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Farnesylated Proteins and Cell Cycle Progression

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Abstract Post-translational modification of proteins by the addition of a farnesyl group is critical for the function of a number of proteins involved in signal transduction. Farnesylation facilitates their membrane association and also promotes protein–protein interaction. Recently, progress has been made in understanding the biological significance of farnesylation. First, effects of farnesyltransferase inhibitors (FTIs) on cancer cells have been examined using a variety of human cancer cells. This study showed that one of the major effects of FTIs is to alter cell cycle progression. Both G0/G1 enrichment and G2/M accumulation were observed depending on the cell line examined. Second, a number of novel farnesylated proteins have been characterized. Of these, Rheb and CENP-E,F are of particular interest. Rheb, a novel member of the Ras superfamily G-proteins, may play a role in the G1 phase of the cell cycle. CENP-E,F are centromere associated motors that play critical roles in mitosis. These results suggest important contributions of farnesylated proteins in the regulation of cell cycle progression. J. Cell. Biochem. Suppl. 37: 64–70, 2001.

Key words: farnesyltransferase inhibitor; cell cycle; Rheb; Ras; Rho; CNEP-E,F

Post-translational modification of proteins by a farnesyl group has emerged as an important event for a number of proteins involved in signal transduction [Sattler and Tamanoi, 1996; Zhang and Casey, 1996]. A variety of proteins including the Ras superfamily G-proteins are farnesylated. Farnesylated proteins undergo further C-terminal modification events that include proteolytic removal of three C-terminal amino acids and carboxyl methylation of the C-terminal cysteine [Sattler and Tamanoi, 1996; Zhang and Casey, 1996]. These C-terminal modifications facilitate their membrane association as well as protein–protein interaction. Farnesylation is catalyzed by protein farnesyltransferase (FTase), a heterodimeric enzyme that recognizes the so-called CaaX motif (C is cysteine, a is an aliphatic amino acid, and X is usually methionine, glutamine, cysteine, serine, or alanine) present at the C-termini of substrate proteins [Zhang and Casey, 1996]. FTase transfers a farnesyl group from farnesyl pyrophosphate and forms a thioether linkage between the farnesyl and the cysteine residue in the CaaX motif (Fig. 1).

Significance of protein farnesylation in cell cycle progression was suggested from the studies utilizing farnesyltransferase inhibitors (FTIs) [Vogt et al., 1996; Barrington et al., 1998]. However, these effects were variable depending on the cell line examined. With some cell lines, enrichment of G0/G1 phase cells was observed, while other cells showed G2/M enrichment. This was in contrast to those observed with geranylgeranyltransferase inhibitors (GGTIs); GGTI consistently induced enrichment of G0/G1 phase cells [Vogt et al., 1996]. Accordingly, more attention has been focused on GGTI than on FTI as far as the cell cycle is concerned.

Recent progress in identifying and characterizing farnesylated proteins led to renewed interest in the effects of FTIs on cell cycle progression. A variety of novel farnesylated proteins have been characterized [Tamanoi et al., 2001]. In addition to Ras proteins that have been extensively studied, other members of Ras superfamily G-proteins have also been shown to be farnesylated. Of particular interest is Rheb [Yamagata et al., 1994] that is conserved from yeast to human. Analysis of *Schizosaccharomyces pombe* Rheb suggested involvement of

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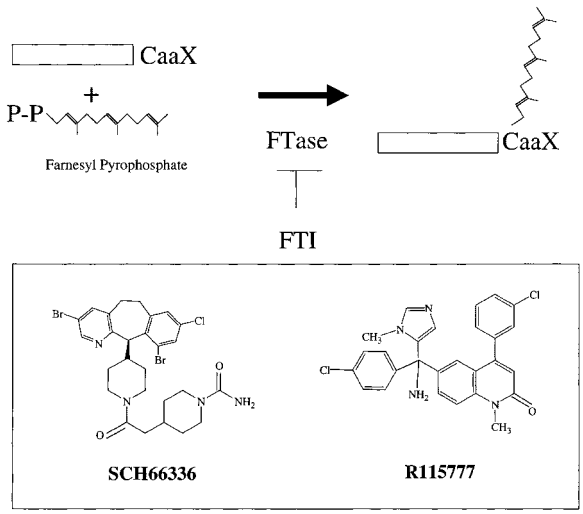

Fig. 1. Function of FTase and FTIs. FTase catalyzes the transfer of a farnesyl group from farnesyl pyrophosphate to a cysteine within the CaaX motif of the substrate protein. FTIs such as SCH66336 and R15777 inhibit this reaction.

this protein in cell cycle progression [Yang et al., 2001]. Another farnesylated protein involved in cell cycle progression is CENP-E,F [Ashar et al., 2000]. In this article, we describe these developments.

