Journal of Cellular Biochemistry Supplement 27:12–19 (1997)

Advances in the Development of Farnesyltransferase Inhibitors: Substrate Recognition by Protein Farnesyltransferase

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Abstract A variety of compounds that show promise in cancer chemotherapy and chemoprevention have been identified as farnesyltransferase inhibitors. These can be classified into mainly two different types of inhibitors, farnesyl diphosphate competitors and CAAX peptidomimetics. The former type acts by competitively inhibiting farnesyltransferase with respect to one of the substrates, farnesyl diphosphate, whereas the latter type acts by mimicking the other substrate, the C-terminal CAAX motif of Ras protein. One example of a farnesyl diphosphate competitor is manumycin, an antibiotic detected in the culture media of a *Streptomyces* strain. The CAAX peptidomimetics were developed based on the unique property of farnesyltransferase to recognize the CAAX motif at the C-terminus of the protein substrate. Our recent studies have focused on understanding the structural basis of this CAAX recognition. By using in vitro mutagenesis, residues of yeast farnesyltransferase important for the recognition of the CAAX motif have been identified. Two of these residues are closely located at the C-terminal region of the β-subunit of farnesyltransferase. These and other results on the structural basis of the CAAX recognition may provide information valuable for structure-based design of farnesyltransferase inhibitors. J. Cell. Biochem. Suppl. 27:12–19. © 1998 Wiley-Liss, Inc.†

Key words: farnesyltransferase; *ras;* manumycin; peptidomimetics; mutational analysis; CAAX recognition

Recent studies on Ras led to the identification of a variety of compounds called farnesyltransferase (FTase) inhibitors [1,2]. These compounds block membrane association of Ras proteins, which play important roles in human tumor formation. Mutations of Ras ,which result in constitutive activation of the protein, have been identified in a wide range of human tumors [3]. However, the mutant Ras cannot transform cells if its membrane association is blocked. Some of these compounds inhibit anchorage-independent growth of *ras*-transformed cells while showing little effect on the growth of untransformed cells. These results raise the possibility that FTase inhibitors are effective in cancer chemotherapy and chemoprevention.

The membrane association of Ras is catalyzed by a series of post-translational modifications that occurs at a conserved motif found at the C-terminus of Ras [3–5]. This sequence is called the CAAX motif (cysteine, aliphatic amino acid, aliphatic amino acid, and the C-terminal amino acid which is usually methionine, alanine, cysteine, glutamine, or serine). The modification events include addition of a farnesyl group to the cysteine in the CAAX motif, removal of the three C-terminal amino acids, and carboxyl methylation of the exposed cysteine. Ras proteins, except K-rasB, undergo further modification by the addition of a palmitoyl group. These subsequent modification events are inhibited when farnesylation is blocked, leading to the accumulation of unmodified Ras proteins in the cytosol.

FTase, which catalyzes the farnesylation of Ras, is a heterodimeric enzyme consisting of α and β subunits [1,6]. This enzyme recognizes the C-terminal CAAX motif of Ras protein and

Contract grant sponsor: NIH, contract grant number: CA41996; Contract grant sponsor: Edwin W. Pauley Foundation; Contract grant sponsor: USPHS; Contract grant number: GM07185.

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Received 6 January 1997; Accepted 1 December 1997

transfers a farnesyl group from farnesyl diphosphate (FPP) to the cysteine in the CAAX motif. The enzyme is found in a wide variety of organisms, and the structure and function of the enzymes from human to yeast are very similar. FTase belongs to the protein prenyltransferase family which also includes geranyl-geranyl transferases (GGTases) I and II. GGTase I modifies proteins ending with the CAAL motif (similar to the CAAX motif except that the C-terminal amino acid is predominantly leucine or phenylalanine) with a geranylgeranyl group. GGTase II modifies proteins ending with CysCys or CysXCys with geranylgeranyl groups. GGTase I substrates include RhoA and Cdc42 proteins, whereas GGTase II substrates include Rab family of G-proteins.

In this paper, we first review the development of a wide variety of compounds that act as FTase inhibitors. Then we discuss our recent study to understand substrate recognition by the FTase enzyme. These structurally based studies may provide additional information for the rational design and development of FTase inhibitors.

