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Chemistry and Biology of Ras Farnesyltransferase

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Mutated forms of *ras* are found in many human tumors and the rate of incidence is significantly higher in colon and pancreatic cancers. The protein product from the *ras* oncogene is a small G-protein, $p21^{ras}$ (Ras) that is known to play a key role in the signal transduction cascade and cell differentiation and proliferation. Mutated Ras is unable to regulate itself and remains constantly activated, leading to uncontrolled cell growth. The function of Ras in signal transduction requires its location near the growth factor receptor at the cell membrane. However, Ras does not have a transmembrane domain. Ras requires farnesylation to increase its hydrophobicity and subsequent plasma membrane association for its transforming activity. This key post-translational modification is catalyzed by the enzyme Ras farnesyltransferase (FTase), which transfers a farnesyl group from farnesylpyrophosphate to the *C*-terminal cysteine of the Ras protein. The requirement has focused attention on FTase as a target for therapeutic intervention. Selective inhibition of FTase will prevent Ras protein from association with the plasma membrane, leading to a disruption of oncogenic Ras function.

Key words: *ras* Oncogene, Ras, Signal transduction cascade, Ras Farnesyltransferase, CAAX motif, Peptidomimetics

INTRODUCTION

Alterations in genes affecting the expression of proteins controlling cell growth and differentiation are considered to be the main cause of cancer. Human cancer develops as a result of genetic disorders, arising from an accumulation of mutations that promote clonal selection of cells with increasingly aggressive behavior (Fearon, 1997). Cancer cells are no longer responsive to the controlling signals and interactions that occur continuously in normal, healthy tissues.

Molecular and cell biology have begun to open the way to the understanding of key cellular processes at the molecular level, therefore, it has become possible to take a more mechanistic approach to the discovery of antitumor agents. There has been substantial progress in many aspects of tumor therapy with the understanding of molecular mechanisms. Currently, the most intensively studied areas are those involved in the signal transduction pathway, the cell cycle and apoptosis, the ubiquitin-proteasome pathway, and tumor angiogenesis.

Many oncogene products and growth factor receptors contain tyrosine kinase domains, which are essential for biological activity. This is consistent with the increased level of tyrosine phosphorylation commonly observed in tumor cells. Meanwhile dramatic progress has been made in our understanding of the precise mechanism by which receptor tyrosine kinases are able to signal to the nucleus. The initial signal is transmitted into cells through the binding of growth factor to tyrosine kinase receptor. This triggers a series of protein-protein interactions with a highly specific phosphotyrosine residue in the ligand-activated, autophosphorylated growth factor receptor. Signal transduction information then flows via a series of protein-protein interactions and phosphorylating events from GRB-2 → SOS → Ras → Raf → MEK → MEK kinase → transcription factors → gene expression. The elucidation of this pathway has yielded many targets for drug discovery (Hinterding *et al.*, 1998).

Among recent cancer-related research in molecular biology, the signal transduction pathways are the first systems to be investigated successfully. In particular, an increased understanding of *ras* oncogenes has led to elucidation of the cytostatic mode of action for anticancer drugs. Mutated forms of *ras* are found in many human tumors and the

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rate of incidence is significantly higher in colon and pancreatic cancers. The protein product from the *ras* oncogene is a small G-protein, $p21^{ras}$, which is known to play a key role in the signal transduction cascade and cell differentiation and proliferation. Mutated Ras is unable to regulate itself and remains constantly activated, leading to uncontrolled cell growth. The function of Ras in signal transduction requires its location near the growth factor receptor at the cell membrane. However, Ras does not have a transmembrane domain. Ras requires farnesylation to increase its hydrophobicity and subsequent plasma membrane association for its transforming activity. This key post-translational modification is catalyzed by the enzyme Ras farnesyltransferase, which transfers a farnesyl group from farnesylpyrophosphate to the C-terminal cysteine of the Ras protein. The requirement has focused attention on FTase as a target for therapeutic intervention. Selective inhibition of FTase will prevent Ras protein from association with the plasma membrane, leading to a disruption of oncogenic Ras function. Thus, FTase inhibitors have the potential to be non-toxic anticancer drugs. This has provided a basis for the more rational design of new anticancer agents (Barinaga, 1997).

Ras oncogenes in human cancer

Human cancer develops as a result of genetic disorders, arising from an accumulation of mutations. Oncogenes belonging to the *ras* family are mutated in approximately 30% of all human tumors. The highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50 %), and the lung (30%) (Barbacid, 1987). The *ras* oncogenes have been the subject of intense investigation for their relation to a variety of human cancers. This work has led to the identification of the genes that are altered in several tumor types and to the elucidation of the role of these genes in carcinogenesis.

In the late 1970s a family of genes was discovered that could cause normal cells to undergo transformation into rapidly dividing tumor cells. These genes are called oncogenes (Greek, *onkos*, mass). Tumor viruses often carry them. The cellular gene, which functions in normal cell physiology, is called a proto-oncogene because it can be converted into an oncogene. When a host cell is infected with tumor viruses, the modified viral gene is inserted in the host genome and may be subsequently expressed. The Harvey and Kirsten murine sacoma viruses were originally identified as genetic elements that induced malignancies in animals. Later, it was found that the commonly observed proto-oncogenes in human tumors are the cellular homology of H-*ras* and K-*ras*. The *ras* family in humans consists of three functional genes, H-*ras*, K-*ras*, and a closely related form, N-*ras*, which has no viral equivalent. Mutations in K-*ras* are predominant in human cancers.

