v) and a flow rate of 2ml.min⁻¹. Retention times for mantGDP and mantGTP were 6 and 13.5min respectively (Figure 2.3a). Purity of synthesised mant nucleotides (section 2.7), e.g. mantGTP vs. mantGDP, was examined using this method.

Non-fluorescent or radiolabelled nucleotides were analysed using a similar method, with a mobile phase of 0.6M monobasic ammonium phosphate (pH4), a flow rate of 1.5ml.min⁻¹, and monitoring UV absorbance at 254nm on an LKB 2158 uvicord absorbance detector and monitoring radioactivity on a Berthold LB 506-C1 HPLC radioactivity monitor. In the case of radiochemical detection, a Berthold LB 5035 HPLC pump was connected between the column and the detector and used to mix liquid scintillant (Zinsser Analytic Quickszint Flow 303) with mobile phase at a flow rate of 2ml.min⁻¹. Retention times for GDP, GTP and GMPPNP were 2.2, 5.3 and 7min respectively (Figures 2.3b,c,d).

When the proportion of mant or non-mant nucleotide bound to protein was being investigated the protein complex was mixed with 10% perchloric acid (50% v/v), to denature the protein and release the nucleotide, and 4M sodium acetate (17% v/v) to increase the pH to 4 to prevent acid hydrolysis of the nucleotide. Typically, 50μl 10% perchloric acid was mixed with 100μl protein complex, before adding 25μl 4M sodium...
Anion exchange chromatography was used for the separation of nucleotides. The HPLC conditions were as follows:

Column: Partisphere SAX 5µm (Whatman)

Mobile Phase: 0.5M ammonium phosphate pH4:methanol (4:1 v/v) (Figure a)

0.6M ammonium phosphate pH4 (Figures b,c,d)

Flow Rate: 2ml.min\(^{-1}\) (Figure a); 1.5ml.min\(^{-1}\) (Figures b,c,d)

a) Separation of mantGDP and mantGTP using fluorescence detection (Ex 365nm; Em 435nm)

b) Separation of \(^{3}\text{H}\)GDP and \(^{3}\text{H}\)GTP using radiochemical detection

c) HPLC Trace showing the retention time of GMPPNP using UV detection at 254nm

d) Separation of GDP and GTP using UV detection at 254nm

Unlabelled peaks represent either the solvent front or non-nucleotide containing contaminants. Nucleotide containing peaks were identified by their characteristic photo diode array spectrum in which they have a peak at 254nm and a shoulder at 276nm.
acetate. Precipitated protein was removed by centrifugation at 10,000g for 2min and the appropriate HPLC method was applied. Typical amounts injected onto the column were 0.5-2nmol for non-fluorescent nucleotides and 30-50pmol for fluorescent or radiolabelled nucleotides. When required, a Waters 996 Photodiode Array Detector (Millipore) was used to identify nucleotide containing peaks based on their characteristic absorbance maximum at 254nm and shoulder at 276nm.

2.10 Stopped-flow Fluorescence Measurements

Stopped-flow experiments were performed using a Hi-Tech SF-61MX stopped-flow fluorimeter operated in the single push mode. Excitation at 366nm was obtained from a mercury-xenon arc lamp and a monochromator, and emitted light was measured through a Wratten 47B bandpass filter. All measurements were carried out in 20mM Tris/HCl pH 7.5, 2mM MgCl₂, 1mM DTT at 20°C. Reagents were loaded via two syringes: one contained Rho.mantGTP (0.2μM) and the other contained RhoGAP (1-32μM). Data were collected and analysed using RK2 software (Hi-Tech Scientific) to give observed rate constants (k₂obs) for the fluorescence intensity changes seen. Multiple traces were collected for each reaction and observed rate constants were averaged.

Data on the effect of concentration of RhoGAP on the observed rate constants for the fluorescence changes were fitted to the following equation (Sermon et al., 1998):

\[ k_{obs} = \frac{k_{max}(P - \sqrt{P^2 - 4[Rho]_o[RhoGAP]_o})}{2[Rho]_o} \]  

(Equation 1)

where \( P = [Rho]_o + [RhoGAP]_o + K_d \), \([Rho]_o = \) total concentration of Rho, \([RhoGAP]_o = \) total concentration of RhoGAP and \( k_{max} \) is the limiting value.
2.11 Quench-flow Measurements

Quench-flow is a technique used to rapidly mix two reagents and quench the reaction within the millisecond to second time range. Quench-flow measurements were carried out using a Hi-Tech RQF-63 Rapid Flow-Quench System. The age time of the reaction upon quenching is determined by the volume of the reaction loop and the flow rate of the reactants in the loop: age time (ms) = loop volume (μL)/flow rate (μL.ms⁻¹). Timepoints up to 165ms were obtained using a combination of loop volumes and flow rates. When timepoints greater than 165ms were required a time-delay mode was used. Here, the two reactants are mixed and held in the loop and, after a predetermined delay, are pushed through the system and mixed with quenching reagent. Before using the system to examine the reaction between RhoGAP and Rho (section 2.11.2), the quench flow apparatus was tested (section 2.11.1) to ensure that the actual quenching times were the same as the calculated quenching times from the rate of flow and volume of tubing.

2.11.1 Testing the Quench-Flow Apparatus

The test reaction used was the alkaline hydrolysis of 2,4-dinitrophenyl acetate (DNPA), which has been used previously for the calibration of quench flow apparatus (Barman and Travers, 1985). DNPA is hydrolysed by NaOH to 2,4-dinitrophenol. DNPA was mixed with a large excess of NaOH and the hydrolysis reaction was quenched with HCl. The extent of hydrolysis can be monitored by following the absorbance spectra and comparing the A_{260} with the A_{294} (see below).

A solution of 9mM 2,4-dinitrophenyl acetate in isopropanol was prepared and then diluted to 0.9mM in 5mM HCl (just prior to use). The start point of the reaction was achieved by mixing 1ml 0.9mM DNPA with 8ml 2M HCl, and then adding 1ml 1M
NaOH. The end point of the reaction was achieved by mixing 1ml 0.9mM DNPA with 1ml 1M NaOH, leaving this solution for five minutes, and then adding 8ml 2M HCl. The absorbance spectra of the start and end points of the reaction were monitored between 220nm and 420nm. The start solution had an absorbance maximum at 244nm, while the end point had a maximum of 262nm with a shoulder at 294nm. The two spectra intersected at 260nm, the isosbestic point, so at this point the absorbance of the solution at any point in the reaction should be the same for solutions containing different ratios of reactant to product. The ratio of the $A_{294}$, which is the point at which there is the largest difference between the start and end solutions, to the $A_{260}$ gives a determination of the extent of DNPA hydrolysis, taking into account differences in the recovery of reactants from the quench flow apparatus.

For the quench flow measurements, one syringe contained 0.9mM DNPA, the second contained 100mM NaOH and the third contained the quenching solution, 200mM HCl. The two reactants were mixed, at 20°C, for between 10.3ms and 10s before quenching, and the absorbance spectrum for each timepoint was recorded. The $A_{294}/A_{260}$ ratio was calculated and used to determine the fraction of DNPA reacted using the following equation: $\text{Ratio}_t/\text{Ratio}_0 = \text{Ratio}_\infty/\text{Ratio}_0$, where $t =$ time, $0 =$ start point and $\infty =$ end point. The fraction reacted was plotted against time (Figure 2.4) and the data fitted to a single exponential to give a first order rate constant of 2.6s$^{-1}$. This corresponds to a second order rate constant of 52M$^{-1}$s$^{-1}$, which is identical or close to the published values of 52M$^{-1}$s$^{-1}$ (Finlayson and Taylor, 1969) and 60M$^{-1}$s$^{-1}$ (Eccleston, 1987), indicating that the quench flow apparatus was calibrated correctly.
Quench-flow measurements of the alkaline hydrolysis of 2,4-dinitrophenyl acetate were performed by mixing equal amounts of 0.9mM 2,4-dinitrophenyl acetate in 5mM HCl with 100mM NaOH. The reaction was quenched at set timepoints with 200mM HCl. For each sample the $A_{294\text{nm}}/A_{260\text{nm}}$ was determined and the fraction of hydrolysed 2,4-dinitrophenyl acetate calculated by reference to samples prepared manually to give $A_{294\text{nm}}/A_{260\text{nm}}$ values at $t = 0$ and $t = \infty$. The fraction of hydrolysed 2,4-dinitrophenyl acetate is plotted against time. The solid line shows the best fit to a single exponential giving a first order rate constant of $2.6s^{-1}$. 
2.11.2 Quench-flow Measurements for the Reaction of RhoGAP and Rho

All measurements were carried out in 20mM Tris/HCl pH 7.5, 2mM MgCl₂, 1mM DTT at 20°C. One syringe contained wild-type RhoGAP (0.4μM), one contained Rho.[³H]GTP (6 or 14μM) and the other contained quenching solution (10% perchloric acid). For each timepoint, 100μl of Rho.[³H]GTP and RhoGAP were mixed. Each reaction was quenched with 100μl of 10% perchloric acid. The final sample was displaced from the collection loop with air. 4M sodium acetate (50μl) was added to each mixture, to increase the pH to 4 and prevent acid hydrolysis of the nucleotide. Samples were centrifuged at 10,000g for 2min to remove denatured protein and the supernatants were analysed by HPLC with radiochemical detection (section 2.9) to determine the proportion of [³H]GTP remaining. Data representing the decline of [³H]GTP were fitted to a single exponential decay curve to give a pseudo first order rate constant for GTP hydrolysis.

2.12 Manual Quench Experiments

All experiments were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT at 20°C. For measurements of intrinsic GTP hydrolysis and mutant RhoGAP catalysed hydrolysis of GTP, 0.2μM Rho.[³H]GTP or Rho.mantGTP and RhoGAP (R282A 0-30μM; R282K 0-64μM) were incubated in a water bath at 20°C. At set timepoints, samples were manually mixed with 10% perchloric acid (50% v/v) in order to denature the protein and 4M sodium acetate (17% v/v) was added to increase the pH to 4. Following centrifugation at 10,000g to remove denatured protein, treated samples were analysed by HPLC with radiochemical or fluorescence detection (section 2.9) to determine the proportion of [³H]GTP or mantGTP remaining. Data representing the decline of [³H]GTP or mantGTP were fitted to single exponential decay curves to give
pseudo first order rate constants for GTP hydrolysis. Data on the effect of the concentration of RhoGAP on the observed rate constants were fitted to equation 1 (section 2.10).

2.13 Fluorescence Anisotropy Measurements

All fluorescence anisotropy measurements were made in an SLM 8000 spectrofluorimeter operated in the T-format so that parallel and perpendicular light could be monitored simultaneously. All measurements were made in quartz cuvettes measuring 4mm by 4mm, at 20°C in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT. Excitation of the mant moiety was carried out at 366nm with emission monitored through Schott KV399 cut off filters.

Rho.mantGMPPNP (400μl of 1μM) or Rho.mantGDP (400μl of 1 or 10μM) was incubated in the cuvette and an initial reading taken. RhoGAP was then titrated into this solution and measurements of parallel and perpendicular emission intensities were made after each addition. Also in the RhoGAP solution was the same concentration of Rho.mant-nucleotide as present in the cuvette. This allowed the [Rho.mant-nucleotide] in the cell to remain constant. When the effects of aluminium fluoride on the binding of Rho.mantGDP to RhoGAP were being monitored, 110μM AlCl₃ (from anhydrous AlCl₃ freshly dissolved in water) and 20mM NaF were present in both the cell solution and the titration solution.
Calculations:

The fluorescence anisotropy ($A$) was calculated using the following equation, where \(I(\text{parallel})\) and \(I(\text{perpendicular})\) are the measured emission intensities:

\[
A = \frac{I(\text{parallel}) - I(\text{perpendicular})}{I(\text{parallel}) + 2I(\text{perpendicular})}
\]  

(Equation 2)

Fluorescence anisotropy was plotted versus \([\text{RhoGAP}]\) and data were fitted using a simple equilibrium model for the bimolecular interaction between Rho and RhoGAP:

\[
Rho + \text{RhoGAP} \rightleftharpoons Rho.\text{RhoGAP}
\]  

(Equation 3)

The dissociation constant \((K_d)\) for the reaction is:

\[
K_d = \frac{[\text{Rho}][\text{RhoGAP}]}{[\text{Rho.}\text{RhoGAP}]}
\]  

(Equation 4)

where \([\text{Rho.}\text{RhoGAP}]\) is unknown. Expressing this unknown in terms of \([\text{Rho}]\), \([\text{RhoGAP}]\) and \(K_d\) gives a quadratic equation, the solution of which is:

\[
[Rho.\text{RhoGAP}] = \frac{P \pm \sqrt{P^2 - 4[Rho][RhoGAP]_0}}{2}
\]  

(Equation 5)

where \(P = [\text{Rho}]_0 + [\text{RhoGAP}]_0 + K_d\), \([\text{Rho}]_0 = \text{total concentration of Rho}\), \([\text{RhoGAP}]_0 = \text{total concentration of RhoGAP}\). Only the negative root from equation 5 is used in subsequent calculations.
The observed anisotropy can be written as follows:

\[
\]  
(Equation 6)

Where \(A_t\) is the total observed anisotropy and \(Q\) is the fluorescence intensity of the Rho.RhoGAP complex relative to the Rho complex. The data were fitted to equation 6 using GraFit software (Erithacus).

2.14 Scintillation Proximity Assay (SPA)

SPAs to measure Q61L Rac.GTP binding to PAK, or either Q61L Rac.GTP, Q63LRho.GTP or Rho.[\(^3\)H]GDP binding to RhoGAP were set up based on methods previously described (Skinner et al., 1994; Gorman et al., 1996). In this assay, GST-PAK(75-132) or GST-RhoGAP (wild-type or mutant), attached via anti-GST to Protein A SPA beads, bind to either Q61L Rac.[\(^3\)H]GTP, Q63L Rho.[\(^3\)H]GTP or Rho.[\(^3\)H]GDP. The scintillation proximity signal produced as a result of this binding was measured using a Wallac 1450 Microbeta Trilux liquid scintillation counter. The standard assay contained, in a final volume of 200μl, 1.25mg of Protein A SPA PVT beads (Amersham Cat No. RPNQ 0019), 3.8μg of anti-GST antibody (Molecular Probes Product No. 7041-1), 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT, 0.2mg.ml⁻¹ BSA, 0.02μM GST-PAK or either 0.005μM, 0.02μM or 0.04μM GST-RhoGAP (wild-type or mutant), and varying concentrations of either Q61L Rac.[\(^3\)H]GTP, Q63L Rho.[\(^3\)H]GTP or Rho.[\(^3\)H]GDP. The reagents were prepared in siliconised glass vials to reduce losses of proteins by adsorption. The plates were sealed, shaken for 30min at room temperature, centrifuged at
840g for 2min, and counted. Control assays were also set up in which GST-protein was omitted.

In initial experiments, measuring Q61L Rac.\([^3H]\)GTP binding to GST-PAK, to optimise concentrations to be used for further experiments either the concentration of GST-PAK was varied at constant concentrations of radiolabelled Rac and anti-GST, or the concentration of anti-GST was varied at constant concentrations of radiolabelled Rac and GST-PAK.

In the SPA assay measuring Rho.\([^3H]\)GDP binding to RhoGAP, 110\(\mu\)M AlCl\(_3\) (from anhydrous AlCl\(_3\) freshly dissolved in water), 20mM NaF or 1mM deferoxamine mesylate (Sigma) were added to the assay when required. In experiments where the concentrations of Rho.\([^3H]\)GDP, MgCl\(_2\), AlCl\(_3\) or NaF were varied, all other concentrations remained constant.

The data obtained from SPAs in which the direct binding of a range of concentrations of radiolabelled protein to GST-protein was monitored, were fitted to an equation describing formation of a binary complex using GraFit to obtain apparent \(K_d\) values. The equation used was:

\[
SPA\ signal = \frac{S_{\text{max}}(P - \sqrt{P^2 - 4A_0B_0})}{2A_0} \quad (\text{Equation 7})
\]

Where \(P = (A_0 + B_0 + K_d)\), \(S_{\text{max}}\) is the maximum SPA signal i.e. signal when GST-PAK/GST-RhoGAP is fully complexed with radiolabelled Rac/Rho, \(A_0\) is the total
concentration of GST protein, $B_0$ is the total concentration of Rac/Rho and $K_d$ is the equilibrium dissociation constant for GST-fusion protein binding to Rac/Rho. In these calculations, the concentrations used were the total concentrations of the reagents present and did not take into account any changes in local concentration due to binding to SPA beads. The estimates of $K_d$ therefore represent apparent rather than absolute values.

The affinities of wild-type Rho.GDP, Rho.mantGDP, Rho.GMPPNP and Rho.mantGMPPNP for RhoGAPs were measured using these proteins as competitive inhibitors of Q61L Rac.$[^3]$H]GTP binding to GST-RhoGAP. The competition assays consisted of the same standard reagents and buffer as described above for the direct binding assay. However, the concentrations of proteins used were 0.02μM GST-RhoGAP (wild-type or mutant), 0.025μM Q61L Rac.$[^3]$H]GTP and competing protein (up to 16μM). Experiments monitoring the effects of aluminium fluoride also contained 110μM AlCl$_3$ and 20mM NaF. SPA data were fitted, using GraFit, to an equation describing competitive binding for two ligands (taken from Thompson et al., 1998), using the $K_d$ values for Q61L Rac.$[^3]$H]GTP binding to wild-type and mutant RhoGAPs:

$$SPA \text{ Signal} = S_{\text{max}} \left( \frac{(K_dP + K_d0)^2 - 4K_i^2A_0B_0}{K_i(P - \sqrt{P^2 - 4A_0B_0})} \right)$$

(Equation 8)

Where $P$, $S_{\text{max}}$, $A_0$, $B_0$ and $K_d$ are as described for equation 7 and $K_i$ is the equilibrium dissociation constant for binding of the inhibitory protein to Rac. This equation does not take into account inhibitor depletion by binding to the protein. Hence, for high-affinity interactions, the $K_i$ value obtained will represent an upper limit of the true value.
Each SPA assay was carried out in duplicate and values were averaged prior to curve fitting. The standards errors quoted are those obtained from these curve fits. The repeatability of the $K_d$ determinations was ascertained by measuring the binding of Q61L Rac.[$^3$H]GTP to GST-wild-type RhoGAP in four independent experiments. The mean $K_d$ value was 0.22μM with a standard deviation of ± 0.04.

2.15 Isothermal Titration Calorimetry (ITC)

ITC was performed in a Microcal MCS calorimeter (Wiseman et al., 1989). Prior to the titration experiment, Rho complexes and RhoGAP in 20mM Tris/HCl pH7.5, 2mM MgCl$_2$, 1mM DTT were thoroughly degassed under vacuum for 15min. The sample cell (volume 1.375ml) was filled with 15μM RhoGAP or buffer (for control) and titrated with a preliminary 2μl injection followed by up to 25 x 10μl injections of Rho complex (180μM) from the injection syringe. Titrations were carried out at 20°C. In the aluminium fluoride experiments, 110μM AlCl$_3$ and 20mM NaF were present in both the injection syringe and the sample cell. Experimental data were analysed using MicroCal Origin software. The endpoint of the titration was adjusted to zero before the resultant data were fitted to a binding isotherm describing binding at a single site (Wiseman et al., 1989). Estimates of the association constant ($K_a$), the enthalpy change ($\Delta H$) and the stoichiometry of the interaction were calculated using Origin. Each individual titration was carried out in duplicate.

2.16 Crystallization Methods

2.16.1 Gel Filtration of Rho.GDP and Wild-type RhoGAP

For crystallization, Rho.GDP and wild-type RhoGAP were further purified using gel filtration in order to maximise the purity of the proteins. A Superdex 75 26/80 column
was equilibrated overnight in 50mM Tris/HCl pH7.5, 50mM NaCl, 3mM DTT (+10mM MgCl₂ for Rho.GDP). Rho.GDP or RhoGAP (20-30mg), obtained from the protein purification procedure described in section 2.5, were loaded onto the column in 1.5-2ml buffer, and the column was run at a flow rate of 1ml.min⁻¹ using the buffer described. Fractions were collected and those containing Rho.GDP or RhoGAP were identified by SDS-PAGE (section 2.6.3). These fractions were pooled and concentrated to >1.2mM using a Centriprep 15ml concentrator with 10kDa molecular weight cut-off (Amicon), centrifuged at 2570g, 4°C. Aliquots were snap frozen in liquid nitrogen and stored at -80°C.

2.16.2 Crystallization of Rho.GDP.RhoGAP Complex

Crystals were grown at 4°C in Linbro 24-well tissue culture plates using the hanging drop vapour diffusion method (Ducruix and Giegé, 1992). The reservoir solution contained 18% PEG2000 MME, 100mM MES/NaOH pH6.2, 10mM MgCl₂, 3mM DTT, 3mM NaN₃ and 114mM (NH₄)₂SO₄. The protein complex stock solution contained 400μM of Rho.GDP and 400μM wild-type RhoGAP in 20mM Tris/HCl pH7.5, 1.5mM MgCl₂, 0.75mM DTT, 2mM deferoxamine and 10mM NaF. The protein complex stock solution was left at 4°C for 30min after mixing, and then spun at 10,000g, 4°C, for 10min before use. The supernatant was used for crystallization.

Silicon grease was used to grease the rims of each well in the Linbro tray. The plate was placed on ice and 1ml of reservoir solution was pipetted into each well. Protein complex stock solution (2μl) was mixed with reservoir solution (2μl) on a siliconised coverslip. The coverslip was then inverted and set on the grease rim of a well in the Linbro tray. The well was sealed by gently pressing the rim of the coverslip. When the required
A number of wells had been set up, the tray was placed in a 4°C room and crystal growth was monitored over a period of several weeks.
CHAPTER 3

The Role of the Conserved Arginine Residue (Arg 282) of RhoGAP in its Interaction with Rho
3.1 Introduction

RhoGAP (sometimes referred to as p50rhoGAP or Cdc42GAP) was the first GTPase-activating protein identified that interacted with and increased the GTPase activity of Rho family proteins (Garrett et al., 1989; Morii et al., 1991; Garrett et al., 1991; Hart et al., 1991; Barfod et al., 1993; Lancaster et al., 1994). Early characterisation showed that RhoGAP increased the GTPase rate of Rho, Rac and Cdc42, with differential activities towards each protein (Lancaster et al., 1994; Self and Hall, 1995b). Many proteins have since been identified with GAP activity towards members of the Rho family (Lamarche and Hall, 1994), and all contain a related Rho-GAP domain that spans approximately 140 amino acids. At the onset of this project there was little published work relevant to the mechanism by which RhoGAP stimulates Rho-GTPase activity. However, amino acid sequence alignment of proteins containing a Rho-GAP domain revealed several invariant residues (Ahmed et al., 1994; Barrett et al., 1997). Using the Rho-GAP, n-chimaerin, Ahmed et al. (1994) showed that deletion of the only invariant arginine together with one amino acid on either side resulted in a mutant defective in GAP activity. The authors suggested that the arginine residue might play a role in GAP activity. Also, mutation of one of two conserved arginine residues in the Ras-GAPs, p120GAP (Skinner et al., 1991; Brownbridge et al., 1993) and NF1 (Gutmann et al., 1993) had been shown to cause a decrease in GAP activity. At the start of this project, it was known, from more detailed studies, that mutation of either of the two conserved arginine residues in NF1 resulted in a reduction in the catalytic parameter, k_{cat}, with only minor effects on the affinity for its substrate (P.N.Lowe and J.F.Eccleston, Pers. Comm.; later published in Sermon et al., 1998). These results were consistent with the proposal, based on the structure of p120GAP, that one arginine had a direct catalytic role and the other had a role in stabilising this catalytic residue (Scheffzek et al., 1996). Based on these pieces of
evidence it was thought that the single conserved arginine in RhoGAP (Arg 282) was likely to be a residue important for its catalytic function.

However, shortly after this project had begun the crystal structure of a 242-residue C-terminal fragment of human RhoGAP was published (Barrett et al., 1997). This fragment contains the catalytic domain and has activity comparable to the full-length 439-residue protein (Lancaster et al., 1994). Of the ten totally conserved residues shown in a sequence alignment of six members of the Rho-GAP family (Figure 1.6), seven are proline or leucine and were proposed to be important for the structural integrity of the molecule, as were two further residues, Asn 385 and Lys 319 (numbered residues 188 and 122 in the crystallized fragment) (Barrett et al., 1997). The conserved arginine, Arg 282 (residue 85 of the crystallized fragment) only interacts with non-conserved residues leading Barrett et al. (1997) to propose that it was likely to be involved in binding to Rho family proteins. Asn 391 (residue 194 of the crystallized fragment) was proposed to be involved in GAP activity due to its absence in p85, a protein lacking GAP activity but containing sequence homology to the catalytic domain of RhoGAP (Barrett et al., 1997).

In order to investigate the role of Arg 282 of RhoGAP in both the catalytic function of RhoGAP and in the binding interaction with Rho, it was decided to use site-directed mutagenesis to mutate arginine to alanine and lysine. Alanine was chosen to examine the effects of removing the side chain, whereas lysine was chosen as it is the closest amino acid to arginine in terms of the length of the side chain and also in terms of the retention of the positive charge. Single turnover kinetic experiments were used to determine the effects of these mutations on the ability of RhoGAP to enhance the Rho-GTPase activity,
and to determine the previously undefined kinetic parameter, $k_{\text{cat}}$, for both wild-type and mutant RhoGAPs.

In addition, the effects of these mutations on the binding interaction between RhoGAP and Rho will be described. It was important to investigate the effect of mutating the conserved arginine in RhoGAP on the binding interaction between RhoGAP and Rho for two reasons: firstly, to determine whether Arg 282 has a role in binding of Rho to RhoGAP, as suggested by Barrett et al. (1997), and secondly to determine whether any effects on catalytic function were due to alterations in binding interactions, for example, a loss in catalytic activity and in binding may suggest a loss of the structural integrity of the protein caused by the mutation. Equilibrium binding constants were determined for wild-type and mutant RhoGAP proteins interacting with Rho bound to the non-hydrolysable GTP analogue, guanylyl imidodiphosphate (GMPPMP). GMPPNP has a NH group bridging the $\beta$- and $\gamma$-phosphates, a position occupied by an oxygen in GTP. As a result, small G proteins cannot cleave the terminal phosphate from GMPPNP thus allowing the binding step of the interaction between Rho and RhoGAP to be isolated from the cleavage step. In some instances the fluorescently modified form of GMPPNP, mantGMPPNP, was used when fluorescence based methods were utilised. Also, the binding affinities for the interaction between wild-type or mutant RhoGAPs and Q63L Rho.GTP or Q61L Rac.GTP were determined. Both the Q63L mutation in Rho and the Q61L mutation in Rac cause the proteins to become constitutively active, that is, their GTPase activity is severely reduced. This allowed measurement of the binding interaction between a GTP-bound form of Rho/Rac and wild-type or mutant RhoGAPs.
3.2 Protein Expression and Purification

Both Rho and RhoGAP were purified as recombinant proteins from an *E.coli* expression system (Garrett *et al.*, 1989; Lancaster *et al.*, 1994). In the case of RhoGAP, the 29kDa C-terminal fragment (amino acids 198-439) originally purified from human spleen by Garrett *et al.* (1991) and cloned by Lancaster *et al.* (1994) was used. The Rho protein, although full-length, had not undergone any post-translational modifications that occur *in vivo* (Brown and Goldstein, 1993) and contained a single point mutation (F25N), shown to improve stability (Paterson *et al.*, 1990; Self and Hall, 1995a).

Wild-type, R282A and R282K GST-RhoGAP proteins were all expressed at the same levels and with the same solubility. The wild-type and mutant RhoGAP proteins were >95% homogeneous by SDS-polyacrylamide gel electrophoresis (an example of a typical gel is shown in Figure 2.1), and electrospray mass spectrometry gave the predicted molecular masses.

3.3 Kinetic Measurements

The fluorescent mant derivatives of guanine nucleotides have previously been shown to behave as close analogues of the untagged nucleotides and have been used in many studies to characterise the interaction of Ras with Ras-GAPs (Eccleston *et al.*, 1993; Moore *et al.*, 1993; Brownbridge *et al.*, 1993; Mittal *et al.*, 1996; Ahmadian *et al.*, 1997a; Ahmadian *et al.*, 1997c; Sermon *et al.*, 1998). The fluorescent label provides a very useful detection method and enables the use of a range of techniques to study protein interactions, such as rapid stopped-flow techniques and fluorescence anisotropy. Initial kinetic measurements were, therefore, carried out using Rho.mantGTP.
3.3.1 Intrinsic GTPase Activity of Rho.mantGTP

The intrinsic GTPase activity was determined with mantGTP bound to Rho. Rho.mantGTP (0.2μM) was incubated at 20°C. Aliquots were removed at intervals and cleavage of bound GTP to GDP by Rho was measured as a function of time using HPLC with fluorescence detection (section 2.9). Data representing the conversion of Rho.mantGTP to Rho.mantGDP were fitted to a single exponential with a rate constant of $3.0 \times 10^{-5} \text{s}^{-1}$ (Figure 3.1).

