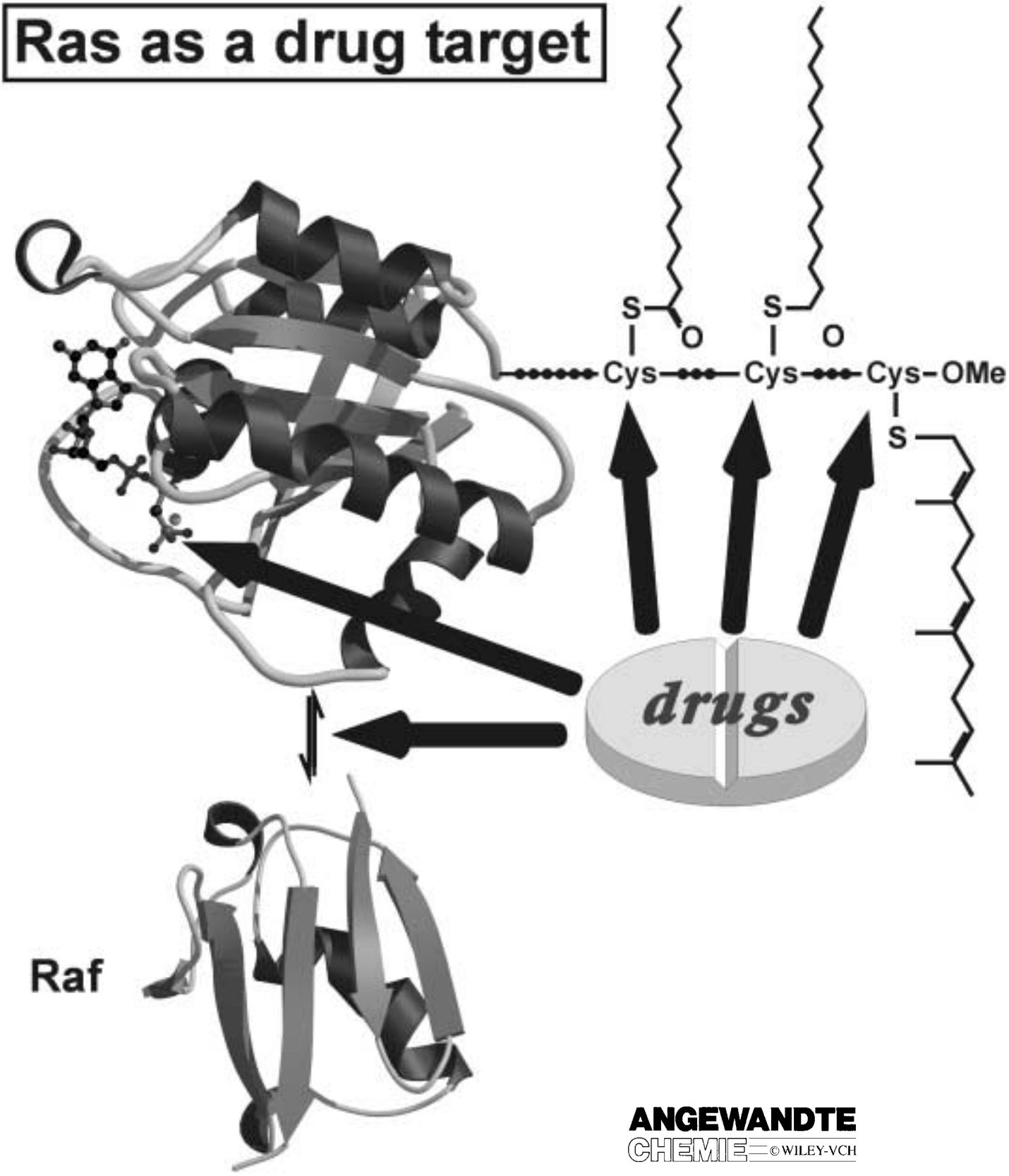
Ras as a drug target



Ras—A Molecular Switch Involved in Tumor Formation

Alfred Wittinghofer* and Herbert Waldmann

Ras, a GTP-hydrolyzing protein, is the product of a proto-oncogene found mutated in about 20–30% of human tumors. It binds GDP/GTP with high affinity and in the presence of a GTPase-activating protein (GAP) has high GTP-hydrolyzing activity. The proto-oncogenic "normal" Ras functions as a regulated molecular switch cycling between a GDP-bound OFF and a GTP-bound ON state and is involved in signal transduction pathways controling cell growth, differentiation, apoptosis, and other events. The oncogenic versions of Ras contain point mutations which block the GTPase activity in the presence and absence of GAP. This process in turn inhibits the cycling of the switch and leads to the accumulation of Ras in the active form and contributes to tumor formation. Substantial effort has been devoted towards understanding the molecular basis for the switch function of Ras proteins and developing Ras-directed antitumor drugs.

Keywords: antitumor agents · drug research · enzyme inhibitors · GTP hydrolysis · proteins

1. Introduction

Ras is the founding member of a superfamily of proteins called Ras-related GTP-binding proteins. Their common property is a molecular mass of 20–25 kDa and the ability to bind guanine nucleotides. They function as molecular switches by cycling between a GTP-bound active state and a GDP-bound inactive state. They regulate a multitude of different cellular reactions but can be grouped into two major categories: the signaling proteins, which act catalytically and are involved in communication between cells and between different cellular compartments, and a second group which acts stoichiometrically as transport factors regulating the delivery of material between cellular compartments.

In this review we will concentrate on the signaling protein Ras, which is a major regulator of many signal transduction processes, such as those regulating growth, differentiation, cell cycle, and apoptosis, some of which are actually seemingly opposing functions. Although we will outline the biological function of Ras, this review focuses on more structural-biochemical and biophysical aspects of the protein and on how this knowledge is applied to the development of potential antitumor drugs.

For more detailed aspects of Ras biology and for earlier references other reviews should be consulted.[1–5]

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2. Ras as a Molecular Switch

2.1. Biological Signal Transduction

There are three human *Ras* genes, *H*-, *N*-, and *K-Ras*, the latter of which is further spliced to produce two different variants, *K-Ras4A* and *K-Ras4B*, which are divergent only in the last 25 residues encoded by the fourth exon. The corresponding H-, N-, and, K-Ras proteins have a molecular mass of 21 kDa. They are posttranslationally modified by the attachment of lipophilic groups to the C-terminus and this modification is necessary for the biological function of the protein.[6] Ras is a molecular switch that cycles between the GDP-bound and the GTP-bound state (Scheme 1). Since the switch-on and the switch-off reactions in the cycle of Ras are intrinsically very slow, the regulatory input by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAP) determines the lifetime of the two states. In the resting state Ras is bound to GDP. It is activated by the action of GEF catalyzing the dissociation of GDP, thus facilitating the loading with GTP, which is the more prevalent guanine nucleotide in the living cell. In the GTP-bound state it interacts with effectors, which are defined as proteins that interact specifically with the GTP-bound state and transmit a signal. The signal is terminated by the GTPase reaction on Ras, which is very slow but is accelerated by Ras-specific GAP. Ras in the GDP-bound state is no longer able to interact with the effectors.

The lifetime of the signal transduced by Ras is determined by the lifetime of the active state. If it is artificially extended, the now unregulated biological response may lead to drastic

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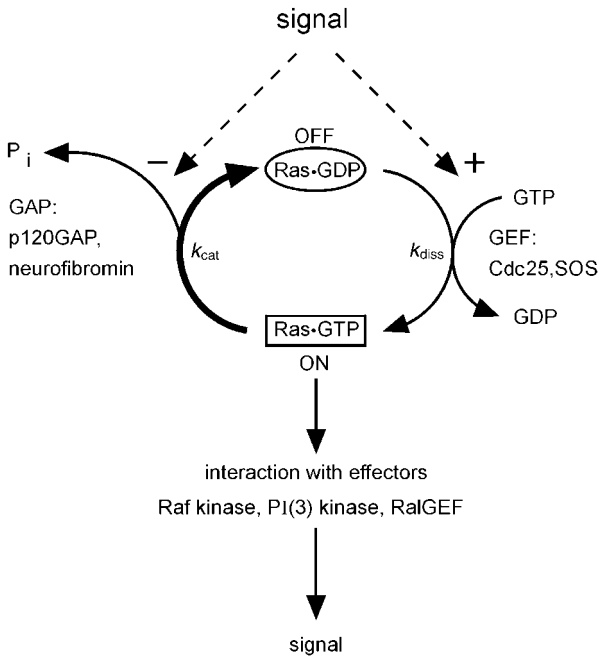

Scheme 1. Cycling of the Ras molecular switch. Ras is inactive in the GDP-bound form. It can be activated by the action of guanine nucleotide exchange factors (GEFs). In the GTP-bound form it interacts with effectors, which are defined as proteins specifically recognizing the GTP-bound form. To complete the cycle, Ras hydrolyzes GTP to GDP and phosphate (P_i). Ras can in principle also be activated by inhibiting the GTPase reaction.

consequences in the cell. Ras was indeed originally found as the active principle of rodent tumor viruses (the name Ras is derived from *ra*t *s*arcoma) and was called an oncogene, a gene that is able to induce tumors in animals or in cell cultures.[2, 5] Later, activated forms of Ras were found in human tumors, and it is now estimated that up to 30% of human tumors carry a mutated Ras oncogene, usually derived from *N*- or *K-Ras*. The activation of the *Ras* gene from a proto-oncogene to an oncogene is the result of a point mutation at either position 12, 13, or 61, the biochemical consequence of which is to render the protein unable to hydrolyze GTP.[2, 5] Since many other genes involved in the Ras signal transduction pathway are also found as oncogenes in human or animal tumors, Ras itself and the Ras pathway are thus considered to be prime targets for antitumor therapy and indeed great progress has been made in that direction.

In the early 1990s, by using information from studies with mammalian cell lines and from *Drosophila melanogaster*[7] and *Caenorhabditis elegans*[8] genetics, a linear signal transduction pathway linking Ras with growth factor receptors and MAP kinase (*m*itogen *a*ctivated *p*rotein kinase) activation was established, and this "Ras pathway" is now considered to be the paradigm for biological signal transduction.[4, 9] The signal is initiated by the binding of a growth factor such as EGF (*e*pidermal *g*rowth *f*actor) or PDGF (*p*latelet-*d*erived *g*rowth *f*actor) to the extracellular domain of their corresponding receptors (Scheme 2). These are transmembrane

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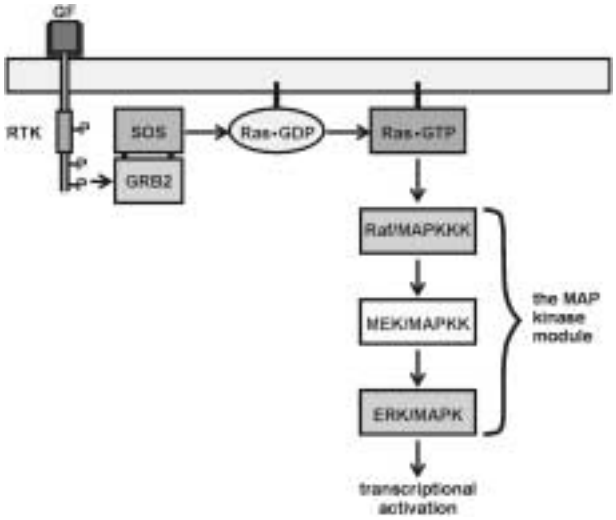
Alfred Wittinghofer was born 1943 in Xanten on the Niederrhein. He studied chemistry in Aachen and made his diploma thesis and his Ph.D. in the laboratory of Prof. Dr. H. Zahn at the Deutsche Wollforschungsinstitut in Aachen, where he synthesized the A chain of insulin with a preformed selective intrachain disulfide bridge in the presence of two additional cysteine residues. He spent three years as a post-doc with Dr. R. Hiskey at the University of North Carolina on the preparation of semisynthetic insulin. He came back to Germany to work a number of years as a postdoc in the Max-Planck-Institut fuer medizinische Forschung in Heidelberg where he studied phosphoryl transfer enzymes. After establishing his own research group there he moved into the field of Ras and other Ras-like GTP-binding proteins on which he has been working ever since. He was appointed Director of the Max-Planck-Institut fuer molekulare Physiologie in 1992.