The high prevalence of mutated *ras* genes in human cancers implicates these mutations as important genetic disorders that promote human oncogenesis. The *ras* gene mutations have been identified in a variety of human tumors and point mutations are different for different tumor types. The frequency of mutation is highly dependent on the specific organ. These mutations are usually detected at a single point and are specific for the mutations in codons 12 (bladder), 13 (myeloid leukemia), 59 (leukemia) and 61 (lung) (Bos, 1989).

The *ras* oncogenes encode four structurally related proteins comprising 188-189 amino acids with molecular weights of 21 kDa ($p21^{ras}$, Ras). The protein encoded by the *ras* oncogene is referred to as the Ras protein. The H-, K-, and N-Ras proteins differ only slightly in their carboxylate termini. In this family, amino acid residues 1-85 are identical in each of the proteins, residues 85-166 contain 85% of the same sequence, and 167-185 is a variable region. As a result of the point mutation of *ras* gene, the Ras protein has an altered amino acid at one of the critical positions 12, 13, 59, and 61. In mutated H-ras, Gly-12 is replaced with Val and Ala-59 is replaced with Thr and in K-ras, Gly-12 is replaced with Ser and Ala-59 is replaced with Thr. In the variable region, the last four amino acid sequences of all Ras proteins are composed of a CAAX motif where C is cysteine, A is valine, isoleucine or leucine and X is methionine or serine (Table I) (Lowy and Willumsen, 1993). The CAAX motif in Ras proteins is recognized by the enzyme Ras Farnesyltransferase and further used in the Farnesyltransferase inhibitors design.

Signal transduction cascade

The Ras proteins are guanine nucleotide-binding proteins (G-proteins). G-proteins are believed to be involved in many cellular processes such as signal transduction, protein transport and secretion, and polypeptide chain elongation. The activity of Ras is similar to that of other G-proteins, and the sequences of Ras proteins are homologous to other G-proteins. Normal Ras is a guanine nucleotide-binding protein that hydrolyzes GTP at rates comparable to other G-proteins and is only active in its GTP-bound form. When the cellular Ras protein is mutated at positions 12 or 61, it still binds to GTP but no longer acts as an effective GTPase. In the absence of the regulatory action of GTP hydrolysis, the mutant Ras remains constantly

Table I. *Amino acid sequences of Ras proteins at C-terminus.*

H-Ras	^{166}HKLRKLNPPDESGPGCMSCK**CVLS**^{189}
K-Ras 4A	^{166}YRLKKISKEEKTPGCVKIKK**CIIM**^{189}
K-Ras 4B	^{166}HKEKMSKDGKKKKKKSKTK**CVIM**^{188}
N-Ras	^{166}YRMKKLNSSDDGTQGCMGLP**CVVM**^{189}

activated.

Over the last years, essentially all of the key players involved in the mitogenic signal transduction cascade have been identified. Many oncogene products and growth factor receptors contain tyrosine kinase domains, which are essential for signal propagation. The intricacies of signal transduction can be seen in the nature of the protein-protein interactions and phosphorylating events, which serve to transmit signals from growth factor receptors to Ras and subsequently from Ras to the nucleus (McCormick, 1993) Within the flow of information from tyrosine kinase to the nucleus, Ras acts as a kind of turnstile through which the signal must pass.

The initial signal is transmitted into cells through the binding of growth factor to tyrosine kinase receptor (Cadena and Gill, 1992). Receptor tyrosine kinases contain extracellular ligand recognition and cytoplasmic protein tyrosine kinase domains. The extracellular domains are responsible for binding to growth factor and, in turn, receiving the external signal. Initial binding of growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) to their receptors induces a conformational change in the receptor, which leads to dimerization and subsequent autophosphorylation of the receptor tyrosine kinase (Heldin and Westermark, 1990). The phosphorylated tyrosine residues provide specific binding sites for signal transduction protein modules containing src homology (SH-2) domains (Pawson, 1993). Growth factor receptor binding protein-2 (GRB-2) is an adapter protein, containing one SH-2. It also has two SH-3 domains, which mediate activation of guanine nucleotide exchange on Ras (Maignan *et al.*, 1995). GRB-2 binds to the receptor through its SH-2 domain and recruits mammalian son of sevenless-1 (m-SOS-1) to the membrane by binding through its two SH-3 domains, to form a stable GRB-SOS complex. m-SOS-1 acts as the guanine nucleotide exchange factor (GEF), and converts Ras from the inactive GDP-bound form to the GTP-bound active conformation by catalyzing the exchange of GDP for GTP. Once Ras is in the active GTP-bound form, it binds to Ras-binding domain of serine/threonine kinase c-Raf and localizes it to the plasma membrane. Once Raf is positioned at the membrane, it initiates a kinase downstream cascade, which is not well understood (McComick, 1994; Moodie and Wolfman, 1994). Active Raf phosphorylates another kinase called mitogen activated protein kinase kinase (MAPKK, also known as MEK) and this then phosphorylates mitogen activated protein kinase (MAPK) on both serine and tyrosine. MAPK translocates to the nucleus where it phosphorylates transcription factors, such as *myc, fos* and *jun* that are believed to be involved in DNA synthesis and ultimately cell growth.