3.3.2 Stopped-Flow Studies of the Interaction of Rho.mantGTP with Wild-type, R282A and R282K RhoGAP

Previous work has established that the fluorescent GTP analogue, mantGTP, shows a decrease in fluorescence on GTP hydrolysis at the active site of Ras (Neal et al., 1990). This property associated with mantGTP has been used to monitor RasGAP-catalysed GTP hydrolysis (Eccleston et al., 1993; Moore et al., 1993; Ahmadian et al., 1997a; Ahmadian et al., 1997c; Sermon et al., 1998). Many of these studies have utilised single-turnover kinetic experiments in which the GTP hydrolysis rate was measured under conditions where the RasGAP (the enzyme) is in molar excess over Ras.mantGTP (the substrate) (Eccleston et al., 1993; Ahmadian et al., 1997c; Sermon et al., 1998). An advantage of this procedure over steady-state kinetic measurements is that the maximally activated rate constant ($k_{\text{cat}}$) can be obtained without relying on an accurate determination of the concentration of GAP (Eccleston et al., 1993). This is important since measurements of the concentration of active protein are difficult to make. Furthermore, the formation and decay of intermediates can be observed during the course of the reaction. Monitoring the single turnover of an enzyme often requires the use of an
Figure 3.1 – Intrinsic GTPase Activity of Rho.[³H]GTP and Rho.mantGTP

Rho.[³H]GTP (0.2μM) or Rho.mantGTP (0.2μM) were incubated in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT at 20°C. At timed intervals, aliquots were removed and the GTP hydrolysis reaction was quenched with perchloric acid. The samples were then analysed using anion exchange chromatography (section 2.9) to determine the percentage of GTP present. The data plotted represents the % of hydrolysable GTP present. Rho had >92% [³H]GTP or >96% mantGTP bound at the start of the reaction and <12% unhydrolysed GTP at the end of the reaction. The solid lines are best fits to single exponentials with rate constants of 1.4 (± 0.1) x 10⁻⁴ s⁻¹ and 3.0 (± 0.4) x 10⁻⁵ s⁻¹ representing the intrinsic GTPase activity of Rho.[³H]GTP (●) and Rho.mantGTP (○), respectively. The errors quoted correspond to the standard errors on the curve fits.
instrument able to take measurements on a milli-second timescale. One such instrument that has been used previously with Ras bound to mant nucleotides to investigate the interaction of Ras and Ras-GAPs is a stopped-flow fluorimeter (Eccleston et al., 1993; Ahmadian et al., 1997c; Sermon et al., 1998).

Previous single-turnover stopped-flow measurements performed for the interaction of Ras.mantGTP and Ras-GAPs (Eccleston et al., 1993; Ahmadian et al., 1997c; Sermon et al., 1998), or Rho.mantGTP and RhoGAP (Ludbrook, 1997) have resulted in biphasic fluorescence changes. In all cases the first fluorescence change has been assumed to represent the binding of Ras/Rho to their GAPs, and the second fluorescence change has been assumed to represent the GTP hydrolysis step. These assumptions have been applied here. The maximum observed rate obtained for the second fluorescence change then represents the catalytic parameter, $k_{cat}$. In addition, these measurements also allow the $K_m$ for the interaction to be determined if these fluorescence changes are monitored at several GAP concentrations. Here, single-turnover stopped-flow experiments were primarily performed in an attempt to determine the $k_{cat}$ for wild-type and mutant RhoGAPs catalysing Rho-GTPase activity.

Rapid mixing of 0.1μM Rho.mantGTP and 3.95μM wild-type RhoGAP in a stopped-flow fluorimeter resulted in an initial decrease ($\approx 20\%$) in fluorescence intensity with a half-time of $0.057s$, followed by an increase ($\approx 22\%$) in fluorescence intensity with a half-time of $0.58s$ (Figure 3.2a). This increase in intensity reached a level slightly higher than that of the starting level in keeping with the observation that the fluorescence of Rho.mantGTP is lower ($\approx 5\%$) than that of Rho.mantGDP (Ludbrook, 1997). Mixing
Figure 3.2 – *Stopped-flow Fluorescence Intensity Changes on Mixing of Wild-type RhoGAP and Rho.mantGTP and Dependence of Changes on GAP Concentration*

a) Rho.mantGTP and wild-type RhoGAP were rapidly mixed in a stopped-flow fluorimeter at 20°C and the fluorescence intensity changes monitored over time. The concentrations after mixing were 0.1μM Rho.mantGTP and 3.95μM wild-type RhoGAP. The solid line is a best fit to a double exponential with rate constants of 12.1s⁻¹ for the initial fast decrease in fluorescence intensity and 1.2s⁻¹ for the slower increase in intensity. The insert is the same data set shown on an expanded timescale.

b & c) Experiments similar to those in a) were performed at final concentrations of wild-type RhoGAP between 0.5 and 15.8μM. When RhoGAP was present at <2μM no second phase was seen so data were fitted to single exponentials. The observed rate constants for the fast-phase and slow-phase from at least four traces were averaged and plotted against RhoGAP concentration. The solid lines represent the best hyperbolic fits to the data giving maximum rate constants of 35.8 (± 4.1) s⁻¹ (b) and 2.3 (± 0.1) s⁻¹ (c) with the half-maximal rates occurring at 6.9 (± 2.4) μM (b) and 2.7 (± 0.5) μM (c) wild-type RhoGAP. The errors quoted correspond to the standard errors on the curve fits. The error bars represent the standard deviation of individual observed rate constants obtained from individual traces.

All measurements were 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT.
0.1μM Rho.mantGTP and 2-15.8μM wild-type RhoGAP gave similar fluorescence changes, with half-times ranging from 0.85s to 0.029s for the fast phase and from 0.83s to 0.37s for the slow phase. These biphasic fluorescence signals are similar to those observed on rapid mixing of N-Ras.mantGTP and the catalytic domain of the RasGAP, NF1 (NF1-344), although opposite in terms of the direction of the fluorescence change (Eccleston et al., 1993; Ahmadian et al., 1997a; Sermon et al., 1998). In addition, the final fluorescence intensity is lower than that of the starting level (Eccleston et al., 1993; Ahmadian et al., 1997a; Sermon et al., 1998), consistent with the observation that there is an overall decrease in fluorescence on cleavage of Ras.mantGTP to Ras.mantGDP (Neal et al., 1990), in contrast to the corresponding increase in fluorescence observed with Rho. These differences between the fluorescence intensity changes seen on interaction of mant nucleotides with either Ras or Rho family proteins suggest that the mant nucleotides may be bound in slightly different conformations or environments.

The biphasic changes in intensity were fitted to double exponentials to obtain two observed rate constants, \( k_{\text{obs}} \) (fast phase) and \( k_{\text{obs}} \) (slow phase). At RhoGAP concentrations of <2μM, only the initial decrease in intensity was observed within the timescale measured (4.5s), which was fitted to a single exponential to give an observed rate constant. Plots of the observed rate constants associated with the rapid decrease and slower increase in fluorescence intensity showed a hyperbolic dependence on RhoGAP concentration (Figure 3.2b,c). The maximum observed rate constant for the decrease in intensity is 35.8s\(^{-1}\) with a half maximal rate at 6.9μM RhoGAP. For the increase in intensity the maximum observed rate constant is 2.3s\(^{-1}\), with a half maximal rate at 2.7μM RhoGAP. Assuming that the slow fluorescence change represents the GTP hydrolysis step, the \( k_{\text{cat}} \) for wild-type RhoGAP is 2.3s\(^{-1}\) and the \( K_m \) is 2.7μM. The latter
fluorescence change may, however, be monitoring the dissociation of the Rho.mantGDP.Pi.RhoGAP complex (k₃ in Scheme 3.1), therefore, must occur at a rate slower or equal to the actual cleavage rate as it can be limited by the preceding step. The kₐₚ obtained here is, therefore, a true estimate or an underestimate of the actual value.

Scheme 3.1

\[
\begin{align*}
    Rho.mantGTP + RhoGAP & \overset{k_1}{\rightleftharpoons} Rho.mantGTP.RhoGAP \overset{k_2}{\rightleftharpoons} Rho.mantGDP.Pi.RhoGAP \\
    & \overset{k_3}{\rightleftharpoons} Rho.mantGDP + Pi + RhoGAP
\end{align*}
\]

Assuming the intensity decrease represents the association of Rho.mantGTP and RhoGAP, a linear dependence of the observed rate constant on RhoGAP concentration would be expected from a simple second-order reaction (Eccleston, 1987). However, the hyperbolic dependence observed here (Figure 3.2) suggests that the interaction is not a single-step binding interaction. The simplest interpretation of this behaviour is that the binding process occurs in two steps. It has been suggested that, with Rho.mantGTP and RhoGAP, there is an initial rapid binding step followed by a conformational change which results in the fluorescence intensity decrease (Ludbrook, 1997).

When 0.1µM Rho.mantGTP was mixed with 3.95µM R282A RhoGAP under the same conditions as those used for wild-type RhoGAP, a decrease (= 26%) in fluorescence intensity was observed with a half-time of 0.074s (Figure 3.3a). Under these conditions, and monitoring timescales up to 50s, no increase in intensity was seen to follow the initial decrease as had been seen with wild-type RhoGAP. Mixing 0.1µM
Figure 3.3 – Stopped-flow Fluorescence Intensity Changes on Mixing of R282A RhoGAP and Rho.mantGTP at 20°C and Dependence of Changes on GAP Concentration

a) Rho.mantGTP and R282A RhoGAP were rapidly mixed in a stopped-flow fluorimeter at 20°C and the fluorescence intensity changes monitored over time. The concentrations after mixing were 0.1µM Rho.mantGTP and 3.95µM R282A RhoGAP. The solid line is a best fit to a single exponential with a rate constant of 9.4s⁻¹.

b) Experiments similar to those in a) were performed at final concentrations of R282A RhoGAP between 0.5 and 15.8µM. The observed rate constants from at least four traces were averaged and plotted against RhoGAP concentration. The solid line represents the best hyperbolic fit to the data giving a maximum rate constant of 13.5 (± 0.5) s⁻¹ with the half-maximal rate occurring at 1.4 (± 0.3) µM R282A RhoGAP. The errors quoted correspond to the standard errors on the curve fits. The error bars represent the standard deviation of individual observed rate constants obtained from individual traces.

All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT.
Chapter 3 – Role of Arg 282 in RhoGAP

(a) Fluorescence intensity over time (seconds) for [R282A RhoGAP] (µM).

(b) Kinetic analysis showing $k_{obs}$ (s⁻¹) as a function of [R282A RhoGAP] (µM).
Rho.mantGTP with 0.5-15.8μM R282A RhoGAP, gave similar fluorescence changes with half-times ranging from 0.21s to 0.055s. The decrease in fluorescence intensity seen with each replicate at each concentration was fitted to a single exponential decay to give an observed rate constant ($k_{obs}$). A plot of $k_{obs}$ versus R282A RhoGAP concentration was fitted to a hyperbola with a maximal observed rate constant of 13.5s$^{-1}$, with the half maximal rate occurring at 1.4μM R282A RhoGAP (Figure 3.3b). As with wild-type, this is demonstrative of a two-step binding interaction. Increasing the temperature from 20°C to 30°C, and mixing 0.1μM Rho.mantGTP with 15.8μM R282A RhoGAP, revealed the biphasic fluorescence changes seen with wild-type RhoGAP (Figure 3.4). However, the increase in fluorescence, assumed to represent hydrolysis, following the initial rapid decrease was much slower than that observed with wild-type RhoGAP, and the full effect was only seen on timescales up to 40s. The data representing the slow phase from six traces, measured at 30°C, were fitted to single exponential decay curves to give observed rate constants. These rate constants were averaged to give an observed rate constant of 0.054 ± 0.006s$^{-1}$. Comparing this value with the average observed rate constant obtained for the interaction of equivalent concentrations of Rho.mantGTP and wild-type RhoGAP, at 20°C, which was 1.9 ± 0.2s$^{-1}$, shows that the observed rate constant obtained with R282A RhoGAP was reduced 35-fold, despite being measured at a higher temperature.

When Rho.mantGTP was rapidly mixed with R282K RhoGAP at 20°C no fluorescence intensity changes were observed. This could be interpreted in two ways: either there was no interaction taking place between R282K RhoGAP and Rho.mantGTP, or there was an interaction with no accompanying fluorescence change. Further experiments showed there was an interaction occurring (section 3.3.4), hence it can be concluded that there is
RhoGAP and Rho.mantGTP at 30°C

Rho.mantGTP and R282A RhoGAP were rapidly mixed in a stopped-flow fluorimeter at 30°C and the fluorescence intensity changes monitored over time. The concentrations after mixing were 0.1μM Rho.mantGTP and 15.8μM R282A RhoGAP. The solid line represents the best fit to a single exponential curve plus a slope, giving a rate constant of 0.054s⁻¹.

All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT.
Fluorescence Intensity

Time (seconds)
no accompanying fluorescence change occurring. These differences in fluorescence changes between wild-type and mutant RhoGAPs illustrate the high level of environmental sensitivity of the mant fluorophore.

The aim of the stopped-flow measurements was to obtain the maximal catalytic rates ($k_{cat}$) for wild-type and mutant RhoGAP proteins. However, due to the absence of any fluorescence intensity changes seen on mixing Rho.mantGTP and R282K RhoGAP, this was not possible with this particular mutant. Furthermore, at 20°C, the second phase of the fluorescence intensity change was absent with the R282A mutant. As a consequence, it was decided to move on to more appropriate methods that allowed the direct measurement of the chemical cleavage of GTP without relying on a fluorescence change.

The fluorescence experiments with Rho.mantGTP and R282A RhoGAP at 30°C suggested that the hydrolysis rate would be on such a timescale that the chemical cleavage step could be measured directly using manual quenching methods (section 2.12), whereas for experiments using wild-type RhoGAP a Rapid Quench Flow instrument (section 2.11) could be used. The methods used to measure the hydrolysis of GTP directly relied on HPLC methods to detect the proportion of GTP and GDP present in a protein sample. Although it was technically more difficult to measure chemical cleavage of GTP rather than monitoring fluorescence changes of mant nucleotides, this method avoids assumptions in relating rates of fluorescence changes to cleavage rates. Furthermore, it enables the use of structurally unmodified nucleotide (tritiated GTP) and allows a direct comparison to be made between modified and non-modified nucleotide.
3.3.3 Intrinsic GTPase Activity of Rho.[$^3$H]GTP

In order to allow a comparison with Rho.mantGTP and also to compare the RhoGAP-catalysed GTPase rates using GTP itself with the uncatalysed rate, the intrinsic GTPase activity of Rho.[$^3$H]GTP was determined. Rho.[$^3$H]GTP (0.2μM) was incubated at 20°C. Aliquots were removed and cleavage of bound GTP to GDP by Rho was measured as a function of time using HPLC with radiochemical detection (section 2.9). Data representing the conversion of Rho.[$^3$H]GTP to Rho.[$^3$H]GDP were fitted to a single exponential with a rate constant of $1.4 \times 10^{-4}$ s$^{-1}$ (Figure 3.1). These data show a 4.7-fold increase in intrinsic GTPase activity when [$^3$H]GTP is bound to Rho rather than mantGTP (section 3.3.1).

3.3.4 Activation of Rho-GTPase by Wild-type, R282A and R282K RhoGAP

This section describes the quench-flow and manual quench experiments used to measure the RhoGAP-catalysed Rho-GTPase activity. These methods allow the cleavage of GTP to be measured directly. In each case, the GTP hydrolysis rate was measured under single turnover conditions where RhoGAP is in excess over Rho.GTP.

To measure the catalytic activity of wild-type RhoGAP, experiments were performed using a Hi-Tech Rapid Quench Flow instrument (section 2.11). Using this instrument, Rho.[$^3$H]GTP (0.2μM) was rapidly mixed with 7μM wild-type RhoGAP and samples were quenched after mixing for between 10ms and 1.5s. HPLC was used to determine the proportion of [$^3$H]GTP remaining in each sample (Figure 3.5a). The data were fitted to a single exponential decay curve giving a pseudo first order rate constant of 5.25s$^{-1}$. This was then repeated using 3μM wild-type RhoGAP, which gave a pseudo first
Figure 3.5 – GTPase Activity of Rho.\[^3\text{H}\]GTP and Rho.mantGTP in the Presence of Wild-type, R282A and R282K RhoGAPs

Wild-type RhoGAP (●), R282A RhoGAP (▲), or R282K RhoGAP (■) were mixed with Rho.\[^3\text{H}\]GTP (a & b) or Rho.mantGTP (c). At intervals aliquots were removed and the GTP hydrolysis reactions were quenched by addition of perchloric acid. The extent of hydrolysis was measured by HPLC (section 2.9). Mixing and quenching were performed manually for mutant RhoGAPs or in a quench-flow apparatus for wild-type RhoGAP.

Time courses for the hydrolysis of GTP were fitted to single exponentials. Panel a) shows data obtained with 7\(\mu\)M wild-type RhoGAP and 0.2\(\mu\)M Rho.\[^3\text{H}\]GTP. The solid line is the best fit with a rate constant of 5.25s\(^{-1}\). Panel b) shows data obtained with 30\(\mu\)M R282A or 30\(\mu\)M R282K RhoGAP and 0.2\(\mu\)M Rho.\[^3\text{H}\]GTP. The solid lines are the best fits with rate constants of 2.9 \times 10^{-2}s\(^{-1}\) for R282A RhoGAP and 9.7 \times 10^{-3}s\(^{-1}\) for R282K RhoGAP. Panel c) shows data obtained with 30\(\mu\)M R282A or 30\(\mu\)M R282K RhoGAP and 0.2\(\mu\)M Rho.mantGTP. The solid lines are the best fits with rate constants of 9.4 \times 10^{-3}s\(^{-1}\) and 6.7 \times 10^{-4}s\(^{-1}\), respectively.

All experiments were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl\(_2\), 1mM DTT at 20\(^\circ\)C. The data plotted represents the % of hydrolysable GTP present. Rho had >92% \[^3\text{H}\]GTP or >96% mantGTP bound at the start of the reaction and <12% (or 25% for quench-flow experiments using wild-type RhoGAP) unhydrolysed GTP at the end of the reaction.
order rate constant of 5.20s\(^{-1}\). In each case the end-points of the reaction were high, with approximately 25% GTP remaining unhydrolysed, and were not the same as the end-point obtained when Rho.\([^3]H\)GTP and wild-type RhoGAP were manually mixed and left for 2min before quenching, when only 8% uncleaved GTP remained. This suggests that a proportion of the Rho.\([^3]H\)GTP in the quench-flow apparatus is not exposed to the RhoGAP and therefore remains unhydrolysed. In this case, the observed rate constants obtained from these experiments may be an underestimate of the actual cleavage rate. These rapid quench-flow experiments proved technically difficult and further points were not obtained. Since the rates at 3 and 7\(\mu\)M are almost identical it can be concluded that saturation has occurred at a concentration of <3\(\mu\)M with a rate of approximately 5s\(^{-1}\). This represents an estimate of the \(k_{cat}\) value. Although a \(K_m\) value can not be obtained from the data, an upper limit can be estimated by visual inspection of the data. Allowing for errors in the data, the estimated \(K_m\) value is \(<1\mu\)M. The hyperbolic curve shown in Figure 3.6a was constructed using values of 5.4s\(^{-1}\) for the \(k_{cat}\) and 0.2\(\mu\)M for the \(K_m\). As these rapid quench-flow experiments proved technically difficult, they have only been performed for the reaction with Rho.\([^3]H\)GTP and not Rho.mantGTP. As initial stopped-flow experiments (Section 3.3.2) provided a lower limit for the cleavage rate of Rho.mantGTP, it was thought that this value was sufficient for comparative purposes against rates obtained with mutant RhoGAPs.

To measure the catalytic activity of R282A RhoGAP manual quenching methods were used (section 2.12), relying on manual mixing and quenching of the sample prior to HPLC analysis. It was possible to use this method for both mutant RhoGAPs as the hydrolysis rates were on a slower timescale than for wild-type RhoGAP. For R282A
Figure 3.6 – Effect of RhoGAP Concentration on the GTPase Activity of Rho.mantGTP or Rho.[^H]GTP

The indicated concentrations of wild-type RhoGAP (●), R282A RhoGAP (▲), or R282K RhoGAP (■) were mixed with Rho.[^H]GTP (a & b) or Rho.mantGTP (c) and time courses of hydrolysis were followed as described in Figure 3.5. The observed pseudo first order rate constants are plotted against concentration of RhoGAP. Panel b) shows the data for the mutant RhoGAPs that is shown in Panel a) but on expanded x and y scales. The inset on panel c) shows the data for R282K RhoGAP on expanded x and y scales. For wild-type RhoGAP, the hyperbolic curve has been constructed using a $K_m$ of 0.2 μM (dashed line) and a $k_{cat}$ of 5.4 s$^{-1}$ (solid line). The solid lines shown for mutant RhoGAPs are fits to hyperbolic curves, with the following parameters: panel b) R282A, $K_m = 0.2 \pm 0.3$ μM and $k_{cat} = 0.025 \pm 0.002$ s$^{-1}$ and R282K, $K_m = 3.9 \pm 1.7$ μM and $k_{cat} = 0.010 \pm 0.001$ s$^{-1}$; panel c) R282A, $K_m = 4.5 \pm 1.7$ μM and $k_{cat} = 0.012 \pm 0.001$ s$^{-1}$ and R282K, $K_m = 25 \pm 7$ μM and $k_{cat} = 0.0011 \pm 0.0001$ s$^{-1}$. All errors quoted correspond to the standard errors on the curve fits.

All experiments were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl$_2$, 1mM DTT at 20°C.
a) Rate Constant (s⁻¹), 0

b) Rate Constant (s⁻¹)

c) Rate Constant (s⁻¹)

[Graphs showing the relationship between [RhoGAP] (μM) and Rate Constant (s⁻¹)]
RhoGAP, manual quench experiments were performed for both Rho.[³H]GTP and Rho.mantGTP. Rho.[³H]GTP or Rho.mantGTP (0.2μM) were mixed with 30μM R282A RhoGAP and the proportion of GTP remaining was monitored with time, using HPLC. The raw data obtained (Figure 3.5b & c) were fitted to single exponential decay curves to give observed pseudo first order rate constants of 2.9 x 10^{-2} s^{-1} and 9.4 x 10^{-3}s^{-1} for Rho.[³H]GTP and Rho.mantGTP, respectively. These experiments were repeated using concentrations of R282A RhoGAP ranging from 1-15μM for measurements with Rho.[³H]GTP or 1-20μM for measurements with Rho.mantGTP. The pseudo-first order rate constants that were obtained ranged from 0.020 to 0.027s^{-1} for Rho.[³H]GTP and 0.002 to 0.011s^{-1} for Rho.mantGTP. Plotting the observed rate constants against [R282A RhoGAP] for experiments with Rho.[³H]GTP (Figure 3.6b) showed that a hyperbolic curve was not well defined at the lower concentrations, as 80% saturation was observed at 1μM RhoGAP, the lowest concentration of RhoGAP used. The RhoGAP concentration could not be reduced to below 1μM as this would make the enzyme concentration less than five-fold greater than the substrate concentration, thereby no longer conforming sufficiently to pseudo first order conditions (Eccleston, 1987). The substrate concentrations could not be reduced further due to the limit of detection of the HPLC method. Bearing in mind these limitations, an upper limit for K_m was estimated as 1μM and a hyperbolic curve was fitted to the data to obtain the maximum rate. The hyperbolic curve shown in Figure 3.6b represents a maximum cleavage rate, k_{cat}, of 0.025s^{-1} and a K_m of 0.2μM. Knowing that wild-type RhoGAP and R282A RhoGAP have similar binding affinities for Rho.GMPPNP (sections 3.4.2.6 and 3.4.3.1; Table 3.3), it is likely that their affinities for Rho.GTP are similar. Therefore, it is likely that, at 1μM wild-type RhoGAP, as with R282A RhoGAP, a high percentage of saturation would have been reached and a hyperbolic curve would not have been very well defined even if more data.
points had been measured on the Rapid Quench-Flow system. Plotting the observed rate constants against [R282A RhoGAP] for experiments with Rho.mantGTP (Figure 3.6c) a hyperbolic dependence of \( k_{\text{obs}} \) on [RhoGAP] was seen. The \( k_{\text{cat}} \) was 0.012 s\(^{-1}\) and the \( K_m \) 4.5 \( \mu \)M indicating a 2-fold lower catalytic activity and at least a 4-fold higher \( K_m \) value when the mant group was attached to GTP. Thus, mutation of Arg 282 to Ala causes a 200- and 190-fold reduction in GAP activity using Rho.GTP and Rho.mantGTP, respectively. With GTP, there is no detectable change in \( K_m \) caused by mutation of Arg 282 to Ala.

Similar experiments to those described for R282A RhoGAP were carried out to measure the catalytic activity of R282K RhoGAP. Again, manual quench experiments to measure the hydrolysis of GTP were performed for both Rho.[\(^3\)H]GTP and Rho.mantGTP. The raw data obtained using 0.2 \( \mu \)M Rho.[\(^3\)H]GTP or Rho.mantGTP and 30 \( \mu \)M R282K RhoGAP are shown in Figures 3.5b & c. These data were fitted to single exponential decay curves to give observed pseudo first order rate constants of \( 9.7 \times 10^{-3} \) s\(^{-1}\) and \( 6.7 \times 10^{-4} \) s\(^{-1}\) for Rho.[\(^3\)H]GTP and Rho.mantGTP, respectively. These experiments were repeated using concentrations of R282K RhoGAP ranging from 1-15 \( \mu \)M for measurements with Rho.[\(^3\)H]GTP or 1-64 \( \mu \)M for measurements with Rho.mantGTP. The pseudo-first order rate constants obtained ranged from \( 1.9 \times 10^{-3} \) s\(^{-1}\) to \( 7.6 \times 10^{-3} \) s\(^{-1}\) for Rho.[\(^3\)H]GTP and 8 x \( 10^{-5} \) s\(^{-1}\) to 8 x \( 10^{-4} \) s\(^{-1}\) for Rho.mantGTP. A hyperbolic dependence of \( k_{\text{obs}} \) on [RhoGAP] was seen with both Rho.[\(^3\)H]GTP and Rho.mantGTP (Figures 3.6b & c). From the curve fits, \( k_{\text{cat}} \) values were 0.010 s\(^{-1}\) and 0.0011 s\(^{-1}\), and the \( K_m \) values were 3.9 \( \mu \)M and 25 \( \mu \)M for Rho.[\(^3\)H]GTP and Rho.mantGTP, respectively. In the case of R282K RhoGAP, the presence of the mant group caused a 10-fold reduction in the \( k_{\text{cat}} \)
and a 6-fold increase in the $K_m$ value. Thus, mutation of Arg 282 to Lys causes a 500- and 2100-fold reduction in GAP activity using Rho.[$^3$H]GTP and Rho.mantGTP, respectively.

These data are summarised in table 3.1.

### 3.4 Binding Measurements

Several methods were used to determine equilibrium binding constants for the interaction between wild-type or mutant RhoGAPs and the triphosphate-bound form of Rho. It was important to obtain these binding affinities in order to determine whether effects on the catalytic function of RhoGAP, caused by the mutations, were the result of a decrease in the binding interaction. Also, the use of these methods has allowed an investigation of the interaction between wild-type or mutant RhoGAPs and Rho bound to either non-modified or fluorescently modified nucleotides.

#### 3.4.1 Fluorescence Anisotropy Measurements

Binding of a fluorophore to a protein can often be monitored by changes in fluorescence anisotropy. These measurements are dependent on the difference between the depolarising effect that a fluorophore has on polarised light when free or bound to a small molecule compared to when bound to a large molecule. When a population of fluorophores are excited with plane polarised light, those molecules that are properly orientated relative to the plane of polarisation will be preferentially excited. The fluorescence emission will also be polarised provided that the fluorophore remains stationary during the lifetime of the excited state. Movement of the excited molecules will reduce the polarisation of the emitted light and will have components that are both
Table 3.1 – Catalytic Constants for Wild-type and Mutant RhoGAPs

Table 3.1 summarises the effects of mutating arginine 282 of RhoGAP to alanine or lysine on the $k_{cat}$ for RhoGAP-catalysed Rho-GTPase activity and the $K_m$ for the interaction of RhoGAP and Rho.GTP. A comparison between Rho.[$^3$H]GTP and Rho.mantGTP has been made and results have also been presented in terms of reduction in GAP activity of mutants compared to wild-type RhoGAP and in terms of the stimulation of the intrinsic GTPase rate of Rho.