FARNESYLTRANSFERASE INHIBITORS

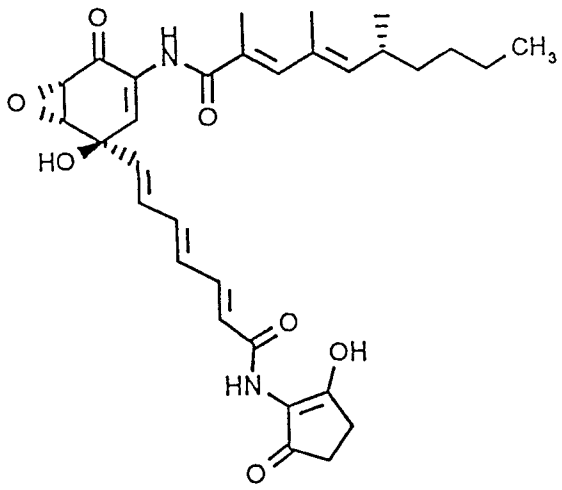
More than sixty compounds have been identified over the years as FTase inhibitors [1]. Some of these compounds are shown in Figure 1. Manumycin was identified by a microbial screen that was developed in 1990 [7,8]. This screen was based on the growth inhibitory activity of the $G_{\beta\gamma}$-complex of yeast heterotrimeric G-protein. Cells with the Gα-subunit disrupted do not grow, since the $G_{\beta\gamma}$-complex is free to exert its growth-inhibitory effect. This inhibition depends on the farnesylation of the Gγ-subunit, Ste18. Thus, inhibition of the prenylation of Ste18 rescues the cells from the growth inhibition. A simple microbial screen based on the above principle enabled the screen of culture media from more than 20,000 organisms. This screen identified manumycin, an antibiotic with long hydrophobic side chains (Fig. 1). Manumycin inhibits FTase with a Ki of 1.2 μM. It is a competitive inhibitor of FTase with respect to one of the substrates, FPP. This is in agreement with the results pointing to the importance of a long side chain for FTase inhibition. Manumycin A, which has a longer side chain than manumycins B and C, exhibits the strongest inhibition among the three. A different screen utilizing an in vitro FTase assay identified tricyclic compound SCH44342 [9]. Results of kinetic analysis are consistent with the idea that this compound acts as a competitive inhibitor of FTase with respect to the protein substrate.

Protein substrates of FTase contain a sequence called the CAAX motif. As discussed before, this motif is recognized by farnesyltransferase, which transfers a farnesyl group to the cysteine within the CAAX motif, forming a thioether linkage. Development of peptidomimetic inhibitors was initiated upon discovering that FTase activity can be inhibited by a tetrapeptide having the CAAX motif [10]. This was followed by the finding that introduction of an aromatic residue such as phenylalanine at the second "A" position of the CAAX tetrapeptide destroys the ability of the peptide to serve as a substrate while maintaining its ability to inhibit FTase reaction [11]. The tetrapeptides, however, need to be further modified to make them resistant to proteases and to enable them to cross the membrane barrier. Approaches taken to overcome these difficulties include reduction of peptide bonds, esterification of the C-terminal carboxylate, and replacement of the central peptide moiety with groups such as benzodiazepine groups or aromatic ring systems [12–15]. Structures of three different peptidomimetics, L739,749, B581, and FTI-276, are shown in Figure 1.

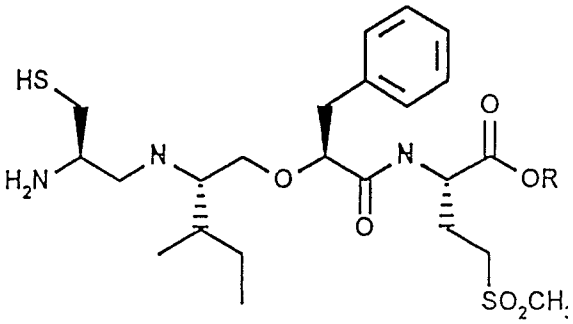
BIOLOGICAL ACTIVITIES OF FARNESYLTRANSFERASE INHIBITORS

Biological activities of manumycin have been investigated in three different systems: the yeast, *C. elegan,* and mammalian cell lines. In yeast, *ras* activation leads to heat shock sensitivity. However; such yeast cells exhibit heat shock resistance when they are grown in the presence of manumycin [16]. In *C. elegans, ras* activation leads to multivulva phenotypes. These phenotypes are suppressed by the addition of manumycin [17]. Finally, manumycin has been shown to inhibit the growth of human colon carcinoma cells, LoVo and hepatoma cell lines, Mahlavu and PLC/PRF/5 [18]. Ras farnesylation in these cells was blocked at 15 μM concentration. Manumycin also inhibited the growth of *ras*-induced tumors transplanted into mice with the T/C value of 0.37 with 6.3 mg/kg dose [7].