The biochemical function of Ras is to transmit growth factor-induced signals from cell surface receptor tyrosine kinases to the cell nucleus. Ras protein acts as a signal mediator for receptor tyrosine kinase and tyrosine-associated receptors. The mitogenic activity of normal Ras is modulated by a GTP-GDP cycle. Signal transmission is normally terminated by GTPase activating proteins (GAPs). When the Ras protein is activated by mutations at position 12 or 61, it can no longer hydrolyze GTP. In the absence of the regulatory action of GTP hydrolysis, the mutant Ras remains activated and lead to uncontrolled cell growth even in the absence of growth factor. This has led to the theory that the biochemical function of mutant Ras proteins in tumor cells is to trigger the oncogenic signaling pathway and to turn on constant cell growth and division.

Biochemical function of Ras

The *ras* oncogene and its protein product, Ras, have been the subjects of intense investigation due to their cellular transforming activities. The Ras proteins play a regulatory role in oncogenic, mitogenic, and developmental signaling pathways. The role of Ras is to transmit growth factor-induced signals from the cell surface receptor tyrosine kinases to the cell nucleus in order to stimulate cell proliferation and differentiation. As previously noted, Ras cycles between its GTP and GDP bound states. This functions as a molecular switch in the signal transduction cascade. Ras proteins are regulated in two ways: (1) by an increase in the rate of guanine nucleotide exchange, which produces the GTP-bound form after cells are stimulated by the ligand, and (2) by an increase in their intrinsic GTPase activity. Guanine nucleotide exchanger factor (GEF) converts membrane associated Ras proteins from the GDP-bound state to the GTP-bound state, turning the "switch" on. GTPase activating protein (GAP) then stimulates the intrinsic Ras GTPase activity and turns it off (McComick, 1989). A key biochemical feature of the Ras proteins is not only its ability to bind guanine nucleotide with high affinity but also its ability to hydrolyze bound GTP to GDP and inorganic phosphate. However, when Ras proteins are mutated at position 12, 13, and 61, GAP no longer activates Ras GTPase activity. Oncogenic mutants are not converted to the GDP-bound state and stay in the active GTP-bound conformation (Fig. 1). Most human carcinomas produce oncogenic Ras proteins that have retarded GTPase activity leading to constant stimulation. The mutated Ras proteins are thus not able to function as on/off switches and remains constantly activated, leading to uncontrolled cell growth (Grand, 1991).

The crystal structures of cellular and mutant Ras proteins complexed with a GTP analog are almost identical, and the only difference is seen in the vicinity of the γ-phosphate of GTP. Biochemical and structural studies suggest that Gln-61 is most important in γ-phosphate binding and GTPase activity. GTP hydrolysis involves Gln-

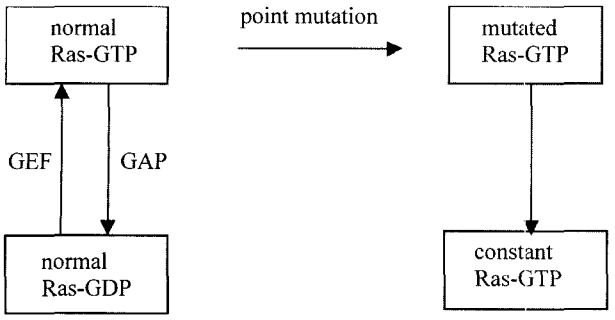

Fig. 1. Normal and abnormal activity of Ras.

61 and Glu-63 as activating species for in-line attack of Wat-175. In the crystal structure, the side-chain of Gln-61 makes (a) contact with Wat-175, which is perfectly placed as the nucleophile in the attack of the γ-phosphate of GTP. This in-line nucleophilic displacement is further facilitated by hydrogen bonds from residues Thr-35, Gly-60 and Lys-16. Based on this crystal structure data, a mechanism for the hydrolysis of GTP has been proposed that the γ-carboxamide of Gln-61 either acts as a general base, polarizing a water molecule for attack on the γ-phosphate of GTP, or stabilizes the incipient transition state during hydrolysis by acting as a hydrogen bond donor (Fig. 2) (Pai *et al.*, 1990). The mutation Gly-12 to Val disrupts the interaction between Gln-61 and the γ-phosphoryl oxygen due to the sterics from the valine side chain. Furthermore, other crystal structures of mutated Ras proteins (Gly-12 to Arg, Gln-61 to His and Leu) confirm the hydrolysis mechanism (Krengel *et al.*, 1990).

The Ras proteins are plasma membrane proteins that bind guanine nucleotides. Originally, Ras is found in the cytosol of cells as a biologically inactive precursor protein. However its ability to play a pivotal role in mitogenic signaling is dependent on localization at the inner surface of the membrane where it can be activated by growth signals from outside the cell. Ras does not possess a transmembrane domain. For membrane localization and proper function, Ras requires posttranslational modification to increase hydrophobicity. Ras must be linked to a fatty (acid) group in order to associate with the membrane lipids. One of the key events in Ras localization to the plasma membrane is farnesylation on a *C*-terminal cysteine residue of the protein. This key posttranslational modification is catalyzed by Ras farnesyltransferase (FTase), which transfers a farnesyl group from farnesylpyrophosphate (FPP) to the cysteine of the CAAX tetrapeptide of Ras.