Key

n.a. - not applicable

* - stopped-flow measurement monitoring fluorescence intensity changes and assuming that they correspond to the actual cleavage rate
<table>
<thead>
<tr>
<th>Rho.mGTP</th>
<th>Rho.&lt;sup&gt;3&lt;/sup&gt;HJTTP</th>
<th>Stimulation of intrinsic GTP hydrolysis (fold increase)</th>
<th>Reduction in GAP activity (fold decrease)</th>
<th>( K_m ) (( \mu \text{M} ))</th>
<th>GTPase rate constant (s(^{-1} ))</th>
<th>GTPase rate (s(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type RhoGAP</td>
<td>R282A RhoGAP</td>
<td>36000</td>
<td>180</td>
<td>4.5</td>
<td>0.025</td>
<td>0.013</td>
</tr>
<tr>
<td>Intrinsic (no RhoGAP)</td>
<td>R282K RhoGAP</td>
<td>≤1</td>
<td>200</td>
<td>70</td>
<td>0.010</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

Table represents data on the stimulation and reduction of intrinsic GTP hydrolysis, reduction in GAP activity, and GTPase rate constant.
parallel and perpendicular to the excitation plane of polarisation. This depolarisation effect is thus dependent on the lifetime of the excited state and the mobility of the fluorophore. Free fluorophores, or those bound to a small molecule, that rapidly rotate within their fluorescence lifetime, lose more of their fluorescence polarisation than when they are complexed to a large molecule (Jameson and Sawyer, 1995). Thus, the mant fluorophore bound to Rho will have more of a depolarising effect than when bound in a complex between Rho and RhoGAP. The relationship between anisotropy and the parallel and perpendicular intensities (equation 2; section 2.13) is such that the more depolarisation that occurs, the lower the anisotropy value.

Provided that the mant fluorophore has restricted local motion when bound to the protein, its fluorescence anisotropy will reflect the overall molecular size of the complex. Time-resolved fluorescence measurements on Ras bound to mant-guanine nucleotides (Hazlett et al., 1993) show that the mant group has little local motion. Fluorescence anisotropy has previously been used to measure the $K_d$ for the interaction between Ras-GAPs and Ras (Brownbridge et al., 1993; Eccleston et al., 1993). Here, using mant derivatives of guanine nucleotides attached to Rho, the interaction of Rho with RhoGAP was monitored by measuring the fluorescence anisotropy changes upon complex formation.

3.4.1.1 Interaction of Rho.mantGMPPNP with Wild-type, R282A and R282K RhoGAP

In order to estimate the binding affinity of RhoGAP to the triphosphate form of Rho, the non-hydrolysable, fluorescently labelled form of a GTP analogue, mantGMPPNP, was used. When RhoGAP was titrated into a solution of Rho.mantGMPPNP an increase in anisotropy was observed, consistent with complex formation (Figure 3.7). The initial
Figure 3.7 – *Fluorescence Anisotropy Titration of Wild-type, R282A or R282K RhoGAP into Rho.mantGMPPNP*

The indicated concentrations of wild-type (●), R282A (▲) or R282K (■) RhoGAP were titrated into a solution of 1µM Rho.mantGMPPNP. Fluorescence anisotropy readings were taken after each addition. The solid lines shown are the best fits to hyperbolic binding curves with $K_d$ values of 3.6 (± 0.6) µM, 21 (± 3) µM and 5.3 (± 1.1) µM for the interaction of Rho.mantGMPPNP with wild-type, R282A and R282K RhoGAP, respectively. The errors quoted correspond to the standard errors on the curve fits. All experiments were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT at 20°C. Each RhoGAP titration solution also contained 1µM Rho.mantGMPPNP in order to keep the concentration of Rho.mantGMPPNP constant.
anisotropy value was 0.192 which increased to 0.236 when 30μM wild-type RhoGAP was added. The data were fitted to a binding isotherm to give a $K_d$ of 3.6μM and a saturating value of 0.250. R282A and R282K RhoGAPs gave similar anisotropy changes, in terms of magnitude, when titrated into Rho.mantGMPPNP (Figure 3.7). The $K_d$ values obtained for R282A and R282K RhoGAPs were 21μM and 5.3μM, respectively. These data indicate that the R282A mutation has caused a 6-fold decrease in affinity for Rho.mantGMPPNP and the R282K mutation has caused a 1.5-fold decrease.

### 3.4.2 Scintillation Proximity Assay (SPA) Measurements

SPA involves the use of scintillant beads (fluomicrospheres) coated with an acceptor molecule to which a ligand will bind selectively. The scintillant beads, developed by Amersham International, are available with a range of acceptor molecules including receptors, antibodies or protein A. Protein A coated onto SPA beads acts as a link between the bead and a selected antibody that then interacts with the ligand. The beads are suspended in normal aqueous buffers and the technique requires the use of a ligand labelled with an isotope that emits low-energy radiation that is dissipated easily into the buffer. $^3$H or $^{125}$I are widely used for this purpose as the average path lengths of their emissions are approximately 4μm and 12μm, respectively (Udenfriend et al., 1987; Bosworth and Towers, 1989). At any point during an assay, bound labelled ligands will be in close proximity to the beads, allowing the emitted energy to activate the fluor and produce light, which can be detected using a scintillation counter. The majority of unbound labelled ligands are too far from the beads to enable transfer of energy. As bound ligands produce light and free ligands do not, this eliminates the need for separation procedures, thus the assays can be carried out under equilibrium binding conditions (Bosworth and Towers, 1989; Takeuchi, 1992). Also, the assay is typically
carried out in 0.2ml of buffer and can be performed in 96-well plates, allowing multiple binding assays to be performed simultaneously and analysed rapidly on a microplate scintillation counter. This technique is widely used in situations where a large number of assays needs to be carried out, such as high-throughput compound screening (Takeuchi, 1992).

Previously a SPA has been used to monitor the interaction of Ras and the RasGAP, NF1 (Skinner et al., 1994). This SPA used scintillant beads coated with protein A to which GST-NF1 bound via an anti-GST antibody. The SPA was used to monitor binding of Q61L Ras\([^{3}\text{H}]\)GTP to GST-NF1 (Skinner et al., 1994). Similar SPAs have been used to measure binding of Ras to Raf (Gorman et al., 1996) and Rac/Cdc42 to PAK (Thompson et al., 1998). This SPA format, utilising anti-GST and GST-fusion proteins, can be used to monitor direct binding of GST-fusion protein to radiolabelled protein (referred to as a direct binding SPA). Alternatively, it can be used to monitor inhibitory effects when another unlabelled protein is added to the assay that competes for binding to either the GST-fusion protein or the radiolabelled protein (referred to as a competitive inhibition SPA). These types of assay formats are represented schematically in Figure 3.8. The calculations used for determination of binding affinities (section 2.14) do not take into account any changes in local concentration due to binding to the SPA beads. The estimates of \(K_d\) therefore represent apparent rather than absolute values.

Initial SPA experiments were based on a method set up to measure binding of Rac to fragments of an effector protein, p21 activated kinase (PAK) (Thompson et al., 1998). In this assay GST-PAK (residues 75-132) is bound to SPA beads via anti-GST antibody. When Q61L Rac\([^{3}\text{H}]\)GTP (mutant Rac unable to hydrolyse GTP efficiently) is
Figure 3.8 – Schematic Representation of the Scintillation Proximity Assay

a) Illustration of the SPA used to measure direct binding of Q61L Rac.[³H]GTP to GST-PAK, or Q61L Rac.[³H]GTP/Q63L Rho.[³H]GTP to GST-RhoGAP (mutant or wild-type). Binding of Rac to GST-PAK or Rac/Rho to GST-RhoGAP brings the radiolabelled protein into close proximity to the scintillant containing SPA bead, thereby causes scintillation (SPA signal).

b) Illustration of the SPA used to measure competitive binding of RhoGAP to Q61L Rac.[³H]GTP. RhoGAP competes with GST-PAK to bind Rac, thereby causing a reduction in the amount of Rac in close proximity to the SPA beads, leading to a reduction in SPA signal.

c) Illustration of the SPA used to measure competitive binding of Rho.nucleotide complex to GST-RhoGAP (mutant or wild-type). Rho.nucleotide complexes compete with Rac to bind GST-RhoGAP, thereby causing a reduction in the amount of Rac in close proximity to the SPA beads, leading to a reduction in SPA signal.
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a)

b)

c)
introduced into the assay it binds to GST-PAK under equilibrium conditions and produces a SPA signal. A non-radiolabelled protein that binds to either GST-PAK or Rac can be added to the assay, and if it competes for binding to the other partner, causes an inhibition of the Q61L Rac.[\(^{3}\)H]GTP/GST-PAK interaction and, therefore, a decrease in the amount of radiolabelled protein in close proximity to the beads. This leads to a decrease in the SPA signal, which can be measured at a range of inhibitor concentrations in order to calculate an apparent $K_i$. RhoGAP can bind to Rac and compete for binding to GST-PAK and hence the interaction of Q61L Rac.[\(^{3}\)H]GTP and wild-type and mutant RhoGAPs was investigated using this competitive inhibition assay. Although all of the kinetic experiments were carried out using Rho, it was thought likely that the effects of mutating RhoGAP on the binding interaction with Rac/Rho may be similar. Since the reagents for this particular assay were available for use this allowed the rapid characterisation of the SPA for use in comparing wild-type and mutant RhoGAPs binding constants. In addition, the use of this SPA involving Rac allowed familiarisation with the assay and the optimal conditions for such an assay. Also, this assay was further developed for use in detecting binding of GST-RhoGAPs to Rho.GMPPNP (section 3.4.2.6).

Prior to measuring the binding of RhoGAP to Rac the optimal concentrations of GST-PAK, Q61L Rac.[\(^{3}\)H]GTP and anti-GST antibody for use in this assay were determined (sections 3.4.2.1-3.4.2.3).

### 3.4.2.1 Variation of GST-PAK

Experiments were first performed in which the concentration of GST-PAK was varied (0-1.28\(\mu\)M) at two different concentrations of both Q61L Rac.[\(^{3}\)H]GTP (0.05\(\mu\)M or 0.25\(\mu\)M)
and anti-GST antibody (23μg.ml⁻¹ or 75μg.ml⁻¹). In each case the SPA signal increased in a linear fashion up to 0.08 or 0.16μM GST-PAK and then decreased as the concentration of GST-PAK was further increased (Figure 3.9). Increasing the concentration of Q61L Rac.[³H]GTP resulted in an increase in the peak signal but the overall shapes of the response were similar. Likewise, increasing the concentration of anti-GST antibody also caused an increase in the peak signal but additionally the maximum signal shifted slightly to a higher GST-PAK concentration. These results are consistent with the hypothesis made by Skinner et al. (1994), who investigated the interaction of GST-NF1 and Q61L Ras.[³H]GTP using a SPA, that with increasing concentration of GST-PAK the signal increases as more Q61L Lac.[³H]GTP.GST-PAK complex is formed. The signal then decreases due to competition between free GST-PAK and GST-PAK complexed to Q61L Lac.[³H]GTP for binding to the limited amount of antibody. At higher concentrations of antibody this competition is noticeable at slightly higher concentrations of GST-PAK. The standard GST-PAK concentration (0.02μM) used for further assays was chosen to be in the region where the signal was increasing linearly with increasing GST-PAK concentration.

3.4.2.2 Variation of Anti-GST Antibody

Experiments were performed in which the concentration of anti-GST antibody was varied (0-150μg.ml⁻¹) at a constant concentration of Q61L Lac.[³H]GTP (0.05μM) and GST-PAK (0.02μM). The signal increased as the antibody concentration increased and saturation occurred at 19μg.ml⁻¹ anti-GST (Figure 3.10). It is assumed that the maximal signal is obtained when the concentration of anti-GST binding sites is the same as the concentration of GST (Skinner et al., 1994). The standard antibody concentration
Figure 3.9 – Effect of Concentration of GST-PAK(75-132) on the SPA Signal

SPAs were performed in which the concentration of Q61L Rac.[³H]GTP was either 0.05μM (○,●) or 0.25μM (□,■) and the concentration of anti-GST antibody was 23μg.ml⁻¹ (○,□) or 75μg.ml⁻¹ (●,■). The concentration of GST-PAK was varied between 0 and 1.28μM. The signal obtained in the wells containing no GST-PAK was subtracted from each data point before plotting. All measurements were carried out in 50mM Tris/HCl pH7.5, 1mM MgCl₂, 2mM DTT.
Figure 3.10 – Effect of Concentration of Anti-GST Antibody on the SPA Signal

SPAs were performed in which the concentration of Q61L Rac[^3H]GTP (0.05μM) and GST-PAK (0.02μM) remained constant. The concentration of anti-GST antibody was varied (0-150μg.ml⁻¹). The signal obtained in the wells containing no anti-GST was subtracted from each data point before plotting. All measurements were carried out in 50mM Tris/HCl pH7.5, 1mM MgCl₂, 2mM DTT.
(19μg.ml⁻¹) used for future assays was such that the amount of antibody used was the lowest concentration of anti-GST required for attainment of the maximum signal.

### 3.4.2.3 Variation of Q61L Rac.[³H]GTP

The effects of varying Q61L Rac.[³H]GTP (0-0.8μM) at a constant concentration of GST-PAK (0.02μM) and anti-GST antibody (19μg.ml⁻¹) are shown in Figure 3.11a. In the absence of GST-PAK, increasing the concentration of Q61L Rac.[³H]GTP produced a linear increase in the SPA signal. This is referred to as the background signal. In the presence of GST-PAK a much larger increase in counts was observed and when the background was subtracted from these data the increase in counts is shown to be saturable (Figure 3.11b). From the lowest concentration of Q61L Rac.[³H]GTP to the highest, the signal to background ratio decreases from 10:1 to 2.5:1. Fitting the data to a binding isotherm gave a calculated apparent $K_d$ of 0.035μM for the interaction of Q61L Rac.[³H]GTP and GST-PAK (Figure 3.11b). This $K_d$ was required for later calculations in order to calculate $K_i$ values for inhibitory proteins. For subsequent competitive inhibition assays the concentration of Q61L Rac.[³H]GTP used was 0.025μM, at which the signal to background ratio is 10:1.

### 3.4.2.4 Binding of Wild-type and Mutant RhoGAPs to Q61L Rac.[³H]GTP using a Competitive Inhibition Assay

Using the optimised GST-PAK/Q61L Rac.[³H]GTP assay, the apparent $K_i$ values for wild-type and mutant RhoGAP were measured. When wild-type, R282A or R282K RhoGAP (0-16μM) were added to an assay containing 0.02μM GST-PAK and 0.025μM Q61L Rac.[³H]GTP a decrease in the SPA signal was detected (Figure 3.12). This was
Figure 3.11 – Effect of Concentration of Q61L Rac.$[^3]H\text{GTP}$ on the SPA Signal in the Presence and Absence of GST-PAK

Anti-GST antibody was present at a concentration of 19μg.ml$^{-1}$. The concentration of Q61L Rac.$[^3]H\text{GTP}$ was varied between 0 and 0.8μM.

a) The concentration of Q61L Rac.$[^3]H\text{GTP}$ was varied in the presence (●) or absence (○) of 0.02μM GST-PAK.

b) The signal obtained in the wells containing no GST-PAK was subtracted from each data point before plotting. The solid line represents the best hyperbolic fit to the data. The calculated apparent $K_d$ for the binding of Q61L Rac.$[^3]H\text{GTP}$ to PAK from the fit was 0.035μM with a standard error of ±0.03. All measurements were carried out in 50mM Tris/HCl pH7.5, 1mM MgCl$_2$, 2mM DTT.
Figure 3.12 – Measurements of Q61L Rac,[³H]GTP Binding to Wild-type and Mutant RhoGAPs using a Competitive Inhibition SPA

The indicated concentrations of wild-type (●), R282A (▲) or R282K RhoGAP (■) were added to a SPA assay containing 0.02μM GST-PAK and 0.025μM Q61L Rac,[³H]GTP, and the SPA signal was measured. The signal obtained from the background wells containing no GST-PAK was subtracted from the data before plotting. Data are plotted as a percentage of the maximum SPA signal obtained in the absence of competing protein, and the solid lines represent the best fit to an equation describing competitive inhibition of binding of radiolabelled Rac to GST-PAK. The apparent Kₗₛ calculated for wild-type, R282A and R282K RhoGAP are 0.63 (± 0.03) μM, 0.16 (± 0.01) μM and 2.8 (± 0.1) μM. All errors quoted correspond to the standard errors on the curve fits. All measurements were carried out in 50mM Tris/HCl pH7.5, 1mM MgCl₂, 2mM DTT.
due to RhoGAP binding competitively to Q61L Rac thereby inhibiting the GST-PAK/Q61L Rac[^3H]GTP interaction and decreasing the amount of radiolabelled protein in close proximity to the beads. The data were fitted to equation 8 (Chapter 2; section 2.15) describing competitive binding for two ligands (using the K_d for the Rac/PAK interaction; section 3.4.2.3) and the apparent K_i's calculated for wild-type, R282A and R282K RhoGAP were 0.63, 0.16 and 2.8 μM, respectively (Figure 3.12). The apparent K_i in this case is equal to the apparent K_d for the RhoGAP/Rac interaction. The affinity of RhoGAP for Q61L Rac[^3H]GTP was increased 4-fold by the R282A mutation and decreased 4-fold by the R282K mutation.

3.4.2.5 Direct Binding of GST-RhoGAP to Q61L Rac[^3H]GTP or Q63L Rho[^3H]GTP

Although the above assay (Rac/PAK) was useful in the rapid characterisation of the binding of Q61L Rac[^3H]GTP to wild-type and mutant RhoGAPs, it could not be used to measure binding of RhoGAP to Rho proteins bound to a range of nucleotides. In order to do this, Rho.nucleotides needed to act as competitive inhibitors in an assay where GST-RhoGAPs bind to Q61L Rac[^3H]GTP directly. Hence, a SPA similar to the Rac/PAK assay but measuring the Rac/RhoGAP interaction was set up.

In this assay GST-wild-type, R282A or R282K RhoGAP (0.02 μM) were substituted for GST-PAK and the concentration of anti-GST antibody remained the same as in the Rac/PAK assay (19 μg.ml^-1). Binding of GST-RhoGAPs to Q61L Rac[^3H]GTP could then be measured directly (Figure 3.8). Q61L Rac[^3H]GTP (0-3.2 μM) was titrated into the assay at a constant concentration of GST-wild-type, R282A or R282K RhoGAP (0.02 μM) and the SPA signals obtained were fitted to hyperbolic binding curves (Figure
The apparent \( K_d \) values for the interaction of wild-type, R282A or R282K RhoGAP to Q61L Rac.\(^3\)H]GTP were found to be 0.2, 0.06 and 1.6\( \mu \)M, respectively. The affinity of RhoGAP for Q61L Rac.\(^3\)H]GTP was increased 3-fold by the R282A mutation and decreased 8-fold by the R282K mutation. These comparative values are similar to those obtained using RhoGAP as a competitive inhibitor in the Rac/PAK assay (Figure 3.12; section 3.4.2.4), although the apparent \( K_d \) values are all 2- to 3-fold lower in the direct binding assay. There are two possible reasons for these differences. Firstly, as previously mentioned (section 2.15), the equation used to calculate the \( K_i \) values does not take into account inhibitor depletion by binding to the protein. Hence, the apparent \( K_i \) values will represent upper limits of the true value. Secondly, GST-RhoGAPs are used in the direct binding assay rather than cleaved RhoGAPs (no GST). The data presented here suggest that, for measuring comparative binding affinities between wild-type and mutant RhoGAPs, the methods of direct binding and competitive inhibition to measure binding affinities are equivalent.

This RhoGAP/Rac SPA was used further to measure the apparent \( K_d \) values of wild-type and mutant RhoGAPs for Rho.GMPPNP, using Rho.GMPPNP as a competitive inhibitor in the assay (section 3.4.2.6). It was also utilised further to examine the interactions of wild-type and mutant RhoGAPs with Rho.GDP (Chapter 4).

When the expression clone for Q63L Rho became available this protein was purified and the \(^3\)H]GTP bound form was substituted for Q61L Rac in the assay described above to measure the direct binding of Q63L Rho.\(^3\)H]GTP to wild-type and mutant RhoGAPs. It was evident from initial experiments using 0.02\( \mu \)M GST-RhoGAPs, as had
Figure 3.13 – Measurements of Wild-type, R282A and R282K RhoGAP Binding to Q61L Rac.[³H]GTP using a Direct Binding SPA

The indicated concentrations of Q61L Rac.[³H]GTP were added to an assay containing 0.02µM wild-type (●), R282A (▲) or R282K (■) GST-RhoGAP and the SPA signal was monitored by scintillation counting. The solid lines represent the best hyperbolic fits to the data with apparent Kₐ values for the interaction of Q61L Rac.[³H]GTP with wild-type, R282A or R282K GST-RhoGAP of 0.2 (± 0.03) µM, 0.06 (± 0.01) µM and 1.6 (± 0.3) µM, respectively. The signal obtained from the control wells containing Rac but no RhoGAP was subtracted from the data before plotting. All errors quoted correspond to the standard errors on the curve fits. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT.
previously been used, that the affinity of Q63L Rho.[\(^3\)H]GTP for RhoGAP was significantly increased compared to Q61L Rac.[\(^3\)H]GTP. Therefore, to improve the data obtained, in the case of wild-type and R282A GST-RhoGAPs, the concentration of GST-RhoGAP was reduced to 0.005\(\mu\)M. SPA signals obtained were fitted to hyperbolic binding curves (Figure 3.14) with apparent \(K_d\) values of 0.016, 0.014 and 0.3\(\mu\)M for the binding of wild-type, R282A and R282K RhoGAP to Q63L Rho.[\(^3\)H]GTP, respectively. The affinities of wild-type and R282A RhoGAP for Q63L Rho.[\(^3\)H]GTP were similar although the R282K mutation cause a 19-fold reduction in affinity.

### 3.4.2.6 Interaction of GST-RhoGAP and Rho.GMPPNP or Rho.mantGMPPNP

**Measured by Competitive Inhibition**

Using the GST-RhoGAP/Rac SPA as detailed in section 3.4.2.5, Rho bound to non-radiolabelled nucleotides could be used in the assay as competitive inhibitors of the RhoGAP/Rac interaction (Figure 3.8). This allowed the affinities of Rho.nucleotide and Rho.mant-nucleotide complexes binding to GST-RhoGAP to be compared in the same assay. The direct measurements of the apparent \(K_d\) values for the interactions between wild-type and mutant RhoGAPs and Q61L Rac.[\(^3\)H]GTP described in section 3.4.2.5 were necessary in order to go on to calculate the apparent \(K_i\) values of any inhibiting proteins added into the assay.

In the case of R282K RhoGAP, the binding to Q61L Rac.[\(^3\)H]GTP was significantly weaker than with wild-type or R282A RhoGAP. At the concentration of Q61L Rac.[\(^3\)H]GTP used (0.025\(\mu\)M) the counts were too low to get the required ratio of signal to background. This assay could, therefore, not be utilised further. Using the wild-type
Figure 3.14 – SPA Measurements of Wild-type, R282A and R282K RhoGAP Binding to Q63L Rho.[³H]GTP

The indicated concentrations of Q63L Rho.[³H]GTP were added to an assay containing 0.005µM wild-type (●), 0.005µM R282A (▲) or 0.02µM R282K (■) GST-RhoGAP and the SPA signal was measured. The signal obtained from the background wells containing no GST-RhoGAP was subtracted from the data before plotting. The solid lines represent the best hyperbolic fits to the data with apparent K_d values for the interaction of Q63L Rho.[³H]GTP with wild-type, R282A or R282K GST-RhoGAP of 0.016 (± 0.001) µM, 0.014 (± 0.001) µM and 0.3 (± 0.01) µM, respectively. All errors quoted correspond to the standard errors on the curve fits. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl_2, 1mM DTT.
SPA Signal (% maximum)

[Q63L Rho.[$^3$H]GTP] (µM)
and R282A RhoGAP/Rac assays, however, the apparent $K_i$ values for any protein competing with Rac for the binding of RhoGAP could be calculated (Figure 3.8). Rho.GMPPNP and Rho.mantGMPPNP (0-16μM) were titrated into the GST-RhoGAP/Rac assays and were effective in inhibiting binding of Q61L Rac.[$^3$H]GTP to wild-type and R282A GST-RhoGAPs and, therefore, reducing the SPA signal (Figure 3.15). The decreases in SPA signal were fitted to equation 8, describing competitive binding for two ligands, and the apparent $K_i$s for the competing proteins were calculated. The apparent $K_i$ values represent the binding constant for the inhibitory protein and GST-RhoGAP. The apparent $K_d$ values for Rho.GMPPNP and wild-type and R282A GST-RhoGAPs were 2.3 and 5.7μM, respectively. The apparent $K_d$s for Rho.mantGMPPNP and wild-type and R282A GST-RhoGAPs were 6.0 and 8.5μM, respectively. These data indicate a decrease in affinity of approximately 2-fold caused by the use of mantGMPPNP rather than GMPPNP. Also, the mutation of Arg 282 to alanine has caused an approximate 2-fold decrease in the affinity of RhoGAP for Rho.GMPPNP or Rho.mantGMPPNP.

The affinity of wild-type RhoGAP for Rho.mantGMPPNP as measured by fluorescence anisotropy was comparable to the result obtained using the SPA. There was only a 1.5-fold difference in the binding affinities obtained despite the use of different techniques. In contrast, the results obtained using fluorescence anisotropy indicate that the Arg to Ala mutation caused a significant reduction in binding of RhoGAP to Rho.mantGMPPNP (6-fold), although as measured by the SPA the reduction is not as significant (1.5-fold decrease). Thus, the results from these two methods were not in complete agreement. Unfortunately, the affinity of R282K RhoGAP and Rho.GMPPNP could not be obtained.

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Figure 3.15 – SPA Measurements of Rho.GMPPNP Binding to Wild-type and R282A RhoGAPs

The indicated concentrations of Rho.GMPPNP (●) or Rho.mantGMPPNP (○) were added to SPAs containing Q61L Rac.[³H]GTP and either wild-type GST-RhoGAP (a) or R282A GST-RhoGAP (b), and the SPA signal was measured. The signal from a blank without GST-RhoGAP was subtracted, and the data plotted as a percentage of the signal in the absence of competing protein. The solid lines represent the best fit to an equation describing competitive inhibition of binding of radiolabelled Rac to GST-RhoGAP.

With wild-type RhoGAP, the apparent K$_d$ values are 2.3 (± 0.4) μM for Rho.GMPPNP and 6.0 (± 0.6) μM for Rho.mantGMPPNP. With R282A RhoGAP, the apparent K$_d$ values are 5.7 (± 1.2) μM for Rho.GMPPNP and 8.5 (± 1.3) μM for Rho.mantGMPPNP.

All errors quoted correspond to the standard errors on the curve fits. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl$_2$, 1mM DTT.
from either the SPA or fluorescence anisotropy measurements. For these reasons, it was, therefore, decided to utilise a further technique to measure binding affinities.

3.4.3 Isothermal Titration Calorimetry (ITC)

ITC is a technique used to detect binding reactions through measuring associated heat changes (reviewed in Ladbury and Chowdhry, 1996; Doyle, 1997). ITC is a true ‘in solution’ equilibrium-binding method. It does not require immobilisation of binding components as in the scintillation proximity assay, nor does it require chemical labelling of reactants as with fluorescence anisotropy. However, a relatively large amount of protein is needed, especially for monitoring weak affinity interactions. In ITC, small aliquots of one reactant are titrated into a cell containing a solution of the second reactant and the absorption or generation of heat is measured. These heat changes are converted to an integrated enthalpy for each step, and used to obtain a binding isotherm for the interaction. This cumulative measurement of heat changes allows an accurate determination of the binding constant, $K_a$, reaction stoichiometry, $n$, and enthalpy, $\Delta H$. Using these experimentally determined $K_a$ and $\Delta H$ values, this technique allows the calculation of $\Delta G$ and $\Delta S$ from the relationship:

$$-RT \ln K_a = \Delta G = \Delta H - T\Delta S$$

However, an evaluation of the thermodynamic properties of the binding interactions between wild-type or mutant RhoGAPs and Rho was not the aim of these studies, and ITC has been used primarily to determine binding affinities using the heat signal produced as a measurement of the binding interaction. In addition the stoichiometry
obtained was used as a measure of the activity of the proteins, allowing a comparison between the activity of wild-type and mutant RhoGAP proteins.