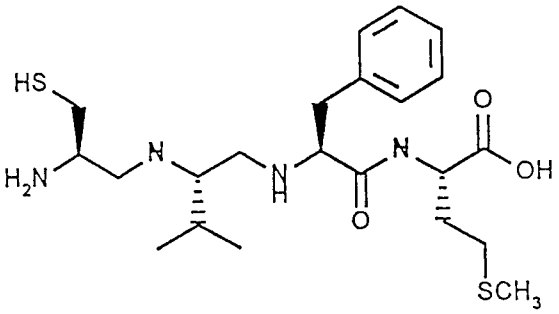
SCH44342 exhibits the ability to cause morphological reversion of cells transformed with H-ras but not with a geranylgeranylated Ras



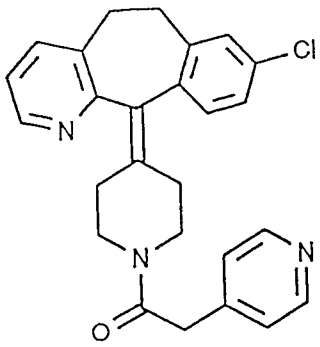
Manumycin A



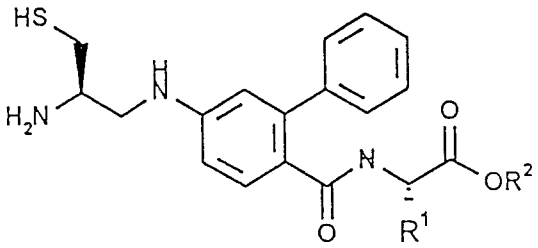
L-739,749 (R=CH_3)



B581



SCH44342



FTI-276 (R^1=CH_2SCH_3, R^2=H)

Fig. 1. Structure of some representative FTase inhibitors.

[9]. Furthermore, this compound does not interfere with PDGF activation of MAP kinase in NIH3T3 cells, suggesting that its effect on untransformed cells is minimal.

Peptidomimetics exhibit remarkable biological activities towards *ras*-transformed cells [12–15, 19–24]. These compounds inhibit Ras processing *in vivo* with concentrations around 1 μM, and inhibit growth of *ras*-transformed cells in soft agar with 1–50 μM concentrations. They also cause morphological reversion of *ras*-transformed cells. K-*ras* transformed cells are relatively resistant to FTase inhibitors and require higher concentrations. Inhibition of the

growth of tumors transplanted in mice is reported with H-ras-transformed NIH3T3 and Rat1 cells. In the case of L744,832, a derivative of L739,749, regression of activated H-*ras*-induced mammary tumors in mice was achieved [24]. The effect was transient, and removal of the compound resulted in the reappearance of tumors, suggesting that the peptidomimetics show reversible effects.

A remarkable feature of the peptidomimetics is their lack of effect on untransformed cells. This is evident from two types of observations [12–15,19–24]. First, untransformed fibroblasts continued to grow in the presence of inhibitors. Second, no abnormalities were detected with animals treated with the peptidomimetics. This was unexpected, since wild-type Ras proteins also require farnesylation for their function. One possible reason why FTase inhibitors exhibit specific effects on transformed cells is that K-rasB and N-ras can be geranylgeranylated in the presence of the inhibitors. Another possibility is that, in transformed cells, unprocessed mutant Ras proteins complexed with their downstream target, Raf, accumulate in the cytosol, and act to exert a dominant inhibitory effect. Such a complex does not accumulate in untransformed cells, because Ras exists mainly in a GDP bound form and the complex readily dissociates.