Ras farnesyltransferase

Myristoylation, palmitoylation, and prenylation are lipid modifications important in the regulation of protein association with membrane lipids and protein-protein interactions. Covalent modification by isoprenoid lipids (prenylation) is now recognized as a mechanism to promote membrane interactions and biological activities of a variety of cellular proteins. Protein prenylation affects about 0.5% of cellular proteins. The prenylated proteins include the nuclear protein lamin B and a number of G-proteins, which are responsible for controlling a wide spectrum of signal transduction pathways (Casey, 1992; Casey and Seabra, 1996). Ras Farnesyltransferase (FTase) is the enzyme that catalyzes the transfer of farnesyl from farnesylpyrophosphate (FPP), an intermediate in the biosynthesis of squalene and isoprenoids, to the cysteine of the CAAX sequence in Ras protein. Farnesylation is required for the localization of Ras protein from the cytosol to the cell membrane. Of particular biochemical interest is the observation that the farnesylation of oncogenic mutants of Ras is absolutely required for the transformation of cells to a tumorgenic state (Casey *et al.*, 1989).

FTase is a heterodimer consisting of two subunits, with molecular weights of 48 kDa (α-subunit) and 46 kDa (β-subunit). FTase is a zinc metalloenzyme that contains one-zinc per protein dimer. Recent X-ray cocrystal evidence indicates that the zinc ion is coordinated by the thiol of the CAAX cysteine residue of the substrate in the active site (Strickland *et al.*, 1998).

The cytoplasmic enzyme FTase recognizes the carboxyl terminus of unprocessed Ras which is characterized by a cysteine residue followed by two aliphatic amino acids

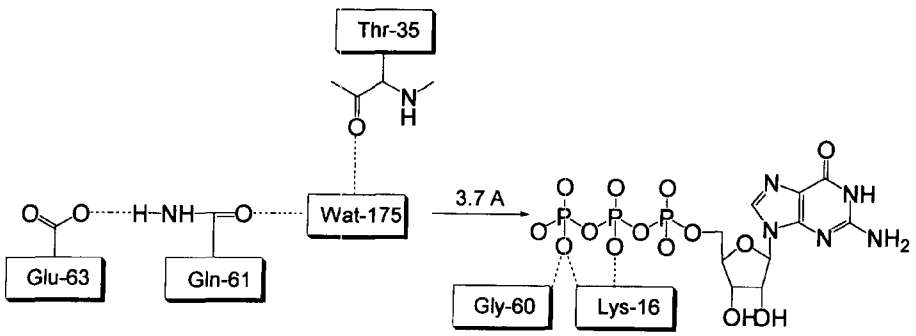

Fig. 2. Schematic drawing of Ras-GTP complex for GTP hydrolysis

(valine, eucine, and isoleucine) and then either serine or methionine. Prior to membrane insertion of farnesylated Ras, the three terminal amino acids are cleaved by CAAX protease. This is followed by methyl esterification of the new C-terminal cysteine residue by protein methyltransferase (Gutierrez *et al.*, 1989). At this stage, the processed Ras protein is able to associate with the plasma membrane. The posttranslational modification of Ras is summarized in Fig. 3. The reason for the proteolytic removal of the AAX residues from farnesylated Ras suggests that the AAX residues hinder the farnesylated protein from inserting into the membrane or possibly that these residues interfere with some other receptor protein. Methylation of the carboxyl terminus serves to increase the hydrophobicity and neutralizes the anionic charge that would be repulsed by the phospholipids (Der and Cox, 1991). After binding to the membrane, Ras proteins are further modified by the addition of palmitate through a thioester linkage to cysteines near the farnesylated *C*-terminal cysteine. For H-, N-, and K-Ras 4A, cysteine 181/180 and cysteine 184 are subjected to further modification. Palmitoylation renders the protein even more hydrophobic and anchors it more tightly to the plasma membrane (Hancock *et al.*, 1989). Of these steps, farnesylation appears to be a necessary and sufficient step for membrane localization, implicating FTase as a preferred target for interrupting oncogenic Ras processing.

FTase is a metalloenzyme containing zinc and magnesium ions (Reiss *et al.*, 1992). The zinc ion in FTase is essential for catalysis and is required for binding of the peptide substrate. Furthermore, mutation of the zinc ligands Asp-297 and His-362 dramatically decreases the ability of the enzyme to bind zinc and to catalyze product formation (Fu *et al.*, 1998). One proposed function of the zinc ion in FTase and other metalloproteins that catalyze alkylation of sulfur is to lower the p*Ka* of the thiol thereby maximizing the amount of thiolate for nucleophilic attack. Indeed, the pH-dependence of peptide binding to FTase suggests that the p*Ka* of the cysteine thiol is lowered from 8.3 for the free peptide (**GCVLS**) to approximately 6.4 upon binding of the peptide to the enzyme (Hightower *et al.*, 1998). Therefore, a variety of evidence indicates that the bound zinc ion coordinates the sulfur of the peptide substrate and facilitates formation of a thiolate nucleophile (Park and Beese, 1997; Matthews and Goulding, 1997; Hightower and Fierke, 1999). It is believed that magnesium ion contributes to the binding and activation of the FPP substrate observed in FPP synthase.