Previously, the interactions between Ras and NF1, Ras and Raf (Lawton et al., 1997) and Cdc42 and PAK (Thompson et al., 1998) have been investigated using ITC. Here, ITC has been used to examine the binding interactions between wild-type or mutant RhoGAPs and Rho.GMPPNP.

3.4.3.1 ITC Measurements of the Interaction of Rho.GMPPNP with Wild-type, R282A and R282K RhoGAP

In a control experiment, titration of Rho.GMPPNP into buffer resulted in small exothermic heat changes representing the heat of dilution (Figure 3.16; Trace 1). When buffer was replaced with wild-type, R282A or R282K RhoGAP, the resultant heat changes were larger and endothermic (Figure 3.16; Traces 2, 3 and 4). As the titration progressed the area under the peaks reduced as saturation of RhoGAP approached until eventually small exothermic heat changes could be seen (Figure 3.16). However, these heats changes seen after Rho.GMPPNP had been saturated with RhoGAP were not always identical to the heat changes in control experiments where Rho.GMPPNP was titrated into buffer alone. The single-site binding model used in the Microcal origin software (Wiseman et al., 1989) relies on a zero baseline after saturation, thus, rather than subtracting the control data from the data with RhoGAP, the end-point of the titrations were adjusted to zero before curve fitting. On fitting the data to single binding site isotherms using the Microcal Origin software, the $K_d$ values for the interactions were obtained. Table 3.2 summarises the data obtained for these interactions. The $K_d$ values
Figure 3.16 – Isothermal Titration Calorimetry Measurements of the Affinity of Rho.GMPPNP for Wild-type, R282A and R282K RhoGAPs

Rho.GMPPNP (0.18mM) was titrated into buffer alone (trace 1), 15µM wild-type RhoGAP (trace 2, ●), 15µM R282A RhoGAP (trace 3, ▲) or 15µM R282K RhoGAP (trace 4, ■) in an isothermal titration calorimeter and heat changes were measured continuously. The raw data from each injection are shown in the top panel. The measured heats from each peak were integrated and the resultant data are plotted in the lower panel as molar ratio of Rho.GMPPNP injected to RhoGAP present against the heat output per mole of injected Rho.GMPPNP. The lines through the points are the best fits to the data assuming a single binding site model with the following parameters: $K_d = 0.8\mu M$, $n = 0.98$ and $\Delta H = 4.1$ kcal.mol$^{-1}$ for wild-type, $K_d = 1.6\mu M$, $n = 0.82$ and $\Delta H = 7.9$ kcal.mol$^{-1}$ for R282A and $K_d = 3.5\mu M$, $n = 0.86$ and $\Delta H = 4.1$ kcal.mol$^{-1}$ for R282K RhoGAP. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl$_2$, 1mM DTT at 20°C. The traces shown are representative of one of two experiments performed for each interaction. The results obtained from duplicate experiments are summarised in table 3.2.
Table 3.2 – Summary of the Parameters Determined by ITC for the Interaction of Wild-type, R282A or R282K RhoGAPs and Rho.GMPPNP

This table shows the experimentally determined values for the association constant ($K_a$), the enthalpy change ($\Delta H$) and the stoichiometry ($n$) obtained from individual ITC experiments. The values for the calculated $K_d$ are also included. Mean values are in bold. These mean values are the values quoted in the text and listed in the summary table (Table 3.3). The errors quoted for $K_a$, $\Delta H$ and $n$ correspond to the standard error on the fit to a single binding site isotherm (Wiseman et al., 1989).

<table>
<thead>
<tr>
<th>RhoGAP</th>
<th>$K_a \times 10^6$ (M$^{-1}$)</th>
<th>$K_d$ (µM)</th>
<th>$\Delta H$ (kcal.mol$^{-1}$)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.83 ± 0.23</td>
<td>1.20</td>
<td>5.98 ± 0.44</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>1.29 ± 0.22</td>
<td>0.78</td>
<td>4.14 ± 0.12</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1.06</td>
<td>0.99</td>
<td>5.06</td>
<td>0.96</td>
</tr>
<tr>
<td>R282A</td>
<td>1.09 ± 0.22</td>
<td>0.92</td>
<td>6.07 ± 0.40</td>
<td>0.89 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>0.64 ± 0.07</td>
<td>1.56</td>
<td>7.94 ± 0.22</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>1.24</td>
<td>7.00</td>
<td>0.86</td>
</tr>
<tr>
<td>R282K</td>
<td>0.36 ± 0.08</td>
<td>2.78</td>
<td>3.99 ± 0.20</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.29 ± 0.05</td>
<td>3.45</td>
<td>4.05 ± 0.31</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.38</td>
<td>3.12</td>
<td>4.02</td>
<td>0.91</td>
</tr>
</tbody>
</table>
for wild-type, R282A and R282K RhoGAP were 1.0, 1.2 and 3.1μM. The substitution of arginine to alanine did not significantly effect the binding interaction, compared to a 3-fold decrease caused by the lysine substitution. The apparent stoichiometry of the interactions varied from 0.86-0.96. As it is known that one mole of a Rho protein interacts with one mole of RhoGAP from structural evidence (Rittinger et al., 1997a), the stoichiometries obtained indicate high activity of the proteins, suggesting that the mutations had no gross affect on protein stability. The results obtained using ITC show that mutation of the arginine to alanine does not affect the binding interaction between RhoGAP and Rho.GMPPNP. In contrast the fluorescence anisotropy results, using mantGMPPNP, (section 4.3.1) show a 6-fold decrease in binding due to the alanine mutation. This difference could be due to differences in the interaction between R282A RhoGAP and Rho.GMPPNP versus Rho.mantGMPPNP caused by the presence of the mant group, although SPA data suggest only a 2-fold difference. Alternatively it could be a result of the different methods used. Unfortunately, Rho.mantGMPPNP could not be substituted for Rho.GMPPNP in the calorimeter to allow a comparison with fluorescence anisotropy data. This was due to the large amounts of mantGMPPNP required for complexing with Rho in order to carry out the required titrations. GMPPNP is expensive to buy and the yield obtained from the preparation of mantGMPPNP is poor, making it costly.

The binding data described here using fluorescence anisotropy, SPA, and ITC are summarised in Table 3.3.
Table 3.3 - Equilibrium Dissociation Constants for Wild-type and Mutant RhoGAPs

Equilibrium dissociation constants were measured using the indicated methods as described in the text.

<table>
<thead>
<tr>
<th>Rho complex</th>
<th>Method</th>
<th>K_d (µM)</th>
<th>K_d (µM)</th>
<th>K_d (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho.GTP Rho.GTP</td>
<td>GTPase</td>
<td>≤1 (^a)</td>
<td>≤1 (^a)</td>
<td>3.9 (^a)</td>
</tr>
<tr>
<td>Rho.mantGTP</td>
<td>GTPase</td>
<td>2.7 (^b)</td>
<td>4.5 (^c)</td>
<td>25 (^c)</td>
</tr>
<tr>
<td>Rho.GMPPNP</td>
<td>ITC</td>
<td>1.0</td>
<td>1.2</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>SPA (^d)</td>
<td>2.3</td>
<td>5.7</td>
<td>e</td>
</tr>
<tr>
<td>Rho.mantGMPPNP</td>
<td>SPA (^d)</td>
<td>6.0</td>
<td>8.5</td>
<td>e</td>
</tr>
<tr>
<td></td>
<td>Anisotropy</td>
<td>3.6</td>
<td>21</td>
<td>5.3</td>
</tr>
<tr>
<td>Q63L Rho.GTP</td>
<td>SPA (^f)</td>
<td>0.016</td>
<td>0.014</td>
<td>0.3</td>
</tr>
<tr>
<td>Q61L Rac.GTP</td>
<td>SPA (^f)</td>
<td>0.2</td>
<td>0.06</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>SPA (^d)</td>
<td>0.63</td>
<td>0.16</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Key**

\(^a\) K_m value (≥ K_d)
\(^b\) Stopped-flow
\(^c\) Quench-flow
\(^d\) Competitive inhibition SPA
\(^e\) SPA signal insufficient to allow K_d determination
\(^f\) Direct binding SPA
3.5 Discussion

Single enzyme turnover experiments have been used with Ras.mantGTP to measure the catalytic rate constant \( k_{\text{cat}} \) of Ras-GAPs (Eccleston et al., 1993; Ahmadian et al., 1997a; Ahmadian et al., 1997c; Sermon et al., 1998). As \( k_{\text{cat}} \) is measured by observing the maximum \( k_{\text{obs}} \) at saturating GAP, these experiments have the advantage over steady-state catalytic measurements in that there is no requirement for precise measurements of the active concentration of GAP. This is illustrated by the fact that, using steady-state kinetic measurements to obtain the \( k_{\text{cat}} \) for NF1-catalysed Ras-GTPase activity, the \( k_{\text{cat}} \) was found to be \( 1.4\text{s}^{-1} \) (Wiesmüller and Wittinghofer, 1992), an order of magnitude less than the value of \( 10\text{s}^{-1} \) that was obtained using single-turnover kinetic measurements (Eccleston et al., 1993). In steady-state experiments, the \( k_{\text{cat}} \) is calculated from the experimentally obtained \( V_{\text{max}} \) and the enzyme concentration. In the measurements by Wiesmüller and Wittinghofer (1992) it was incorrectly assumed that the GAP protein was pure and fully active, leading to an inaccurate estimation of the enzyme concentration. It was later reported that, using an improved purification procedure to obtain a more active preparation of NF1, the \( k_{\text{cat}} \) was indeed similar to that obtained by Eccleston et al. (1993) (Ahmadian et al., 1996).

Using single-turnover techniques similar to those used for Ras-GAPs (Eccleston et al., 1993; Ahmadian et al., 1997a; Ahmadian et al., 1997c; Sermon et al., 1998) the \( k_{\text{cat}} \) values for wild-type and mutant RhoGAPs catalysing the hydrolysis of GTP or mantGTP by Rho were measured. The \( k_{\text{cat}} \) for catalysis of Rho-GTPase activity by wild-type RhoGAP has been estimated to be \( 5\text{s}^{-1} \). Wild-type RhoGAP catalyses Rho-mantGTPase activity with a \( k_{\text{cat}} \) of \( 2.3\text{s}^{-1} \), as measured by the fluorescence change assumed to be accompanying hydrolysis. If the fluorescence change is, however, measuring the
dissociation of RhoGAP and Rho.GDP (k₃ in scheme I; section 3.3.2) that takes place after GTP hydrolysis, then the rate of fluorescence change may be lower than the rate of cleavage. In any case, the kₕ value referred to here will only be a true estimate or underestimate of the actual value. These values represent a 36,000-fold or 77,000-fold stimulation of the intrinsic GTP or mantGTP hydrolysis rates, respectively (Rho.GTP 1.4 x 10⁻⁴ s⁻¹; Rho.mantGTP 3 x 10⁻⁵ s⁻¹). These levels of stimulation are similar to those seen with p120GAP and neurofibromin (NF1) on Ras.mantGTP when measured using single-turnover experiments (Eccleston et al., 1993; Ahmadian et al., 1997c, Sermon et al., 1998).

Using Rho.GTP, the kₕ for R282A RhoGAP was 0.025 s⁻¹ and for R282K RhoGAP was 0.010 s⁻¹. These values represent a reduction in the ability of RhoGAP to stimulate Rho-GTPase activity by 200- and 500-fold, respectively. In terms of stimulation of intrinsic GTPase activity this corresponds to a 180- and 70-fold stimulation by R282A and R282K RhoGAP, respectively, compared to a 36,000-fold stimulation by wild-type RhoGAP.

Using Rho.mantGTP, the kₕ for R282A RhoGAP was 0.012 s⁻¹ and for R282K RhoGAP kₕ was 0.0011 s⁻¹. These represent a reduction in the ability of RhoGAP to stimulate Rho-mantGTPase activity by 190- and 2100-fold, respectively. In terms of stimulation of intrinsic GTPase activity this corresponds to a 400- and 35-fold stimulation by R282A and R282K RhoGAP, respectively, compared to a 77,000-fold stimulation by wild-type RhoGAP. In all cases the kₕ values measured when Rho was bound to mantGTP, are lower (≤9-fold) than those measured when GTP itself is bound. However, in both cases the data show that neither alanine nor lysine could substitute for arginine and maintain efficient catalysis. Early investigations using Cdc42 also revealed that mutation of Arg 282 in RhoGAP to either alanine or lysine resulted in a protein no longer able to stimulate
Cdc42-GTPase activity as efficiently as wild-type protein (Graham et al., 1997). The objective of mutating arginine to lysine was to see if substitution with another basic side chain could retain the catalytic function of RhoGAP. However, since substitution with lysine severely impaired the catalytic activity of RhoGAP, specific properties of the arginine side chain must be required, e.g. charge distribution, steric qualities or bonding capabilities. Similarly, one of the two conserved catalytic arginines in Ras-GAPs (R1276 in NF1; R789 in p120GAP) could not be replaced with lysine and maintain efficient catalysis of Ras-GTPase activity (Ahmadian et al., 1997c).

As it was found here that mutation of the conserved arginine resulted in a loss of activity, it was important to determine effects on binding to the triphosphate form of Rho to examine whether the loss of catalytic function was due to alterations in the binding interactions. The $K_m$ values of wild-type and mutant RhoGAPs for Rho.GTP were estimated from the single-turnover kinetic experiments. If the initial binding step is a rapid equilibrium relative to $k_{cat}$, as has previously been assumed when measuring RasGAP kinetics (Ahmadian et al., 1997a; Sermon et al., 1998), $K_m$ will equal $K_d$. In any case, the true $K_d$ for the interaction with Rho.GTP will be less than or equal to this measured $K_m$. The $K_m$ values for both wild-type and R282A RhoGAP interacting with Rho.GTP were estimated to be $\leq 1 \mu M$. From the data obtained it is difficult to say definitively what effect the mutation of arginine to alanine had on this binding parameter. With Rho.mantGTP the $K_m$ values were similar for wild-type and R282A RhoGAP (wild-type 2.7$\mu M$; R282A 4.5$\mu M$). However, the $K_m$ values obtained for R282K were 3.9$\mu M$ with Rho.GTP and 25$\mu M$ with Rho.mantGTP, indicating that the arginine to lysine substitution had a more detrimental effect on the $K_m$. Using the single turnover experiments, $K_m$ values were determined rather than $K_d$ values and even these $K_m$ values...
were difficult to accurately obtain. Therefore, the binding interaction was investigated independently of the catalytic activity using equilibrium binding methods in order to determine \( K_d \) values. In order to determine the \( K_d \) values of wild-type and mutant RhoGAPs for the triphosphate-bound form of Rho, the affinities of RhoGAPs for Rho.GMPPNP and for Q63L Rho.GTP, neither of which show any significant hydrolysis during the time course of the experiments, were measured directly using ITC and SPA experiments (Table 3.3). Additionally, the affinity of wild-type and mutant RhoGAPs for Q61L Rac.[\(^3\)H]GTP were obtained from SPA experiments. Although ITC would be expected to give the true \( K_d \) values and SPAs measure an apparent \( K_d \) (due to calculations for determining binding affinity not taking into account any changes in local concentration due to binding to the SPA beads), these two methods gave similar values, although in general, the affinity measured by ITC was about 2 to 5-fold higher than that determined by the SPA. The measured affinity of wild-type RhoGAP for Rho.GMPPNP reported here (ITC 1.0\( \mu \)M; SPA 2.3\( \mu \)M) is similar to published values of 1.2\( \mu \)M and 2.5\( \mu \)M that were obtained using assays to measure the inhibition of GAP activity by Rho.GMPPNP (Self and Hall, 1995b; Zhang and Zheng, 1998). The affinity of wild-type RhoGAP for Q63L Rho.GTP was at least 50-fold higher than that for Rho.GMPPNP. This is consistent with previous reports that the Q61L Ras and Q63L Rho bind to their GAPs with higher affinity than wild-type proteins, although the reasons for this are unclear (Bollag and McCormick, 1991; Self and Hall, 1995b). The alanine mutation resulted in a small decrease (2 to 3-fold) in the affinity of RhoGAP for Rho.GMPPNP, no measurable difference in the affinity for Q63L Rho.GTP and a 4-fold increase in the affinity of RhoGAP for the GTP form of Q61L Rac. The lysine mutation resulted in a more significant impairment of binding (3 to 19-fold decrease in affinity) to Rho.GMPPNP, Q63L Rho.GTP and Q61L Rac.GTP, suggesting that this mutation has
caused some structural perturbation that has affected the binding interaction and has likely contributed to the reduction in catalysis. Using the SPA assay to measure the $K_d$ of wild-type and R282A RhoGAP for Rho.mantGMPPNP gave similar values for both interactions (wild-type 6.0μM; R282A 8.5μM), with the presence of the mant group causing a slight reduction in the affinity of these RhoGAPs for Rho.GMPPNP. Using fluorescence anisotropy measurements there was a more significant reduction in binding affinity when arginine was mutated to alanine (wild-type 3.6μM; R282A 21μM). Since the affinities of wild-type and R282A RhoGAP for Rho.GMPPNP are similar as measured by ITC and SPA, it appears that the data obtained using fluorescence anisotropy methods with Rho.mantGMPPNP do not reflect the true effects of the Arg to Ala mutation. It may be that this is a consequence of the method rather than the presence of the mant group, although this has not been investigated.

ITC measurements of the interaction of wild-type and mutant RhoGAPs with Rho.GMPPNP gave a measure of the enthalpy changes of the interaction. The enthalpy changes ($\Delta H$) associated with the interactions of wild-type, R282A or R282K RhoGAP with Rho.GMPPNP are positive (endothermic) and from the relationships $-RT \ln K_a = \Delta G = \Delta H - T\Delta S$, $\Delta S$ values are also positive. This indicates that the binding interactions are entropically driven. The $\Delta H$ value associated with the interaction between R282A RhoGAP and Rho.GMPPNP is higher than those associated with the interaction of either wild-type or R282K RhoGAP binding to Rho.GMPPNP (Figure 3.16). The $K_a$ values for wild-type and R282A RhoGAPs are similar and so, therefore, are the free energy changes, the $\Delta G$ values. The similar $\Delta G$ and different $\Delta H$ values mean that the $\Delta S$ values will be different, with the $\Delta S$ value for the mutant protein being more positive. These results indicate that the binding interaction between R282A RhoGAP and
Rho.GMPPNP results in a larger increase in entropy than that between wild-type RhoGAP and Rho.GMPPNP. This difference is likely to be due to some structural rearrangement. From the structure of Cdc42.GMPPNP complexed to wild-type RhoGAP (Rittinger et al., 1997a) the Arg 282 residue is known to hydrogen bond to Gly 12 of Cdc42. The loss of the arginine residue in the R282A mutant is likely to result in the loss of this interaction. It is possible that this results in a less rigid structure associated with a larger entropy.

In summary, Arg 282 contributes 200-fold out of the total 36,000-fold catalytic stimulation by RhoGAP, but, in contradiction to the hypothesis of Barrett et al. 1997, is not required for binding of RhoGAP to the triphosphate-bound conformation of Rho. This arginine residue, therefore, plays an important role in the activation of the hydrolysis of GTP by RhoGAP. The mechanism by which this residue contributes to the catalytic function of RhoGAP is discussed in later chapters. The involvement of this conserved arginine in the catalytic function of RhoGAP is in qualitative agreement with reported data on the role of the conserved arginine in the Rho-GAP, n-chimaerin (Ahmed et al., 1994), and reports published subsequent to the work performed here on the Rho-GAPs, myr5 (Müller et al., 1997), Cdc42GAP (synonymous to RhoGAP) (Hoffman et al., 1998; Leonard et al., 1998), and p190GAP (Li et al., 1997).

The catalytic role of Arg 282 in RhoGAP is also similar to the role of a conserved arginine residue in GAPs for Ras family proteins. Unlike the single conserved arginine residue in Rho-GAPs, there are two conserved arginine residues in Ras-GAPs (R789 and R903 in p120GAP; R1276 and R1391 in NF1). Mutation of either of these residues in p120GAP and NF1 can cause a reduction in the GAP activity (Skinner et al., 1991;
Brownbridge et al., 1993; Gutmann et al., 1993; Ahmadian et al., 1997c; Sermon et al., 1998). Although the majority of the stimulation of intrinsic GTPase activity has been attributed to R1276 in NF1 or R789 in p120GAP, it was also shown that the second residue in Ras-GAPs (R1391 in NF1; R903 in p120GAP) contributed to the GAP induced GTPase activity (Ahmadian et al., 1997c; Sermon et al., 1998). Structures of p120GAP alone and in complex with Ras (Scheffzek et al., 1996; Scheffzek et al., 1997) show that two arginines are situated close to each other and it was proposed that R789 performs the catalytic role whilst R903 stabilises the orientation of this catalytic arginine. The catalytic function of either R1276 in NF1 or R789 in p120GAP could not be replaced by lysine (Ahmadian et al., 1997c). This parallels the data presented in this thesis showing that the catalytic arginine of RhoGAP, Arg 282, cannot be substituted for lysine and still maintain catalytic activity. However, the stabilising arginine residue of NF1 or p120GAP could be replaced by lysine. In RhoGAP it is likely that a similar stabilising role is carried out by the conserved lysine residue, Lys 319 (residue 122 in the crystallized fragment of RhoGAP (Barrett et al., 1997)). This conserved lysine residue located in the B-helix, close to the RhoGAP/small G protein interaction site, is solvent exposed, and is involved in a main-chain hydrogen bonding network with a number of residues including Arg 282 (Barrett et al., 1997; Rittinger et al., 1997a). Mutation of Lys 391 in RhoGAP or the corresponding residue in p190GAP, Lys 1321, to alanine has been shown to result in a loss of GAP activity, similar to that seen on mutation of the conserved arginine residue (Li et al., 1997). It is therefore, apparent that two conserved basic residues in both Ras-GAPs and Rho-GAPs are important for their catalytic function. The absolute requirement for arginine as the catalytic residue is likely to be due to the strict stereochemical requirements for enzyme catalysis. The role of the stabilising residue seems only dependent on the placement of a positive charge near to this catalytic residue.
The data presented here and the data published on mutagenesis of Ras-GAPs (Ahmadian et al., 1997c; Sermon et al., 1998) support the mechanism by which the GAP protein supplies a catalytic residue to the small G protein thereby stabilising the transition state of the GTP hydrolysis reaction. It is evident also that mutation of the catalytic arginine residue in RhoGAP does not completely abolish all of the GAP-enhanced GTPase activity and it is likely that the GAP also stabilises the small G protein into a catalytically competent state. This will discussed in more detail in Chapter 6.
CHAPTER 4

*Aluminium Fluoride Mediated Complexes between RhoGAP and Rho*
4.1 Introduction

In 1958, Rall and Sutherland first described the stimulation of adenylate cyclase by the hormones adrenaline and glucagon, and the subsequent production of cyclic AMP. Their studies also revealed that NaF was an activator of adenylate cyclase, although the mechanism of fluoride stimulation of this enzymatic activity remained unknown for many years. The target of F⁻ was identified as a guanine nucleotide-binding regulatory component of adenylate cyclase (Ross and Gilman, 1977), later identified as the α-subunit of the heterotrimeric G protein (Gₐ) that couples the hormone-activated receptor to the adenylate cyclase (Northup et al., 1983). Fluoride activation of the Gₐ subunit and the resulting production of cAMP was shown to be dependent on the presence of Mg²⁺ and ATP (Sternweis et al., 1981). However, the requirement for nucleotide to achieve similar levels of activation was sporadic. Investigation into the cause of these variable results revealed that they were due to contaminating Al³⁺ present either in the reagents, due to leaching from glass tubes, or in the nucleotide preparation (Sternweis and Gilman, 1982). Under the conditions used, i.e. 5mM NaF and 10mM MgCl₂, the presence of contaminating aluminium was found to be essential for fluoride activation. Further investigation revealed that the fluoride-mediated activation of the Gₐ subunit was specific for Al³⁺ or Be²⁺, and the activating species at 5mM NaF were suggested to be AlF₄⁻ or BeF₃⁻ (Sternweis and Gilman, 1982).

In an analogous system, fluoride was shown to stimulate the production of cGMP by photoreceptor phosphodiesterase via activation of the α-subunit of the heterotrimeric G protein, transducin (Stein et al., 1985). Using this system, it was shown that fluorides in the millimolar range mimicked the effects of GTP or hydrolysis-resistant GTP analogues (Northup et al., 1983; Stein et al., 1985). Further studies demonstrated that one molecule
of aluminium fluoride activates one transducin\(_\alpha\),GDP (T\(_\alpha\)) molecule and aluminium fluoride had no effect on T\(_\alpha\) when the nucleotide site was empty or contained GDP\(\beta\)S (Bigay et al., 1985). This evidence led to the proposal that aluminium fluoride might have a tetrahedral coordination and act as an analogue of the \(\gamma\)-phosphate of GTP (Bigay et al., 1985; Bigay et al., 1987).

Whilst characterising the stabilising effects of aluminium fluoride and beryllium fluoride on microtubules bound to GDP or ADP, Combeau and Carlier (1989) found that both fluoride complexes bound to GDP and ADP with an affinity higher than the natural ligand, Pi. This led the authors to speculate that these complexes were forming transition state analogues. Indeed, the X-ray structures of G\(_{\text{tot}}\).GDP.AlF\(_4^-\) and G\(_{\text{tot}}\).GDP.AlF\(_4^-\) revealed that the bound AlF\(_4^-\) was octahedrally coordinated with the fluorines in a planar configuration (Coleman et al., 1994; Sondek et al., 1994). By analogy to the transition state of the phosphoryl transfer reaction, in which phosphate adopts a trigonal bipyramidal arrangement, this configuration led to the proposal that the complexes were in fact analogues of the transition state (Coleman et al., 1994; Sondek et al., 1994).

Despite the structural and biochemical similarities between heterotrimeric G proteins and small G proteins (Bourne et al., 1991), until recently it was thought that aluminium fluoride could not bind to members of the Ras superfamily of small G proteins. This was shown to be true for Ras, Rap, Rab and Arf proteins (Kahn, 1991). However, Mittal et al. (1996) demonstrated that aluminium fluoride could bind to GDP-bound Ras and form a stable complex, provided that Ras-GAPs were present. This ability of aluminium fluoride to induce high affinity complex formation was later shown to be a general phenomenon for members of the Ras superfamily of small G proteins and their respective
GAPs (Ahmadian et al., 1997b). Furthermore, X-ray crystallography studies of the aluminium fluoride-mediated complexes between Ras.GDP and RasGAP (Scheffzek et al., 1997), Rho.GDP and RhoGAP (Rittinger et al., 1997b) and Cdc42.GDP and Cdc42GAP (analogous to RhoGAP) (Nassar et al., 1998) have shown that the coordination of aluminium is similar to that found in the $G_\alpha$ structures (Coleman et al., 1994; Sondek et al., 1994). Hence, these structures have been proposed to be transition state analogues.

The X-ray structure of the proposed transition state complex between RhoGAP, Rho.GDP and AlF$_4^-$ (Rittinger et al., 1997b), shows that the conserved Arg 282 residue of RhoGAP is placed directly into the active site of Rho. Arg 282 interacts with the $\beta$-$\gamma$ bridging oxygen and one of the fluorides of the AlF$_4^-$ (Rittinger et al., 1997b). This structural evidence suggests a direct role for Arg 282 in the stabilisation of the transition state. Here, the properties of the aluminium fluoride-mediated Rho.GDP.RhoGAP complex and the role of Arg 282 in the formation of this complex have been examined biochemically. In experiments requiring the presence of aluminium fluoride, 110µM AlCl$_3$ and 20mM NaF were present. In aqueous solutions of NaF, aluminium has been reported to form predominantly hexacoordinated complexes with $F^-$, H$_2$O or OH$, whose average stoichiometry depends on the pH and on the excess concentration of fluoride ions (Martin, 1988; Martin 1996; Martinez et al., 1996). It has been proposed that, at 20mM NaF, the predominant species at pH 7.5 is (H$_2$O)$_2$AlF$_4^-$ (Martin, 1988; Martin, 1996), however, it is impossible to predict that this species alone binds to these proteins in solution. Therefore, although the species seen in the X-ray structure of the aluminium fluoride-mediated complex of Rho.GDP and RhoGAP (Rittinger et al., 1997b) was
interpreted as $\text{AlF}_4^-$, the exact bound species present on the protein in solution is not known and therefore will be referred to as $\text{AlF}_x$.