FTIs AND THEIR EFFECTS ON CANCER CELLS

Recent development of FTIs has provided a valuable tool to examine the consequences of inhibiting protein farnesylation in mammalian cells. These small molecular weight compounds are developed as anti-cancer drugs [Sattler and Tamanoi, 1996; Gibbs and Oliff, 1997; Tamanoi et al., 2001]. A number of different approaches, including the use of substrate analogues, screening of natural compounds, and screening of chemical libraries, led to the identification of FTI compounds [Sattler and Tamanoi, 1996; Gibbs and Oliff, 1997; Tamanoi et al., 2001]. Two compounds, SCH66336 and R115777, currently used in clinical trials are shown in Figure 1. In preclinical studies using transgenic mice and xenografts, FTIs showed the ability to inhibit the growth of tumors or even cause regression of tumors [Kohl et al., 1995]. Cell cycle changes and apoptosis induction accompanied these effects [Barrington et al., 1998]. Importantly, no major abnormalities were observed with mice treated with FTI [Kohl et al., 1995]. The success of the preclinical studies led to phase I clinical studies that defined maximum tolerated doses [Zujewski et al., 2000]. Currently, FTIs are being evaluated in phase II and III clinical trials.

One of the Major Effects of FTIs Is Alteration of Cell Cycle Progression

In the past, we have carried out experiments to define biological effects of FTIs using a variety of cancer cell lines. A number of different effects have been observed. First, FTIs inhibit anchorage-independent growth of a wide range of cancer cell lines. A survey of over 40 human cancer cell lines showed that more than 70% of cell lines tested were sensitive to FTI [Sepp-Lorenzino et al., 1995]. Second, FTIs cause changes in cell cycle progression [Vogt et al., 1996; Suzuki et al., 1998a; Ashar et al., 2001]. Third, FTIs are capable of inducing apoptosis when the cells are exposed to low serum conditions [Suzuki et al., 1998b].

Of the above FTI effects, cell cycle effect is of particular interest. Our recent experiments carried out with different FTI compounds showed that the extent of cell cycle effects correlate well with the degree of the inhibition of farnesylation. We have observed enrichment of G0/G1 phase cells with concomitant decrease of S-phase cells with a number of cancer cell lines. These cell lines include a prostate cancer cell line LNCaP, a breast cancer cell line MDA-MB468, and an ovarian cancer cell line PA1. Similar observations have been reported [Vogt et al., 1996; Edamatsu et al., 2000; Ashar et al., 2001].

Effects of FTIs on cell cycle related proteins have also been investigated. FTIs induce hypophosphorylation of retinoblastoma protein (pRb), a key player in the transition from the G1 to S phase [Sepp-Lorenzino and Rosen, 1998; Edamatsu et al., 2000]. Two types of proteins, cyclins and cyclin-dependent kinase inhibitors, regulate the activity of cyclin-dependent kinase that is responsible for the phosphorylation of pRb. While the effect of FTIs on cyclins is unclear, it has been consistently observed that FTIs are capable of upregulating $p21^{Waf1/Cip1}$ [Feldkamp et al., 1999; Sepp-Lorenzino and Rosen, 1998; Jiang and Tamanoi, unpublished communications]. This $p21^{Waf1/Cip1}$ upregulation appears to be at the transcriptional level, as FTIs increase expression of p21-luciferase construct [Sepp-Lorenzino and Rosen, 1998; Jiang and Tamanoi, unpublished communications]. The $p21^{Waf1/Cip1}$ activation in MCF-7 cells was

dependent on p53 [Sepp-Lorenzino and Rosen, 1998], however, p53-independent activation of $p21^{Waf1/Cip1}$ was also observed with other cell lines [Feldkamp et al., 1999].

FTIs also cause enrichment of G2/M phase cells. This was observed with cell lines such as lung cancer cell lines A-549 and Calu-1, a colon cancer cell line HCT116 and pancreatic cancer cell lines MIA PaCa-2 and Colo357. Accumulation of prophase and metaphase cells was detected with A-549 cells treated with FTI [Ashar et al., 2000; Crespo et al., 2001]. Examination of M-phase chromosomes in these cells revealed an unusual configuration in which microtubules radiated from the center (termed rosetta configuration) [Crespo et al., 2001]. Effects of FTIs on M-phase progression is of particular interest in light of the observations that FTIs synergize with microtubule disrupting agents [Moasser et al., 1998; Suzuki et al., 1998a]. FTI synergized with vincritine to enrich G2/M phase cells in *v*-K-*ras* transformed normal rat kidney cells [Suzuki et al., 1998a]. FTI and taxol synergized to inhibit the growth of MCF-7 cells and arrested them in metaphase [Moasser et al., 1998].