Herbert Waldmann, born in 1957, received his Ph.D. in 1985 (Universität Mainz, H. Kunz). After postdoctoral studies (1985–1986, Harvard University, George Whitesides) and habilitation (1991, Universität Mainz) he accepted a professorship at the Universität Bonn in 1991. In 1993 he moved to the Universität Karlsruhe as Full Professor of Organic Chemistry. In 1999 he was appointed as Director at the Max Planck Instiut of Molecular Physiology, Dortmund (Department of Chemical Biology) and as Full Professor of Biochemistry at the University of Dortmund. Herbert Waldmann has been the recipient of the Friedrich Weygand Award for the advancement of peptide chemistry, of the Carl Duisberg Award of the Gesellschaft Deutscher Chemiker, and the Steinhofer Award of the Steinhofer Foundation. His current research interests include bioorganic chemistry and natural product synthesis as well as biocatalysis, stereoselective synthesis, and combinatorial chemistry. A major focuss of the reasearch activities is on the combination of organic chemistry, biophysics, and biology for the synthesis and biological evaluation of peptide and protein conjugates which are involved in biological signal transduction processes. Most recently syntheses of natural products and natural product derived compound libraries on polymeric supports have been investigated by the Waldmann group (for further information see: www.chemie.uni-karlsruhe.de/OC/akwa/ak.htm).

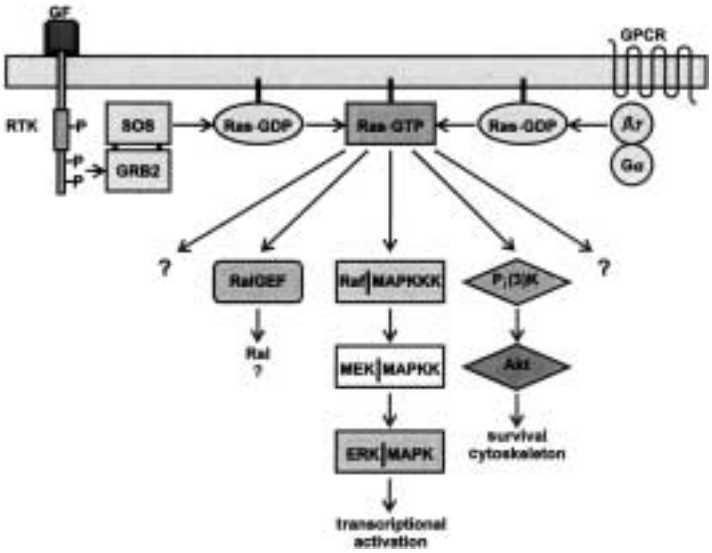


Scheme 2. Simple version of the Ras signal transduction pathway as described in the text. Recruitment of the RasGEF SOS to the plasma membrane by activated, growth factor bound RTKs (receptor tyrosine kinases) leads to activation of Ras. It in turn activates a cascade of protein kinases, MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), MAP kinase (MAPK), whereby the upstream kinases activates the next in-line kinase by phosphorylation. Phosphorylated MAPK (= Erk) is transported into the nucleus to phosphorylate transcription factors which mediate the cellular response to the original signal, the growth factor (GF).

proteins with a cytoplasmic tyrosine-specific protein kinase domain called receptor tyrosine kinases (RTKs). The binding of ligand induces dimerization of the receptor and subsequent transphosphorylation on tyrosine residues in the cytoplasmic part of the receptor, both in the kinase domain and other regions. Phosphorylation activates the receptor and generates binding sites for proteins containing SH2 (*s*rc *h*omology 2, where *src* is an oncogene originally isolated from a *Sarc*om) domains, which are protein modules specifically recognizing phosphorylated tyrosine residues in a sequence-specific context. One such protein is Grb2 (*g*rowth factor *r*eceptor *b*inding), which is complexed to the Ras guanine nucleotide exchange factor RasGEF SOS (from the *Drosophila* gene *son-of-sevenless*). The translocation of SOS to the plasma membrane is believed to activate Ras by bringing both proteins close to each other and thus favoring the interaction.

Once activated Ras interacts with the downstream target or effector, the Ser/Thr-specific protein kinase Raf—itself originally found as an oncogene (*ra*pid *f*ibrosarcoma). The terms upstream and downstream in signal transduction derives from a (now obsolete) concept of a linear hierarchical order of reactions where one reaction leads to (upstream) and one from (downstream) the protein in question. Translocation of Raf kinase to the plasma membrane somehow activates the kinase activity by a process that is not completely understood, but involves allosteric regulation as well as participation of other kinases and regulatory proteins such as 14-3-3.[1, 10–12] Once activated Raf in turn activates the protein kinase MEK by phosphorylation, and this in turn activates the protein kinase ERK. These three in-line kinases are also called MAP kinase (MAPK, mitogen activated protein kinase), MAP kinase kinase (MAPKK), and MAP kinase kinase kinase (MAKKK), and constitute a so-called MAP kinase module.[13] Such kinase modules are found in a large variety in all higher eucaryotic cells and are sometimes (maybe always) bound to so-called scaffold proteins, which help to organize the module and possibly guarantee the specificity of signal transmission.[14] Ras signaling is terminated by the action of GAP, which may also be recruited to the plasma membrane for more efficient interaction. The prototype p120GAP is in fact an SH2-containing protein and has been shown to be recruited to activated receptors.[15–19] The signal is also terminated by MAPK phosphorylating SOS, which in turn is no longer able to interact with Grb2.[20–23]

The elucidation of the linear signal transduction cascade through the MAP kinase module was a landmark achievement performed in several laboratories and was believed to explain the biological function of Ras. However, in recent years it has become clear that the different functions of Ras are unlikely to be explained by a single linear MAP kinase cascade and that different signals upstream and downstream of Ras more likely have to be invoked to explain the complexities of Ras biology (Scheme 3). Ras was shown to



Scheme 3. Involvement of Ras in more than one signal transduction event. Ras is activated by more than one upstream signal and relays the signal to more than one effector. The outcome of the cellular response depends on the number of signals and their integration by the target protein(s), that is, transcription factors.

be activated not only by receptor tyrosine kinases (RPTKs) but also by cytoplasmic tyrosine kinases such as Src and by G-protein-coupled receptors (GPCRs), to name just a few. Similarly it was shown that Ras induces different signals presumably by interacting with more than one effector. In a landmark experiment it was in fact shown by White et al.[24] that one can dissect different downstream signaling pathways of Ras by introducing mutations into the effector region which block a certain set of pathways without touching others,[24–28] and that more than one pathway has to be switched on for full transformation by Ras.

In the meantime, by using the newly developed techniques of the two-hybrid analysis, a large number of actual or potential Ras effectors have been identified as molecules which interact with the GTP-bound form—but not the GDP-

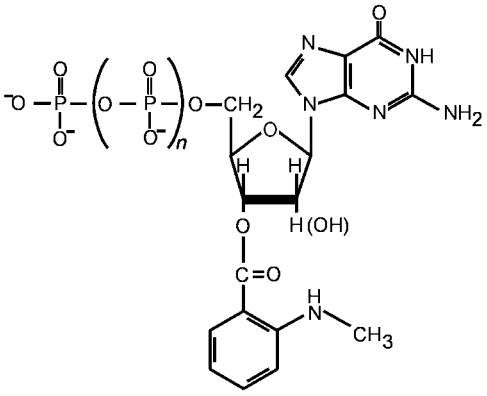
bound form—of Ras. The most important among these are the various isoforms of the lipid kinase phosphatidylinositol-3′-kinase (PI3K)[29–31] and of RalGEF[32–34], which is a nucleotide exchange factor (GEF) for the Ras-related protein Ral, of which several isoforms (Rgl, Rlf/RalGEF2[35, 36]) have been described. Another complication in unraveling the complicated network of interactions is the fact that certain Ras-like proteins such as Rap (four isoforms) and R-Ras (three isoforms) also interact with the same set of effectors in a GTP-dependent manner.

Although it is tempting to speculate that particular pathways are switched on for one particular biological effect, this appears not to be the case. Rather, we may assume that Ras always induces more than one pathway at a time and that the final signal output depends on a multitude of Ras-dependent and Ras-independent parallel and interfering (crossover) signaling pathways which are different in different types of cells. These signals are finally integrated by a complex downstream machinery to produce the cell-type-specific biological response.

2.2. Biochemistry and Structure of Ras

Most of the biochemical and structural studies have been done with H-Ras (Ras, for short) for no obvious reason and its properties will thus be discussed here, but it is clear that the biochemical and structural properties of the other isoforms are similar, even though they are not completely identical in sequence. Ras consists of 189 amino acids with five conserved sequence elements which are also found in other Ras-related proteins and are necessary for binding guanine nucleotides and for the hydrolysis of GTP. The last four amino acids (CVLS) constitute what is called a CAAX box, where C stands for cysteine, A for an aliphatic, and X for any amino acid. CAAX is the signal recognition sequence for the enzyme farnesyltransferase which thioalkylates the cysteine residue with a C15 prenyl group (see Section 3). This modification is the first in a series of posttranslational modifications which is responsible for anchoring Ras into the plasma membrane, a process which is necessary for its biological activity.[37–40] It has been shown that the first 166 residues of Ras are necessary and sufficient for its biochemical properties[41] and that the C terminus is thus only necessary for localization in the plasma membrane and is not involved in any interaction. Correspondingly, for technical reasons, many biochemical and structural studies on Ras have been performed with versions with truncated C termini (amino acids 1 – 166 or 1 – 172) of the protein.

Ras binds guanine nucleotides with high specificity and affinity. The affinity for GDP/GTP is so high that no equilibrium method can be used for its measurement. The affinity is instead determined by measuring both association and dissociation rate constants, which is most conveniently done by using fluorescently labeled methylanthraniloyl (mant- or m- in brief) nucleotides (Scheme 4). These nucleotides have been proven to be very versatile for the study of the biochemistry of Ras proteins.[42–44] The rates thus measured allow the calculation of an equilibrium dissociation constant



Scheme 4. Examples of fluorescent guanosine nucleotides which are used in the biochemical analysis of Ras proteins. They carry a fluorescent *N*-methylanthraniloyl group on the 2′ or 3′-position of the ribose group. The 2′-deoxy mGDP ($n=1$) and 2′-dmGTP ($n=2$) nucleotides are shown. In the case of the ribose derivatives, a mixture of 2′,3′ analogues is commonly used.

in the order of 10 pM for GDP and GTP.[43–45] The binding affinity is strongly dependent on the presence of Mg^{2+} ions. In their absence the dissociation rate constant increases by several hundredfold with a concomitant increase in the dissociation constant.[46, 47]

The GTPase reaction is very slow, of the order of 0.02 min^{-1}, and is strongly stimulated by GTPase-activating proteins (see Sections 2.5 and 3.4). Like any other biological phosphoryl transfer reaction it is strictly dependent on the presence of Mg^{2+} ions. The specificity for binding guanine nucleotide is very high, and no other standard nucleotide shows easily measurable binding. The affinity for adenine nucleotides has been estimated from kinetic methods to be lower by at least six orders of magnitude.[48] Small changes of the guanine ring system led to drastic changes in affinity: xanthosine, which has the 2-amino group replaced by an oxygen atom, binds with a 280-times lower affinity, and an inosine nucleotide diphosphate, which misses the 2-amino group, shows a similarly reduced binding.[48] The β-phosphate group is necessary for high affinity binding as shown by GMP, which has an affinity six orders of magnitude lower.[43–48]

The three-dimensional structure of Ras has been determined from two truncated versions of the H-Ras protein that terminate either at position 166[49, 50] or at 171.[51, 52] The structure of full-length proteins has never been solved, although crystals of the protein have been reported.[52] The fold of the protein consists of a six-stranded mixed β-sheet and five α-helices on both sides of the sheet (Figure 1 A). This fold is found in all standard GTP-binding proteins, including the much larger heterotrimeric G proteins or biosynthesis elongation factor Tu (EF-TU; the first GTP-binding protein whose structure was solved), and is called the G-domain fold.