Several mechanisms have been proposed for the reaction catalyzed by FTase. Substitution of electron-withdrawing fluorine on the methyl group attached to C(1) of FPP decreases the reaction rate for yeast FTase (Dolence and Poulter, 1995). This indicates that farnesylation proceeds with some ionic character. However, peptide farnesylation by both yeast and human FTase occurs with inversion of configuration at C(1), which is consistent with an associative mechanism but cannot completely rule out a dissociative ion-pair mechanism (Mu *et al.*, 1996; Edelstein *et al.*, 1998). The measured secondary kinetic isotope effect near unity also provides evidence for associative character in the transition state (Weller and Distefano, 1998). Therefore, the most likely catalytic mechanism for FTase

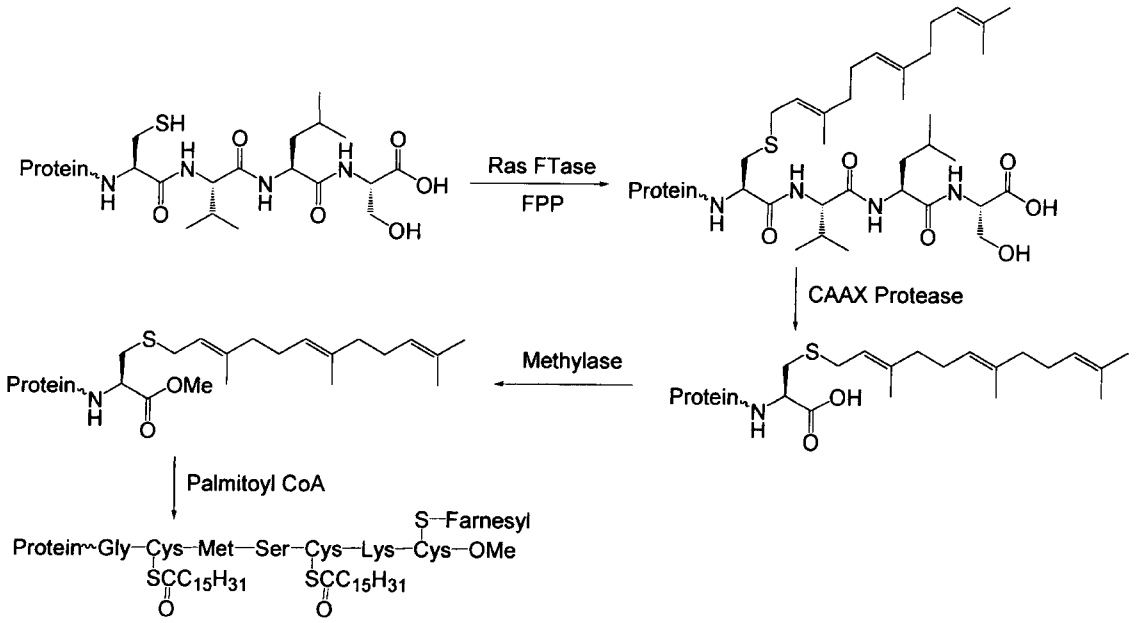

Fig. 3. Posttranslational modification of H-Ras

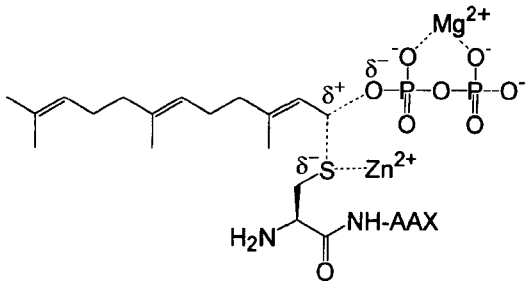

Fig. 4. Proposed associative transition state for farnesylation

involves a transition state with partial positive charge on C(1) and partial negative charge on the peptide cysteine residue (Fig. 4).

In 1998, the X-ray crystal structure of a ternary complex of rat FTase-CAAX-FPP analog had been reported to a resolution of 2.5Å (Strickland *et al.,* 1998). Rat FTase shares 93% homology with the human FTase. Presently, several X-ray structures are available, including FTase (Park *et al.,* 1997; Dunten *et al.,* 1998) and FTase-FPP complex (Long and Casey, 1998). The crystal structure of FTase reveals the zinc-binding site, as well as the overall structure of the enzyme. The secondary structure of the α-subunit includes 15 α-helices, four 3_{10} helices and a β-strand. The β-subunit contains 14 α-helices, seven short 3_{10} helices and three β-strands. Two six parallel helices, but antiparallel to each other, are folded into an α-α barrel structure. One end of the barrel is open to the solvent, forming a deep cleft in the center of the barrel, which is believed to be an enzymatic active site.

The active site is formed by two clefts that intersect at a bound zinc ion. One cleft is lined with highly conserved aromatic residues appropriate for binding the farnesyl isoprenoid with required specificity. The binary complex suggests that the depth of the hydrophobic binding cavity acts as a ruler discriminating between isoprenoids of different lengths. The other cleft contains a nine-residue peptide that may bind to the Ras substrate. The catalytic zinc ion is coordinated by β-subunit residues Asp-297 (2.1, 2.5 Å), Cys-299 (2.3 Å), and His-362 (2.4 Å) and a well-ordered water molecule. Asp-297β forms a bidentate ligand, resulting in distorted pentacoordinate geometry. In the FTase ternary complex, the peptide sulfur displaces the water molecule and coordinates the zinc (Fig. 5). The *C*-terminal carboxylate group of the peptide forms a hydrogen bond to the side chain amino group of Gln-167 of the β-subunit. The sulfur atom of the peptide cysteine residue is 2.5 Å from the zinc, suggesting it is coordinating to the zinc ion.

Ras farnesyltransferase inhibitors

Ras cycles between its GTP-bound "on state" and GDP-bound "off state" to regulate the transduction of biological information from the membrane to the nucleus. The crucial role played by Ras in signal transduction is dependent on its plasma membrane association. Since farnesylation catalyzed by the enzyme FTase is the required step for Ras membrane localization and subsequent transforming activity, inhibition of FTase provides an attractive strategy for developing new anticancer drugs. The *C*-terminal cysteine of Ras is critical for membrane association as shown from site-directed mutagenesis studies. When Cys-186 (in H-Ras) is mutated to serine, oncogenic Ras remains in the cytosol and is inactive (Willumsen *et al.*, 1984). Thus selective inhibition of FTase will prevent Ras protein from association with the plasma membrane, leading to a disruption of oncogenic Ras function. Recent interest in the development of inhibitors of FTase has been attributed to their ability to inhibit the growth of malignant cells.