At an early stage in the project, a complex of Rho bound to the fluorescently modified nucleotide, mantGDP, was used and fluorescence anisotropy measurements were made to assess the effect of $\text{AlF}_x$ on the interaction of Rho.mantGDP with wild-type, R282A and R282K RhoGAPs. Originally, Rho bound to mant nucleotides were intended to be used to characterise the effects of mutating the conserved arginine on the catalytic function of RhoGAP and its binding capabilities, as mant nucleotides had previously been shown to behave as good analogues of GTP and GDP (Neal et al., 1990). As detailed in Chapter 3, it was not possible to study the catalytic function of both mutants by monitoring fluorescence changes, therefore, methods that did not rely on fluorescence properties were used, allowing native nucleotides to be used also. Therefore, in addition to using fluorescence anisotropy methods, ITC and SPA binding experiments were used in order to assess the effects of $\text{AlF}_x$ on the interaction of Rho bound to GDP itself with wild-type and mutant RhoGAPs.

4.2 Fluorescence Anisotropy Measurements of the Interaction between Wild-type, R282A or R282K RhoGAP and Rho.mantGDP in the Absence or Presence of $\text{AlF}_x$

When wild-type RhoGAP was titrated into a solution of Rho.mantGDP in the absence of $\text{AlF}_x$, a gradual increase in anisotropy was observed, with saturation still not reached at a RhoGAP concentration of 80$\mu$M (Figure 4.1). Fitting the data to a binding isotherm gave a $K_d$ of 75$\mu$M. The initial anisotropy value was 0.198 and the fitted curve indicated a saturating anisotropy value of 0.251. The amplitude of the change is similar to that
Figure 4.1 – Fluorescence Anisotropy Titration of Wild-type RhoGAP into Rho.mantGDP

The indicated concentrations of wild-type RhoGAP were titrated into a solution of 10μM Rho.mantGDP. Fluorescence anisotropy measurements were taken after each addition. The solid line represents the best fit to a hyperbolic binding curve, with a $K_d$ value of 75μM and a standard error of 25μM for the interaction of Rho.mantGDP and wild-type RhoGAP. The RhoGAP titration solution also contained 10μM Rho.mantGDP in order to avoid dilution of the Rho.mantGDP on addition of RhoGAP solution. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl$_2$, 1mM DTT at 20°C.
observed when the interaction of RhoGAP and Rho.mantGMPPNP was monitored using this method (section 3.4.1.1). A comparison of the affinities of wild-type RhoGAP for Rho.mantGDP or Rho.mantGMPPNP, as measured by anisotropy, show a 21-fold higher affinity for the GMPPNP form of Rho, indicating differential binding between GMPPNP- and GDP-bound forms of Rho.

When wild-type RhoGAP was titrated into Rho.mantGDP in the presence of AlFx, a hyperbolic increase in fluorescence anisotropy was observed with a $K_d$ for the interaction of 2.3μM (Figure 4.2). The initial anisotropy value was 0.197 and the saturation value, as calculated from the fit, was 0.247. A comparison of the calculated $K_d$s of wild-type RhoGAP for Rho.mantGDP in the absence or presence of AlFx shows that AlFx caused a 30-fold increase in the affinity of wild-type RhoGAP for Rho.mantGDP.

When wild-type RhoGAP was substituted for R282A or R282K RhoGAP, the starting anisotropy values were similar to those seen with wild-type RhoGAP, however, at 30μM mutant RhoGAPs the anisotropy values were <0.21, compared to 0.245 with wild-type RhoGAP (Figure 4.2). Assuming identical saturating anisotropy values to those obtained with wild-type, the anisotropy changes obtained with mutant RhoGAPs can be fitted to hyperbolic binding curves with binding affinities of >40μM. However, it may be that there is a different maximum anisotropy response with the mutant RhoGAPs compared to wild-type. A lower maximum signal could result from an increase in the local motion of the mant fluorophore that only occurs in the presence of the mutant RhoGAPs, which would contribute to the lower anisotropy values. Later data, using the SPA shows that this is likely not to be the case as similar, low binding affinities were obtained.
Figure 4.2 – Fluorescence Anisotropy Titrations of Wild-type, R282A or R282K RhoGAP into Rho.mantGDP in the Presence of Aluminium Fluoride

The indicated concentrations of wild-type (●), R282A (▲) or R282K (■) RhoGAP were titrated into a solution of 1μM Rho.mantGDP. Both solutions contained 110μM AlCl₃ and 20mM NaF. The RhoGAP titration solution also contained 1μM Rho.mantGDP in order to prevent dilution of Rho.mantGDP on addition of RhoGAP solution. Fluorescence anisotropy readings were taken after each addition and plotted versus RhoGAP concentration. The solid lines represent the best fits to hyperbolic binding curves. The $K_d$ for the interaction of Rho.mantGDP and wild-type RhoGAP in the presence of AlF₃ is 2.3 (± 0.1) μM. The R282A and R282K RhoGAP data have been fitted assuming a level of saturation identical to that of wild-type RhoGAP. The $K_d$ values for R282A and R282K RhoGAP with Rho.mantGDP in the presence of AlF₃ are 41 (± 17) μM and 75 (± 66) μM, respectively. The errors quoted correspond to the standard errors on the curve fits. Titrations were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT at 20°C.
Anisotropy

\[ \rho_{\text{GAP}} \] (pM)
Therefore, the significantly weaker affinities obtained with both mutant RhoGAPs compared to wild-type, suggest that Arg 282 is required for the formation of the Rho.mantGDP.AlF₃.RhoGAP complex.

4.3 ITC Measurements of the Interaction between Wild-type and Mutant RhoGAPs and Rho.GDP in the Presence of AlF₃

To examine the effects of AlF₃ on the interaction between RhoGAP and Rho bound to non-modified GDP rather than the fluorescently modified mantGDP, the method of ITC was utilised. ITC experiments in the presence of AlF₃ showed that titration of Rho.GDP into wild-type or R282A RhoGAP at 20°C was associated with an endothermic heat change (Figure 4.3; Traces 3 & 4). A control experiment in which RhoGAP was replaced with buffer produced a much smaller endothermic heat change representing the heat of dilution (Figure 4.3; Trace 1). These heat changes were not identical to the heat changes seen on saturation of Rho.GDP with RhoGAP (Traces 3 & 4) and, therefore, this control data was not subtracted from the experimental data. For curve fitting purposes the endpoints of the titrations were adjusted to zero. On fitting the data to single binding site isotherms using Microcal Origin software, the Kₐ values for the interactions were obtained. The Kₐ values calculated for wild-type and R282A RhoGAP binding to Rho.GDP with AlF₃ were 0.5 and 1.0μM, respectively, showing that the arginine to alanine mutation resulted in only a 2-fold decrease in affinity, in contrast to the results obtained with Rho.mantGDP using fluorescence anisotropy methods. The stoichiometries were 0.96 and 0.94 for Rho.GDP binding to wild-type or R282A RhoGAP, respectively. As it is known that 1 mol of Rho interacts with 1 mol of RhoGAP, in the presence of AlF₃ (Rittinger et al., 1997b), the stoichiometries obtained
Figure 4.3 – Isothermal Titration Calorimetry Measurements of Binding of Rho.GDP to Wild-type, R282A and R282K RhoGAPs in the Presence of Aluminium Fluoride

Experiments were performed in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT at 20°C in the presence of 110μM AlCl₃ and 20mM NaF (in cell and syringe). Rho.GDP (0.18mM) was titrated into buffer alone (trace 1), 15μM wild-type RhoGAP (trace 3, ●), 15μM R282A RhoGAP (trace 4, ▲) or 15μM R282K RhoGAP (trace 2, ■), and heat changes were measured continuously with time on a Microcal isothermal titration calorimeter. The raw data from each injection are shown in the top panel. The measured heats from each peak were integrated and the resultant data are plotted in the lower panel as molar ratio of Rho.GDP injected into RhoGAP against heat output per mole of injected Rho.GDP. The solid lines through the points are the best fits to the data assuming a single binding site model with the following parameters: $K_d = 0.4\mu M$, $n = 1.0$ and $\Delta H = 13.9\text{ kcal.mol}^{-1}$ for wild-type RhoGAP and $K_d = 0.9\mu M$, $n = 0.9$ and $\Delta H = 12.5\text{ kcal.mol}^{-1}$ for R282A RhoGAP. The traces shown are representative of one of two experiments performed for each interaction. The results obtained from duplicate experiments are summarised in table 4.1.
Heat Output vs. Time (min)

Heat Output (kcal/mole of Rho.GDP)

Molar Ratio
indicate high activity of the proteins. Table 4.1 summarises the data obtained for duplicate experiments performed for the interactions of wild-type and R282A RhoGAP with Rho.GDP in the presence of AlF₃. No heat changes were observed when Rho.GDP was titrated into R282K RhoGAP, hence no conclusions regarding the binding can be made.

Unfortunately, using the same experimental conditions as those in the presence of AlF₃, titration of Rho.GDP into wild-type RhoGAP in the absence of AlF₃ produced no significant heat changes when compared to the heat of dilution resulting from the addition of Rho.GDP to buffer (data not shown), therefore, no measurement of binding could be carried out. The absence of heat changes may be due to absence of binding or binding with little or no resultant net heat change.

These ITC measurements suggest that formation of the Rho.GDP.AlF₃.RhoGAP complex is not dependent on Arg 282, as there is only a 2-fold decrease in affinity of Rho.GDP for R282A RhoGAP compared to the affinity for wild-type RhoGAP. Attempts were made to substitute Rho.GDP for Rho.mantGDP allowing a comparison to be made with the fluorescence anisotropy data which suggested that the arginine was required for Rho.mantGDP.AlF₃.RhoGAP formation. However, no heat changes were observed with Rho.mantGDP.
Table 4.1 – Summary of the Parameters Determined by ITC for the Interaction of Wild-type, R282A or R282K RhoGAPs and Rho.GDP in the Presence of AlF₃

This table shows the experimentally determined values for the association constant (Kₐ), the enthalpy change (ΔH) and the stoichiometry (n) obtained from individual ITC experiments. The values for the calculated Kₐ are also included. Mean values are in bold. These mean values are the values quoted in the text and listed in the summary table (Table 4.2). The errors quoted for Kₐ, ΔH and n correspond to the standard error on the fit to a single binding site isotherm (Wiseman et al., 1989).

<table>
<thead>
<tr>
<th>RhoGAP</th>
<th>Kₐ x 10⁶ (M⁻¹)</th>
<th>Kₐ (µM)</th>
<th>ΔH (kcal.mol⁻¹)</th>
<th>n</th>
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<tr>
<td>Wild-type</td>
<td>2.33 ± 0.32</td>
<td>0.43</td>
<td>13.92 ± 0.25</td>
<td>1.0 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1.63 ± 0.33</td>
<td>0.61</td>
<td>15.47 ± 1.47</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td></td>
<td><strong>1.68</strong></td>
<td><strong>0.52</strong></td>
<td><strong>14.70</strong></td>
<td><strong>0.96</strong></td>
</tr>
<tr>
<td>R282A</td>
<td>1.17 ± 0.07</td>
<td>0.85</td>
<td>12.49 ± 0.12</td>
<td>0.91 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.94 ± 0.05</td>
<td>1.06</td>
<td>14.35 ± 0.46</td>
<td>0.96 ± 0.02</td>
</tr>
<tr>
<td></td>
<td><strong>1.06</strong></td>
<td><strong>0.96</strong></td>
<td><strong>13.42</strong></td>
<td><strong>0.94</strong></td>
</tr>
</tbody>
</table>
4.4 SPA Measurements of the Interaction between Wild-type and Mutant RhoGAPs and Rho.GDP or Rho.mantGDP in the Absence or Presence of AIF₆

A comparison of the results obtained from the ITC and the fluorescence anisotropy show that R282A RhoGAP can form a tight complex, in the presence of AIF₆, with Rho.GDP, but not with Rho.mantGDP. As it was not certain as to whether this was due to the different methods used, SPA methods were utilised, which allowed the comparison of Rho.GDP and Rho.mantGDP under the same assay conditions. The SPA used was a competition assay in which Rho.GDP or Rho.mantGDP were competing ligands in an assay containing fixed concentrations of GST-RhoGAP and Q61L Rac.[³H]GTP. Q61L Rac.[³H]GTP binds to GST-RhoGAP and produces a SPA signal. The Rho.GDP/Rho.mantGDP compete with Q61L Rac.[³H]GTP for binding to GST-RhoGAP and any competition is seen as a decrease in SPA signal. Measurements were only made for wild-type and R282A RhoGAP, as a SPA assay utilising R282K RhoGAP had high signal to background ratios as a result of its weak binding to Q61L Rac.[³H]GTP (discussed in section 3.4.2.6).

Using this SPA assay the affinities of Rho.GDP and Rho.mantGDP for wild-type and R282A RhoGAP in the absence of AIF₆ were determined. When Rho.GDP and Rho.mantGDP (0-16μM) were titrated into the GST-wild-type or GST-R282A RhoGAP/Q61L Rac.[³H]GTP assays they showed little inhibition of the Rac/RhoGAP interaction (approximately 20% inhibition at 16μM) (Figure 4.4). Due to the low level of inhibition it was difficult to calculate the apparent Kᵢ accurately. Assuming that the signal would eventually reach background levels the decreases in SPA signal were fitted to equation 8 describing competitive binding for two ligands (section 2.14), and the
Figure 4.4 - SPA Measurements of Rho.GDP and Rho.mantGDP Binding to Wild-type and R282A RhoGAP in the Presence and Absence of Aluminium Fluoride using a Competitive Inhibition Assay

The indicated concentrations of Rho.GDP or Rho.mantGDP were added to SPAs containing Q61L Rac.[3H]GTP and either wild-type GST-RhoGAP (a) or R282A GST-RhoGAP (b), and the SPA signal was measured. The signal from the background without GST-RhoGAP was subtracted and the data were plotted as a percentage of the signal without added Rho.GDP or Rho.mantGDP. The solid lines represent the best fit to an equation describing competitive inhibition of radiolabelled Rac binding to GST-RhoGAP. With wild-type RhoGAP (a) the apparent $K_i$ values are 32 (± 5) μM for Rho.GDP without AlF$_x$ (O) and 1.1 (± 0.1) μM with AlF$_x$ (●), and 40 (± 6) μM for Rho.mantGDP without AlF$_x$ (Δ) and 1.9 (± 0.3) μM with AlF$_x$ (▲). With R282A RhoGAP (b), the apparent $K_i$ values are 34 (± 6) μM for Rho.GDP without AlF$_x$ (O) and 3.7 (± 0.5) μM with AlF$_x$ (●), and 45 (± 9) μM for Rho.mantGDP without AlF$_x$ (Δ) and 34 (± 7) μM with AlF$_x$ (▲). The errors quoted correspond to the standard errors on the curve fits. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl$_2$, 1mM DTT.
The apparent $K_i$s for the competing proteins were calculated to be $\geq 30\mu M$. The apparent $K_i$ values represent the apparent $K_d$ values for Rho.GDP/RhoGAP interactions. When compared to the calculated apparent $K_d$ values for the Rho.GMPPNP/RhoGAP interaction (section 3.4.2.6) these data indicate differential binding between the GDP and GMPPNP forms of Rho, as seen with the fluorescence anisotropy measurements (section 4.2).

Rho.GDP or Rho.mantGDP (0-16μM) were also titrated into the wild-type or R282A GST-RhoGAP/Q61L Rac.[$^3H$]GTP assays in the presence of AlF$_x$. In both wild-type and R282A RhoGAP assays, Rho.GDP, in the presence of AlF$_x$, caused a high level of competitive inhibition of the RhoGAP/Rac interaction (>90% or >60% inhibition at 16μM in the wild-type and R282A RhoGAP assays, respectively). The apparent $K_i$s measured using Rho.GDP in the presence of AlF$_x$ were calculated as 1.1 and 3.7μM for wild-type and R282A RhoGAP respectively (Figure 4.4). These results agree with the ITC results in that the arginine to alanine mutation does not abolish formation of the AlF$_x$ induced complex between Rho.GDP and RhoGAP. When Rho.GDP was replaced with Rho.mantGDP in the wild-type RhoGAP assay a similar level of competitive inhibition of the RhoGAP/Rac interaction was seen. However, in the R282A RhoGAP assay, a level of inhibition was seen that was similar to Rho.mantGDP in the absence of AlF$_x$, hence, indicating that the presence of AlF$_x$ did not have any effect on the interaction between Rho.mantGDP and R282A RhoGAP. The apparent affinity for the interaction of wild-type RhoGAP with Rho.mantGDP and AlF$_x$ was only $<2$-fold lower ($K_d = 1.9\mu M$) than that with Rho.GDP and AlF$_x$. However, the apparent affinity of R282A RhoGAP with Rho.mantGDP and AlF$_x$ was reduced $>8$-fold ($K_d = \geq 30\mu M$), compared to that with Rho.GDP and AlF$_x$. This indicates a difference in the interaction between R282A
RhoGAP and Rho bound to non-mant and mant nucleotides, with AlF₃ having no effect on the interaction of R282A RhoGAP and Rho.mantGDP, but increasing the affinity of R282A RhoGAP for Rho.GDP. The lack of effect seen with Rho.mantGDP is similar to the lack of binding observed when fluorescence anisotropy was monitored.

In order to quantify the requirements for fluoride-mediated effects on complex formation of Rho.GDP and RhoGAP (discussed in Chapter 5) a direct binding SPA was set up using GST-wild-type or GST-R282A RhoGAP and Rho.³[H]GDP. Using this assay, measurements of the apparent affinities of wild-type or R282A RhoGAP for Rho.GDP in the presence of AlF₃ were carried out using the same aluminium and fluoride concentrations as used in the competitive inhibition SPA described above. When Rho.³[H]GDP was titrated into an assay containing either GST-wild-type RhoGAP or GST-R282A RhoGAP, in the presence of AlF₃, a hyperbolic response was observed (Figure 4.5). The data were fitted to hyperbolic binding curves to give apparent Kₐ values of 0.48μM for wild-type RhoGAP and 1.4μM for R282A RhoGAP. These values represent 2 to 3-fold tighter binding when compared to apparent Kₐ values obtained using the competitive inhibition assay, but are almost the same as the KₐS measured using ITC. This 2 to 3-fold difference between these two types of SPA assay, measuring Rho.GDP and RhoGAP interactions, is consistent with the 2 to 3-fold difference seen between similar assays used to measure Q61L Rac and RhoGAP interactions (see sections 3.4.2.4 & 3.4.2.5).

Comparing wild-type with mutant, the difference between the Kₐ values are very similar with both assays. As discussed in Chapter 3 (section 3.4.2.5), the difference between the values obtained from a competitive inhibition assay and a direct binding assay are most likely due to the fact that the values obtained from the
The indicated concentrations of Rho.[³H]GDP were added to SPAs containing either wild-type GST-RhoGAP (●) or R282A GST-RhoGAP (▲). The signal from a blank without GST-RhoGAP was subtracted, and the data were plotted as a percentage of the maximum signal. The solid lines represent the best hyperbolic fits to the data. The apparent $K_d$ values for the interaction of Rho.GDP and wild-type or R282A RhoGAP were 0.48 (± 0.07) μM and 1.4 (± 0.04) μM, respectively. The errors quoted correspond to the standard errors on the curve fits. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT, 110μM AlCl₃ and 20mM NaF.
Chapter 4—Aluminium Fluoride Complexes

![Graph showing SPA Signal (% maximum) vs [Rho.\[^3\]H\]GDP (\mu M)]]
competitive inhibition assay represent upper limits of the true value for the calculated $K_i$, as the equation does not take into account inhibitor depletion.

The equilibrium binding constants obtained in this chapter are summarised in table 4.2.

**4.5 Discussion**

It has been reported previously that Ras.GDP stably associates with RasGAP only in the presence of aluminium fluoride (Mittal *et al.*, 1996). Similarly, Cdc42.GDP and RhoGAP form an aluminium fluoride-mediated ternary complex (Ahmadian *et al.*, 1997b). Using fluorescence anisotropy, ITC and SPA methods, it has been demonstrated here that AlFx also induces the formation of a high affinity complex between Rho.GDP or Rho.mantGDP and wild-type RhoGAP. The $K_d$ values measured for the interaction of Rho.GDP and wild-type RhoGAP ranged from 0.4-1.1μM compared to approximately 30μM for the interaction in the absence of AlFx. The presence of the mant fluorophore attached to the GDP caused a slight increase in the $K_d$ values for the interaction in the presence of AlFx to values of 1.9-2.3μM. Although a range of methods were used, the binding affinities are consistent and within 3-fold of each other.

It has been proposed that AlFx-mediated complexes between small G proteins and their GAPs represent analogues of the transition state of the GTPase reaction (Scheffzek *et al.*, 1997; Ahmadian *et al.*, 1997b; Rittinger *et al.*, 1997b). Biochemical evidence presented in Chapter 3 shows that the conserved arginine residue in RhoGAP, Arg 282, is essential for efficient catalysis of the GTPase activity of Rho but not for the binding interaction between RhoGAP and Rho. Also, Ahmadian *et al.* (1997c) and Sermon *et al.* (1998)
Table 4.2 - Equilibrium Dissociation Constants for Wild-type and Mutant RhoGAPs

Binding to Rho.GDP in the Presence or Absence of Aluminium Fluoride

Equilibrium dissociation constants were measured using the indicated methods as described in the text.

<table>
<thead>
<tr>
<th>Rho complex</th>
<th>Method</th>
<th>Wild-type Kd (μM)</th>
<th>R282A Kd (μM)</th>
<th>R282K Kd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho.GDP + AlF₅⁻</td>
<td>ITC</td>
<td>0.5</td>
<td>1.0</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>SPA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1</td>
<td>3.7</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>SPA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.48</td>
<td>1.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rho.mantGDP + AlF₅⁻</td>
<td>Anisotropy</td>
<td>2.3</td>
<td>&gt;40&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&gt;40&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SPA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9</td>
<td>≥30</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rho.GDP</td>
<td>SPA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≥30</td>
<td>≥30</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rho.mantGDP</td>
<td>SPA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≥30</td>
<td>≥30</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Anisotropy</td>
<td>75</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<sup>a</sup> No Significant Heat Change  
<sup>b</sup> Competitive Inhibition SPA  
<sup>c</sup> Direct Binding SPA  
<sup>*</sup> Assuming the same Δ anisotropy as for wild-type  
N.D. not determined
show that a conserved arginine residue in Ras-GAPs is essential for efficient catalysis of the GTPase activity of Ras but not for the protein:protein interaction. It has been proposed that the role of these residues is to stabilise the transition state of the GTP hydrolysis reaction. Furthermore, structural evidence is available to further substantiate the role of GAPs in transition state stabilisation. The structures of Cdc42.GMPPNP.RhoGAP, proposed to be the ground state (substrate/enzyme complex) of the GTP hydrolysis reaction (Rittinger et al., 1997a), and the structure of the transition state analogue, Rho.GDP.AIF₄.RhoGAP (Rittinger et al., 1997b) have been published. In the Cdc42.GMPPNP.RhoGAP complex (Rittinger et al., 1997a), the conserved Arg 282 residue of RhoGAP does not contact the active site, whereas an overlap of the two G proteins in the structures shows a 20° rotation of RhoGAP resulting in the interaction of the conserved arginine with the nucleotide and aluminium fluoride moiety (Rittinger et al., 1997b). It is evident from the structure of Rho.GDP.AIF₄.RhoGAP that RhoGAP directly contributes the Arg 282 residue into the active site of Rho (Rittinger et al., 1997b). Also, structural studies of AIF₄-complexes have shown that, in the case of the transition state analogue of Goα1 (Coleman et al., 1994), Gαα (Sondek et al., 1994) and H-Ras.GDP binding to the RasGAP, p120GAP (Scheffzek et al., 1997), an arginine residue interacts with the aluminium fluoride moiety that occupies the position of the terminal phosphate. The contribution of this single amino acid (Arg 178 in Goα1; Arg 174 in Gαα; Arg 789 in p120GAP; Arg 282 in RhoGAP) has been described as an ‘arginine finger’ pointing directly into the GTP binding site of the GTPase and stabilising the transition state (Bourne, 1997).

Surprisingly, considering this structural evidence suggesting that the Arg 282 is important in transition state stabilisation (Rittinger et al., 1997b), R282A RhoGAP also effectively
forms the Rho.GDP.AlF₅₆.RhoGAP complex with an affinity only slightly lower than seen with wild-type RhoGAP (R282A ITC 1.0µM, SPA 1.4 or 3.7µM; wild-type ITC 0.5µM, SPA 0.48 or 1.1µM). This appears to be in direct contradiction to the recent report concluding that the same arginine residue (referred to as R305 of Cdc42GAP) was required for formation of the AlF₅₆-mediated complex (Hoffman et al., 1998). Hoffman et al. (1998) reported that mutation of the conserved arginine resulted in a 50-fold loss of affinity of Cdc42GAP for Cdc42.mantGDP in the presence of AlF₅₆, and hence concluded that this arginine was required for formation of the AlF₅₆-mediated complex. These contrasting conclusions may be due to the differing nucleotides used. When Rho.GDP was used in the presence of AlF₅₆, R282A RhoGAP was found to form the Rho.GDP.AlF₅₆.RhoGAP complex. However, using SPA and fluorescence anisotropy measurements with Rho.mantGDP in the presence of AlF₅₆, R282A RhoGAP appeared not to form the Rho.mantGDP.AlF₅₆.RhoGAP (SPA ≥30µM; Anisotropy 75µM). Thus, if Rho.mantGDP alone had been used, the conclusion would have been as Hoffman et al. (1998), i.e. that Arg 282 is required for Rho.GDP.AlF₅₆.RhoGAP complex formation. However, perhaps due to the precise steric constraints required for formation of the AlF₅₆-mediated complex, replacement of arginine with alanine interferes with formation of the Rho.mantGDP.AlF₅₆.RhoGAP, but not with the Rho.GDP.AlF₅₆.RhoGAP complex.

This biochemical data presented here does not provide any clues as to what the structural differences are between the AlF₅₆-mediated Rho.GDP complexes with wild-type and R282A RhoGAP. The arginine/nucleotide interaction seen in the X-ray structure of Rho.GDP.AlF₅₆.RhoGAP (Rittinger et al., 1997b), which was proposed to be significant for stabilisation of this complex, is lost with the R282A mutant yet little change in affinity is seen. It has been shown that Arg 282 is required for efficient catalysis (Chapter
and the likely role of arginine is to stabilise the transition state. However, the X-ray structure of Cdc42.GDP.AlF₃.R305A Cdc42GAP has recently been published (Nassar et al., 1998). Cdc42GAP is analogous to RhoGAP, and Arg 305 in Cdc42GAP is analogous to Arg 282 in RhoGAP. In the wild-type structure, Arg 305 is coordinated to Gln 61 of Cdc42 via its main chain carbonyl group, and also interacts with a fluorine atom. In the R305A Cdc42GAP.Cdc42.GDP structure the main chain carbonyl of alanine makes the coordination with Gln 61, and Tyr 32 from Cdc42 interacts with the fluorine atom that is bonded to the arginine in the wild-type structure (Nassar et al., 1998). Thus, in the R305A complex, the loss of binding energy caused by loss of the arginine/nucleotide interaction appears to be compensated for by rearrangement of the hydrogen bonds. It may be that with R282A RhoGAP, these structural changes can take place when interacting with Rho.GDP, but due to the precise steric requirements needed for the Tyr residue to interact with the AlFₓ moiety, when Rho.mantGDP is used this interaction is not possible.
CHAPTER 5

Magnesium Fluoride Mediated Complexes between Rho and RhoGAP
5.1 Introduction

As previously mentioned, heterotrimeric G proteins are potently activated by fluoride in the presence of low concentrations of aluminium (Sternweis and Gilman, 1982). It has also been shown that heterotrimeric G proteins can be activated by fluoride in the absence of aluminium. Sternweis and Gilman (1982) showed that beryllium can also co-activate with fluoride. Later, Higashijima et al. (1987) showed that the fluorescent enhancement seen on activation of Goα with either GTPγS or Al3+, Mg2+ and F− could also be seen at high concentrations of Mg2+ and F− in the apparent absence of Al3+. This latter observation was extended by Antonny et al. (1990; 1993) who observed that activation of transducin in the presence of 2mM MgCl2 was also apparent using concentrations of NaF above 3mM with no added Al3+. The activation dose-response curve representing the fluorescence enhancement seen on Gα activation as a function of [F−] in the presence of 2mM Mg2+ showed cooperativity with a Hill coefficient of 3 (Antonny et al., 1990). It was, therefore, proposed that at least three fluoride atoms were implicated in this magnesium-dependent activation process. As in the case of Al3+-dependent fluoride activation, Mg2+-dependent activation was shown to be dependent on the presence of GDP (Antonny et al., 1993). In addition, using 31P NMR spectroscopy the bound magnesium was shown to influence the chemical shifts of the α and β phosphorous of the GDP suggesting the location of a magnesium at the γ phosphorus site (Antonny et al., 1993). It was proposed that Mg2+ may associate with three F− to form MgF3−, and mimic the γ-phosphate of GTP (Antonny et al., 1990; Antonny et al., 1993).