Perhaps the most surprising observation concerning FTase inhibitors is the lack of correlation between the state of *ras* mutation and the sensitivity to FTase inhibitors. A large number of cancer cell lines have been examined for their sensitivity to the FTase inhibitor, L-744,832 [25]. While the soft agar growth of over 70% of all tumor cell lines tested was inhibited by the inhibitor, the response to the drug was observed irrespective of whether the cell contained mutated *ras*. This, together with other observations that biological effects of the inhibitors were seen at concentrations of inhibitors not sufficient to inhibit modification and membrane association of K-rasB protein, and the time course of biological effects of the FTase inhibitors did not correlate with the time course of the inhibition of Ras modification, led to the idea that FTase inhibitors may reverse cellular transformation by preventing farnesylation of another target(s) (target X) required for tumorigenesis [26–28]. Several proteins for this target X have been proposed. One is the Rho-family of small GTP binding proteins, which control cell morphology. It has been shown that FTase inhibitors block farnesylation of RhoB both in vivo and in vitro and that the inhibitors suppress RhoB-dependent cell growth [29]. In addition, RhoD, which controls actin cytoskeleton and endocytosis, contains the CAAX motif [30], and RhoE is farnesylated [31]. Other candidates for target X include another G-protein, Rheb [32]; a farnesylated tyrosine phosphatase, PRL-1 [33]; and type I inositol phosphate 3,5-phosphatase [34]. Work is in progress to investigate the extent of activity of FTase inhibitors on these target X candidates.

STRUCTURAL BASIS OF THE CAAX RECOGNITION BY FTASE

As described above, studies on FTase led to the development of FTase inhibitors. In particular, understanding the unique property of FTase to recognize the CAAX motif was critical in the development of peptidomimetics. To further understand the structural basis of this recognition, we sought to identify residues of FTase involved in the recognition of the CAAX motif. The approach we developed was to mutagenize FTase and alter its ability to recognize the CAAX motif. We took advantage of the fact that GGTase I, which is closely related to FTase, recognizes a similar but distinct motif, the CAAL box. The difference between the CAAX motif and the CAAL motif is in the C-terminal amino acid. (The C-terminal residue of the CAAX motif is predominantly serine, methionine, cysteine, alanine, or glutamine, whereas the C-terminal residue of the CAAL motif is leucine or phenylalanine.) We also took advantage of the fact that FTase and GGTase I share the same α-subunit and their β-subunits are approximately 30% homologous, and hypothesized that we could mutagenize the β-subunit of FTase to identify mutant β subunits that could function as a GGTase Iβ. As shown in Figure 2, we have been able to obtain mutant FTase β which can recognize the CAAL motif. Although these FTase mutants transfer a farnesyl group to GGTase I substrates, studies in yeast [35] suggest that the farnesylated GGTase I substrates are functional in vivo.

We used the yeast system for this analysis, since this system provides a powerful genetic screen [36,37]. Yeast FTase consists of Ram2 and Dpr1/Ram1 as α- and β-subunits, respectively. The yeast GGTase I, on the other hand,