Although some of the sequence conservation of GTP-binding proteins (Figure 1 B) had been observed before the first three-dimensional structure had been solved[53] the role of the conserved sequence elements could only be appreciated afterwards. Not surprisingly, they are invariably involved in the binding of guanine nucleotides, hydrolysis of GTP, and/or in the mechanism of conformational change. They have been

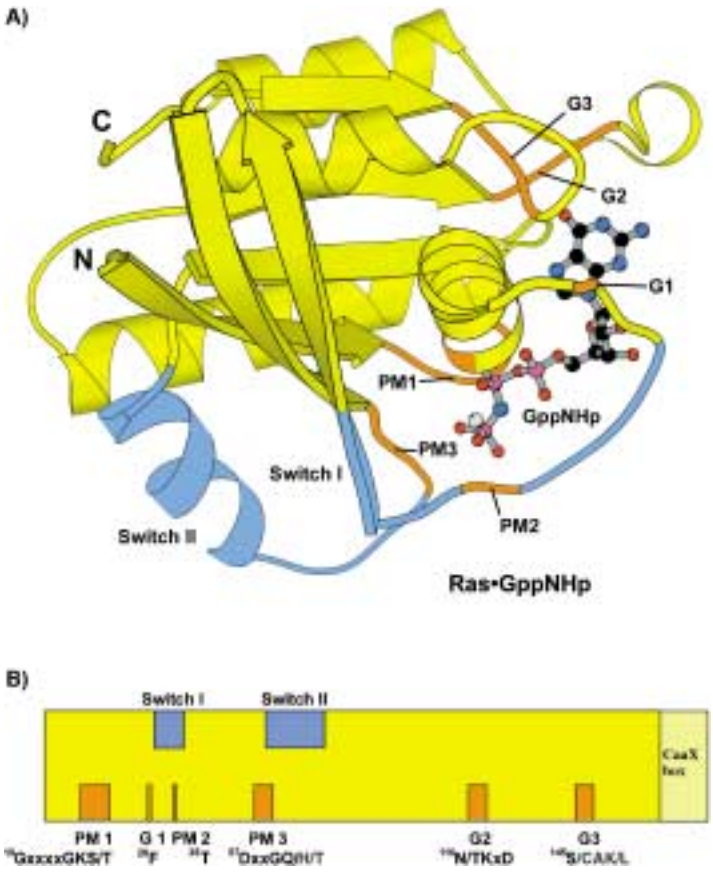
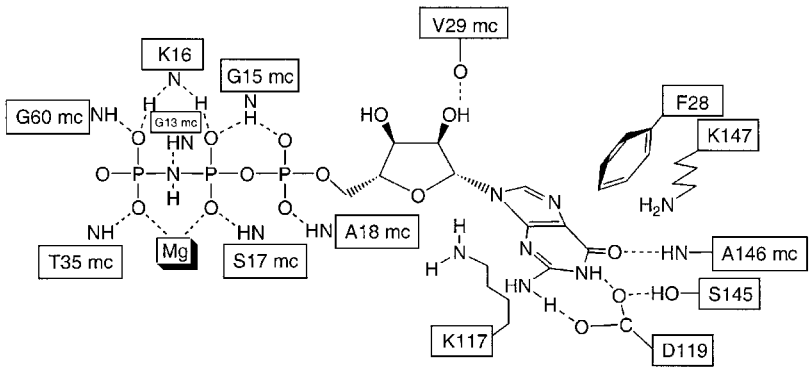


Figure 1. Primary and tertiary structure of Ras. A) A ribbon plot of Ras in the triphosphate form (as GppNHp complex), with the conserved sequence elements of all GTP-binding proteins (brown) located around the nucleotide. The two regions in Ras which change their structure between the GTP- and GDP-bound forms, the Switch I and II regions, are shown in blue. The N and C termini of the polypeptide chain are indicated. B) Linear schematic representation of Ras, with the location of conserved sequence elements, and Switch regions (color coding as in A). The structure in A is only that of the actual G domain which misses the C-terminal extension with the CAAX box which is absolutely essential for the biological function but is not necessary for any of the structural and biochemical properties discussed here.

called the G motifs (G1 to G4)[54, 55] or, according to their function, PM and G motifs which describe their involvement in the binding of either phosphate and Mg^{2+} ions or the guanine base, respectively.[56] The network of interactions in the nucleotide binding site is shown in Scheme 5.



Scheme 5. Interactions of the Ras-bound triphosphate analogue GppNHp with selected, usually conserved residues in Ras. mc = peptide main chain.

The PM1 motif ^{10}GxxxxGKS/T forms a loop (L1 in Ras), also called the P loop, which is involved in binding the charged phosphate groups. Through its totally conserved lysine it forms a ringlike structure that wraps tightly around the β-phosphate of GDP/GTP. The main-chain nitrogen atoms of residues 13–16 point towards the negatively charged phosphate groups and, together with the side chain of Lys16, create a positively polarized environment for these charges. The serine/threonine residue following the lysine coordinates to the important Mg^{2+} cofactor in both the GDP- and GTP-bound state. Its substitution by Ala or Asn severely modifies the interaction between the protein, metal ion, and nucleotide.[57–59] As a result of this the affinity to guanine nucleotides is very weak and in turn the protein has a relatively high affinity (relative to GDP/GTP) to guanine nucleotide exchange factors. The GEFs are then sequestered by the mutated Ras proteins. Therefore such a mutation is called dominant negative, because it inhibits signaling through the GEFs.

Although various consensus motifs have been proposed around this conserved threonine (^{35}T) in the PM2 motif, none of these residues except Thr are actually conserved. This threonine is a direct ligand of Mg^{2+} in the GTP-bound state, binds the γ-phosphate of GTP, and is a key residue that triggers the conformational change after GTP hydrolysis.[60] Mutation of Thr35 to alanine reduces the affinity to nucleotide and the mutant protein can no longer be activated by GAP, probably as a result of the incorrect coordination of Mg^{2+} ions.[59]

In the ^{57}DxxGQ/H/T (PM3) motif the aspartate group is involved in binding Mg^{2+} ions through a water molecule. The glycine residue is coordinated by a main-chain hydrogen bond to the γ-phosphate group, another interaction crucial for the conformational change after GTP hydrolysis. Most GTP-binding proteins contain a Gln residue crucial for GTP hydrolysis. This residue is replaced by Thr in Rap proteins and by His in elongation factors.

The Phe or Tyr residue constituting the G1 motif is only conserved in proteins belonging to the Ras superfamily. It is located perpendicular to the guanine base and creates strong hydrophobic interactions between the protein and base together with the conserved lysine from the G2 motif. Mutating this residue to leucine weakens the nucleotide binding drastically by a corresponding increase in the dissociation rate.

In ^{116}N/TKxD (G2) the guanine base of the nucleotide is located in a hydrophobic pocket. Specific hydrophobic contacts and ionic interactions and hydrogen bonds stabilize its binding. Both the Asn and Lys are involved in linking together various subregions of the nucleotide binding site. The aspartate residue is involved in a double hydrogen bond to the guanine base and is the major element responsible for the high specificity of the protein for guanine nucleotides. Many residues in the G2 motif have been mutated in Ras-related proteins. The effect of the mutations is invariably to increase the rate of dissociation of the nucleotides from the protein. Such mutants

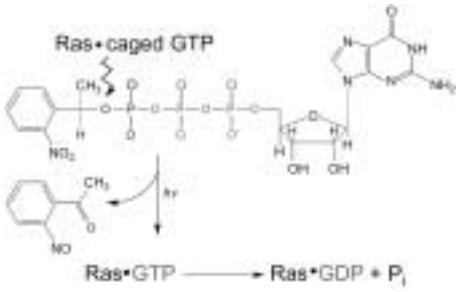
are permanently activated as a consequence of the fast exchange reaction and/or are dominant negative. Mutation of Asp to Asn reduces the affinity for guanine nucleotides several hundredfold, but shows a similarly increased affinity for xanthosine nucleotides,[45, 61, 62] since binding of the latter preserves the double hydrogen-bond system. These types of mutants have been useful in studying the role of EF-Tu,[63] Ras,[45, 64] Rab5,[65] and FtsY.[66]

The G3 motif ^{145}S/CAK/L is, with the exception of the alanine residue, not totally conserved in the Ras superfamily. It seems to serve as a helper function in the binding of the guanine base since its side chains do not participate in any direct interaction with the base. Instead the Lys or Leu residue is involved in stabilizing the position of Phe 28 residue (the G1 motif) by a hydrophobic interaction and Ser 145 forms a hydrogen bond with Asp 119. The nitrogen atom on the Ala 146 residue of the main chain makes a strong hydrogen bond with O6 of the base and this interaction is another factor responsible for the specificity of guanine nucleotide binding; adenine would not be accommodated for steric reasons.

The structure of Ras was solved both in the triphosphate and diphosphate form,[49–52, 67] by using GTP analogues such as GppNHp[49, 50] and Gpp(CH_2)p[51] (two non- or slowly hydrolyzing analogues), where the β-γ-bridging oxygen atom is replaced by an NH or CH_2 group. These studies showed that the structural changes are confined to only two small areas which have been defined as Switch I and II.[51] The structures showed how the conformational change is triggered since both switch regions are bound to the γ-phosphate through two invariant residues: Switch I by Thr 35 and Switch II by Gly 60.[60] Removal of the γ-phosphate group in the course of GTP hydrolysis allows these regions to relax and adopt a new conformation in what may be termed a loaded spring mechanism.[68]

The structure of Ras was also solved by using various analogues of guanine nucleotides such as the fluorescent derivative mantGppNHp[69] (Scheme 4), and caged GTP,[67] which carries a photolabile protecting group on the γ-phosphate residue (Scheme 6). Crystals of the Ras · caged GTP complex were obtained and the protecting group was removed by flash photolysis. The Ras · GTP structure then obtained represents a true enzyme · substrate complex.[67, 70] In an extension of these studies, Ras · GTP was allowed to hydrolyze to Ras · GDP in the crystal.

A number of mutants of Ras were also structurally investigated, most notably oncogenic mutants of Ras, such as G12V, G12R, G12D, and Q61L.[52, 71, 72] They showed a similar overall structure as the wild-type. Although small changes around the γ-phosphate group were observed in all of these structures the analysis did not provide any consistent explanation for the inability of these mutants to hydrolyze GTP.

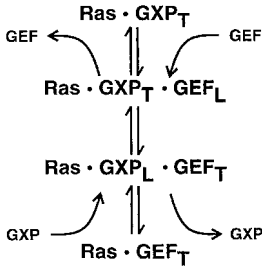


Scheme 6. Structure of caged GTP. Crystals containing Ras · caged GTP are photoloyzed and then used to determine the crystal structure of Ras · GTP, which represents the Michaelis–Menten complex of the GTPase reaction.

2.3. The Switch-On Mechanism

Although Ras can in principle be switched ON by inhibiting the GAP-mediated GTPase reaction[73] the prevalent mode of activation is by interaction with GEFs. A number of specific GEFs for Ras have been identified which can be grouped into two main categories (for a review, see ref. [74]). The first is SOS, short for the *son-of-sevenless* mutation in *Drosophila*, with two mammalian isoforms which contain polyproline motifs necessary for its interaction with SH3 domains, such as those contained in the adaptor protein Grb2.[75–79] These exchange factors are activated by recruitment of the SOS · Grb2 complex to tyrosine-phosphorylated receptors. The second class of RasGEF, with three isoforms and several splice variants, contains a number of domains which are believed to bind to and are activated by second messengers, such as Ca^{2+} and Ca^{2+}-calmodulin.[80, 81] A recently identified member of this group is activated by diacylglycerols.[82, 83] Both types of GEFs contain a Cdc25-like domain, which was first identified in the yeast protein Cdc25. This domain is necessary and sufficient for fast guanine nucleotide exchange.[84] A second domain, REM (*r*as *e*xchange *m*otif), is found at a variable distance from the CDC25 domain.[85] As the structure has shown (see below), the latter is mostly responsible for the stability of the catalytic domain.

GEFs increase the dissociation rate dramatically, by several orders of magnitude in the case of Ras and Ran where it has been measured under saturating conditions, by reducing the affinity appropriately. The nucleotide thus comes off in less than a second.[84, 86, 87] A minimal model for the reaction (Scheme 7) involves GEF approaching the Ras · GDP binary complex and forming a ternary complex.[84, 86–90] Thereafter the nucleotide binding pocket goes from a tight (GXP_T) to a loose binding conformation (GXP_L), which results in a fast release of nucleotide from the ternary Ras · GDP · GEF complex and formation of the Ras · GEF complex. Ras · GTP is generated by binding GTP (the dominant nucleotide in the cell), formation of a ternary complex, and release of GEF.



Scheme 7. The GEF-catalyzed exchange reaction on Ras. The Ras · GXP_T-nucleotide complex, where the nucleotide is still tightly bound, interacts with GEF to form a ternary complex with loosely (L) bound GEF. This complex undergoes a conformational change to a state with loosely bound nucleotide and tightly bound GEF, which is now able to relax into a nucleotide-free binary complex. The reversal of the reaction reforms the Ras · nucleotide complex, which under the conditions in the cell would mostly be Ras · GTP.

It should be stressed that the guanine nucleotide exchange reaction can in principle proceed in both directions (GDP versus GTP exchange or the reverse), since all the partial reaction steps are very fast in each direction.[84, 86, 87] In the case of EF-Tu (R. Goody, unpublished results) or Ran,[86, 87] the reaction is actually faster in the direction where the GDP-bound state is formed. Since GEF merely works as a catalyst, and in the absence of other restraints that drive the reaction towards the GTP-bound state—such as incorporation of the products into the membrane—the outcome of the reaction is solely determined by the relative affinity of the GTP-binding proteins, the concentration of GDP versus GTP in the cell (or in the particular compartment of the cell where the reaction occurs), and the concentration of components that bind either the GDP- or the GTP-bound state.