Characterization of the FTI Effects Points to the Involvement of Multiple Farnesylated Proteins

Another key observation concerning FTIs is that not all the FTI effects could be explained by the inhibition of Ras farnesylation. While the effects of FTIs on cells with H-*ras* activation were largely due to the inhibition of farnesylation of H-Ras protein [Feldkamp et al., 2001], FTI effects observed with cells harboring K-*ras* activation or with wild type *ras* appear not to involve Ras proteins. This was somewhat surprising, since FTIs were developed with the idea to inhibit Ras protein, an oncoprotein activated in a wide range of human tumors. One explanation for the inability of FTIs to inhibit Ras is that there is an isoform of Ras, K-Ras4B, that is particularly resistant to the action of FTI. This isoform has a stretch of basic amino acids adjacent to the farnesylation site, and this polybasic domain promotes alternative modification by the addition of a geranylgeranyl group [Whyte et al., 1997]. It is worth pointing out that the effects of FTI to enrich G2/M phase cells were revealed using cell lines harboring K-*ras* activation. Thus, it is likely that this effect does not involve Ras protein. The FTI-induced expression of $p21^{Waf1/Cip1}$ is also unlikely to be due to the inhibition of Ras, as Ras functions to stimulate $p21^{Waf1/Cip1}$ expression. These observations point to the involvement of farnesylated proteins other than Ras.

RECENT PROGRESS IN THE IDENTIFICATION OF FARNESYLATED PROTEINS

Significant progress has been made in identifying and characterizing farnesylated proteins. These proteins were identified by the presence of the CaaX motif at their C-termini. As mentioned before, this motif is recognized by FTase. Farnesylation was confirmed by labeling these proteins with radioactive mevalonic acid or by their ability to serve as substrates of FTase. A list of some representative farnesylated proteins identified in mammalian cells is shown in Table I [for details see, Tamanoi et al., 2001]. Of these, we will focus on the Ras superfamily G-proteins and CENP-E,F, as they are implicated in cell cycle.

Ras and Rho Proteins

Involvement of Ras and Rho proteins in cell cycle progression has been extensively documented [Pruitt and Der, 2001]. Analysis of these proteins in the regulation of $p21^{Waf1/Cip1}$ expression in fibroblasts showed that Ras activates $p21^{Waf1/Cip1}$ expression, while Rho functions to inhibit the expression of $p21^{Waf1/Cip1}$. The involvement of Rho proteins in the expression of $p21^{Waf1/Cip1}$ is of particular interest, as a number of farnesylated Rho proteins have recently been identified. As shown in Table I, RhoB, RhoD, RhoE, Rho6, Rho7, and TC10 are farnesylated. RhoB ends with an unusual CaaX motif, CKVL, and could exist as either a farnesylated or a geranylgeranylated form [Lebowitz and Prendergast, 1998]. On the other hand, RhoD, RhoE, Rho6, Rho7, and TC10 end with a typical CaaX motif and RhoE was shown to be exclusively farnesylated [reviewed in Tamanoi et al., 2001]. RhoB, RhoD, and RhoE are ubiquitously expressed, while Rho6, Rho7, and TC10 appear to be expressed in a tissue specific manner. It has been reported that FTI causes RhoB to be exclusively geranylgeranylated and that the expression of a geranylgeranylated form of RhoB causes induction of $p21^{Waf1/Cip1}$ [Lebowitz and Prendergast, 1998]. It will be interesting to examine whether other farnesylated Rho proteins are also involved in the regulation of $p21^{Waf1/Cip1}$.