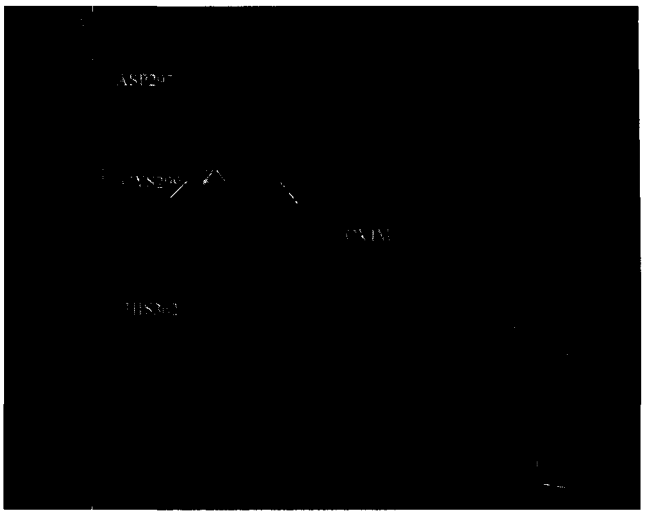

Fig. 5. Zinc coordination in ternary FTase complex (FPP analog is omitted for clarity)

A key property of FTase is its ability to recognize both Ras proteins and short tetrapeptides corresponding to the CAAX sequence (Reiss *et al.*, 1990). The optimal sequence of these short tetrapeptides has been extensively examined and it has been shown that the cysteine is a requirement for successful binding. While the capacity for a high-affinity interaction of the CAAX peptides with FTase generally reflects the abilities of these peptides to serve as alternate substrates for the enzyme, variants of these peptides have been identified as true competitive inhibitors of the FTase without themselves being farnesylated. For example, the **CVIM** tetrapeptide (in K-Ras 4B) inhibits FTase, but also acts as a substrate for farnesylation. However, the incorporation of an aromatic amino acid into the A_2 position of CA_1A_2X (such as **CVFM**) prevents the tetrapeptide from serving as a substrate (Brown *et al.*, 1992). This provides a valuable starting point for the design of inhibitors that could serve to block protein farnesylation.

Before a crystal structure of FTase was available, most

molecular targets were designed on the basis of a proposed mechanism for the enzyme reaction. Site-directed mutagenesis experiments, photoaffinity labeling studies, and NMR studies of bound inhibitors have provided information on what features are necessary for tight binding to the active site of FTase. Later, a catalytic mechanism was proposed and was supported by the X-ray data. It is envisioned that the donor FPP is bound in the enzyme's active site via hydrophobic interactions with the isoprene portion and ionic interactions with the highly charged diphosphate moiety. The cysteine sulfur coordinates to the zinc ion, perhaps with assistance of a nearby general base. The coordination aids in the formation of thiolate ion, thereby increasing the nucleophilicity of cysteine thiol. The methionine residue sits in a binding pocket that provides a key selection for farnesylation versus geranylgeranylation, and presumably forms an electrostatic interaction to a positively charged region of the active site. However, the exact role of methionine side chain in the enzyme-bound conformation is still unknown. The two central residues in Cys-AA-Met presumably lie in a hydrophobic binding pocket.

Many groups have focused on developing inhibitors of FTase as potential antitumor agents. These inhibitors can be divided into four groups based on their mechanism of action (1) peptidomimetic inhibitors based on the CAAX motif; (2) inhibitors of FPP; (3) bisubstrate inhibitors; (4) natural products (Buss and Marsters, 1995; Gibbs *et al.*, 1997; Leonard, 1997).

The use of FTase as a target in volume screening has led to the identification of a variety of microbial natural products, which inhibit FTase. Several natural inhibitors of FTase have been reported, but in general these have been less potent than the CAAX-based peptidomimetics. Some are competitive with FPP, including **manumycin** and **chaetomellic acid B**, while another inhibitor such as **pepticinnamin**, is competitive with the Ras peptide. **Zaragozic acid**, previously disclosed as a potent inhibitor of squalene synthase, has also been identified as a FTase inhibitor. The remaining inhibitors are noncompetitive with neither FPP nor Ras peptide, and their mechanism of inhibition is not known. Examples of such inhibitors are **10-desmethoxystreptonigrin** and **gliotoxin**. **PD-083176** (Leonard *et al.*, 1997) was recently identified as a potent selective Ras FTase inhibitor through compound library screening. A common feature among many of the natural inhibitors is a hydrophobic side chain and is best exhibited in **manumycin**, **chaetomellic acid B**, and **zaragozic acid**. However, compounds **10-desmethoxystreptonigrin**, **pepticinnamin**, and **PD-083176** share the highly conserved aromatic residues (Fig. 6). Synthetic analogs of FPP, CAAX protease and methyl transferase inhibitors have been reported (Bolton *et al.*, 1994). However, they have received little attention, perhaps due to the involve-