It has only been in more recent years that aluminium-dependent fluoride mediated complexes between small G proteins and their GAPs have been reported (Mittal et al., 1996; Ahmadian et al., 1997b; Rittinger et al., 1997b; Hoffman et al., 1998; Nassar et al.,
1998). As well as this aluminium-dependent complex formation, an Al$^{3+}$-independent
effect of fluoride has also been observed for the interaction of a small G protein and its
GAP (Vincent et al., 1998). Vincent et al. (1998) have shown qualitatively that
aluminium is not required for formation of a fluoride-mediated complex between
Rho.GDP and p190RhoGAP and this Al$^{3+}$-independent binding was attributed to the
formation of a complex containing magnesium fluoride. It therefore appears that neither
the fluoride-dependent activation of G$\alpha$ subunits (Higashijima et al., 1987; Antonny et
al., 1990; Antonny et al., 1993) nor the high affinity fluoride-dependent complex
formation between the GDP forms of small G proteins and their respective GAPs (Mittal
et al., 1996; Ahmadian et al., 1997b; Hoffman et al., 1998) requires the presence of
aluminium.

To confirm that, in the absence of aluminium, magnesium can support fluoride-mediated
binding of Rho.GDP to RhoGAP, SPA measurements have been made. Also, as the only
existing report of aluminium-independent fluoride-mediated complex formation between
a small G protein and its GAP was qualitative in nature (Vincent et al., 1998), these
requirements have been quantified. In addition, in collaboration with the Protein
Structure Division at N.I.M.R, structural studies have been undertaken in order to
elucidate the structure of an aluminium-free fluoride-mediated complex between
RhoGAP and Rho.GDP.

5.2 Formation of a High Affinity Fluoride-Dependent Complex Between Rho.GDP
and RhoGAP in the Absence of Aluminium

Whilst investigating the effects of aluminium fluoride on the interaction between
Rho.GDP and RhoGAP (Chapter 4), experiments were also performed in the presence of
NaF but absence of AlCl₃. Equilibrium-binding measurements of Rho.GDP to RhoGAP were made using SPAs in which Q61L Rac.[³H]GTP was bound to GST-RhoGAP which was attached to SPA beads via anti-GST. Rho.GDP was used as a competitive inhibitor for the Rac/RhoGAP interaction, thereby causing the SPA signal to reduce. In the presence of 2mM MgCl₂, 20mM NaF and 0.11mM AlCl₃ the apparent Kₐ was 1.1μM compared to ≥30μM in the absence of NaF and AlCl₃ (Figure 4.4). Similar results were obtained in the presence of 2mM MgCl₂ and 20mM NaF but absence of added AlCl₃, where the apparent Kₐ was 1.0μM for the interaction between Rho.GDP and RhoGAP compared to ≥30μM in the absence of NaF (Figure 5.1). These results indicate that a high affinity fluoride-dependent complex could be formed between Rho.GDP and RhoGAP in the absence of added aluminium.

Sternweis and Gilman (1982) originally described how contaminating aluminium was responsible for the activation of heterotrimeric G proteins by fluoride. Thus, it was important to demonstrate that the high affinity complex formation observed in the absence of added aluminium between Rho.GDP and RhoGAP was not mediated by trace amounts of aluminium. Firstly, the aluminium content of the buffers was determined using optical emission spectrometry carried out by S.Francis (Glaxo Wellcome) on a Varian Liberty 220 ICP-OE spectrometer using a wavelength emission of 396.152nm. The aluminium content of the buffers was found to be undetectable (<0.05μM). Secondly, the aluminium chelator, deferoxamine, was added to all further assays to ensure that any effects could not be attributed to free Al³⁺. Deferoxamine is a powerful chelator of aluminium with a Kₐ of ~10⁻²⁰M (Evers et al., 1989).
Figure 5.1 - SPA Measurements of Rho.GDP Binding to RhoGAP in the Presence and Absence of Sodium Fluoride

The indicated concentrations of Rho.GDP were added to SPAs containing Q61L Rac.[³H]GTP and wild-type GST-RhoGAP, and the SPA signal was measured. The signal from the background without GST-RhoGAP was subtracted and the data were plotted as a percentage of the signal without added Rho.GDP. The solid lines represent the best fit to an equation describing competitive inhibition of binding of radiolabelled Rac binding to GST-RhoGAP. The apparent Kᵢ values are 26 (± 5) μM for Rho.GDP without NaF (O) and 1.0 (± 0.1) μM with 20mM NaF (●). The errors quoted correspond to the standard errors on the curve fits. All measurements were carried out in 20mM Tris/HCl pH7.5, 2mM MgCl₂, 1mM DTT.
A simplified version of a SPA to investigate the aluminium-independent fluoride-mediated complex formation between Rho.GDP and wild-type RhoGAP was set up that measured direct binding between Rho.\([^3]H\)GDP and RhoGAP. The affinity of Rho.\([^3]H\)GDP for RhoGAP in the presence of 2mM MgCl₂, 20mM NaF and 0.11mM AlCl₃ was first determined using this method. The concentration of Rho.\([^3]H\)GDP was varied (0-4μM) at a fixed concentration of GST-RhoGAP and a hyperbolic increase in the SPA signal was observed giving an apparent Kₐₜ of 0.48μM (Figure 5.2). The experiment was repeated in the absence of added aluminium and presence of 1mM deferoxamine. Again, a hyperbolic increase in the SPA signal was observed, giving an apparent Kₐₜ of 0.28μM (Figure 5.2). These results prove that, under these conditions, aluminium is not required for fluoride-mediated binding of the GDP form of Rho to RhoGAP.

5.3 Requirements for Fluoride-Dependent High Affinity Binding of Rho.GDP to RhoGAP in the Absence of Aluminium

Although qualitative data had been published describing aluminium-independent fluoride-mediated effects between the Rho-GAP, p190GAP, and Rho.GDP (Vincent et al., 1998), no quantitative data had been published in the literature describing the requirements of such an interaction between a small G protein and its GAP. Therefore, the requirements for fluoride-mediated binding in the absence of aluminium (presence of 1mM deferoxamine) were examined.

Using a SPA in which a fixed amount of Rho.\([^3]H\)GDP and GST-RhoGAP were present, the MgCl₂ concentration in the assay was varied from 0.01-8mM in the presence of 20mM NaF and 1mM deferoxamine. Complex formation between Rho.\([^3]H\)GDP and
Figure 5.2 - The Aluminium Chelator, Deferoxamine, Does Not Affect Binding of Rho.GDP to RhoGAP in the Presence of NaF and MgCl₂

SPAs were performed in which the concentration of Rho.[³H]GDP was varied in the presence of 0.04µM GST-RhoGAP. Experiments were performed in the presence of 0.11mM AlCl₃ and absence of deferoxamine (●), or absence of AlCl₃ and presence of 1mM deferoxamine (▲). Measurements were carried out in 20mM Tris/HCl, pH7.5, 2mM MgCl₂, 20mM NaF and 1mM DTT. The SPA signals from assays in which GST-RhoGAP was omitted, were subtracted from those in their presence and these values are plotted against the added concentration of radiolabelled Rho.GDP. The solid lines show the best fits to binding isotherms, with apparent Kₐ values of 0.48 (± 0.07) µM and 0.28 (± 0.03) µM for the interaction of Rho.GDP and wild-type RhoGAP in the absence and presence of deferoxamine, respectively. The errors quoted correspond to the standard errors on the curve fits.
RhoGAP showed a hyperbolic dependence upon the concentration of MgCl\(_2\) with an apparent \(K_d\) of 1.1mM (Figure 5.3). This experiment verified that this aluminium-independent complex formation seen at 20mM NaF is dependent on magnesium. The NaF dependency for the formation of the magnesium fluoride-mediated complex was investigated using the SPA described above but in the presence of 2mM MgCl\(_2\) and varying the NaF concentration from 0-50mM. Unlike the hyperbolic dependence on MgCl\(_2\) seen at a fixed concentration of NaF, at fixed concentrations of MgCl\(_2\), a sigmoidal dependence of Rho.GDP/RhoGAP complex formation on the concentration of NaF was observed (Figure 5.4). These data were fitted to the Hill equation (SPA signal = \(S_{max}[\text{NaF}]^n/(K^n + [\text{NaF}]^n)\); where \(S_{max}\) = maximum SPA signal, \(K\) = concentration for half-maximal binding and \(n\) = Hill coefficient), giving a Hill coefficient of 2.2 and a concentration for half-maximal binding of 19mM. Binding of Rho.[\(^3\)H]GDP to GST-RhoGAP (Figures 5.3 and 5.4) was only detectable when both MgCl\(_2\) and NaF were present. These data show that high-affinity complex formation of Rho.GDP with RhoGAP, in the absence of aluminium, requires both fluoride and magnesium.

5.4 Requirements for High Affinity Binding of Rho.GDP to RhoGAP in the Presence of Aluminium and 10\(\mu\)M MgCl\(_2\)

Since it was shown that magnesium is required for fluoride-dependent high affinity complex formation in the absence of aluminium (Figure 5.3), and addition of aluminium at high magnesium concentration did not greatly affect complex formation (Figure 5.2), it was addressed whether aluminium could promote complex formation at low magnesium concentrations. Small G proteins are unstable in the absence of magnesium, therefore, experiments were performed at 10\(\mu\)M MgCl\(_2\), a concentration which is sufficiently low
Figure 5.3 - Effect of MgCl$_2$ Concentration on Binding of Rho.GDP to RhoGAP in the Presence of NaF and Deferoxamine

SPAs were performed in which the concentration of MgCl$_2$ was varied in the presence of 0.04μM GST-RhoGAP and 0.25μM Rho.[$^3$H]GDP. Measurements were carried out in 2mM MgCl$_2$, 20mM NaF and 1mM deferoxamine. The SPA signals from assays in which GST-RhoGAP was omitted were subtracted from those in their presence and these values are plotted against the added concentration of MgCl$_2$. The solid lines show the best fits to binding isotherms with an apparent $K_d$ of 1.1mM and a standard error of 0.1mM for Rho.GDP binding to wild-type RhoGAP.
Figure 5.4 - Effect of NaF Concentration on Binding of Rho.GDP to RhoGAP in the Presence of either MgCl₂ plus Deferoxamine or AlCl₃

SPAs were performed in which the concentration of NaF was varied in the presence of 0.04μM GST-RhoGAP and 0.25μM Rho.[³H]GDP. Measurements were carried out in 2mM MgCl₂ and 1mM deferoxamine (▲) or 10μM MgCl₂ and AlCl₃ (●). The solid line is the best fits to the Hill Equation, giving a Hill coefficient of 2.2 (± 0.3) and a concentration for half-maximal binding of 19 (± 2) mM. Data below 1.5mM NaF are not plotted as the blanks were high compared to the signal. The errors quoted correspond to the standard errors on the curve fit.
for no significant complex formation to occur (Figure 5.3), but high enough to give a stable SPA signal for several hours. In the presence of 20mM NaF, there was a hyperbolic dependence of Rho.GDP/RhoGAP complex formation on AlCl₃ concentration (Figure 5.5), showing that aluminium can promote complex formation at low concentrations of magnesium. The apparent $K_d$ for aluminium was calculated to be 0.19mM. When 1mM deferoxamine was included in the assay, complex formation was prevented at concentrations of aluminium up to 0.5mM (Figure 5.5). Thus, aluminium can promote complex formation of Rho.GDP with RhoGAP at low concentrations of magnesium. This demonstrates that aluminium does not bind to the complexes with extremely high affinity such that it is not removed by deferoxamine, therefore, any complex formation due to contaminating aluminium will be prevented by 1mM deferoxamine.

To address the dependence of AlFx-mediated complex formation on NaF concentration the NaF concentration was varied at 10μM MgCl₂ (Figure 5.4). However, as the concentration of NaF was reduced below 1.5mM the control SPA signal in the absence of GST-RhoGAP became markedly increased, very likely caused by insolubility of aluminium hydroxide, such that the interpretation of assays was unclear. At concentrations of NaF above 20mM there was a decline in the GAP dependent signal. The concentration of NaF required for half maximal effect could be estimated as about 2.5mM, with saturation at less than 6mM. These values are less than those determined for magnesium-dependent complex formation (section 5.3; Figure 5.4).
Figure 5.5 - Effect of AlCl₃ Concentration on Binding of Rho.GDP to RhoGAP in the Presence of 10μM MgCl₂ and NaF

SPAs were performed in which the concentration of AlCl₃ was varied in the presence of 0.04μM GST-RhoGAP, 0.25μM Rho.[³H]GDP and 10μM MgCl₂. Experiments were performed in the absence (●) or presence of 1mM deferoxamine (▲). The solid line shown for data in the absence of deferoxamine is the best fit to a binding isotherm, with an apparent Kₐ of 0.19mM and a standard error of 0.03mM.
In the SPA measurements described in Figure 5.2 used to determine the affinity of Rho.GDP for RhoGAP in the presence of aluminium and fluoride, excess magnesium was also present. This magnesium may have contributed to the fluoride-mediated binding. Thus, similar experiments were carried out in the presence of 20mM NaF, 0.11mM AlCl₃, but with 10μM MgCl₂. The apparent Kₐ of Rho.GDP for RhoGAP under these conditions was 0.17μM (Figure 5.6).

5.5 Fluoride-Mediated Interaction between Rho.GDP and Mutant R282A RhoGAP

In order to investigate the role of Arg 282 in the formation of the aluminium-independent magnesium fluoride-mediated complex formation between Rho.GDP and RhoGAP experiments similar to those in Figure 5.2 were used to determine binding affinities of GST-R282A RhoGAP and Rho.GDP (Figure 5.7). Experiments were also performed with aluminium in order to make a direct comparison between aluminium-dependent and magnesium-dependent complex formation. The apparent Kₐ in the presence of 0.11mM AlCl₃ and 2mM MgCl₂ was 1.4μM, in the presence of 2mM MgCl₂ and 1mM deferoxamine was 4.9μM and in the presence of 0.11mM AlCl₃ and 10μM MgCl₂ was 0.92μM.

5.6 Crystallization and X-ray Structure of a Complex of Rho.GDP and RhoGAP in the Presence of MgCl₂, NaF and Deferoxamine

As the crystal structure of Rho.GDP.AlF₄⁻ .RhoGAP had been solved in house at N.I.M.R. it was decided to attempt to crystallize the fluoride-mediated complex of Rho.GDP and RhoGAP in the absence of aluminium under similar conditions. Crystals were grown at 4°C using the hanging drop vapour diffusion method. The reservoir solution contained
Figure 5.6 – SPA Measurements of Fluoride-Mediated Binding of Rho.GDP to RhoGAP in the Presence of Aluminium and Low Concentrations of Magnesium

SPAs were performed in which the concentration of Rho.\(^{3}H\)GDP was varied in the presence of 0.04μM GST-RhoGAP. Experiments were performed in the presence of 0.11mM AlCl\(_3\) and 10μM MgCl\(_2\). Measurements were carried out in 20mM Tris/HCl, pH7.5, 20mM NaF and 1mM DTT. The SPA signals from assays in which GST-RhoGAP was omitted, were subtracted from those in their presence and these values are plotted against the added concentration of radiolabelled Rho.GDP. The solid line shows the best fit to a binding isotherm with an apparent K\(_d\) of 0.17μM and a standard error of 0.02μM.
Figure 5.7 - SPA Measurements of Fluoride-Mediated Binding of Rho.GDP to R282A RhoGAP in the Presence and Absence of Aluminium

SPAs were performed in which the concentration of Rho.[\(^3\)H]GDP was varied in the presence of 0.04μM R282A GST-RhoGAP. Experiments were performed in the presence of 0.11mM AlCl\(_3\) and 2mM MgCl\(_2\) and absence of deferoxamine (●), in the presence of 0.11mM AlCl\(_3\) and 10μM MgCl\(_2\) (■), or in the absence of AlCl\(_3\) and presence of 2mM MgCl\(_2\) and 1mM deferoxamine (▲). Measurements were carried out in 20mM Tris/HCl, pH7.5, 2mM MgCl\(_2\), 20mM NaF and 1mM DTT. The SPA signals from assays in which GST-RhoGAP was omitted, were subtracted from those in their presence and the data plotted as percentage of the maximum obtainable signal. The solid lines show the best fits to binding isotherms, with apparent K\(_d\)s for Rho/RhoGAP of 1.4 (± 0.04) μM (0.11mM AlCl\(_3\) and 2mM MgCl\(_2\)), 0.92 (± 0.06) μM (0.11mM AlCl\(_3\) and 10μM MgCl\(_2\)) and 4.9 (± 0.6) μM (2mM MgCl\(_2\) and 1mM deferoxamine). The errors quoted correspond to the standard errors on the curve fits.
Chapter 5 - Magnesium Fluoride Complexes

18% PEG2000 MME, 100mM MES/NaOH pH6.2, 10mM MgCl₂, 3mM DTT, 3mM NaN₃ and 114mM (NH₄)₂SO₄. The protein complex stock solution contained 400µM of Rho.GDP and 400µM wild-type RhoGAP in 20mM Tris/HCl pH7.5, 1.5mM MgCl₂, 0.75mM DTT, 2mM deferoxamine and 10mM NaF. Under these crystallisation conditions, crystals grew to a size of approximately 120 x 20 x 30µm over a two week period (Figure 5.8). The crystals appeared identical to those obtained for the Rho.GDP.AlF₄⁻.RhoGAP complex. They were orthorhombic with one complex per asymmetric unit.

The X-ray structure of the complex between Rho.GDP and RhoGAP in the presence of fluoride but absence of aluminium was solved by S.J. Gamblin, K. Rittinger and S.J. Smerdon, Protein Structure Division, N.I.M.R, using the crystals described above. The crystals were flash frozen in glycerol using liquid nitrogen and transported to Daresbury for collection of diffraction datasets. The dataset used for the final structure was taken to 1.8Å. Molecular replacement was used, using the atomic model of the Rho.GDP.AlF₄⁻.RhoGAP structure. The molecular replacement was carried out separately for the two protein components with the AlF₄⁻ moiety and all the water molecules excluded from the model. Initial molecular replacement showed that the two proteins adopted the same arrangement as in the aluminium complex. The overall structure is shown in Figure 5.9. Automated model refinement, with water building produced a map with clear electron density for the metallo-fluoride moiety. The map clearly showed a metal ion coordinated by three equatorial fluoride ligands, as opposed to four equatorial fluoride ligands seen in the structure of Rho.GDP.AlF₄⁻.RhoGAP (Rittinger et al., 1997b) (Figure 5.10), and by a β-phosphate oxygen and a water molecule in the apical positions. The refined structure
Figure 5.8 – Crystals of a Complex between Rho.GDP and RhoGAP in the Presence of Fluoride but Absence of Aluminium

Crystals obtained for the complex between Rho.GDP and RhoGAP in the presence of magnesium and fluoride, but absence of aluminium. Crystals were grown in hanging drops by vapour diffusion using equal amounts of reservoir solution (containing 18% PEG2000 MME, 100mM MES/NaOH, pH 6.2, 10mM MgCl₂, 3mM DTT, 3mM NaN₃, 114mM (NH₄)₂SO₄) and stock solution of the protein complex at 400μM in 20mM Tris/HCl, pH 7.5, 0.75mM DTT, 1.5mM MgCl₂, 2mM deferoxamine and 10mM NaF. Crystals were approximately 100μm x 20μm x 30μm in size.
Figure 5.9 – Overall View of the Complex between Rho.GDP and RhoGAP in the Presence of Magnesium Fluoride

The structure of Rho.GDP.MgF$_3$.RhoGAP complex viewed along the protein-protein interface. Rho.GDP is coloured turquoise and RhoGAP is purple. The interconnecting loops are shown in orange. The nucleotide, nucleotide-associated Mg$^{2+}$, and the MgF$_3$ are shown in red. The P-loop, switch I and switch II regions of Rho are labelled.
View of a portion of the electron density map around the nucleotide binding site in the Rho.GDP.AlF$_4$.RhoGAP (upper figure) (Rittinger et al., 1997b) and the Rho.GDP.MgF$_3$.RhoGAP (lower figure) structures. The electron densities for Arg 282 from RhoGAP and the aluminium- or magnesium- fluoride moieties are shown. The upper figure clearly shows four ligands around the metal ion, in a square planar arrangement, whereas the lower figure shows three ligands around the metal ion, in a trigonal planar arrangement. The nucleotide (GDP) is also shown, along with the nucleotide-associated Mg$^{2+}$. Metal-fluoride moieties, purple; oxygen, red; nitrogen, blue; carbon, yellow.
shows that, as well as having the same overall structure as the Rho.GDP.AlF₄⁻.RhoGAP complex, the key interactions are also maintained (Figure 5.11). Specifically the catalytic arginine, Arg 282 (labelled as R85, numbered from the first residue of the crystallized fragment), still interacts via its main-chain carbonyl with Gln 63 from Rho, and its guanidinium group is positioned over the metallo-fluoride moiety (Figures 5.10 & 5.11). Although the geometry of the trigonal arrangement of fluorides around the magnesium centre is different to the square planar arrangement around aluminium, it results in a single major change in bonding interactions. In the AlF₄⁻ complex one of the fluoride ligands, F1, interacts with Gly 62 from Rho (G62Rho) and another, F4, interacts with Lys 18 from Rho (K18Rho). In the MgF₃ complex F1 makes both of these interactions (Figure 5.11).

5.7 Discussion
AlF₃ mediates high-affinity binding of Rho.GDP to RhoGAP (Figures 5.2 and 5.5; Rittinger et al., 1997b) and of Ras.GDP to NF1 (Mittal et al., 1996; Ahmadian et al., 1997b; Scheffzek et al., 1997). However, a similar affinity for the interaction of Rho.GDP and RhoGAP was found here even when no aluminium was added. Fluoride-dependent activation of heterotrimeric G proteins was previously attributed to contamination by aluminium (Sternweis and Gilman, 1982). It was therefore, important to demonstrate that this was not the case here. The following evidence suggested that the binding observed here was not due to contaminating aluminium: (i) Direct measurement of the aluminium concentration in the buffers showed that it was <0.05μM (ii) Addition of 1mM deferoxamine, which chelates aluminium with extremely high affinity (∼10⁻²⁰M) (Evers et al., 1989), did not block fluoride-mediated complex formation at high
Schematic representations of the interactions between either MgF$_3$ or AlF$_4^-$ and Rho.GDP and RhoGAP in the Rho.GDP.MgF$_3$.RhoGAP or Rho.GDP.AlF$_4^-$.RhoGAP complexes, respectively. The numbering used is such that the first residue in the fragment that was crystallized corresponds to residue 198 in full-length RhoGAP. Therefore, R85$_\text{GAP}$ is equivalent to R282 of RhoGAP. The single major difference in the bonding interactions are, in the AlF$_4^-$ complex F1 interacts with G62$_\text{Rho}$ and F4 interacts with K18$_\text{Rho}$, whereas in the MgF$_3$ complex F1 makes both of these interactions.
Coordination of GDP.MgF$_3^-$

Coordination of GDP.AIF$_4^-$
magnesium concentrations (Figure 5.2) (iii) Deferoxamine can chelate aluminium from the Rho.GDP/RhoGAP/AlF$_3$ complex (Figure 5.5). The data shown in Figures 5.3 and 5.4 demonstrate that fluoride-mediated, aluminium-independent complex formation of Rho.GDP with RhoGAP requires the presence of fluoride and high concentrations of magnesium. These data support the conclusion of Vincent et al. (1998) that fluoride-mediated Rho.GDP binding to p190GAP (GAP domain homologous to that in RhoGAP) did not absolutely require aluminium but was supported by magnesium. It has also been found that the requirement for aluminium for the fluoride-mediated binding of Ras.GDP to its GAP, NF1, can be replaced by magnesium (Graham et al., 1999b), suggesting that this is a general phenomenon for the interaction of small G proteins and GAPs. As heterotrimeric G proteins can also be activated by fluoride in a magnesium-dependent, aluminium-independent manner (Higashijima et al., 1987; Antonny et al., 1990; Antonny et al., 1993), magnesium fluoride activation of other guanine nucleotide-binding proteins, and indeed other nucleotide-binding proteins, may well be possible.

The use of equilibrium-binding assays has allowed quantification of the requirements for fluoride-mediated binding of the GDP form of Rho to RhoGAP. Although these systems with many reactants and complex solution chemistry (Martin, 1996; Martinez et al., 1996) prevent a complete analysis, many significant conclusions can be made. At 20mM NaF, MgCl$_2$ promoted binding of Rho.GDP to RhoGAP (Figure 5.3) with an apparent K$_d$ value of 1.1mM. At the same concentration of NaF, in the presence of 10μM MgCl$_2$, AlCl$_3$ promoted binding of Rho (Figure 5.5) with an apparent K$_d$ value of 0.19mM. The concentrations of fluoride required for half-maximal effect were much higher for magnesium-dependent (19mM) than for aluminium-dependent (2.5mM) binding (Figure 5.4). In the absence of aluminium, the apparent affinity of Rho.GDP for RhoGAP, in the
presence of approximately half-saturating concentrations of NaF (20mM) and of MgCl_2 (2mM), was found to be 0.28μM (Figure 5.2). With 10μM MgCl_2, 20mM NaF and 0.11mM AlCl_3, the affinity of Rho for its GAP was slightly higher (Kd 0.17μM). In conclusion, high concentrations of magnesium and of fluoride are required to obtain fluoride-mediated response in the absence of aluminium. In contrast the aluminium-mediated response requires lower concentrations of both aluminium and of fluoride. It should be noted, however, at approximately half-saturating concentrations of magnesium fluoride or of aluminium fluoride the affinities of Rho.GDP for RhoGAP are close to each other. Thus, either aluminium or magnesium is able to effectively mediate complex formation in the presence of fluoride.

The results seen here are qualitatively similar to those seen with Gα. With each protein, aluminium allows activation with much lower concentrations of fluoride than that required to obtain magnesium-dependent effects, and for the latter millimolar concentrations of MgCl_2 are required (Figures 5.3 and 5.4; Higashijima et al., 1987; Antonny et al., 1990; Antonny et al., 1993). However, there are quantitative differences in that the concentrations of aluminium required for half maximal activation of Gα are 1-5μM (Sondek et al., 1994), about two orders of magnitude less than for Rho.

In the absence of aluminium, fluoride-dependent complex formation of Rho.GDP with RhoGAP showed a simple, hyperbolic dependence upon the concentration of magnesium (Figure 5.3). However, the dependence upon concentration of fluoride was sigmoidal (Figure 5.4). The data was fitted to the Hill equation with a Hill coefficient of 2.2. Such behaviour was not previously studied with Rho (Vincent et al., 1998), but is similar to that reported for fluoride-dependent activation of transducin (Antonny et al., 1990).
the latter case the Hill coefficients obtained, taken in conjunction with kinetic measurements (Antonny et al., 1993), were interpreted as supporting a model in which there is formation of a GDP.MgF$_3$ complex, mimicking the $\gamma$-phosphate of GTP.

Over the past few years the X-ray structures of several different protein.nucleotide complexes have been reported. The structures of NDP kinase (Xu et al., 1997), UMP kinase (Schlichting and Reinstein, 1997), Ras.RasGAP (Scheffzek et al., 1997) and Cdc42.Cdc42GAP (Nassar et al., 1998), each contain a penta-coordinated aluminium with three equatorial fluorine atoms, an axial oxygen atom from the $\beta$-phosphate of the nucleoside diphosphate and an axial water molecule. This trigonal bipyramidal geometry is that expected for the true transition state of the phosphoryl transfer reaction. In contrast, structures of transducin (Sondek et al., 1994), G$_{ia}$ (Coleman et al., 1994), myosin subfragment 1 (Fisher et al., 1995) and Rho.RhoGAP (Rittinger et al., 1997b) contain an octahedrally coordinated aluminium atom. Here, the X-ray diffraction structure of the complex of Rho.GDP and RhoGAP in the presence of fluoride and magnesium, but absence of aluminium, has been described. This shows a central metal ion coordinated with three equatorial fluorine atoms, with a $\beta$-phosphate oxygen and a hydrolytic water molecule in the apical positions. As in the AlF$_3$ structures, this trigonal bipyramidal arrangement of ligands is that expected for the true transition state. The observed trigonal bipyramidal magnesium is unusual because this metal often adopts six coordination geometry. Nevertheless, magnesium compounds with a coordination number of five have been described, although they predominantly have a square bipyramidal configuration (Carugo et al., 1993; Hollaway and Melnik, 1994). Examples have been described where magnesium adopts a distorted trigonal bipyramidal geometry, although in each case one of the ligating atoms is carbon (Carugo et al., 1993; Hollaway
and Melnik, 1994). The trigonal bipyramidal coordination of magnesium seen here is very unusual, and it is possible that the protein stabilizes this unusual form, just as it is designed to stabilize the structurally-related transition state itself.