TABLE I. Farnesylated Proteins in Mammalian Cell

Protein	C-terminal sequence	Function	Localization
H-Ras	CLVS	Proliferation, differentiation	Plasma membrane
N-Ras	CVVM	Proliferation, differentiation	Plasma membrane
K-Ras	CVIM	Proliferation, differentiation	Plasma membrane
Rheb	CSVM	Cell cycle (G1/S)?	Plasma membrane
Rap2A	CNIQ		Golgi
RhoB	CKVL	Endocytosis, transcriptional regulation, and apoptosis	Endosomes
RhoD	CVVT	Cytoskeleton and endocytosis	Endosomes, plasma membrane
Rho6/Rnd1	CSIM	Cytoskeleton and adhesion	Adhesive junction
Rho7/Rnd2	CNLM	Cytoskeleton	
RhoE/Rho8/Rnd3	CTVM	Cytoskeleton and adhesion	Plasma membrane, intracellular structure
TC10	CLIT	Cytoskeleton and transformation	Cell membrane
CENP-E	CKTQ	Cell cycle (M), Centromere binding	Centromere
CENP-F	CKVQ		Centromere
PTPCAAX1/hPRL1	CCIQ	Tyrosine phosphatase	Endosomes, plasma membrane
PTPCAAX2/hPRL2/OV-1	CCVQ		Endosomes, plasma membrane
hPRL3	CCVM		Endosomes, plasma membrane
$InsP_3$ 5-phosph.I	CVVQ	Inositol signaling	Plasma membrane
$InsP_3$ 5-phosph.IV	CSVS		Golgi
Hdj2	CQTS	Molecular chaperone	Cytosol
Nuclear lamin A	CSIM	Nuclear lamins	Nucleus
Nuclear lamin B	CAIM	Nuclear lamins	Nucleus

Rheb GTPase

Rheb, a novel member of the Ras-superfamily G-proteins, was initially identified as a GTPase whose expression is increased in rat hippocampal granule cells in response to maximal electoconvulsive seizures and *N*-methyl-D-aspartic acid-dependent synaptic activity in the long-term potentiation paradigm [Yamagata et al., 1994]. Other treatments such as epidermal growth factor and fibroblast growth factor stimulation also upregulate Rheb expression [Yamagata et al., 1994]. Subsequently, a human homologue of Rheb was identified and was shown to be ubiquitously expressed [Gromov et al., 1995]. Elevated expression of Rheb in transformed cells has been reported [Gromov et al., 1995]. Rheb is localized in the plasma membrane with an enrichment of expression in apparent membrane ruffles [Clark et al., 1997]. This Rheb localization was similar to that of H-Ras and distinct from that of K-Rev. FTI decreases membrane association and increases the amount of Rheb in the cytosol [Clark et al., 1997].

Rheb interacts with Raf-1 as demonstrated by the use of the yeast two-hybrid assay and by GST-pull down experiments [Gromov et al., 1995; Yee and Worley, 1997]. The interaction occurs at the N-terminal region of Raf-1. Yee and Worley [1997] showed that Raf-1 phosphorylated by protein kinase A preferentially interacts with Rheb, as treatments that increase cAMP level led to increased binding of Raf-1 to Rheb in PC12 cells [Yee and Worley, 1997]. In addition, Rheb synergized with Raf-1 to transform NIH3T3 cells [Yee and Worley, 1997]. However, Clark et al. [1997] reported that Rheb antagonizes the action of Ras to transform cells and to activate MAP kinase in a *Xenopus* oocyte lysate system. Further investigation is needed to understand how Rheb affects Raf-1.

We have identified Rheb homologues in a variety of organisms including two yeasts (*S. cerevisiae* and *S. pombe*), fruit fly, zebra fish, and sea squirt [Urano et al., 2000]. Comparison of their sequences revealed unique features shared by the members of this family. First, they contain a conserved arginine residue at the position corresponding to the 12th amino acid of Ras. This residue in Ras is involved in GTP hydrolysis and replacement of this residue with an amino acid other than glycine or proline leads to a decrease in the intrinsic GTPase activity and increased resistance to GTPase activating proteins. The presence of arginine in Rheb at this position may suggest that Rheb GTPase is regulated by a mechanism different from that used by Ras. Second, they share similar effector domain sequences. A consensus sequence of FV(E/D)SY(Y/D)PTIEN(E/T)F(T/S/N)(R/K) is present at this position. Effector domain is a region of Ras-superfamily G-protein

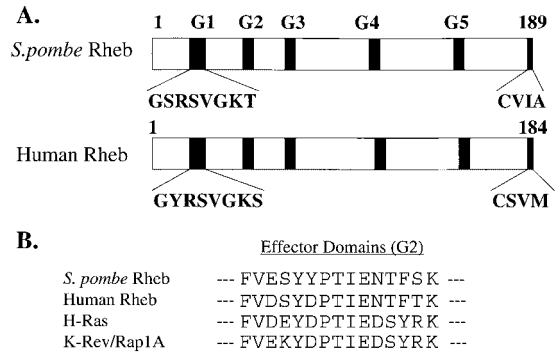

Fig. 2. Structure of *S. pombe* and human Rheb proteins. **A**: Comparison of *S. pombe* and human Rheb proteins. The G boxes (G1–5) are indicated. Note that both proteins contain arginine in the G1 box and carry the CaaX motif at their C-termini. **B**: Similarity in the effector domain (G2) sequence among the two Rheb proteins, H-Ras and K-Rev proteins.

where the interaction with its downstream effector molecules takes place. Finally, they all end with the CaaX motif, suggesting that they are farnesylated. Comparison of the structures of *S. pombe* and human Rheb proteins is shown in Figure 2.