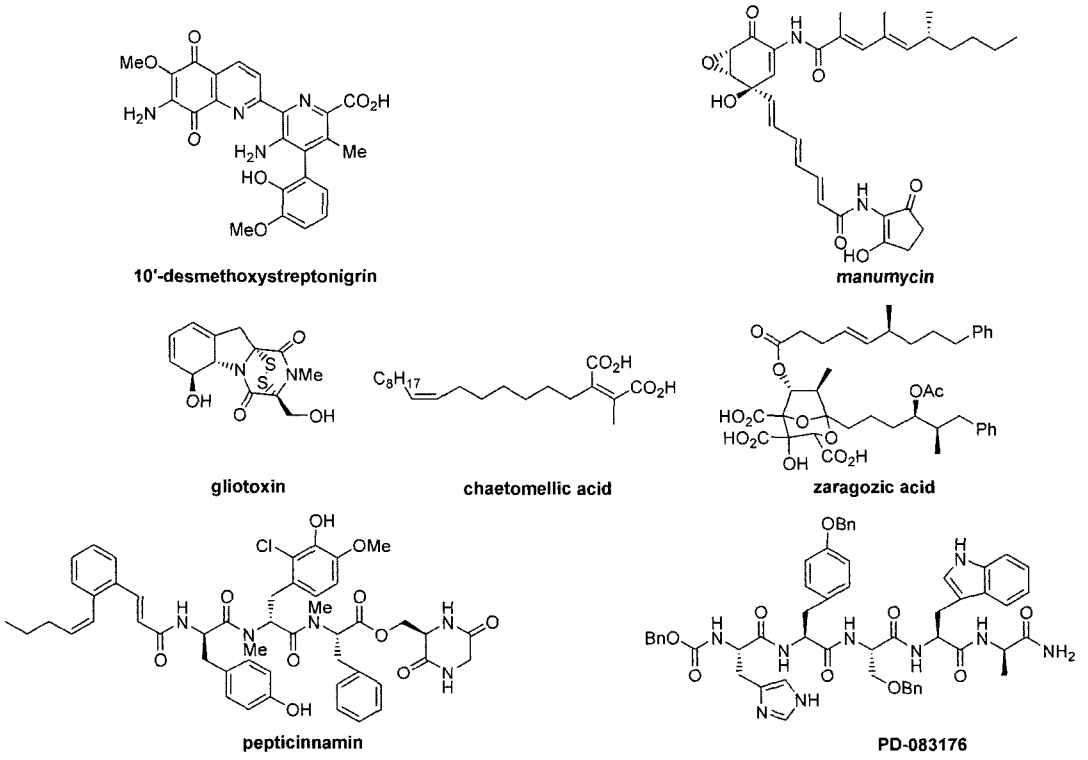

Fig. 6. Natural Ras FTase inhibitors

ment of these substrates in other biological pathways.

Simple tetrapeptides corresponding to the CAAX sequence can act as effective inhibitors of Ras FTase. Tetrapeptides such as **CVIM** can inhibit FTase *in vitro* (IC_{50} = 150 nM) but has no effect on whole cells, presumably due to poor membrane permeability and susceptibility to proteolysis. In order to enhance cellular uptake and improve proteolytic stability, we and several other investigators have focused on peptidomimetics, which probe the hydrophobic binding pocket in FTase and the maximizing of binding of the inhibitors.

Representative structures of CAAX-based peptidomimetics are shown in Fig. 7. These demonstrate modifications of the central part of the CAAX tetrapeptides with reduced amide, olefin, and hydrophobic linkages. Their structural similarity to the C-terminal CAAX sequence strongly suggests that they are associated with a hydrophobic binding pocket and replace the two central aliphatic amino acids. The central linkers play a key role in appropriately positioning the cysteine moiety for binding by zinc coordination and providing the affinity for FTase. In fact, inhibitory potency is highly dependent on the nature of the spacer group. Most of the peptide mimics retain the methionine carboxyl and cysteine thiol groups of the original tetrapeptide. To further improve the metabolic stability, the cysteine amide bond can be reduced to a methylene amino group as a peptide isostere. The reduction of this amide bond leads to an increased inhibition potency against FTase *in vitro* as well as Ras processing *in vivo.*

Recent efforts have shifted to non-thiol tetrapeptides such as, **BMS-193,269** (Hunt *et al.*, 1996), because of the disadvantage of the highly oxidizable thiol functionality *in vivo.* Bisubstrate inhibitors of Ras FTase incorporate the structural motifs of both FPP and CAAX tetrapeptide. It would be interesting to determine whether a bisubstrate would selectively bind FTase over GGTase-I. The phosphinate inhibitors, **BMS-184,467** and **-185,878**, have shown about 2000-fold higher affinity for FTase versus GGTase-I (Patel *et al.*, 1995).

There has been considerable debate over the bioactive conformation of the CAAX peptides. The conformation of the CAAX-based inhibitors has been studied by NMR spectroscopy, and early research suggested the possibility of a "turn-like" backbone conformation of **L-739,750** in the presence of FTase enzyme by the transferred nuclear Overhauser effects (trNOEs) (Kobran *et al.*, 1995). It has been also proposed that the bound zinc facilitates a turn arrangement of the peptide **BZA2B** through coordination of the cysteine thiol and methionine carboxylate (Masrers *et al.*, 1994). However, the potency of inhibitor **FTI-276** argues against a β-turn, as its rigidity makes it impossible for this molecule to adopt a β-turn conformation (Hamilton and Sebti, 1995). More recent evidence (Clerc *et al.*, 1995; Burn *et al.*, 1997), particularly that from conformationally