Very little information on the kinetic mechanism of the GEF reaction has been obtained so far. The existence of a binary complex between GEF and the GTP-binding protein, anticipated from the EF-Tu · EF-Ts complex described earlier, was demonstrated for Ras · Cdc25.[84, 88–90] It has been postulated that the rate-limiting step of the overall exchange reaction on Ras mediated by Cdc25 is the conformational change of the nucleotide on Ras from a tight to a loose binding conformation.[84] The rate of this reaction is very similar to that of the second step of the two-step binding reaction of a nucleotide to Ras alone, where the initially formed complex with loosely bound nucleotide ($K_D \approx 10\ \mu M$) isomerizes to a tightly-bound complex ($K_D \approx 10$ pM).[43] This mechanism enables a similar rearrangement of the structure when the nucleotide binds to empty Ras or the binary Ras · GEF complex. The studies with Cdc25 have also shown that the affinity of the Cdc25 domain for RasGDP is of the order of several hundred micromolar,[84] which explains why no futile nucleotide exchange occurs unless the exchange factor is co-localized with Ras in the membrane.

The structure of a Ras · SOS complex has been solved by Boriak-Sjodin et al.[91] and it gave important insights into the mechanism of Ras activation (Figure 2). The minimal domain of Cdc25, and presumably of SOS, responsible and sufficient for efficient catalytic activity contains 248 residues (Cool and Wittinghofer, unpublished results) and most of the detailed kinetic studies have been performed with fragments of a similar size.[84, 89, 90] The fragment of SOS used for the crystallization was 485 residues long, which corresponds to residues 564 – 1049 from human SOS. This fragment also contains the REM motif,[85] also called CR0 (*c*onserved *r*egion *0*[91]). Sequence analysis showed this region to be conserved in Cdc25-like Ras exchange factors, but is found at different distances relative to the catalytic domain in different Ras, Ral, and Rap exchange factors. In the structure this region apparently supports a protruding helical hairpin from the catalytic domain and is probably responsible for its stability, since smaller fragments of SOS, and also probably of Cdc25, tend to be labile. The catalytic domain containing the sequence-conserved regions SCR 1 – 3 is almost completely α-helical (Figure 2).

The catalytic domain of SOS is described as an oblong bowl, in the center of which binds the Ras. The structure of the Ras · SOS complex shows that the P loop, the Switch I and II regions, and the region around β3 of Ras are involved in the interaction, as predicted from a number of mutational studies in many laboratories.[92, 93] The most prominent, although not necessarily most important, interactions between Ras and SOS in terms of binding energy for the binary complex involve Switch I and the protruding helical hairpin. The Switch I region is completely flipped out of its normal position such that the residues, such as Phe 28 and Thr 35, which contact the base and the phosphate groups are removed from their binding site positions and stabilized by the helical hairpin of SOS. The other important features of the formation of the binary complex involve almost all the residues from the Switch II region forming direct contacts with helices B, D, E, and G from SOS. The Switch II region is very well structured in the Ras · SOS complex and shows features that are incompatible with nucleotide binding. These features are the intrusion of a glutamic acid (Glu 942) and a hydrophobic leucine (L 938) into the Mg^{2+} and phosphate binding area, occupation of the Mg^{2+} binding site by the carbonyl oxygen atom from Ala 59, and the complete rearrangement of the β3-α2 loop such that Gly 60 and Lys 16 are involved in a tight interaction with Glu 62 of Ras. This residue has been shown to be crucial for the exchange reaction with Cdc25 and Sdc25.[94] Lys16 is particularly important for the binding of phosphates, as it contacts the β-phosphate group in the GDP- and the $\beta\gamma$-phosphate group in the GTP complex. It is totally invariant in all P-loop proteins,[95] and its mutation in Ras reduces drastically their affinity to guanine nucleotides.[96] The structure of the Ras · SOS binary complex shows that the base-binding area on Ras is open, while binding to the phosphate binding area is hindered by many interactions with SOS. Thus, in order for the nucleotide to rebind to Ras, it would have to enter through the base-binding site, and induce a conformational change which releases SOS and also induces tight binding in the phosphate binding area.[91, 97] Such a structural change might correspond to the rate-limiting first-order reaction identified in kinetic experiments.[84]

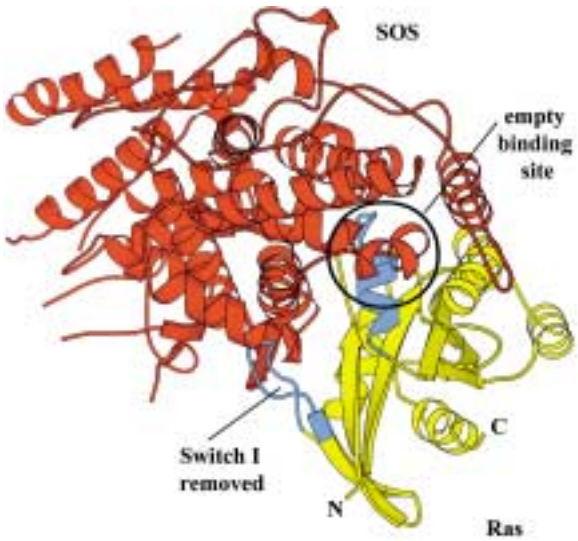


Figure 2. Ribbon representation of the binary Ras · SOS complex, an intermediate in the exchange reaction shown in Scheme 7. Ras is coloured as in Figure 1, SOS is shown in red, and the location of the nucleotide binding site on Ras is indicated.

2.4. Interaction with Effectors

Once activated, Ras · GTP interacts with effectors. The first one to be isolated was the protein kinase Raf, of which three isoforms Raf-1, A-, and B-Raf exist. They are 70 – 80 kDa proteins with a C-terminal kinase domain and an N-terminal regulatory region consisting of many modules which somehow, by a mechanism that is not completely understood, keep the kinase in an inactive state.[10–12] The best understood module in the regulatory N-terminus is the Ras binding domain (RBD) which consists of 80 residues[98] and is a stable, independently folding domain.[99, 100] It is necessary and sufficient for the interaction with Ras in the GTP-bound form. Residues in the effector region whose mutation have been shown to block the biological activity of Ras are at the same time unable to interact with the RBD, thus strengthening the assumption that the isolated domain retains the same properties as in the intact protein.[98, 101–104] It is believed that the main function of Ras is to recruit Raf, through RBD, to the plasma membrane where in a chain of events that include allosteric interactions, phosphorylations, and interactions with other proteins such as 14-3-3, the protein kinase somehow becomes activated.[11, 12] Another regulatory domain in the N-terminus of Raf is the cysteine-rich domain (CRD), a zinc-binding domain which is also believed to interact with Ras, as well as with 14-3-3 proteins and lipids, and whose role in activation and/or down-regulation of the kinase activity is disputed.[105–112]

The structure of the Ras-binding domain of Raf-1 alone has been determined with NMR spectroscopy[113, 114] and in its complex with the Ras homologue Rap1A by X-ray crystallography.[115] Rap1A is a close homologue of Ras, with an overall sequence identity of 50 % and with almost identical relevant residues in the effector region. The structure showed that RafRBD has the same fold as ubiquitin—apparently a stable fold that can be used for many purposes. The interaction with Ras involves only the Switch I region and not the Switch II region (Figure 3 A). An interprotein β-sheet is formed between the two proteins, with some main-chain interactions between the strands, but the majority of interactions are through hydrophilic side chains, whereby Rap is mostly negatively and RafRBD is mostly positively charged. Mutants in the effector region that had been shown to block the biological activity of Ras were indeed involved in forming the interface of the complex. The structure of a complex between RafRBD and a mutant of Rap which even more closely resembles Ras (Raps; Figure 3 A) showed that the major difference between the Ras-Raf and Rap-Raf interaction and the reason for weaker affinity between Rap and Raf[116, 117] is a charge reversal in the effector region: a glutamic acid in Ras and a lysine in Rap1A.

The CRD structure was solved by NMR spectroscopy.[118] As expected from the conspicuous location of cysteine residues, CRD is a Zn-coordinating protein with high structural similarity to the diacylglycerol binding domain found in the classical protein kinase C.[119]

RalGEF and its isoforms were also investigated structurally and biochemically in more detail. They are approximately 600 – 800 residues large and contain at the C terminus a motif

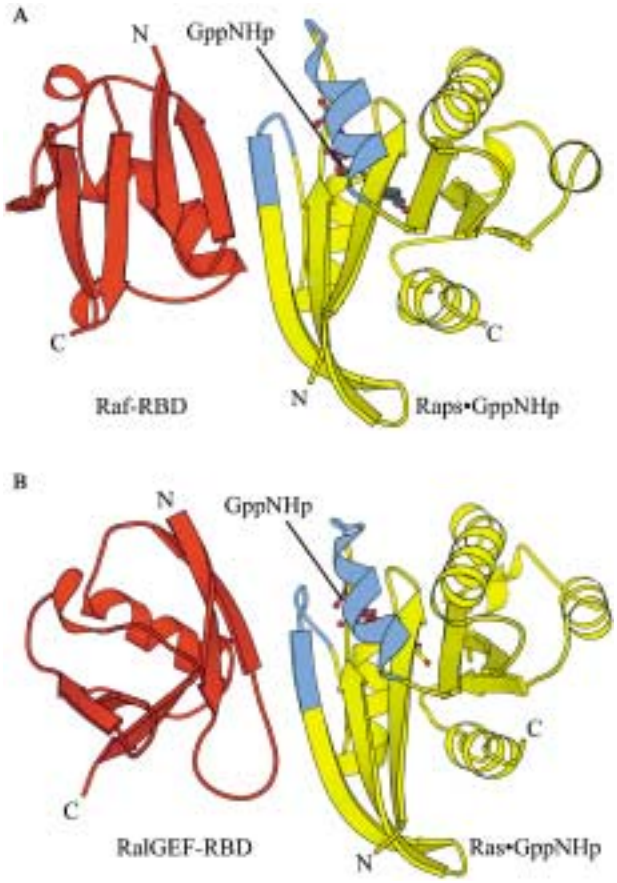


Figure 3. A) Ribbon diagram of the Raps · Raf-RBD complex, where Raps is a close homologue of Ras with identical residues at the interface with the complex, and Raf-RBD is the Ras binding domain of the Ras effector Raf kinase. B) Ribbon diagram of the Ras · RalGEF-RBD complex. Although there is no sequence homology between the two Ras-binding domains from different effectors, they have a similar tertiary structure and interact with Ras in a similar way, albeit using somewhat different residues on Ras. Effectors are colored red.

that binds to Ras · GTP.[32–34] It was shown that a small 100-residue module from the C terminus is necessary and sufficient to bind to Ras in a GTP-dependent manner.[116] It was also shown that this Ras binding domain from RalGEF binds with high affinity to Rap · GppNHp and with lower affinity to Ras · GppNHp and that again residue 31 in Rap/Ras is responsible for most of the specificity.[116] The structure of the domain was solved by NMR spectroscopy[120] and by X-ray crystallography.[121] The structures showed RalGEF · RBD to have the same ubiquitin fold as Raf-RBD, even though it does not show significant sequence homology. The structures of the Ras-binding domains of Rlf[122] and Rgl[123] have additionally been determined and, not surprisingly, show the same structural topology.

The structures of complexes of RalGEF-RBD with either a Ras mutant[124] or Ras itself[125] were solved by X-ray crystallography, and show the same type of interaction through an interprotein β-sheet as observed in Ras · RafRBD (Figure 3 B). However different residues of Ras are involved. This observation was not totally unexpected as residues in the effector region had been identified whose mutation specifically block the interaction with a subset of

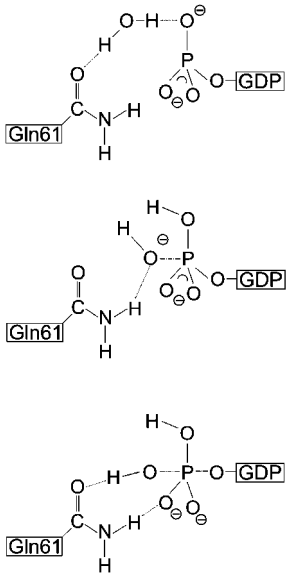
effectors without greatly disturbing those of others. Although these partial loss-of-function mutations are not totally specific, it is generally believed that the E37G mutation blocks Ras-Raf interaction, the Ras-T35S mutation blocks the Ras-RalGEF interaction, and Y40C blocks both interactions without disturbing the PI(3)K.[27, 126–131] The structure shows a weak hydrogen bond between Glu 37 of Ras and RalGEF and none between the Ras-E31K mutant and RalGEF. However, E37 shows a strong hydrogen bond with Raf-RBD, which supports the biological data obtained. Tyr 40 of Ras forms a considerable part of the binding interface of Ras · RalGEF, thus explaining the loss in affinity, and seems to be indirectly involved in the Ras · Raf-RBD interface (Vetter et al., unpublished results). The specificity of Ras versus Rap binding in the Ral signal transduction pathway seems to be a result of a charge repulsion between Glu 31 and a number of acidic residues on RalGEF-RBD.[124, 125] A mutational analysis shows the distinction between Ras and Rap is abolished by mutation of one of these acidic residues.[132]

Not much is known about the binding interaction with other effectors. However, it is evident from mutational data that PI(3)K also interacts through the effector region, although in a different mode, as evidenced from partial loss-of-function mutations. Although the Ras binding region and interacting residues on the catalytic p110 subunit on PI(3) kinases were identified, a stable,[128, 133] independently folding domain could not be expressed. The structure of the p110 catalytic subunit of the PI(3) kinase in the γ isoform shows a Ras binding domain highly homologous to that of Raf and RalGEF. Computer simulations suggest a similar mode of interaction.[134] However, the structure also shows that the RBD is tightly coupled to the kinase domain, which suggests there is an allosteric control of the enzymatic activity by Ras. AF6, another putative effector,[135, 136] has been shown to bind in a similar way as Raf and RalGEF, as evidenced from ^{31}P NMR data, where AF6 induces a similar conformation of the mobile effector loop on binding to Ras · GppNHp.[137]

2.5. The Switch-Off Reaction

In terms of the physiological and medical relevance the most important aspect of Ras biochemistry is the GTPase reaction, as highlighted by the fact that oncogenic versions of Ras are blocked in both the intrinsic and—even more importantly—in the GAP-mediated GTPase reaction. For this reason its reaction mechanism has been the focus of a heated debate.