Schlichting and Reinstein (1999) have recently demonstrated a direct affect of pH on the coordination number of a metal atom, proposed to be aluminium, in UMP kinase when crystallized in the presence of 0.8mM AlCl₃, 30mM MgCl₂ and 4mM NaF. They have also classified the available crystal structures proposed to contain aluminium fluoride according to the crystallization conditions used. Almost all structures fall into two classes; those determined below pH7 contain an octahedrally coordinated metal atom with four fluoride ligands in a square planar arrangement, proposed to be AlF₄⁻, while those crystallised above pH7 contain penta-coordinated metal atom with three equatorial fluoride ligands in a trigonal planar arrangement, proposed to be AlF₃. Interestingly the X-ray structure of Cdc42.GDP.Cdc42GAP complex crystallized in the presence of aluminium fluoride (Nassar et al., 1998) does not obey this rule, however that of Rho.GDP.RhoGAP in the presence of aluminium fluoride (Rittinger et al., 1997b) does. Cdc42 and Rho are highly homologous proteins and the domains of Cdc42GAP and RhoGAP used for these studies were identical as was the pH of crystallization. Thus, it may have been anticipated that the structures around the conserved active site would be near identical. However the Cdc42-containing structure contained a central metal ion coordinated to three equatorial fluorine atoms, an axial oxygen atom from the β-phosphate of the nucleoside diphosphate, and an axial water molecule; this was interpreted as AlF₃. In contrast, in the Rho-containing aluminium fluoride structure the metal ion is octahedrally coordinated with four equatorial fluoride ions and this was interpreted as AlF₄⁻. For the crystallization of the Cdc42.GDP.AlF₃.Cdc42GAP complex
high concentrations of magnesium (100mM) and low aluminium (0.08mM) were used (Nassar et al., 1998). In the light of the results presented here, it would appear that under these conditions it is likely that the complexes formed do not contain AlF$_3$ as originally assumed but in fact contain MgF$_3^-$. 

As discussed by Schlichting and Reinstein (1997; 1999), AlF$_3$ is not the most abundant aluminium fluoride species under the crystallisation conditions used to obtain the AlF$_3$ complexes discussed. However, it was proposed that the enzyme precisely fits only the AlF$_3$ species and enforces this form at the active site. However, high millimolar concentrations of magnesium were present in all cases, and hence it seems quite plausible that some (or even all) of the structures proposed to contain AlF$_3$ might in fact contain a MgF$_3^-$ species. The concentration of fluoride that is required for maximal aluminium-mediated binding is very much lower than that for magnesium-mediated binding. Hence higher fluoride concentrations will increase the likelihood of magnesium fluoride rather than AlF$_x$ binding, though the species bound will be dependent upon its affinity for the protein. Furthermore, it is likely that the structure of magnesium fluoride activated G$\alpha$ (Higashijima et al., 1987; Antonny et al., 1990; Antonny et al., 1993), which has not yet been determined, would show a trigonal pyramidal arrangement rather than the previously proposed tetrahedral arrangement (Antonny et al., 1990). It is noteworthy that such MgF$_3^-$ complexes would be expected to mimic the transition state of hydrolysis very closely both in charge and geometry.

In conclusion, it has now been demonstrated that both heterotrimeric G proteins and small G proteins can form aluminium-independent, magnesium-dependent complexes with fluoride, suggesting this might be a more general property of nucleotide-binding
proteins. Furthermore it is possible that some X-ray structures previously interpreted as containing aluminium fluoride might in fact contain magnesium fluoride.
CHAPTER 6

Final Discussion
The experiments described in this thesis have examined the role of the conserved arginine residue of RhoGAP in the interaction with Rho. The role of this residue has been investigated using single turnover kinetic experiments to determine effects on the catalytic rate, and equilibrium-binding methods have been used to determine effects on the binding affinity of RhoGAP for Rho. Also, transition state analogues have been used to further investigate the role of the conserved arginine residue in transition state stabilisation.

ITC, SPA and fluorescence anisotropy methods have been used to obtain the $K_d$ values for the interaction between wild-type or mutant RhoGAPs and Rho bound to a range of nucleotides. One single method could not be used for the reasons discussed here. ITC is an ‘in solution’ equilibrium-binding method that does not require immobilisation of binding components and can be used with native nucleotides such as GTP. This method is capable of giving precise measurements of the $K_d$ and the stoichiometry of the interaction. However, this technique requires an accurate determination of the protein concentration and also requires a relatively large amount of protein, especially so for measurements of weak affinity interactions. Moreover, as binding is monitored by associated heat changes, those interactions that give low or no heat changes cannot be detected. For this reason it was not possible to use this single method for determining equilibrium dissociation constants for every interaction investigated. SPA proved to be the most versatile of the methods and required small amounts of proteins. However, one of the interacting components is effectively immobilised on the SPA beads and thus apparent $K_d$ values only can be obtained. In addition, the interaction with the SPA beads requires the use of a GST-tagged protein, the properties of which may not always represent the properties of the un-tagged protein. The apparent $K_d$ values obtained here,
using both the direct binding SPA assay and the competitive inhibition SPA assay, were similar in magnitude to those obtained by ITC, suggesting that deviations caused by the local concentration of GST-fusion protein on the SPA beads are small. This also suggests that any effects caused by the presence of the GST-tag must be small. The third method used, fluorescence anisotropy, relied on the use of mant-labelled nucleotides bound to Rho. Although previously mant-nucleotides have been found to behave as good analogues of native nucleotides and have been used to investigate the interaction of Ras and Ras-GAPs (Eccleston et al., 1993; Moore et al., 1993; Ahmadian et al., 1997a; Ahmadian et al., 1997c; Sermon et al., 1998) it has been shown here that the presence of the mant group interferes with the interaction between R282A RhoGAP and Rho.GDP in the presence of AlF₄⁻. The use of the fluorescence anisotropy method, therefore, did not always give results that were representative of the interaction that takes place in the absence of the mant fluorophore.

The binding experiments undertaken here have allowed a comparison of the interaction between wild-type RhoGAP and Rho bound to either GDP or the GTP analogue, GMPPNP, i.e. in the ‘inactive’ or ‘active’ state. Conflicting data exist in the literature as to whether or not, like Ras-GAPs, RhoGAP binds preferentially to the GTP-bound form of Rho family proteins over the GDP-bound form. Early estimates of the binding affinities of RhoGAP for Rho family proteins relied on a competition assay in which the RhoGAP-stimulated Rho-GTPase activity was inhibited by the addition of Rho, Rac or Cdc42 bound to either non-hydrolysable GMPPNP or GDP (Lancaster et al., 1994; Self and Hall, 1995b). The concentration at which there was 50% inhibition of activity was taken as an estimate of the binding affinity. These experiments indicated that the GMPPNP-bound forms of Rho, Rac and Cdc42 bound to RhoGAP with similar
micromolar affinities, and the GDP-bound forms of all three proteins bound to RhoGAP with only a 2 to 5-fold reduction in affinities (Lancaster et al., 1994; Self and Hall, 1995b). In contrast, it has been reported that the Ras-GAP, p120GAP, binds to Rho.GDP with an affinity 100-fold less than to Ras.GMPPNP (Brownbridge et al., 1993). Another group, using a competition assay similar to that used by Lancaster et al. (1994) and Self and Hall (1995) to calculate binding affinities, reported RhoGAP to have at least a 180-fold greater affinity for the GMPPNP-bound form of Cdc42 compared to the GDP-bound form (Zhang et al., 1997). Similarly, using fluorescence anisotropy measurements, a large difference in the anisotropy changes monitoring the interaction of Cdc42GAP (identical to RhoGAP) with Cdc42.mantGMPPNP or Cdc42.mantGDP was observed, indicating that the mantGMPPNP-bound Cdc42 had a much higher affinity for Cdc42GAP than the GDP-bound form (Leonard et al., 1998). In contrast, using a competition activity assay, only a 3- or 6-fold greater affinity for RhoGAP was seen for the GMPPNP-bound forms of Rho and Rac, respectively, compared to the GDP-bound forms of the proteins (Zhang et al., 1998; Zhang and Zheng, 1998). However, in the case of Rac the binding affinity reported for the GMPPNP-bound form (Zhang et al., 1998) is 15-fold weaker than that previously reported (Lancaster et al., 1994; Self and Hall, 1995b). Here, using equilibrium binding measurements, the affinities for the interaction between wild-type RhoGAP and Rho bound to either GMPPNP or GDP were determined (Tables 3.3 & 4.2). The data presented here show that the affinity of RhoGAP for Rho.GMPPNP, as measured by SPA, or Rho.mantGMPPNP, as measured by fluorescence anisotropy, is at least 13-fold or 21-fold greater than that for Rho.GDP or Rho.mantGDP, respectively, indicating that there is differential binding between RhoGAP and GMPPNP- and GDP-bound forms of Rho. In addition, a comparison of the $K_m$ values obtained for the interaction with Rho.GTP with the $K_d$ values obtained for the
interaction with Rho.GMPPNP (Table 3.3) suggest that the GMPPNP-bound Rho may bind slightly weaker to RhoGAP, therefore, the difference in affinities between Rho.GMPPNP and Rho.GDP may be an underestimate of the differential binding between Rho.GTP and Rho.GDP.

The data presented here clearly show that the conserved arginine residue, Arg 282, in RhoGAP is essential for efficient catalysis and not for the binding interaction between RhoGAP and the triphosphate-bound conformation of Rho. The turnover numbers for wild-type, R282A and R282K RhoGAPs were 5s⁻¹, 0.025s⁻¹ and 0.010s⁻¹, respectively. These values for the mutant RhoGAPs represent a 200- and 500-fold reduction in the ability of RhoGAP to stimulate Rho-GTPase activity, showing that neither alanine nor lysine could substitute for arginine and maintain efficient catalysis. Since substitution with the basic side chain of lysine severely impaired the catalytic activity of RhoGAP, specific properties of the arginine side chain must be required. Similarly, mutation of a conserved arginine in the Ras-GAPs, NF1 (Arg 1276) and p120GAP (Arg 789) results in an enzyme no longer able to efficiently catalyse Ras-GTPase activity (Mittal et al., 1996; Ahmadian et al., 1997c; Sermon et al., 1998). Uncovering the role of these conserved arginine residues in catalysis has been greatly aided by structures of the complexes between RhoGAP or p120GAP and the GDP-bound forms of Rho or Ras, respectively, in the presence of aluminium fluoride. There has been a large amount of evidence to support G protein.GDP.AlF₄⁻ complexes being transition state analogues of the phosphoryl transfer reaction. Originally, it was found that the GDP-bound form of the Gα subunit of heterotrimeric G proteins was activated by aluminium fluoride (Sternweis and Gilman, 1982). As aluminium fluoride was thought to be tetrahedrally coordinated, it was initially proposed that it acted as an analogue of the γ-phosphate of GTP (Bigay et
al., 1985; Bigay et al., 1987). However, the X-ray structures of $G_{\text{G1}}, GDP\cdot AlF_4^{-}$ and $G_{\text{G}, GDP\cdot AlF_4^{-}}$ revealed that the bound $AlF_4^{-}$ was octahedrally coordinated with the fluorines present in a planar configuration (Coleman et al., 1994; Sondek et al., 1994), leading to the proposal that the complex was a transition state analogue. This was further supported by the fact that a comparison of the $G_{\text{G1}}, GDP\gamma S$ and the $G_{\text{G}, GDP\cdot AlF_4^{-}}$ structures (Coleman et al., 1994) revealed that there was a structural rearrangement in the latter complex, resulting in the positioning of an arginine residue, mutation of which had been shown to reduce catalytic function (Freissmuth and Gilman, 1989), into an appropriate conformation for catalysis (Coleman et al., 1994). The structure of the transition state analogue, Rho.GDP.AlF_4^-RhoGAP (Rittinger et al., 1997b), shows that Arg 282 plays a direct role in coordinating the aluminium fluoride moiety. In addition, the conserved glutamine residue, Gln 63, in Rho is stabilised by an interaction with the Arg 282, which in turn positions a water molecule directly in line with the $\beta$-phosphate oxygen (Rittinger et al., 1997b). In contrast, the structure of Cdc42.GMPPNP.RhoGAP, that has been described as an analogue of the ground state of the reaction, shows that the Arg 282 is not in a position that would support catalysis as it has no interactions with the active site of Cdc42 (Rittinger et al., 1997a). A comparison of the two structures shows a $20^\circ$ rigid body rotation of the two proteins relative to each other resulting in the placement of Arg 282 into the active site (Rittinger et al., 1997b). Thus, the biochemical data that has been reported here together with these structural studies provide strong evidence that the role that Arg 282 plays in catalysis by RhoGAP is to stabilise the transition state of the reaction. Similarly, the structure of Ras.GDP.AlF_3^-p120GAP (Scheffzek et al., 1997) shows that Arg 789 coordinates to aluminium fluoride, showing that the conserved arginine residues in Ras-GAPs and RhoGAPs have similar roles in transition state stabilisation. These catalytic arginines have become known as 'arginine
fingers'. In qualitative agreement with the data reported here, there have been several reports indicating a catalytic role for the conserved arginine in \( n \)-chimaerin (Ahmed et al., 1994), myr5 (Müller et al., 1997), Cdc42GAP/RhoGAP (Graham et al., 1997; Hoffman et al., 1998; Leonard et al., 1998), and p190GAP (Li et al., 1997). As well as the catalytic arginine residues identified in Rho-GAPs and Ras-GAPs, an essential catalytic arginine in \( G_\alpha \) subunits of heterotrimeric proteins has also been identified (Freissmuth and Gilman, 1989; Coleman et al., 1994; Sondek et al., 1994). In all cases the guanidinium group of the arginine residue interacts with the aluminium fluoride moiety in the transition state analogues (Coleman et al., 1994; Sondek et al., 1994; Scheffzek et al., 1997; Rittinger et al., 1997b), suggesting that the role of these residues is similar between these different families of G proteins.

During phosphoryl transfer reactions, a partial negative charge develops. If this is on the \( \gamma \)-phosphate, the reaction is associative and goes through a pentavalent phosphorous intermediate. If the charge is on the leaving group, which in the hydrolysis of GTP is the \( \beta,\gamma \)-phosphate-bridging oxygen atom, the mechanism is dissociative and shows a metaphosphate-like transition state (Maegley et al., 1996). This is shown in the scheme below (taken from Maegley et al., 1996).

![Diagram showing the dissociative and associative pathways for phosphoryl transfer reactions.](image-url)
It has been suggested that GAPs stabilise the transition state by neutralising these developing charges and thus catalyzing the phosphoryl transfer reaction (Scheffzek et al., 1997). On the basis of the arguments put forward by Maegley et al. (1996), that residues that neutralise the developing negative charge on the \( \gamma \)-phosphate would act anticatalytically in a dissociative mechanism, Scheffzek et al. (1997) proposed that the GAP most likely stabilises an associative transition state. This was because Arg 789 of the Ras-GAP, p120GAP, in the Ras.GDP.AlF_3.p120GAP structure has a stronger interaction with a fluorine atom, representing an oxygen of the \( \gamma \)-phosphate, than with the \( \beta \)-\( \gamma \) bridging oxygen. In addition, the bond lengths between aluminium and the leaving group and nucleophilic water are more representative of an associative mechanism (Scheffzek et al., 1997). However, Rittinger et al. (1997b) concluded that the positioning of Arg 282 of RhoGAP in the Rho.GDP.AlF_4.RhoGAP structure, where it interacts with a fluorine group and the \( \beta \)-\( \gamma \) bridging oxygen, means that both mechanisms would be enhanced by the interaction of the conserved arginine with the transition state. However, they also concluded that the bond length between the apical oxygen ligand and the aluminium implies that the reaction is associative (Rittinger et al., 1997b).

Conserved arginine residues are present in GAPs for Ras, Rho, Ran and Arf families of small G proteins (Ahmadian et al., 1998). The best characterised ‘arginine fingers’ are those in Rho-GAPs and Ras-GAPs and their role in GTPase activation is strongly supported by structural and biochemical data. The similar characteristics of the role of the catalytic arginine in the GAP mechanism of Ras-GAPs and Rho-GAPs may be common to other GAPs for small G proteins. Indeed, the conserved arginine in the ArfGAP, ARD1, has recently been shown to be critical for GAP activity and not for the interaction with its substrate, p3, suggesting that it may act as an ‘arginine finger’ (Vitale
et al., 1998). Ahmadian et al. (1998) have identified that the catalytic arginine residues in Rho-GAPs and Ras-GAPs and the conserved arginine residue in Rap-GAPs are preceded by an aromatic amino acid residue (phenylalanine or tyrosine), known from structural data with RhoGAP and p120RasGAP to play a role in balancing the orientation of the catalytic arginine (Scheffzek et al., 1996; Barrett et al., 1997). However, assuming an 'arginine finger' is involved, this stabilisation effect is not likely to take place in all GAPs as in Ran-GAPs and Arf-GAPs there is no aromatic amino acid preceding an invariant arginine residue (Ahmadian et al., 1998). One role for the arginine residues in RhoGAP and p120GAP seems to be to stabilise the conserved glutamine residue in Rho or Ras into a position where it is able to orientate the hydrolytic water molecule into a position in line with the β-phosphate oxygen. When these glutamine residues are mutated in both Rho and Ras, they are no longer able to be downregulated by their GAPs (Bollag and McCormick, 1991). Rap proteins belong to a sub-group of the Ras family. A striking feature of these proteins is that, although they have regions of complete sequence identity to Ras, such as the effector domain, they have a threonine residue in the position normally occupied by the conserved glutamine (Pizon et al., 1988). Despite this, their GTPase activity can be activated by Rap-GAPs, and Rap-GAP activity is not changed when the threonine of Rap proteins is mutated to glutamine (Maruta et al., 1991; Rubinfeld et al., 1992). This evidence suggests that the Rap-GAP mechanism is likely to be different to the Rho-GAP and Ras-GAP mechanisms, and may not involve a conserved arginine.

Although the $k_{\text{cat}}$ for catalysis of the Rho-GTPase activity by RhoGAP is severely reduced by the mutation of Arg 282 to alanine, there still remains a GTP hydrolysis rate that is 180 times faster than the intrinsic rate in the absence of GAP. As discussed, Arg 282 contributes to catalysis by stabilising the transition state, resulting in a lowering of the
activation energy. As R282A RhoGAP still increases the level of Rho-GTPase by 180-fold, it can be concluded that transition state stabilisation by Arg 282 contributes about 50% of the reduction in activation energy by RhoGAP and other factors account for the remaining half of its catalytic power. Similarly, the Ras-GAP, NF1, without the catalytic arginine stimulates Ras-GTPase by at least 100-fold (Sermon et al., 1998). In both RhoGAP and Ras-GAPs, catalytic enhancement is likely to arise both from stabilisation of a conformation most complementary to the transition state and also from ground-state destabilisation, both of which will result in a decrease in activation energy. As discussed, the catalytic arginine acts to stabilise the transition state conformation, however, GAPs could also stabilise this conformation by performing an allosteric function thereby stabilising the small GTP-binding protein in a catalytically competent conformation. There is evidence to support that GAPs stabilise a catalytically competent conformation, and also that they may act to destabilise the ground state. Firstly, Ras and Rho proteins hydrolyse GTP at a rate of <0.03 min⁻¹ (Eccleston et al., 1993; Sermon et al., 1998; Zhang and Zheng, 1998, Graham et al., 1999a). In contrast, Gα subunits, that have an inbuilt ‘arginine finger’, hydrolyse GTP at about 2-4 min⁻¹ (Bourne et al., 1991). In the presence of their respective GAPs (RhoGAPs, Ras-GAPs or RGS GAPs), Rho, Ras and Gα all hydrolyse GTP at a rate of between 10² and 10³ min⁻¹ (Sprang, 1997). The structure of the Gαi1 GAP, RGS4, in a complex with AlF₄⁻ and Gαi1 has been solved (Tesmer et al., 1997) and it shows that RGS4 stimulates Gαi1 even though it appears to provide no catalytic residues to the transition state complex. It appears that the increase in catalysis is due to the stabilisation of the switch regions of Gαi1 (Tesmer et al., 1997). In structures of small G proteins in the absence of GAPs (Pai et al., 1990; Schlichting et al., 1990; Tong et al., 1991; Hirshberg et al., 1997; Wei et al., 1997) the switch II regions are highly mobile, however, in the small G protein/GAP transition state structures these regions become less
flexible, suggesting that the GAP stabilises these regions (Scheffzek et al., 1997; Rittinger et al., 1997b). Secondly there is also evidence that Ras-GAPs and RhoGAPs support ground state stabilisation, as catalytically inactive NF1 and n-chimaerin, in which the conserved arginine residues have been mutated, bind with increased affinity to their G protein partners (Ahmed et al., 1994; Morcos et al., 1996). It has also been observed here that R282A RhoGAP binds to Q61L Rac with a higher affinity than wild-type RhoGAP.

In accordance with transition state theory, there are groups on enzymes that interact better with the transition state than with unaltered substrate (Fersht, 1984; Wells and Fersht, 1995). The improvement in binding energy by going from the substrate to transition state lowers the activation energy of the reaction and so increases catalytic rate. As discussed by Fersht (1984), under conditions of saturating substrate, catalysis occurs when the binding energy is realised only in the transition state complex; i.e. its energy is lowered while that of the enzyme-substrate remains the same. This lowers the activation energy of $k_{cat}$ and so increases the rate of reaction. Removal of a side chain which only binds the substrate in the transition state should therefore leave $K_d$ for substrate binding unaltered and just lower $k_{cat}$ (Wells and Fersht, 1995). Removal of Arg 282 in RhoGAP reduces $k_{cat}$ by 200-fold, but with a negligible effect on substrate binding consistent with Arg 282 only binding the substrate in the transition state. The apparent binding energy for binding of the arginine side chain to the transition state is 3.1 kcal.mol$^{-1}$, from the equation $\Delta G = -RT \ln(k_{cat}^{mutant}/k_{cat}^{wild-type})$ (Wells and Fersht, 1995). Conversely, the R282A mutant RhoGAP should bind 200-fold weaker to the transition state. RhoGAPs and Ras-GAPs form AlF$_x$-mediated ternary complexes with the GDP forms of their respective small G protein partners, which have been proposed to represent analogues of the transition state of the Rho- or Ras-GTPase reaction (Mittal et al., 1996; Rittinger et al., 1997b;
Scheffzek et al., 1997; Ahmadian et al., 1997c; Hoffman et al., 1998). AlF₃ has been shown to increase the affinity of binding of Rho.GDP to RhoGAP by approximately 30-fold (Table 4.2), consistent with the formation of a complex closer to the transition state. If the complex precisely mimics the transition state, it was anticipated that the R282A RhoGAP would bind with a 200-fold reduced affinity for Rho.GDP in the presence of AlF₃. However, it was shown that R282A RhoGAP also effectively forms the Rho.GDP.AlF₃.RhoGAP complex with an affinity similar to wild-type. Thus, Arg 282 is not required for formation of this complex. This suggests that arginine is not fulfilling the same role in stabilising the Rho.GDP.AlF₃.RhoGAP complex as it is predicted to do in the transition state.

The data presented here show that the affinity of wild-type RhoGAP for Rho.GDP in the presence of AlF₃ is approximately the same as that for Rho.GTP and for Rho.GMPPNP (Tables 3.3 & 4.2). It has been concluded above that at least 200-fold of the catalytic rate enhancement is likely due to transition state stabilisation, and hence, transition state theory predicts that GAP would bind at least 200-fold tighter to the transition state than to the ground state of the substrate. Thus, also in this feature, the AlF₃ complex does not have the anticipated properties of the transition state itself.

The structures of the Ras.GDP.AlF₃.RasGAP and Rho.GDP.AlF₄⁻.RhoGAP complexes have many of the properties anticipated for the transition state (Rittinger et al., 1997b; Scheffzek et al., 1997). They resemble the Gα structures in that the aluminium atom is coordinated by apical oxygens and by planar equatorial fluorines. Further, at least with RhoGAP, there is a conformational rearrangement versus the ground state with the movement of the catalytic arginine to form a close interaction with AlF₄⁻ (Rittinger et al.,
However, it was clearly recognised (Rittinger et al., 1997b) that the octahedral \( \text{AlF}_4^- \) configuration could only represent an approximation of the trigonal bipyramidal transition state. Mutation of the catalytic arginine of NF1 either to lysine (Ahmadian et al., 1997c) or alanine (P.N. Lowe, Pers. Comm.) effectively prevented formation of the AlF
\text{x}-mediated Ras.mntGDP or Ras.GDP complexes, respectively, as contrasted to an only 2 to 3-fold reduction in affinity when Arg 282 in the RhoGAP system is mutated. It may be that the structural rearrangement that has been shown to take place in the complex between R305A Cdc42GAP and Cdc42.GDP in the presence of aluminium fluoride, resulting in a residue from Cdc42 coordinating the aluminium fluoride, does not take place in Ras-GAPs.

Several other X-ray diffraction structures of protein.nucleotide complexes reported to contain aluminium fluoride have now been published (Fisher et al., 1995; Scheffzek et al., 1997; Rittinger et al., 1997b; Xu et al., 1997; Schlichting and Reinstein, 1997; Nassar et al., 1998). In each of these structures, there is either a trigonal bipyramidal or octahedral arrangement of the ligands around the central metal atom consistent with them being analogues of their transition states. Some structures, such as Ras.RasGAP (Scheffzek et al., 1997) and Cdc42.Cdc42GAP (Nassar et al., 1998), have been interpreted as containing AlF_3, however, other structures such as Rho.RhoGAP (Rittinger et al., 1997b) and G_\alpha (Sondek et al., 1994; Coleman et al., 1994) were proposed to contain AlF_4-. As previously discussed, phosphoryl transfer reactions proceed by either a dissociative or associative mechanism, or a mixture of both. It has been proposed that the likely mechanism that Ras-GAPs and RhoGAPs uses most resembles this associative mechanism which involves the development of the largest negative charge on the planar fluoride ligands (Scheffzek et al., 1997; Rittinger et al., 1997b). Therefore, it could be
debated that the trigonal bipyramidal configuration of AlF$_3$ best represents the configuration expected of the true transition state of the enzyme, however, AlF$_4^-$ best approximates the expected charge.

The X-ray structure of Rho.GDP.MgF$_3^-$RhoGAP reported in Chapter 5 contains a magnesium atom coordinated to three planar fluorines with two apical ligands, the β-phosphate bridging oxygen and the attacking water molecule. That is, the MgF$_3^-$ exists in a trigonal bipyramidal configuration, representing the configuration of the γ-phosphate in the true transition state of the phosphoryl transfer reaction. Also the MgF$_3^-$ has a portion of the negative charge expected of the true transition state. Thus, the Rho.GDP.MgF$_3^-$ .RhoGAP complex would be expected to mimic the transition state of hydrolysis very closely both in charge and geometry.

It was suggested above that the AlF$_x$-mediated complex between Rho.GDP and RhoGAP did not have all of the thermodynamic properties expected of the true transition state though it appears to be a close structural analogue. This was based on two observations. Firstly, RhoGAP bound to Rho.GDP, in the presence of AlF$_x$ with similar affinity as to Rho.GTP. Secondly, removal of the catalytic arginine residue of RhoGAP reduced catalysis by 200-fold but AlF$_x$-mediated binding of the mutant RhoGAP to Rho.GDP was only reduced 2- to 4-fold. The apparent affinity of the magnesium fluoride-mediated complex is not very different from the AlF$_x$-mediated complex, suggesting that it is not a dramatically closer thermodynamic mimic of the transition state. However, the R282A mutation caused a 17-fold decrease in binding affinity for magnesium fluoride complex formation but only 5-fold for AlF$_x$-mediated complex formation, suggesting that the MgF$_3^-$ species might be a slightly closer approximation to the transition state. However,
irrespective of whether mediated by MgF\textsubscript{x} or by AlF\textsubscript{x}, the R282A mutant does not show the loss of affinity to Rho.GDP that might be anticipated from the catalytic enhancement provided by Arg 282 if these fluoride complexes are close thermodynamic transition state analogues.