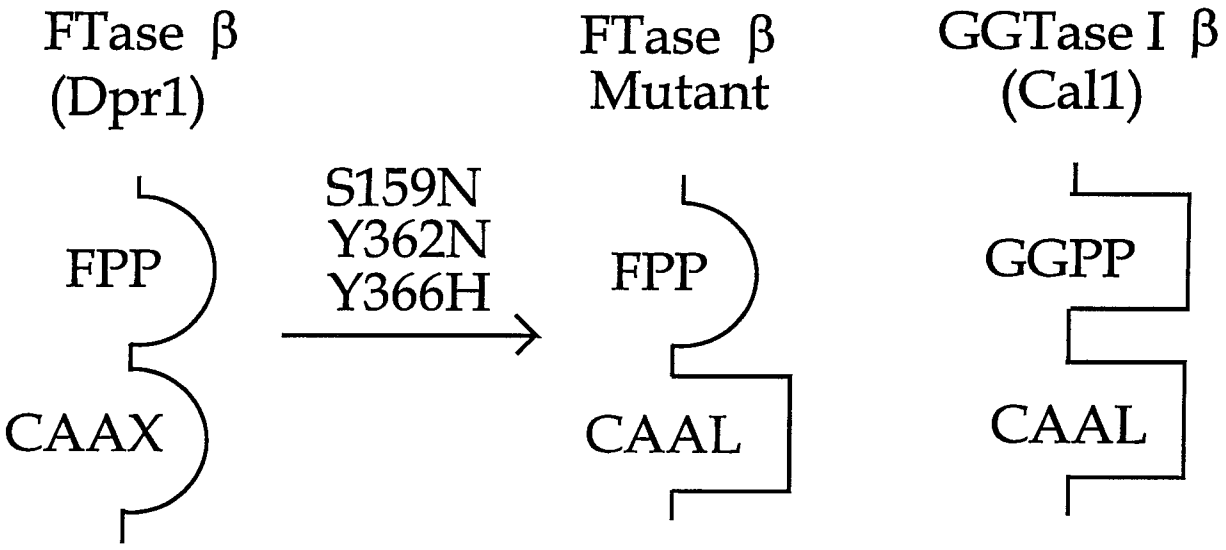


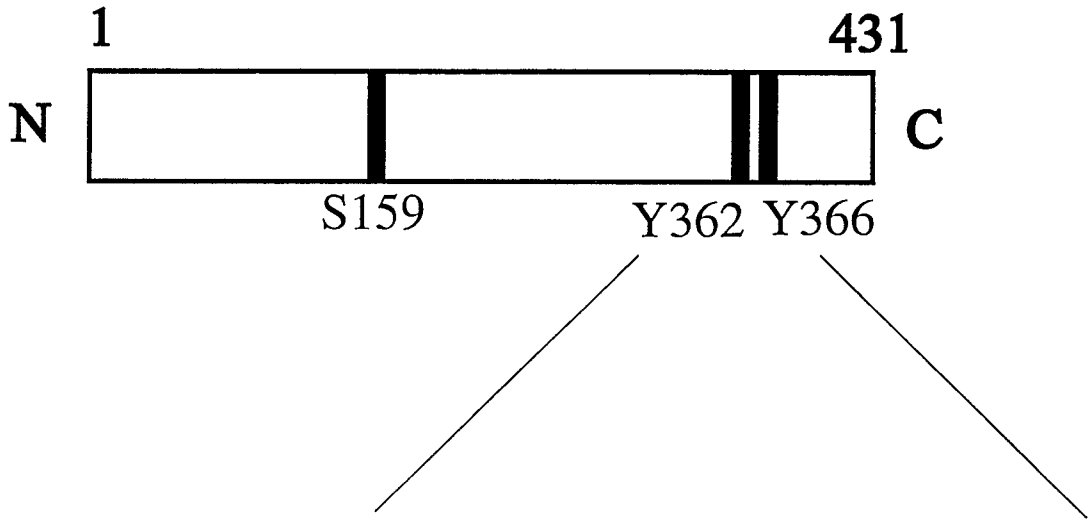
Fig. 2. Conversion of the CAAX recognition of FTase by single amino acid changes.

consists of Ram2 and Cal1/Cdc43 subunits. A temperature sensitive mutant *cal1* carries a mutation in the β-subunit of GGTase I. Failure to modify GGTase I substrates such as Rho1 and Cdc42 at the non-permissive temperature results in temperature sensitive growth. The *DPR1* gene was randomly mutagenized by PCR and introduced into the *cal1* strain to screen for mutant *DPR1* genes that could suppress the temperature sensitive growth of the *cal1*. Extensive screening led to the identification of three residues of Dpr1 (S159, Y362, Y366) involved in recognition of protein substrates (Fig. 2) [36,37]. A single amino acid change in any one of these residues is sufficient to suppress the temperature sensitive growth of the *cal1* strain. Characterization of purified mutant proteins revealed that the mutant enzymes have increased affinity for the CAAL motif and are capable of farnesylating the CAAL-containing substrate proteins. The kcat/Km value for the CAAL motif was about 20-fold higher for the S159N mutant than the wild type, while the kcat/Km value for the CAAX motif was 3-fold lower for the mutant than the wild-type enzyme.

Further insight into the role of these residues in recognition of the CAAX motif was obtained when residues 159 and 366 were each changed to all possible amino acids [37]. With residue 159, asparagine and aspartic acid mutants were the only amino acids which could suppress the temperature sensitivity of the *cal1* strain. The aspartic acid mutant was less efficient than the asparagine mutant. In the case of residue 362, leucine, methionine, and isoleucine were the only amino acids that showed efficient suppression of the *cal1* mutant. It is interesting that asparagine and aspartic acid have similar van der Waals volume, and leucine, methionine, and isoleucine have identical van der Waals volume. These results suggest that an introduction of a particular size residue at this site converts CAAX recognition to that of CAAL recognition. In the case of residue 362, the hydrophobic character of leucine, methionine, and isoleucine may also be important in the recognition of the CAAL motif.