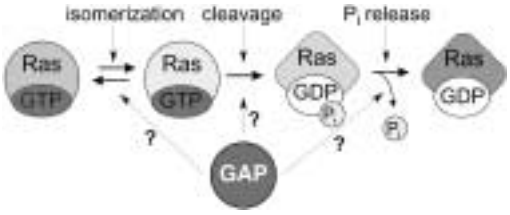
In the high-resolution structure of Ras · GppNHp a nucleophilic water molecule was found close to the γ-phosphate group and is considered to represent the attacking nucleophile.[50] It was also found that Gln 61 is close to the γ-phosphate group and that in turn a carboxylate group was in hydrogen-bonding distance from the side chain of Gln 61. This observation lead to the hypothesis that Gln 61 might serve as a general base for activating the nucleophile.[50] Some experimental evidence apparently supports this view.[138, 139] However, based on theoretical considerations[140] and by introducing an unnatural glutamine analogue with an even lower pK_a value into the Gln 61 position,[141] as well as from mutational analysis of the carboxylate group[142] this interpretation appeared unlikely. Model calculations suggested that the γ-phosphate group, with a calculated pK_a value of approximately 3, might itself function as a base (Scheme 8).[140] Experimental studies by mutagenesis and NMR spectroscopy showed that this suggestion of a substrate-assisted catalysis is likely to be valid. A linear free energy relationship was in fact established between the pK_a value of the γ-phosphate group and the rate of GTP hydrolysis, thus supporting the concept.[142–144] The same was also postulated for the GTPase reaction of the α subunit of a heterotrimeric G protein.[145]



Scheme 8. Proposed substrate-assisted mechanism for the intrinsic GTPase reaction of Ras, where the γ-phosphate group itself activates the attacking nucleophilic water molecule, and Gln 61 stabilizes the transition state.

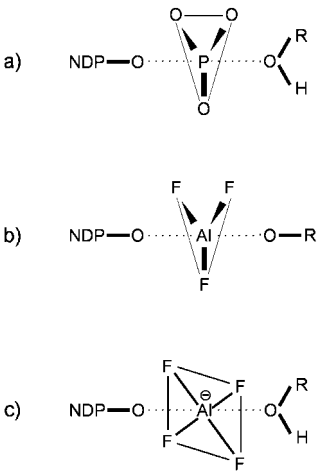
Since the slow switch-off by the intrinsic GTPase was considered physiologically less important than the GAP-mediated reaction, the latter mechanism was of considerable interest. GAP proteins were first discovered by Trahey and McCormick in a landmark paper where they showed that while recombinant Ras · GTP in vitro has a half-life of about 30 minutes it is quickly hydrolyzed after microinjection into cells.[146] The protein responsible was then shown to indeed stimulate the hydrolysis of wild-type but not of oncogenic Ras. Since then several Ras-specific GAPs (RasGAPs) have been isolated and cloned, the most important and best studied are p120GAP[147, 148] and the product of the neurofibromin gene responsible for the genetically determined disease neurofibromatosis (type I).[149–152] All RasGAPs are different proteins of varying length and complexity and contain a domain of approximately 330 residues, which alone is able to induce rapid GTP hydrolysis.[153, 154] It was shown for neurofibromin that a domain of approximately 220 residues seemed to be required and sufficient for inducing GTP hydrolysis.[155]

For the GAP-mediated GTPase reaction of Ras it was shown by saturating GAP or neurofibromin with increasing concentrations of Ras and treating the data with the Michaelis – Menten equation that the k_{cat} value is between $5-20\ s^{-1}$, that is, about 10^5 times higher than the unstimulated reaction.[154, 156, 157] Since the overall reaction is stimulated by such a large factor it was discussed which of the partial reaction steps was rate limiting and was stimulated by GAP (Scheme 9). Original data seemed to suggest that a conformational change in the intrinsic reaction determined the overall rate[44] and it was therefore intriguing to assume that GAP was stimulating this conformational change. This assumption would have meant that Ras was in principle a fast GTPase enzyme and just needed GAP to become activated. Data



Scheme 9. Schematic overview of the partial steps of the GTPase reaction of Ras. It is now generally believed that the chemical cleavage itself is rate-limiting in the intrinsic GTPase reaction of Ras and is stimulated by GAP.

supporting and questioning this assumption were published.[158, 159] In another school of thought it was proposed that Ras was an imperfect enzyme and needed the presence of chemical groups on GAP for fast hydrolysis.[142, 159] Support for this proposal came from studies with aluminum fluoride, which mimicks the transition state of the phosphoryl transfer reaction (Scheme 10) in many enzymatic systems.[160] It was found that Ras binds aluminum fluoride only in the presence of stoichiometric amounts of GAP and that the presence of an invariant Arg residue on GAP is necessary for this effect.[161] Furthermore stopped-flow single turnover experiments demonstrated that the catalysis by GAP, but not its binding to Ras, required the presence of this arginine residue, which suggests that catalysis does indeed need the participation of at least one residue from GAP.[155, 162]



Scheme 10. Aluminum fluoride complexes are believed to be (stable) mimics for the transferred phosphate group in the transition state of (enzymatic) phosphoryl transfer reactions. Both planar AlF_3 and AlF_4^- configurations with two axial oxygen atoms from the nucleophile and the leaving group have been observed in crystallographic structures. NDP = nucleotide diphosphate.

The issue was finally solved independently by solving the structure of the complex between Ras · GDP and GAP 334 in the presence of aluminum fluoride.[163, 164] It indeed shows an arginine residue from GAP penetrating the active site of Ras and contacting the aluminum fluoride, which is supposed to mimic the phosphoryl group to be transferred (Figure 4). This structure was interpreted to mean that the phosphoryl transfer reaction is associative and accumulates negative charge in the transition state of the GTPase reaction, and that the guanidinium side of Arg 789 was neutralizing this negative

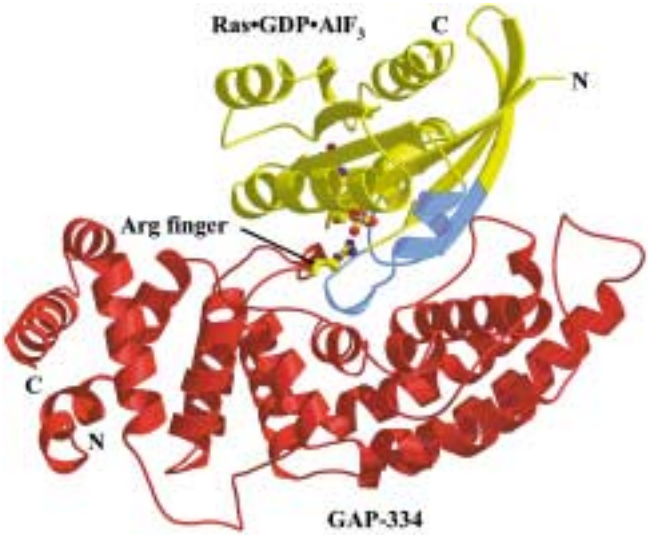


Figure 4. Ribbon plot showing the complex between GAP-333 and Ras-GDP in the presence of aluminum fluoride. The structure represents a mimic of the transition state of the GAP-catalyzed reaction and shows an arginine residue (the arginine finger) contacting AlF_3, which mimics the transferred phosphate.

charge and thereby stabilizing the transition state of the reaction. This interpretation supported the kinetic data which showed that mutation of this Arg residue reduced the k_{cat} value in both p120GAP and neurofibromin 2000-fold.[162, 165]

The structure further showed that Gln 61 from Ras stabilizes the transition state by making a bidentate hydrogen bond to both the fluoride ion and the attacking water molecule. This observation explained the finding that Gln 61, which is found mutated in many tumors, is also a very important chemical group for catalysis. Its mutation to alanine reduces the catalytic efficiency of the Ras · RasGAP system by five orders of magnitude (Ahmadian et al., unpublished results). The structure also showed why mutations of Gly 12 to any other amino acid inhibits the GAP-mediated GTP hydrolysis, since they would sterically clash with many atoms in the active site of the transition-state mimic. Since Gly 12 mutants have been shown to bind to GAP[156, 157, 166, 167] we can assume that the ground-state Ras-RasGAP complex is different from the transition-state complex seen in the structure. The mechanism of GTPase activation by GAPs was structurally and biochemically confirmed for the interaction between Rho-type GTP-binding proteins and their specific Rho-GAPs[168–174] and might thus constitute a universal principle for the GTPase activation of small GTP-binding proteins.[175]

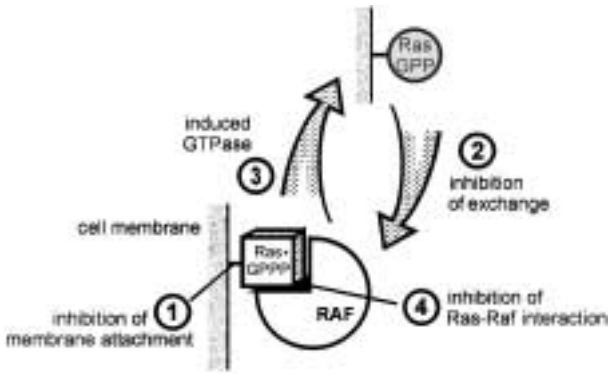
3. The Ras Protein as a Molecular Target for Antitumor Drugs

The crucial role of the Ras proteins in maintaining the regular life cycle of cells and the extensive involvement of mutated Ras in the development of numerous cancers has sparked the idea that influencing the function of Ras might open up new opportunities for the development of alternative antitumor drugs.[176, 177] Disruption of the aberrant growth

signals of oncogenic Ras proteins provides an example of what is now recognized as "signal transduction therapy".[178]

At least four distinct approaches have been pursued for targeting the transforming actions of mutated Ras proteins (Scheme 11):

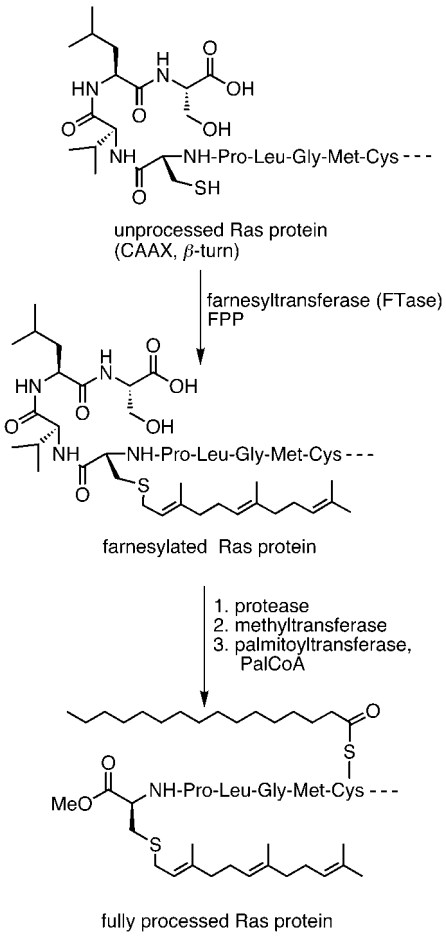
1. Ras proteins must be localized at the plasma membrane in order to perform their biological function in both the normal and the transformed state of the cell. Covalently attached lipids are the major driving force for membrane localization. Nonlipidated Ras is cytosolic and biologically inactive. Several posttranslational modifications are required at the carboxyl terminus of Ras before the initially biosynthesized precursor protein matures into a biologically active protein localized to the plasma membrane. Interfering with these processing steps might disrupt the aberrant growth signals of oncogenic Ras proteins.
2. Ras proteins are activated by the exchange of GDP for GTP. Prevention of this exchange reaction has been considered by some as an opportunity to block Ras action.
3. Oncogenic mutants of Ras contain mutations that impair their sensitivity to RasGAPs, thus preventing the Ras protein from being switched off again. Compounds that stimulate the intrinsic GTPase activity of oncogenic Ras proteins would convert them back into inactive proteins and terminate the growth-promoting signal.
4. Upon activation, Ras relays the stimulus by activating further proteins such as the Raf, RalGEF, and PI(3) kinase. Thus antagonizing its interactions with downstream effectors would prevent the activated Ras from relaying its signal.