In conclusion, the conserved arginine is not essential for formation of the AlF\textsubscript{x}- or MgF\textsubscript{x}-mediated complexes, although it is essential for catalysis. The biochemical evidence presented here suggests that, although the transition state analogues are close structural representatives of the true transition state of the phosphoryl transfer reaction, they do not have the thermodynamic properties expected of the true transition state.

As mentioned in the introduction, oncogenic mutants of Ras are found in about 25-30\% of human tumours. Most of them have an impaired intrinsic GTPase and are insensitive to GAPs, thus making them 'constitutively active' and able to continuously transmit downstream signals. Aberrant activation of Rho and Rac have also been implicated in tumour formation and some of their guanine nucleotide exchange factors are known to act as oncogenes. Pharmaceutical intervention that facilitated the inactivation of these proteins by increasing the GTPase activity of these proteins may have therapeutic benefits in the treatment of such tumours. The biochemical studies presented here and those on Ras-GAPs (Ahmadian \textit{et al.}, 1997c; Sermon \textit{et al.}, 1998), together with the structural data available (Scheffzek \textit{et al.} 1997; Rittinger \textit{et al.}, 1997b) has provided convincing evidence for the role of a conserved arginine in the catalytic GTPase-enhancing activity of GAPs. An understanding of the mechanism has led to suggestions that a small molecule able to mimic the effects of this 'arginine finger' and increase the GTPase
activity of these small G proteins may have some therapeutic benefits in the treatment of cancers resulting from the malfunction of these proteins.

A significant understanding of the role or the conserved arginine, Arg 282, in RhoGAP has been gained from these studies. It is now clear that the mechanism by which RhoGAP stimulates the GTPase activity of RhoGAP is similar to that seen for stimulation of Ras-GTPase activity by the Ras-GAPs, NF1 and p120GAP. Further work is needed to establish whether this mechanism is similar for other families of GAPs. It would be especially interesting to elucidate the RapGAP-activated GTPase mechanism of Rap, which does not require the conserved glutamine residue, that is one of the conserved features of the mechanism for both Ras and Rho.
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Appendix 1 - Publications arising from this work


The Conserved Arginine in Rho-GTPase-Activating Protein Is Essential for Efficient Catalysis but Not for Complex Formation with Rho-GDP and Aluminum Fluoride

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ABSTRACT: The Rho family of small GTP-binding proteins are downregulated by an intrinsic GTPase, which is enhanced by GTPase-activating proteins (GAPs). RhoGAPs contain a single conserved arginine residue that has been proposed to be involved in catalysis. Here, the role of this arginine has been elucidated by mutation followed by determination of catalytic and equilibrium binding constants using single-turnover kinetics, isothermal titration calorimetry, and scintillation proximity assays. The turnover numbers for wild-type, R282A, and R282K RhoGAPs were 5.4, 0.023, and 0.010 s⁻¹, respectively. Thus, the function of this arginine could not be replaced by lysine or alanine. Nevertheless, the R282A mutation had a minimal effect on the binding affinity of RhoGAP for either Rho-GTP or Rho-GMPPNP, which confirms the importance of the arginine residue for catalysis as opposed to formation of the protein−protein complex. The R282A mutant RhoGAP still increased the hydrolysis rate of Rho-GTP by 160-fold, whereas the wild-type enzyme increased it by 38000-fold. We conclude that this arginine contributes half of the total reduction of activation energy of catalysis. In the presence of aluminum fluoride, the R282A mutant RhoGAP binds almost as well as the wild type to Rho-GDP, demonstrating that the conserved arginine is not required for this interaction. The affinity of wild-type RhoGAP for the triphosphate form of Rho is similar to that for Rho-GDP with aluminum fluoride. These last two observations show that this complex is not associated with the free energy changes expected for the transition state, although the Rho-GDP-AlF₃−−RhoGAP complex might well be a close structural approximation.

Rho, Cdc42, and Rac are members of the Rho family of small GTP-binding proteins that control key cellular processes, including actin organization, cytokinesis, transcription, secretion, and endocytosis (1–3). Rho family proteins cycle between an inactive GDP-bound form and an active GTP-bound form. The GTP form binds protein effectors which transmit downstream signals. They are downregulated by an intrinsic GTPase, which stimulates their GTP-bound form. RhoGAPs are GTPase-activating proteins (GAPs), which stimulate the intrinsic GTPase activity of Rho family proteins, including bcr, n-chimaerin, p190, and RhoGAP, all contain a homologous catalytic domain (3). Within this domain is a single conserved arginine residue (4); in the case of RhoGAP, it is Arg-282 [identical to Arg-85 of the crystallized RhoGAP (5)]. X-ray crystallography studies of the complex between RhoGAP, Rho-GDP, and AlF₃−, which was proposed to be an analogue of the transition state, have shown that this residue is placed directly into the active site of Rho, suggesting a direct role for Arg-282 in the hydrolysis of GTP by RhoGAP (6). This role is similar to that proposed for invariant arginines in Ras-GAPs (7–10) and in Ga (11, 12).

Although experimental data supporting the role of this conserved arginine in catalysis by RhoGAPs have been recently published (13–18), hitherto the effect of mutagenesis of this residue on the turnover number (kₐₚ) has not been available. Therefore, we have measured the effect of mutation to either alanine or lysine on the kₐₚ for enhancement of Rho-GTPase and on the affinity for binding to substrate. These data showed that the basic center of arginine is indeed essential for efficient catalysis and cannot be replaced by that of lysine. We also examined the properties of the AlF₃−-mediated Rho-GDP-RhoGAP complex and the role of the conserved arginine in its formation. Perhaps unexpectedly, on the basis of existing literature, the replacement with alanine did not affect formation of a complex with Rho-GDP and AlF₃−. We show that the Rho-GDP-AlF₃−RhoGAP complex is not associated with the free energy changes expected for the transition state, although it might well be a close structural approximation.

EXPERIMENTAL PROCEDURES

Proteins and Nucleotide Complexes. Plasmids expressing GST−RhoGAP or GST−RhoA [the latter containing a point
mutation (F25N) to improve stability (19) were generous gifts from A. Hall. Arg-282 of GST—RhoGAP (C-terminal fragment residues 198–439, numbered from the first residue of full-length p50 RhoGAP) (4) was mutated to alanine and lysine using the Stratagene QuikChange mutagenesis kit. The plasmids containing GST—RhoGAP (wild-type or mutant) and GST—Rho were transformed into Escherichia coli DH5α and BL21, respectively. Proteins were expressed as GST fusions and were purified with and without the GST tag on glutathione agarose columns as previously described (20). Proteins were finally dialyzed against 20 mM Tris-HCl (pH 7.5), 2 mM MgC\textsubscript{2}O\textsubscript{4}, and 1 mM DTT. All proteins had the anticipated M\textsubscript{r} when they were analyzed by electrospray mass spectrometry. Protein concentrations were calculated from the A\textsubscript{280} using extinction coefficients of 14 420 M\textsuperscript{-1} cm\textsuperscript{-1} for RhoGAP and 26 430 M\textsuperscript{-1} cm\textsuperscript{-1} for Rho, calculated from the extinction coefficients of Tyr and Trp (21) and that of GTP (7950 M\textsuperscript{-1} cm\textsuperscript{-1}). Rho—\textsuperscript{3}H]GTP, Q61L Rac—\textsuperscript{3}H]GTP, and Q63L Rho—\textsuperscript{3}H]GTP were prepared as described for Q61L Ras–\textsuperscript{3}H]GTP (22). Rho—GMPPNP was prepared as described for Rac/Cdc42—GMPPNP (23). Rho—mant nucleotide complexes were prepared as described previously (20, 24).

Measurements of GTPase Activity. All experiments were carried out in 20 mM Tris-HCl (pH 7.5), 2 mM MgC\textsubscript{2}O\textsubscript{4}, and 1 mM DTT at 20 °C. For measurements of intrinsic GTPase activity, or mutant RhoGAP-catalyzed GTPase activity, 0.2 \mu M Rho—\textsuperscript{3}H]GTP was incubated with 0–30 \mu M RhoGAP. At intervals, 100 \mu L samples were mixed with 50 \mu L of 10% perchloric acid and then adjusted to pH 4 by addition of 17 \mu L of 4 M sodium acetate. For measurements of wild-type Rho—\textsuperscript{3}H]GTP-catalyzed GTPase activity, Rho—\textsuperscript{3}H]GTP (0.2 \mu M) was mixed with wild-type RhoGAP in a Hi-Tech Rapid Quench Flow system using perchloric acid to quench, and then samples were removed and adjusted to pH 4 with sodium acetate. Samples were analyzed by anion exchange HPLC with radiochemical detection (Berthold) using a Partisphere SAX column (Whatman) with a mobile phase of 0.6 M NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} (pH 4). The decrease in the amount of \textsuperscript{3}H]GTP was fitted to a single-exponential decay curve to give a pseudo-first-order rate constant for GTP hydrolysis. Data on the effect of concentration of RhoGAP on this rate constant were analyzed as described in ref 20, taking the K\textsubscript{m} for Q61L Rac—\textsuperscript{3}H]GTP binding to wild-type and R282A GST—RhoGAPs to be 0.2 and 0.06 \mu M, respectively. The affinities of Q61L Rac—\textsuperscript{3}H]GTP and of Q63L Rho—\textsuperscript{3}H]GTP were measured by direct titration SPAs with 0.02 \mu M GST—RhoGAP (wild-type or mutant) as described in ref 23.

RESULTS

Catalytic Activity of Wild-Type, R282A, and R282K RhoGAP. Single-turnover kinetic experiments (9, 10, 20), in which the GTP hydrolysis rate was measured under conditions where RhoGAP (the enzyme) is in molar excess over Rho—\textsuperscript{3}H]GTP (the substrate), were used to determine the kinetic parameters for activation of Rho-GTPase by wild-type, R282A, and R282K RhoGAPs. An advantage of this procedure over steady-state kinetic measurements is that the maximally activated rate constant (k\textsubscript{cat}) is independent of the concentration of GAP (20). An estimate of K\textsubscript{m} can also be obtained. Although it was technically more difficult, we decided to directly measure cleavage of GTP itself rather than monitoring fluorescence changes of mant nucleotides as has previously been done in such experiments (9, 10, 20, 26). This was done because, in specific circumstances, mant nucleotides were not perfect analogues of native nucleotides (see below) and also to avoid any assumptions in relating rates of fluorescence changes to cleavage rates.

Time courses of hydrolysis at near-saturating, fixed concentrations of wild-type and mutant RhoGAPs (panel A) and the effect of concentration of RhoGAP on the pseudo-first-order rate constant for hydrolysis (panels B and C) are shown in Figure 1. k\textsubscript{cat} was found to be 5.4 s\textsuperscript{-1} for wild-type, 0.023 s\textsuperscript{-1} for R282A, and 0.010 s\textsuperscript{-1} for R282K RhoGAPs (Figure 1). Thus, there is a 230- and 540-fold reduction in GAP activity caused by mutation of Arg-282 in R282A and R282K RhoGAPs, respectively. The reduction in GAP activity caused by mutation of Arg-282 in R282A and R282K RhoGAPs is hyperbolic in its dependence on Rho concentration and will be described in detail elsewhere.
activity of the proteins, suggesting that the mutations had no gross affect on protein stability. The $K_d$ values calculated for wild-type, R282A, and R282K RhoGAP were 0.8, 1.6, and 3.5 $\mu M$, respectively. Thus, the substitution of lysine for arginine has resulted in an approximate 4-fold decrease, whereas the alanine substitution causes an only 2-fold decrease in binding affinity.

It has previously been observed that aluminum fluoride mediates the formation of a stable complex between RhoGAP and Rho-GDP (6) or Cdc42-GDP (17, 27). We have investigated the contribution of the conserved Arg-282 residue in the formation of this complex. ITC experiments in the presence of AlF$_3$ showed that titration of Rho-GDP into wild-type or R282A RhoGAP was associated with a strongly endothermic heat change, which fitted well to a single-binding site isotherm (Figure 3) with a stoichiometry of 0.9—1.0. The $K_d$s for wild-type and R282A RhoGAP binding to Rho-GDP with AlF$_3$ were calculated as 0.4 and 0.9 $\mu M$, respectively, showing that the arginine to alanine mutation resulted in an only 2-fold decrease in binding affinity. No heat changes were observed when Rho-GDP was titrated into R282K RhoGAP in the presence of AlF$_3$, hence, no conclusions regarding binding could be made.
 Interaction between Wild-Type and Mutant RhoGAPs with Rho Complexed with mant Nucleotides. Hoffman et al. (17) reported that mutation of the conserved arginine resulted in a 50-fold loss of affinity for Cdc42-mantGDP in the presence of AlF₃, and hence concluded that this arginine was required for formation of the AlF₃-mediated complex. This contrasts with our data, using GDP itself showing an only 2–3-fold decrease in affinity (Table 1). Although we and others have found that mant nucleotides can behave as good analogues of GTP or GDP, the following experiments suggested that this discrepancy was related to the use of mantGDP rather than GDP. SPA experiments were performed to measure affinities for Rho-mantGDP in the presence of AlF₃. The Kₐ for wild-type RhoGAP binding to Rho-mantGDP was 1.9 μM, similar to that for binding to Rho-GDP (1.1 μM) (Figure 4 and Table 1). However, using R282A RhoGAP, Rho-mantGDP bound extremely poorly (Kₐ ≥ 30 μM), whereas we showed above that Rho-GDP binds with high affinity (Kₐ = 3.7 μM). We also used fluorescence anisotropy, the technique used in ref 17, and found that any change in anisotropy upon mixing R282A RhoGAP with Rho-mantGDP in the presence of AlF₃ was barely detectable, whereas wild-type RhoGAP gave a hyperbolic increase in anisotropy with a Kₐ of 2.3 μM (data not shown). Therefore, we believe that, perhaps due to the precise steric constraints required for formation of the AlF₃-mediated complex, replacement of arginine with alanine interferes with formation of the Rho-mantGDP but not with formation of the Rho-GDP complex.

DISCUSSION

Single-enzyme turnover experiments have been used previously to measure Kₐ values of RasGAP-catalyzed Ras-mantGTPase (9, 10, 20). Here, we have determined Kₐ for catalysis of Rho-GTPase by wild-type RhoGAP to be 5.4 s⁻¹. This represents a 38000-fold stimulation over the intrinsic Rho-GTP hydrolysis rate (1.4 x 10⁻⁴ s⁻¹). This level of stimulation is similar to those seen with p120-GAP and neurofibromin on Ras-mantGTP (10, 20).

The kₐ for R282A RhoGAP was 0.023 s⁻¹ and for R282K RhoGAP was 0.010 s⁻¹. These values represent a reduction in the ability of RhoGAP to stimulate Rho-GTPase by 230- and 540-fold, respectively, showing that neither alanine nor lysine could substitute for arginine and maintain efficient catalysis. Since substitution with the basic side chain of lysine severely impaired the catalytic activity of RhoGAP, specific properties of the arginine side chain must be required.

The Kₐ values of wild-type and mutant RhoGAPs for wild-type Rho-GTP were estimated from single-turnover kinetic experiments (Figure 1 and Table 1). If the initial binding step is a rapid equilibrium relative to kₐ, as has previously been assumed in measuring RasGAP kinetics (10, 20), Kₐ
Table 1: Catalytic and Equilibrium Dissociation Constants for Wild-Type and Mutant RhoGAP

<table>
<thead>
<tr>
<th>Rho Complex</th>
<th>Wild-Type RhoGAP</th>
<th>R282A RhoGAP</th>
<th>R282K RhoGAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ ($\mu$M)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_d$ ($\mu$M)</td>
</tr>
<tr>
<td>Rho-GTP</td>
<td>$\geq 0.5^a$</td>
<td>5.4</td>
<td>$\geq 0.5^a$</td>
</tr>
<tr>
<td>Rho-GMPPNP</td>
<td>ITC</td>
<td>0.8</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>SPA</td>
<td>2.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Rho-GDP</td>
<td>SPA</td>
<td>$\geq 30$</td>
<td>$\geq 30$</td>
</tr>
<tr>
<td>Rho-GDP and AlF$_4^-$</td>
<td>ITC</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>SPA</td>
<td>1.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Rho-mantGDP and AlF$_4^-$</td>
<td>SPA</td>
<td>1.9</td>
<td>$\geq 30$</td>
</tr>
<tr>
<td>Q63L Rho-GTP</td>
<td>SPA</td>
<td>0.016</td>
<td>0.014</td>
</tr>
<tr>
<td>Q61L Rac-GTP</td>
<td>SPA</td>
<td>0.2</td>
<td>0.06</td>
</tr>
</tbody>
</table>

$^a$ Catalytic and equilibrium dissociation constants were measured using the indicated methods as described in Experimental Procedures and in the legends of Figures 1–4. $^b$ $K_a$ value ($\geq K_d$). $^c$ SPA signal insufficient to allow $K_d$ determination. $^d$ No heat change seen.

**Figure 4:** SPA measurements of Rho-GDP and Rho-mantGDP binding to wild-type and R282A RhoGAP in the presence and absence of aluminum fluoride. The indicated concentrations of Rho-GDP or Rho-mantGDP were added to SPAs containing Q61L Rac-[$^3$H]GTP and either wild-type GST—RhoGAP (A) or R282A GST—RhoGAP (B), and the SPA signal was measured. The signal from a blank without GST—RhoGAP was subtracted, and the data were plotted as a percentage of the signal without added Rho-GDP or Rho-mantGDP. The solid lines represent the best fit to an equation describing competitive inhibition of binding of radiolabeled Rac to GST—RhoGAP. With wild-type RhoGAP, the apparent $K_q$ values are 32 $\mu$M for Rho-GDP without AlF$_4^-$ (O) and 1.1 $\mu$M with AlF$_4^-$ (●) and 40 $\mu$M for Rho-mantGDP without AlF$_4^-$ (△) and 1.9 $\mu$M with AlF$_4^-$ (●). With R282A RhoGAP, the apparent $K_q$ values are 34 $\mu$M for Rho-GDP without AlF$_4^-$ (O) and 3.7 $\mu$M with AlF$_4^-$ (●) and 45 $\mu$M for Rho-mantGDP without AlF$_4^-$ (△) and 34 $\mu$M with AlF$_4^-$ (●).

will equal $K_d$. In any case, the true $K_d$ for interaction with Rho-GTP will be less than or equal to this measured $K_m$. The $K_d$s of RhoGAPs for Rho-GMPPNP and for Q63L Rho-GTP and Q61L Rac-GTP complexes, none of which show significant hydrolysis during the time course of the experiments, were measured directly from ITC and SPA experiments (Table 1). These methods gave comparable values for $K_d$, although the affinity measured by ITC was about 2–3-fold higher than that estimated by the SPA. The $K_d$ for wild-type RhoGAP binding to Rho-GMPPNP reported here is similar to that previously published (30, 31). The alanine mutation resulted in a small decrease (2–3-fold) in the affinity of RhoGAP for Rho-GMPPNP and no measurable difference in the affinity for wild-type or Q63L Rho-GTP and actually increased its affinity for the GTP form of Q61L Rac (Table 1 and ref 14). The lysine mutation resulted in a more significant impairment of binding (≥4-fold decrease) to both Rho and Q61L Rac (Table 1), suggesting a slightly greater structural perturbation than in the alanine mutation.

Thus, Arg-282 contributes 230-fold out of the total 38000-fold catalytic stimulation by RhoGAP but is not required for binding of RhoGAP to the triphosphate-bound conformation of Rho. This conclusion is in qualitative agreement with reported data on the role of the conserved arginine in n-chimaerin (13), myr5 (15), Cdc42GAP/RhoGAP (14, 17, 18), and p190GAP (16).

As R282A RhoGAP still increases the level of Rho-GTPase by 160-fold, we conclude that transition-state stabilization by Arg-282 contributes about 50% of the reduction in activation energy by RhoGAP and other factors account for the remaining half of its catalytic power. Similarly, RasGAP without the catalytic arginine stimulates Ras-GTPase by at least 100-fold (10). In both RhoGAP and RasGAPs, catalytic enhancement is likely to arise both from stabilization of a conformation most complementary to the transition state (6, 10, 32) and also from ground-state destabilization. The latter is supported by the observation of catalytically inactive RasGAP and n-chimaerin mutants which bind with increased affinity to the triphosphate forms of their respective G protein partners (13, 33).

In accordance with transition-state theory, there are groups on enzymes which interact better with the transition state than with the unaltered substrate (34, 35). The improvement in binding energy by going from the substrate to transition state lowers the activation energy of the reaction and so increases the catalytic rate. As discussed by Fersht (35), under conditions of saturating substrate, catalysis occurs when the binding energy is realized only in the transition-state complex; i.e., its energy is lowered while that of the enzyme–substrate complex remains the same. This lowers the activation energy of $k_{cat}$ and so increases the rate of
reaction. Removal of a side chain which only binds the substrate in the transition state should therefore leave $K_a$ for substrate binding unaltered and just lower $k_{cat} (34)$. Removal of Arg-282 reduces $k_{cat}$ by 230-fold, but with a negligible effect on substrate binding. The apparent binding energy for binding of the arginine side chain to the transition state is 3.2 kcal/mol, from the equation $\Delta G = -RT \ln(k_{cat}/k_{cat, wild-type}) (34)$. Conversely, the R282A mutant RhoGAP should bind 230-fold weaker to the transition state. RhoGAPs and RasGAPs form A1F$^-\text{mediated ternary complexes with the GDP forms of their respective small G protein partners, which have been proposed to represent analogues of the catalytic arginine to form a close interaction with A1F$^-$ (5, 6). However, it was clearly recognized (6) that the octahedral A1F$^-\text{configuration could only represent an approximation of the trigonal bipyramidal transition state. Mutation of the catalytic arginine of NF1 RasGAP either to lysine (9) or to alanine$^2$ effectively prevented formation of the A1F$^-\text{mediated Ras-mantGDP or Ras-GDP complexes, respectively, as contrasted to an only 2—3-fold reduction in affinity when Arg-282 in the RhoGAP system is mutated. This may be due to the different coordinations of the aluminum between the Ras-GDP-A1F$^-\text{RhoGAP (8) and the Rho-GDP-A1F$^-\text{RhoGAP complexes (6). In summary, the data reported here show that the major constituent of the Rho-GDP-A1F$^-\text{RhoGAP complex in solution does not show the thermodynamic stabilization expected of the transition state, likely because of the different chemistry of the A1F$^-\text{complex and that of the true transition state, even though it might well be structurally a very close approximation.}

ACKNOWLEDGMENT

We thank A. Hall for expression plasmids for RhoGAP and Rho, G. Thompson for purified Rac protein, S. Graham for mass spectrometry analysis, M. Goggin for large-scale fermentation, and R. Leatherbarrow and D. Trentham for critical evaluation of the manuscript.

REFERENCES


2 P. N. Lowe, unpublished observations.

The structures of Ras-GDP-A1F$^-RasGAP and Rho-GDP-A1F$^-RhoGAP complexes have many of the properties anticipated for the transition state (6, 8). They resemble the $G_a$ structure in that the aluminum atom is coordinated by apical oxygens and by planar equatorial fluorines. Further, at least with RhoGAP, there is a conformational rearrangement versus the ground state with the movement of the catalytic arginine to form a close interaction with A1F$^-$. However, it was clearly recognized (6) that the octahedral A1F$^-\text{configuration could only represent an approximation of the trigonal bipyramidal transition state. Mutation of the catalytic arginine of NF1 RasGAP either to lysine (9) or to alanine$^2$ effectively prevented formation of the A1F$^-\text{mediated Ras-mantGDP or Ras-GDP complexes, respectively, as contrasted to an only 2—3-fold reduction in affinity when Arg-282 in the RhoGAP system is mutated. This may be due to the different coordinations of the aluminum between the Ras-GDP-A1F$^-\text{RhoGAP (8) and the Rho-GDP-A1F$^-\text{RhoGAP complexes (6). In summary, the data reported here show that the major constituent of the Rho-GDP-A1F$^-\text{RhoGAP complex in solution does not show the thermodynamic stabilization expected of the transition state, likely because of the different chemistry of the A1F$^-\text{complex and that of the true transition state, even though it might well be structurally a very close approximation.}

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REFERENCES


2 P. N. Lowe, unpublished observations.
Essential Catalytic Arginine in RhoGAP

The Rho family of small GTPases, including Rho, Rac and Cdc42, are involved in the control of cell shape and motility via regulation of the actin cytoskeleton [1]. Rac also has a role in the activation of an NADPH oxidase in phagocytic cells [1]. These proteins cycle between an inactive GDP-bound form and an active GTP-bound form. They are down-regulated by GTPase activating proteins (GAPs), which stimulate their intrinsic GTPase activity.

Rho family GAPs contain a catalytic domain within which there is a sequence containing a conserved arginine [2] (Figure 1). The structure of this GAP domain of Rho-GAP has recently been published [3] and this arginine 282 residue has been located on the surface of the protein and has been proposed to be involved in binding of Rho-GAP to small GTPases. It has also been reported that specific conserved arginine residues in Ras-GAPs play an essential role in GTP hydrolysis of Ras [4,5]. We have investigated the role of arginine 282 in the ability of Rho-GAP to bind Rho family proteins and increase their GTPase activity.

Site-directed mutagenesis of Rho-GAP (C-terminal fragment residues 198-439) at the conserved arginine 282 residue was carried out using the Stratagene quick change mutagenesis kit. The resulting mutant plasmids containing alanine or lysine substitutions were transformed into E.coli DH5α cells and expressed and purified as GST fusion proteins which were cleaved with thrombin. The proteins were all homogeneous by SDS-polycrylamide gel electrophoresis and had the anticipated Mr when analysed by electrospray mass spectrometry.

The affinity of wild-type, R282A and R282K Rho-GAP for the Rho family protein Rac was estimated using a scintillation proximity assay (SPA) [6]. In this assay Rac (Q61L)[3H]GTP binding to GST-PAK (PAK, p21 activated kinase) can be monitored by scintillation counting. Rho-GAP added to the assay, binds competitively to Rac and the SPA signal decreases (Figure 2). The apparent Kₗₜₜ for wild-type, R282A and R282K Rho-GAP are 0.63, 0.16 and 2.8μM, respectively, indicating that the affinity of Rho-GAP for Q61L Rac was increased approximately 4-fold by the R282A mutation and decreased approximately 4.5-fold by the R282K mutation.

![Figure 1. Amino acid sequence comparison of a region within the GAP domain of Rho-GAP family proteins.](image-url)

**Figure 1.** Amino acid sequence comparisons of a region within the GAP domain of Rho-GAP family proteins. Semi-conserved regions are shown in bold and the conserved arginine residue (that has been mutated in Rho-GAP) is boxed.

![Figure 2. Inhibition of Rac binding to PAK by wild-type and mutant Rho-GAPs analysed by SPA](image-url)

**Figure 2.** Inhibition of Rac binding to PAK by wild-type and mutant Rho-GAPs analysed by SPA (▲ Rho-GAP Wild-type, ● Rho-GAP R282A, ■ Rho-GAP R282K; GST-PAK 0.02μM; Rac[3H]GTP 0.025μM).

**Figure 3.** Effect of wild-type and mutant Rho-GAPs on GTP hydrolysis of Cdc42.[3H]GTP (▲ Rho-GAP Wild-type, ● Rho-GAP R282A, ■ Rho-GAP R282K; Rho-GAPs (0.1μM) incubated at 4°C with 2.5μM Cdc42.[3H]GTP and samples analysed by HPLC with radiochemical detection).

The effects of the R282A and R282K mutant Rho-GAP proteins on catalysis of Cdc42.GTP hydrolysis has been investigated at a single concentration of Cdc42 and Rho-GAP (Figure 3). The R282A and R282K mutants had at least a 15-fold and 87-fold decrease in rate of GTP hydrolysis, respectively, when compared to the wild-type protein.

The results reported here show that the conserved arginine 282 is not essential for binding of Rho-GAP to Rho proteins but appears to be important in catalysis.

The interaction of wild-type, R282A and R282K Rho-GAPs with Rho family proteins will be further investigated using transient kinetic methods to measure precisely the effects on binding and catalysis.

Acknowledgements: This work was supported by the MRC, U.K. We are grateful to Mr Gladstone Thompson for providing Rac and PAK and Mr Steve Graham for analysing proteins by mass spectrometry.