While S159 residue is located at the N-terminal portion of the protein molecule, the other two residues are closely located in the C-terminal region of the molecule (Fig. 3). The sequence of the C-terminal region encompassing residues 362 and 366 is shown in Figure 3, along with the sequence of corresponding regions in FTases and GGTases from a variety of species. It is interesting that the two tyrosine residues, Y362 and Y366, are conserved in all FTase molecules. In addition, they are located exactly four residues apart in all FTase subunits. Furthermore, the sequence surrounding these residues is highly conserved in FTase proteins but is different from the corresponding sequence of GGTase I. Such sequence conservation is consistent with its possible involvement in the CAAX recognition.

Recently, the three-dimensional structure of Rat FTase has been determined [38]. Both the



FTase β	S.c. (Dpr1)	350	GLRDKPGAHSDF**Y**HTN**Y**CLLGLAVAES
	Human	349	GLLDKPGKSRDF**Y**HTC**Y**CLSGLSIAQH
	Bovine	349	GLLDKPGKSRDF**Y**HTC**Y**CLSGLSIAQH
	Rat	349	GLLDKPGKSRDF**Y**HTC**Y**CLSGLSIAQH
	Pea	346	GLRDKPGKRRDH**Y**HSC**Y**CLSGLSLCQY
GGTase-Iβ	S.c. (Cal1)	328	GFSKNDEEDADL**Y**HSC**L**GSAALALIEG
	Human	308	GFAKWPDSHPDA**L**HAY**F**GICGLSLMEE
	Rat	308	GFAKWPDSHPDA**L**HAY**F**GICGLSLMEE
	Drosophila	298	GFAKWPQATPDP**F**HTY**L**GLCGLAFTGE
	S. pombe	299	GFSKTPGEFPDV**L**HSA**L**GLYAMAYQDD

Fig. 3. Amino acid residues of FTase β involved in the recognition of the CAAX motif. Dpr1 protein consisting of 431 amino acids is at the top.

α and β-subunits are largely composed of α-helices. While the β-subunit forms a barrel-like structure, the α-subunit has an overall crescent-shaped structure that envelops part of the β-subunit. The exact position of the CAAX binding site has not been determined from this structural study, since co-crystals with the substrate protein have not yet been obtained. However, a speculation has been made based on the binding of a peptide with a structure similar to that of the CAAX peptide [38]. This speculation agrees with our assignment of residues 361 (corresponds to residue 362 of the yeast enzyme) and 365 (corresponds to residue 366 of the yeast enzyme) as key residues involved in the recognition of the CAAX box, since these residues are located close to the predicted CAAX binding site as well as to the position of the catalytic zinc atom.

FUTURE PROSPECTS

As described in this paper, a variety of FTase inhibitors have been developed and may provide valuable tools in cancer chemotherapy and chemoprevention. Further characterization of these compounds should provide information concerning their effects on tumor growth and formation. In addition, studies on the target of these compounds, FTase, may lead to further development of FTase inhibitors. Our mutational analysis focused on CAAX recognition by this enzyme. This analysis led to the identification of amino acid residues involved in CAAX recognition. Photo-affinity labeling studies using derivatives of the CAAX peptide may also provide information on other residues that may be involved in substrate recognition. A specific region, Asp110-Arg112, of the α-subunit of Rat

FTase, has been involved in the recognition of the CAAX motif [39]. Furthermore, Zn^{2+} appears to play an important role in the recognition of the CAAX motif, since the replacement of Zn^{2+} with Cd^{2+} leads to an alteration of CAAX recognition [40]. Finally, mutational analyses in the predicted substrate binding pocket of FTase should enable us to gain a clearer understanding of the structural basis of CAAX recognition. The information obtained from these studies should be valuable in improving the designing of structure-based inhibitors of FTase.

ACKNOWLEDGMENTS

We thank Drs. Isabel Sattler, Patrick Poullet, and Dominique Dorin for discussions. This work is supported by NIH grant CA41996. W.Y. is supported by the Edwin W. Pauley Foundation and J.U. is supported by USPHS National Research Service Award GM07185.

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