Scheme 11. Schematic view of the possible approaches for developing anti-Ras drugs for use in antitumor therapy (see text).

3.1. Inhibition of Posttranslational Modification of the Ras Proteins

The Ras proteins are synthesized as biologically inactive, cytosolic precursor proteins. They are then modified by several posttranslational processing steps at the carboxyl terminus and thereby converted into biologically active proteins localized at the plasma membrane. The cysteine residue of the C-terminal CAAX sequence (C is cysteine, A is generally an aliphatic amino acid, and X is methionine, serine, alanine, or glutamine) is first enzymatically S-farnesylated, the AAX part is then cleaved off by a specific protease, and the free C-terminal cysteine is finally converted into a methyl ester (Scheme 12). Additional cysteine residues in the immediate vicinity of the CAAX moiety in H- and N-Ras are lipidated by formation of palmitic acid thioesters. The anchoring of the Ras proteins to the plasma membrane is



Scheme 12. Posttranslational modification of Ras proteins with lipid groups (FPP = farnesylpyrophosphate, PalCoA = palmitoyl CoA).

mediated by the lipid groups. Genetic experiments have shown that farnesylation is essential to Ras function.[40] Palmitoylation appears not to be an absolute necessity for Ras cell-transforming activity but is important for conferring full biological activity on Ras.[179] The K-Ras4B protein compensates for the absence of a hydrophobic palmitoyl moiety by having a positively charged lysine-rich region near the CAAX box to enhance membrane interactions with negatively charged phospholipids.

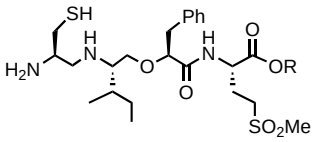
The finding that lipid modification of Ras, in particular farnesylation, is crucial to its biological activity led to the idea that blocking the enzymes responsible for the modification would in turn inhibit Ras function and provide new approaches for developing potent antitumor drugs.

3.1.1. Inhibition of Ras Farnesyltransferase (FTase)

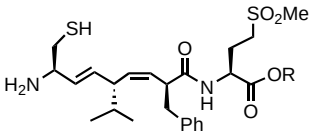
FTase is a heterodimeric protein composed of an α and a β subunit. Zinc and magnesium ions are required for its

activity.[180, 181] A closely related heterodimeric enzyme is geranylgeranyltransferase I (GGTase I). It also recognizes proteins with a CAAX box when X is leucine. Both enzymes share the same α subunit and the requirement for Zn^{2+} and Mg^{2+} ions, but the β subunits are different. The similarity of these enzymes highlights the importance of selectivity of FTase inhibitors.

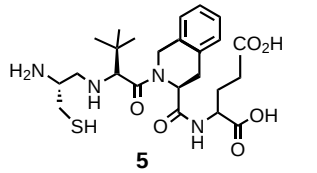
Since the discovery that the Ras proteins are farnesylated numerous inhibitors of FTase have been developed. This field has been reviewed in detail and only a brief overview is given here.[38, 176, 177, 182–184] Initial studies on inhibitor development relied on information about substrate specificity as well as structural studies of the substrate both in solution and bound to the enzyme (obtained from NMR spectroscopic investigations) where the bound ligand Cys-Val-Trp-Met forms a β loop of type III (Scheme 12). The crystal structure of the free enzyme at 2.25 Å resolution[185] shows the active site consists of two clefts which are at the junction of a bound zinc ion. The zinc ion coordinates the thiol group of cysteine in a ternary complex. One of the clefts was found to be a hydrophilic surface groove near the subunit interface. It could correspond to the site of binding of the Ras protein. The second cleft is a region of aromatic residues and of a size that would accommodate the farnesyl moiety, but not the longer geranylgeranyl pyrophosphate. In addition the structure of the rat enzyme in the complex with farnesyl pyrophosphate was solved at 3.41 Å resolution.[186] The three-dimensional information obtained from the X-ray structures should now allow for the development of more efficient inhibitors.



1 R = H, L-739,749
2 R = CH_3, L-739,750



3 R = H, B 956
4 R = CH_3, B 1086



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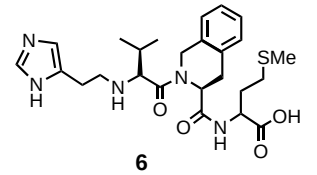

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Scheme 13. FTase inhibitors in which amide bonds were replaced by isosteric amines and ethers, and which incorporate non-natural amino acids.

3.1.2. Inhibitors Based on the CAAX Motif

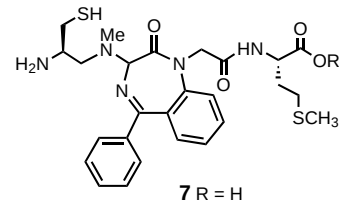
Peptide analogues were designed on the basis of the CAAX motif in which peptide amide bonds were replaced by amine and ether groups.[187] In particular the β-turn mimetic **1** (Scheme 13) inhibits the FTase in vitro with an IC_{50} value of 1.8 nM and shows highly specific activity compared to the inhibition of GGTase I. The less-polar methyl ester prodrug **2** showed better results in vivo and inhibits both farnesylation of the Ras protein and growth of Ras-transformed cells, whilst proliferation of Raf- or Mos-transformed cells was not influenced. Growth of human pancreatic adenocarcinoma cells with mutated K-Ras, c-Myc, and p53 genes was inhibited by the application of **2**. If the compound is administered over a period of five days to mice with implanted Ras-dependent tumors, tumor growth can be reduced by up to 66% compared to untreated mice, whereas application of the antitumor antibiotic doxorubicin only resulted in a 33% reduction under the same conditions. It is particularly noteworthy that treatment with the β-turn mimetic—in contrast to treatment with doxorubicin—was without any visible side effects, such as weight loss.

However, **2** also affected the regulation of the formation of the actin stress fiber.[188] Rho proteins are involved in the regulation of various cytoskeletal structures, and RhoB is believed to be one of the prime targets of FTase inhibitors. RhoB is apparently both geranylgeranylated and farnesylated.[189, 190] Vesicular localization of RhoB was inhibited when cells were treated with **2**. Thus **2** may also inhibit the farnesylation of RhoB and thereby interfere with formation of the actin stress fiber.[191]

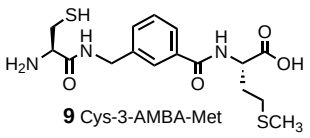
Further modifications of the CAAX tetrapeptide structure led to inhibitor **3**, which blocked H-Ras farnesylation with an $IC_{50} = 11$ nM.[192] Tumor cell lines expressing mutant H- and N-Ras were most sensitive against this compound, which inhibits tumor growth of EJ-1 human bladder carcinomas by about 60% at a dose of 100 mg kg^{-1}. The in vivo results and particularly tumor regression induced by the CAAX mimetics thus described clearly demonstrate the efficacy of FTase inhibitors as antitumor agents.

High activity and selectivity was also achieved by replacing the phenylalanine residue in Cys-Val-Phe-Met with 1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Tic) and modifying the peptide backbone to give **5** (Scheme 13).[193] This compound displayed an IC_{50} value of 2.8 nM against rat FTase and was 500-times more selective than against GGTase I. In compound **6** the terminal cysteine residue was replaced by a 4-imidazole group and gave an increase in activity ($IC_{50} =$ 0.79 nM).[194]

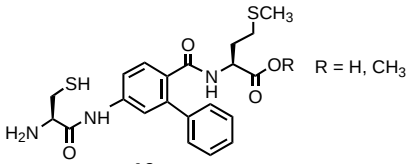
Replacement of the two aliphatic amino acids in the CAAX motif was achieved with benzodiazepines such as **7** and **8** (Scheme 14). The central unit of these peptidomimetics imitates a β-turn and brings the NH_2 terminus of the cysteine analogue and the COOH terminus of the methionine group into spatial proximity; these then can complex the Zn^{2+} ion which is essential for FTase activity.[195] The free acid **7** inhibits the enzyme with an IC_{50} value of 1 nM, whilst in intact cells the methyl ester **8**, despite its weaker in vitro activity, is significantly more potent because it can penetrate the plasma membrane better as a result of its lower polarity. This property can be used to convert the morphology of H-Ras-transformed cells back into the normal form and to inhibit growth of these cells. The substance shows no effect on Src-transformed and untransformed rat fibroblasts. The inhibitor therefore acts selectively on Ras-transformed cells and does not influence growth of normal cells. This result is noteworthy because farnesylation of the wild-type H-Ras protein is inhibited by the benzodiazepine, and H-Ras is involved in growth of normal fibroblasts. Detailed examination of this unexpected result shows that inhibitor **8** reduces the amount of farnesylated H-Ras protein in both nontransformed and



7 R = H
8 R = CH_3



9 Cys-3-AMBA-Met

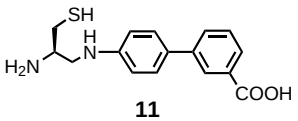


10

Scheme 14. FTase inhibitors in which the AA dipeptide was replaced.

H-Ras-transformed cells. However, only in the transformed cells does this lead to the expected reduction of enzyme activity in the Ras cascade: both the amount of phosphorylated Raf and the activities of MEK-1, MEK-2, and MAP kinase were reduced in the tumor cells.[196] In addition, stimulation of the Ras signal pathway was not influenced by EGF in normal cells or in Src-transformed cells. Normal cells apparently have the ability to activate the signal pathway independently of Ras-inhibitory drugs. In fact, further experiments have shown that farnesylation of the K-Ras protein by the inhibitors is considerably less influenced than that of H-Ras. K-Ras is also a substrate of GGTase I and can therefore be geranylgeranylated if activity of the FTase is reduced. Normal cells and Src-transformed cells, in contrast to H-Ras-transformed cells, are able to compensate for the loss of a Ras protein or an enzyme important for normal cell growth by activation of alternative pathways.

The two aliphatic amino acids have also been replaced by the hydrophobic spacers 3-(aminomethyl)benzoic acid (3-AMBA, **9**, Scheme 14)[197] and 3- and 4-aminobenzoic acid (3- and 4-ABA) as well as 2-phenylaminobenzoic acid (**10**).[198, 199] These compounds displayed IC_{50} values in the nanomolar range, and **10** (R = H) blocked the growth in nude mice of a human lung carcinoma expressing oncogenic Ras.[200]



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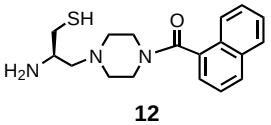


12

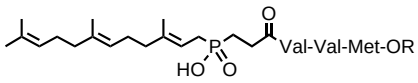
Scheme 15. FTase inhibitors in which the AAX tripeptide was replaced.

Further design led to the replacement not only of the central AA dipeptide but of the AAX tripeptide. Thus, biphenyl derivative **11**[201] and piperazine analogue **12**[202] were developed (Scheme 15). They display IC_{50} values in the nanomolar range, whereas geranylgeranyltransferase was blocked with micromolar IC_{50} values. Both compounds disrupt Ras processing in cells, and piperazine derivative **12** suppresses tumor growth by 75 % at a dose of 40 mg kg^{-1}.

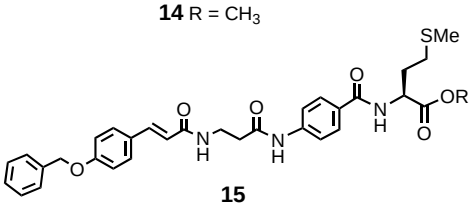
3.1.3. Bisubstrate Inhibitors

The design of FTase inhibitors based on the structure of farnesyl pyrophosphate has been pursued with less intensity as a consequence of the possible nonselective effects of competing with other enzymes, such as squalene synthetase, which also accept farnesyl pyrophosphate as a substrate.[38, 176, 177, 182–184]

However, bisubstrate inhibitors incorporating a farnesyl and a CAAX mimetic are very promising since one can expect that they will display enhanced activity and selectivity. The phosphinic acids **13** (R = H) and **14** (R = Me, Scheme 16) are examples of bisubstrate analogues. Indeed, **13** is an effective



13 R = H
14 R = CH_3



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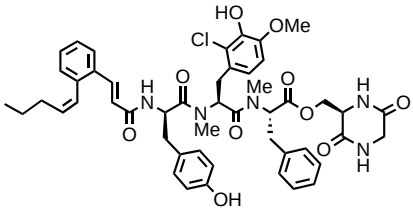


16 pepticinnamin E

Scheme 16. Bisubstrate inhibitors of FTase.