Characterization of Rheb in *S. pombe* points to its involvement in cell cycle progression at the G0/G1 phase [Yang et al., 2001]. Rheb is an essential gene in *S. pombe*, as no haploid cells with *rheb* disruption could be obtained. Conditionally shutting down *rheb* expression by the use of the *nmt1* promoter in haploid cells showed that inhibition of the Rheb function leads to cell cycle arrest at the G0/G1 phase. Cells with a DNA content of 1 N accumulated. Morphology of the accumulated cells resembled those of cells after nitrogen starvation [Mach et al., 2000]. In fact, inhibition of *rheb* expression led to the expression of *fnx1* and *mei2*, two genes whose expression is stimulated by nitrogen starvation [Mach et al., 2000]. The *fnx1* gene encodes a protein with sequence similarity to the proton-driven plasma membrane transporters from the multidrug resistance group of the major facilitator superfamily of proteins. These results suggest that Rheb plays a critical role at a checkpoint that determines cell cycle progression or to move into the G0 phase. The function of Rheb to maintain cell cycle progression requires its farnesylation, as a mutant form of Rheb defective in farnesylation was incapable of supporting this function [Yang et al., 2001]. Furthermore, *S. pombe* mutant defective in FTase exhibited enrichment of G0/G1 phase cells and this phenotype was complemented by the expression of a mutant form of Rheb that could bypass farnesylation [Yang et al., 2001].

The study on *S. pombe* Rheb raises the possibility that mammalian Rheb plays a role in cell cycle regulation. In support of this idea, we have shown that human Rheb can replace the function of *S. pombe* Rheb [Yang et al., 2001]. However, characterization of Rheb in mammalian cells is needed to investigate this point. It will also be interesting to examine whether mammalian Rheb protein plays a role in the effects of FTI to enrich cells in the G1 phase of the cell cycle.

CENP-E,F Proteins

CENP-E,F proteins have been identified from the survey of proteins ending with the CaaX motif [Ashar et al., 2000]. This was carried out using the FINDPATTERNS computer program. Although their CaaX motif is somewhat unusual (CKTQ for CENP-E and CKVQ for CENP-F), farnesylation of these proteins was established using labeling with radioactive mevalonic acid. These proteins function as centromere-associated kinesin motor and play critical roles in the progression of cells during the M-phase of the cell cycle [Abrieu et al., 2000]. CENP-E,F proteins contain two microtubule binding motifs, one at the N-terminus and the other at the C-terminus. FTI inhibits farnesylation of these proteins and inhibits their association with microtubules [Ashar et al., 2000]. On the other hand, FTI does not alter the association of these proteins with the centromere. As mentioned above, FTIs cause accumulation of G2/M phase cells with cell lines such as a lung cancer cell line A-549 [Ashar et al., 2000; Crespo et al., 2001]. CENP-E,F proteins may be implicated in these FTI effects.

CONCLUSION AND FUTURE PROSPECTS

Farnesylated proteins play important roles in cell cycle progression. Ras and Rho proteins are involved in the regulation of cyclins and cyclin-dependent kinase inhibitors. Recent studies added another Ras-superfamily G-proteins, Rheb, as a potential candidate involved in cell cycle. This protein may affect the Ras signaling pathway, as Rheb is found to bind Raf-1. Further experiments, however, are needed to gain insight into a potential interplay between Ras and Rheb. Insights into the

mechanism of the action of Rheb may also be obtained by exploiting the genetically amenable *S. pombe* system. Other interesting farnesylated proteins that are involved in cell cycle progression are CENP-E,F, centromere-associated kinesin motors required for progression through the M-phase. Characterization of these CENP proteins may provide novel insight into how farnesylation could influence cell cycle progression. In the future, we may see yet more farnesylated proteins involved in cell cycle. Currently, a genome-wide search for the proteins ending with the CaaX motif is ongoing, and this may yield additional farnesylated proteins. Further characterization of these proteins may lead to a deeper understanding of the significance of farnesylation in cell cycle progression.

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