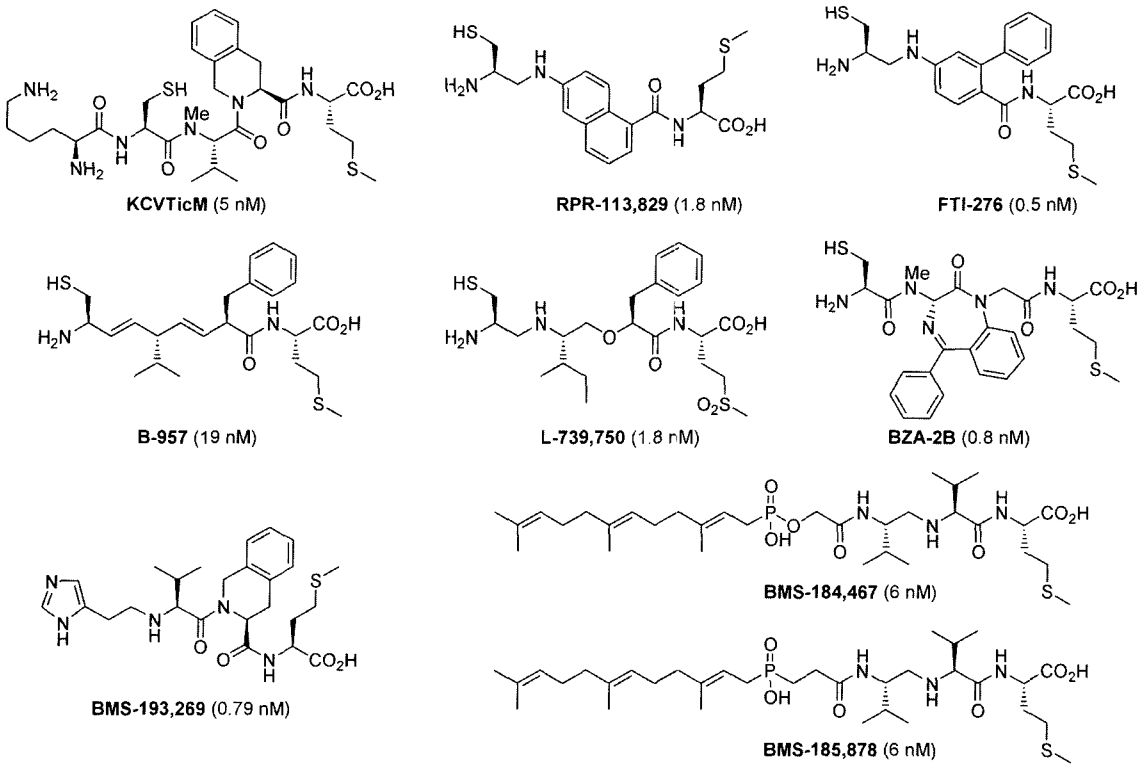

Fig. 7. Peptidomimetic FTase inhibitors

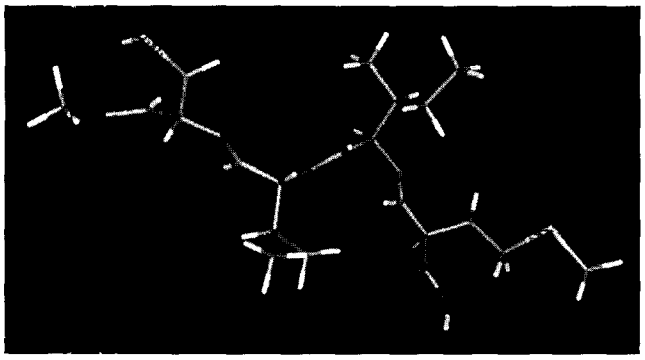

Fig. 8. Enzyme-bound conformation of ***N*-Ac-CVIM(Se)**

constrained CAAX mimetics such as, **KC(*N*-Me)VTicM** and **RPR 113829**, has led to the suggestion that an "extended" conformation is more favorable. The recent disclosure of the ternary complex of the FTase provides insight into the extended conformation of the CAAX peptidomimetics (Fig. 8).

Ras proteins have been the subjects of intense study due to their oncogenic transforming activities. This perspective has emerged from inhibition of Ras processing as a novel method toward therapeutic intervention. The requirement that Ras must be farnesylated and associated with the plasma membrane for its transforming activity has prompted the use of FTase inhibitors as anticancer agents. A number of FTase inhibitors based on the CAAX sequence have been reported. These mainly include conformationally constrained tetrapeptides and tetrapeptidomimetics, bisubstrate analogs based on transition state mimics, and natural compounds isolated through library screening. Many of these compounds are effective inhibitors of both the FTase enzyme *in vitro* and cellular Ras processing. Animal studies have shown that FTase inhibitors have low toxicity and are highly active against tumors. Although no results on humans are available, at least two FTase agents are in clinical trials now.

Recent efforts show very perplexing results (Cox and Der, 1997). The use of Ras inhibitors has resulted in an unexpected discovery: some inhibitors' activity is not limited to tumors with *ras* mutations. FTase inhibitors obviously block FTase, but K-Ras, the form of Ras that is by far the most often mutated in human tumors, can escape the farnesylation block and receive a fatty chain from an alternative enzyme. This discovery suggests that blocking FTase should not stop K-Ras-driven tumors. In the cancer cell lines, FTase inhibitors block the growth of some tumors with a mutant K-*ras* gene, some with other *ras* mutations, and even some that have no *ras* mutations at all. This implies that there is another target besides Ras that is farnesylated and is very important for the growth of these tumors. Because FTase modifies more than 20 proteins, it will be difficult to find that key target.

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