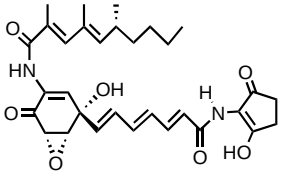
in vitro inhibitor and the prodrug **14** has activity in H-Ras- and—to a lesser extent—K-Ras-transformed cells.[203] Prodrug **14** also inhibits growth of malignant cells of the neurofibromatosis type I which presumably contain overactive wild-type Ras as a result of the absence of active neurofibromin GAP.[204] These highly promising results are tempered, however, by the lower bioavailability and in vivo activity in comparison to the peptidomimetics. Recently, benzyloxycinnamoylamide **15** and two analogues thereof were reported as bisubstrate inhibitors of yeast FTase,[205] where the benzyloxycinnamoyl group mimics the farnesyl moiety. These compounds displayed IC_{50} values in the low micromolar range.

The natural product pepticinnamin E (**16**) is also considered a bisubstrate inhibitor of FTase. The successful synthesis of pepticinnamin E sets the stage for preparing analogues of

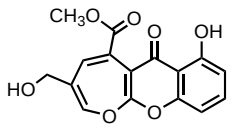
this peptidic compound and to delineate the important structural parameters responsible for its FTase-inhibiting activity. Initial studies revealed that the central tripeptide part and, in particular, the absolute configuration of the central chlorinated amino acid are decisive for the inhibitory activity.[206] The bisubstrate inhibitors developed so far (as well as some of the CAAX mimetics, see Section 3.1.2) have the advantage that they do not contain a free cysteine SH group and are thus more stable.

3.1.4. Farnesyltransferase Inhibitors from Natural Sources and Compound Libraries

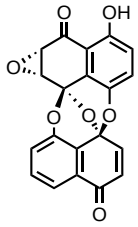
In addition to pepticinnamin E several natural products have been identified as FTase inhibitors, including for example, manumycin (**17**) and analogues,[207] fusidienol (**18**)[208] and the preussomerins, for example, **19**[209] (Scheme 17; for further examples see ref. [182]). In general, these compounds have been less potent than the CAAX-based mimetics, typically displaying IC_{50} values in the low micromolar or the high nanomolar range. High-throughput



17 manumycin A



18 fusidienol

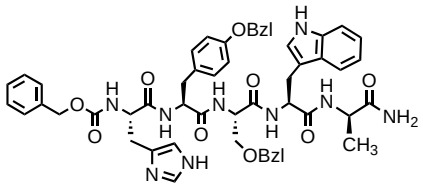


19 preussomerin G

Scheme 17. FTase inhibitors from natural sources.

screening of compound libraries was used to identify further inhibitors of farnsyltransferase. Thus a highly potent pentapeptide **20** was identified (Scheme 18) that inhibited FTase with $IC_{50} = 17$ nM and that antagonized Ras in *Xenopus* oocytes.[210] Systematic derivatization and truncation of the peptide backbone finally led to a dipeptide derivative **21** that inhibited Ras processing in cells and increased the life span of tumor-bearing nude mice by 35% at a dosage of 200 mg kg^{-1}.[182, 211]

In particular, a series of completely nonpeptidic non-sulfhydryl FTase inhibitors **22**–**24** was uncovered (Scheme 18). These chlorobenzocycloheptapyridines display pronounced selectivity for FTase over GGTase I. They show improved in vivo antitumor activity and pharmacokinetic profiles in mice when administered orally and inhibit H-Ras processing in Cos monkey kidney cells.[212, 213] Extensive variation of the substituents on the piperidine moiety and the tricyclic ring system led to the development of compound



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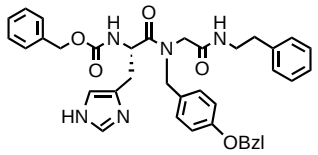

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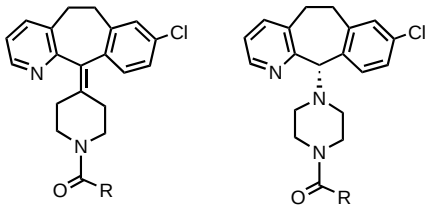
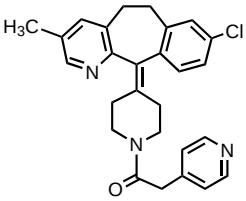


22 **23**



24

Scheme 18. FTase inhibitors identified from compound libraries.

23, which inhibits FTase with $IC_{50} = 40$ nM and is inactive toward GGTase I.

3.1.5. Lack of Toxicity to Normal Cells

Although FTase inhibitors influence the farnesylation of Ras they are likely to interfere with the posttranslational modifications of other CAAX-containing proteins as well. Apart from the approximately 20 farnesylated proteins that are known today, farnesylation is also required for normal Ras function, which in turn is critical for normal cell viability. For these reasons farnesyltransferase inhibitors were thought to be potentially toxic. However, these drugs proved to be surprisingly nontoxic and did not display inhibitory activity against rodent fibroblast cells.[214] Farnesyltransferase inhibitors not only inhibited the growth of transformed cells in culture far more than the growth of normal cells, but also showed surprisingly few side effects in the treatment of tumor-bearing animals, with no obvious toxicity in normal tissues following treatments of up to six weeks.

There are several possible explanations to account for this apparent lack of toxicity. Some geranylgeranylated Ras-related proteins might compensate for the loss of Ras function.[215] Alternatively, inhibition of farnesyltransferase

may reduce Ras activity below the level required for transformation, yet still allow sufficient Ras activity to maintain normal cell viability.[216] Alternatively, a different signaling pathway may be activated when Ras is not anchored to the plasma membrane. Toxicity and effectivity studies have often been performed in rodent fibroblast cells containing oncogenic H-Ras. However, prenylated K-Ras4B and N-Ras are not as effectively blocked by the farnesyltransferase inhibitors as H-Ras (see Section 3.1.6).[217] Thus normal cells may be less sensitive to these drugs because they express K-Ras4B and N-Ras. In this context it should be noted that H-Ras mutations are relatively uncommon in human tumors.[218] Rather, the *K-Ras* gene is the most frequently mutated in solid human cancers, whereas the *N-Ras* gene is prevalent in leukemias. Thus the preclinical evaluation of the farnesylation inhibitors has yet to be critically re-evaluated for trials in humans.

3.1.6. Mechanism of Action

Intensive investigations of a variety of FTase inhibitors has clearly revealed that these drugs demonstrate antiproliferative activity in cell cultures against a variety of transformed cell lines and tumors in animals. In particular, cells with a mutant H-Ras protein are sensitive against these drugs. Although the growth of K-Ras4B-transformed cells is also blocked, much higher concentrations are needed for this effect since this protein is a much better substrate for FTase than H-Ras.[219] Furthermore, the K-Ras4B protein can more easily be geranylgeranylated by GGTase, thus compensating for its lack of farnesylation.[220, 221] Since geranylgeranylated forms of Ras proteins can also potently transform cells, farnesyltransferase inhibitors are not expected to inhibit the growth of tumors with mutant K-Ras4B. However, growth inhibition is observed despite this alternative prenylation. Clearly blocking the farnesylation of one or more proteins other than Ras is responsible for, or at least contributes to, the observed effect. This proposal is supported by the finding that growth of fibroblast cells transformed by an N-terminal myristoylated (and thereby membrane anchored) Ras that cannot become farnesylated or geranylgeranylated is inhibited by FTase inhibitors as well.[222]

These findings have raised the questions as to which additional proteins are the molecular targets of FTase inhibitors. One class of candidates involves members of the Rho family of proteins, in particular RhoB. RhoB is a particularly attractive target as Rho proteins have been shown to be required for Ras transformation and since RhoB is an immediate early gene response protein activated by growth factor signaling. RhoB is targeted by FTase inhibitors (see Section 3.1.2 and ref. [223]). Another novel Ras-related farnesylated protein, Rheb, may also be a target. Rheb modulates Ras function and is an antagonist of Ras transformation. Its processing and membrane localization can be inhibited by a FTase inhibitor.[224]

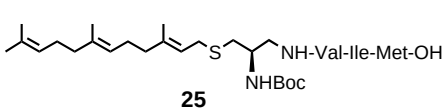
Many of the biological studies with FTase inhibitors suggested that these agents are cytostatic and suppress cell growth, rather than being cytotoxic in patients. However, farnesyltransferase inhibition was also observed to induce tumor regression of mammary and salivary carcinomas in a mouse tumor model.[214] The mechanism of shrinkage was unclear, but a recent study showed that a FTase inhibitor can induce apoptosis (cellular suicide) in cell cultures, and is independent of the p53 tumor suppressor protein (a mediator of apoptosis).[225] These observations may be clinically important because resistance to standard anticancer agents is often conferred by alterations in p53. Thus FTase inhibitors may still be efficacious against tumors containing mutant p53.

Many of the questions concerning the biological activity of FTase inhibitors have been addressed, however numerous questions remain to be answered. In particular the consequences of the K-Ras4B geranylgeranylation bypass and the uncertainty about the cellular targets of these drugs deserve intensive investigation. Nonetheless it is clear and accepted that FTase inhibitors have proven effective and shown significant antitumor activity against Ras-dependant tumors with little or no whole animal toxicity in many animal tumor models. They have now reached the stage of human clinical evaluation.[211, 226] The clinical trials will provide answers to questions about efficacy, toxicity, development of resistance, and mechanism of action of these compounds such that their full therapeutic potential can be appreciated.

3.2. Carboxyl Terminal Protease, Methyltransferase, and Palmitoyltransferase/esterase as Targets

In addition to farnesylation, complete processing of Ras is required for the protein to have full biological activity. Thus, the protease, the methyltransferase, and the protein that introduces the palmitic acid thioester could be alternative targets for new anti-Ras drugs. The endoproteinase can be inhibited by analogues of the C-terminal CAAX tetrapeptide in which the cysteine amide group is reduced to a methyleneamine or to a hydroxymethyleneamine.[227, 228]

Compounds **25** and **26** (Scheme 19) displayed K_i values in the 60–80 nM range. These inhibitors could serve as starting points for the design of new drug candidates. This opportunity is further strengthened by the recent isolation and molecular cloning of the Rce1 protein from *Saccharomyces cerevisiae* as the CAAX protease.[229] It is localized at the membrane and its deletion leads to defects in Ras localization. The methyltransferase that methylates Ras has been identified in several mammalian tissues and can be inhibited by simple farnesylcysteine analogues such as farnesylthioacetic acid.[230] The use of methyltransferase inhibitors for interfering with Ras function has, however, not been investigated.



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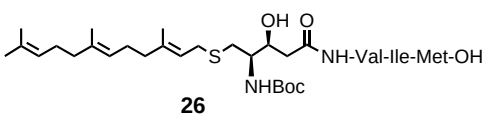

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Scheme 19. Inhibitors of Ras carboxyl terminal protease. Boc = *tert*-butoxycarbonyl.

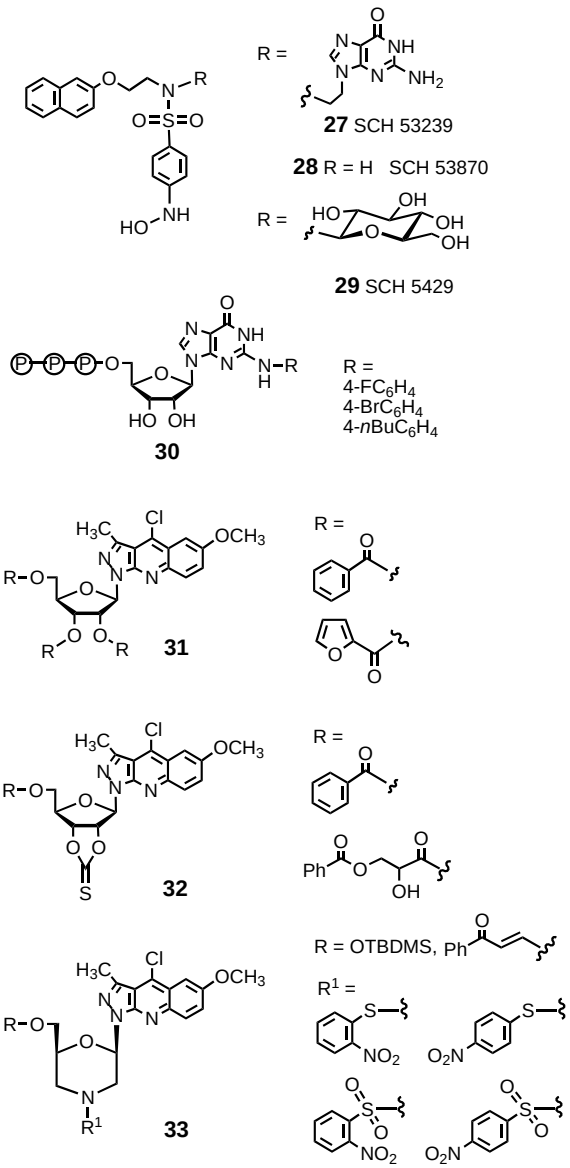
The possible involvement of a putative palmitoyltransferase in the final processing of H- and N-Ras has attracted considerable interest[231] since these Ras-protein isoforms exert their full biological function only if they are palmitoylated. Indeed, an enzymatic activity was described that palmitoylates Ras,[232] and this later turned out to be a thiolase. Until now the long-searched-for transferase has not been isolated. However, a protein palmitoyl thioesterase has been identified that processes Ras.[233] The enzyme was described as "the first bona fide player" in Ras palmitoylation, and inhibiting its function may open up new alternatives for regulating the localization at the plasma membrane and thereby the biological activity of H- and N-Ras.

3.3. Inhibition of Nucleotide Exchange

The rationale to search for inhibitors of the GDP-GTP exchange reaction is not entirely clear, since oncogenic versions of Ras are mostly bound to GTP in the cell.[146] Nevertheless, attempts to identify such compounds have been undertaken. They could potentially be useful in cases where upstream components of the Ras pathway rather than Ras itself are activated. On the basis of molecular modeling studies compounds SCH 53239 (**27**), SCH 53870 (**28**), and SCH 54292 (**29**) were designed to bind competitively with GDP in the nucleotide binding site (Scheme 20).[234] Indeed these compounds inhibited nucleotide exchange on Ras with IC_{50} values of 0.5 – 0.7 μM, but they do not compete with GDP binding. NMR analysis of SCH 54292 bound to Ras revealed that it binds to the Switch II region of Ras and not to the nucleotide binding site. The Switch II region (residues 60 – 70) is involved in the interaction of Ras with nucleotide exchange factors. When the PC 12 cells stimulated with nerve growth factor were treated with SCH 53870 the Ras signaling was blocked. Glycoside SCH 54292 did not show this effect, probably because of reduced membrane permeability.

A series of nucleoside triphosphates and nucleoside analogues was investigated for their ability to interfere with GDP binding to the Ras proteins. Several GTP analogues with alterations in the ribose part displayed drastically decreased binding affinity to both normal and mutated Ras.[235] Methylation of N-7 or replacement of N-7 by a CH group were also ineffective. However, several N-2-substituted GTPs **30** (Scheme 20) showed affinities higher than that of GDP itself, and the 4-(*n*-butyl)phenyl derivative bound to the oncogenic mutant twice as strongly as to the cellular protein. Pyrazolo[3,4-*b*]quinoline ribosides **31** and **32** as well as the morpholino analogues **33** were found to be moderate in vitro inhibitors of the nucleotide exchange process of oncogenic Ras.[236] Surprisingly, fully protected triesters **31** and the corresponding thionocarbonates **32** were the most effective ribo compounds, displaying IC_{50} values in the low μM range. A slight improvement was recorded in the morpholines **33**.

These first examples demonstrate that one can, in principle, inhibit the exchange of GDP for GTP on Ras and thus prevent its activation. However, rather than trying to inhibit nucleotide exchange itself or inhibit the Ras-GEF interaction, it might be more reasonable to develop noncompetitive inhibitors of the GEF-catalyzed nucleotide exchange, similar to the action of Brefeldin A. The latter compound stabilizes the abortive ArfGDP-Sec7 domain complex.[237, 238] A similar noncompetitive approach for the Ras·GEF complex would be to inhibit its interaction with effectors that form an overlapping interface.

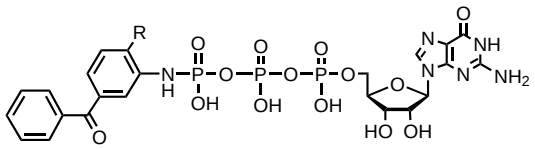


Scheme 20. Inhibitors of Ras GDP-GTP exchange. TBDMS = *tert*-butyldimethylsilyl.

3.4. Ras GTPase as a Target

Since Ras is an oncogene and the major problem with oncogenic Ras is its inability to hydrolyze GTP, it was always considered a possibility that stimulating the GTPase of oncogenic Ras would be a valuable target for the development of anti-Ras drugs. The discovery that the (apparently simple) introduction of an arginine residue into the active site of Ras stimulates the GTPase very efficiently has fueled this idea considerably, although GAP itself is unable to act on

oncogenic Ras. The idea of GTPase inducers has gained further support by some recent data with the GTP analogue DABP-GTP (**34**; Scheme 21).[239, 240] Substitution of the β-phosphate by any ester or amide normally completely blocks the hydrolysis of that compound. In the case of DABP-GTP it was found, first for G_α subunits of heterotrimeric G proteins



34 R = NH_2: DABP-GTP

35 R = H: MABP-GTP

Scheme 21. Structure of DABP-GTP and MABP-GTP—GTP analogues that are hydrolyzed by oncogenic Ras.

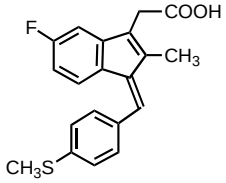
and then in a more thorough study on Ras, that this analogue is efficiently hydrolyzed on the proteins. The aromatic amino group is mostly responsible for this effect, since MABP-GTP (**35**) is only very slowly hydrolyzed. Furthermore, Gln 61 is not necessary for this effect and actually all oncogenic mutants of Ras including those of Gly 12 hydrolyze DABP-GTP more efficiently than the wild-type.[241] This is good news for drug development since it shows that in principle oncogenic Ras can be induced to break the β,γ phosphate bond efficiently if the appropriate chemistry is supplied to the active site. Clearly such GTP analogues themselves are not good lead compounds, since the high affinity for Ras and the high concentration of GTP in the cells renders this approach unfeasible, but the search for small compounds homing into the active site of Ras and carrying reactive chemical groups as indicated by the GAP and DABP-GTP studies makes this approach a valid one. The structure of DABP-GppNHp bound to Ras[241] should be helpful in designing the proper scaffold for a GTPase-directed lead compound.

3.5. Inhibition of the Interaction of Ras with Effector Proteins

A key event in Ras-mediated signal transduction involves the interaction of Ras with downstream effector targets. A critical effector is the Raf serine/threonine kinase, but it is clear that Ras function is mediated through its interaction with various effectors. Inhibition of the interaction between Ras and one or several of its downstream effectors could open up new alternatives for interfering with and regulating Ras signaling. Although the functionally different effectors have a common fold, they lack sequence homology and use different residues for the interaction with Ras. Accordingly, it appears possible to develop selective inhibitors.

That this is indeed possible was shown by employing peptides corresponding to the Ras-effector region or to the Ras-binding domains of the downstream targets. The interaction between c-Raf-1 and Ras · GTP was successfully inhibited by pentadecapeptides[242] as well as hepta- and octapeptides derived from the Ras binding domain of Raf-1.[106, 243] Some of these compounds inhibited Ras-Raf binding with IC_{50} values as low as 7 μM (observed for one of the heptapeptides) and blocked the Ras-mediated activation of MAP-kinase. In many cases, the reason for the inhibition is not obvious, since these peptides were not derived from the interface of the Ras · RafRBD complex determined from the three-dimensional structure.[115, 117] A series of 13 – 28 amino acid peptides derived from the effector-binding region of H-Ras and bearing various amino acid substitutions were investigated as inhibitors of the association of Ras with different effectors.[244] The peptide corresponding to the original H-Ras sequence inhibited Ras association with three different effectors at K_i values of 1 – 10 μM. Introducing amino acid substitutions corresponding to Ras mutations led, however, to peptides that selectively inhibited one or two Ras-effector interactions. Thus, the specificity observed with the whole Ras protein was retained in the effector-region peptide. These results suggest that, in principle, it should be possible to develop small molecules as inhibitors of Ras-mediated protein – protein interactions. However, this process is generally considered to be difficult because of the fact that the interface usually covers a large nonsolvent-accessible surface area between 1000 – 5000 Å^2 (in the case of the Ras · RafRBD: 1300 Å^2). Protein – protein interfaces are additionally rather flat and do not contain deep water-filled pockets as found in enzyme active sites. Furthermore, mutational studies have shown that residues in the interface of a protein · protein complex do not contribute equally to the binding energy and that sometimes 1 – 3 residues are responsible for most of the binding energy, in a way that is not at all obvious from the structure.[245–247] It might thus be necessary to construct new types of scaffolds specifically designed to cover a large surface area of the protein in question.

A successful candidate to inhibit the Ras-Raf interaction is the nonsteroidal anti-inflammatory drug (NSAID) sulindac sulfide (Scheme 22).[248] Sulindac itself has been used in the therapy of tumors in patients with the inherited cancer disposition Familial Adenomatous Polyposis[249, 250] and seems to target tumors carrying a Ras oncogene.[251] Recent biochemical and cell biology studies suggest that sulindac sulfide inhibits Ras transformation apparently by blocking the Ras-Raf interaction.[248] Although the affinity is presently too low to be a meaningful drug, it constitutes a promising lead compound.



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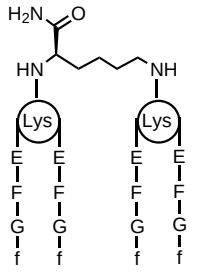
Scheme 22. Sulindac sulfide, an inhibitor of the Ras-Raf interaction.

3.6. Miscellaneous Approaches to Interfering with Ras Function

In addition to the strategies summarized in Sections 3.1 to 3.5 a few other approaches have been taken to interfere with Ras. For instance, RNA aptamers were selected against the farnesylated peptide Boc-Cys-Lys-Ser-Lys-Thr-Lys-Cys(Farn)-OH, which is modeled on the C terminus of K-Ras.[252] The peptide was immobilized on a column chro-

matography matrix through the N-terminal cysteine by means of a disulfide bridge. After ten rounds of selection the binding RNA aptamers were analyzed to reveal two consensus sequences: GGGUGGG and GGGAGGG. They showed binding affinities of 139 and 930 nM for the farnesylated peptides, respectively, and bound to the non-farnesylated peptide at least ten times more weakly. Such high-affinity aptamers that specifically recognize the hydrophobic farnesylated C terminus of K-Ras could be useful in interfering with the protein's function. The aptamers could be employed to specifically complex with farnesylated Ras and thereby prevent its access to cell membranes, thus resulting in a loss of Ras function. In a related approach a library of branched peptidic molecules was generated and selected for its ability to bind to the non-farnesylated C terminus of H-Ras.[253] From the entire library, which consisted of more than 150000 compounds, individual members were identified and investigated for their ability to prevent the farnesylation of Ras-peptides and GFP-CAAX fusion proteins (GFP = *g*reen *f*luoreszent *p*rotein). Compound **37** (Scheme 23) displayed sequence selectivity and inhibited the farnesylation of an H-Ras peptide with an IC_{50} value of 100 μM. These findings show that it is also possible to prevent Ras processing not by interfering with the enzyme but by blocking the enzymes's access to its substrate by using a synthetic molecule to mask the substrate.



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Scheme 23. A branched peptide identified from a library that binds to H-Ras peptides and GFP-CAAX fusion proteins (GFP = green fluorescent protein) and prevents their farnesylation.

In a further approach antisense oligonucleotides have been designed that interact with H-Ras mRNA codon 12, that is, the site at which the oncogenic mutation occurs, and thereby inhibit the expression of mutant H-Ras.[254] After absorption to polymeric nanoparticles these oligonucleotides inhibited neoplastic growth of HBL100Ras1 in nude mice.

4. Conclusion

Many signaling pathways from the outside to the interior of cells use the Ras protein for the transduction of the signal. Ras is a GTP-binding protein which is attached to the plasma membrane through mandatory posttranslational modifications. It is activated by recruitment of guanine nucleotide exchange factors to the plasma membrane and is thus converted into the active GTP-bound form. It interacts with different effectors which all contain a Ras binding domain with a similar structure. As the effectors have no sequence homology they use different residues to interact with Ras. To complete the cycle Ras is switched off by the action of GTPase-activating proteins which accelerate the intrinsic GTPase reaction by complementing the active site.

Since Ras is the most frequent oncogene found in human tumors it has long been considered an attractive target for anticancer therapy. Potential targets for the interference of the Ras function are its interaction with regulators and effectors, posttranslational modification, and the GTPase reaction. Here we have shown that various approaches have been undertaken to inhibit Ras action in the cell. The most promising and advanced of these currently is the inhibition of the enzyme farnesyltransferase, which attaches the lipophilic isoprenyl group to the C-terminal cysteine residue of the mature protein and is thus vital for Ras function. Although FTase inhibitors are nontoxic to normal cells and are in various phases of clinical trials, their efficacy and mechanism of action is still not completely understood.

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