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In Vitro Prenylation of the Small GTPase Rac13 of Cotton[1]

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Previous work (D.P. Delmer, J. Pear, A. Andrawis, D. Stalker [1995] Mol Gen Genet 248: 43–51) has identified a gene in cotton (*Gossypium hirsutum*), *Rac13*, that encodes a small, signal-transducing GTPase and shows high expression in the fiber at the time of transition from primary to secondary wall synthesis. Since *Rac13* may be important in signal transduction pathway(s), regulating the onset of fiber secondary wall synthesis, we continue to characterize *Rac13* by determining its ability to undergo posttranslational modification. In animals Rac proteins contain the C-terminal consensus sequence CaaL (where "a" can be any aliphatic residue), which is a site for geranylgeranylation (B.T. Kinsella, R.A. Erdman, W.A. Maltese [1994] J Biol Chem 266: 9786–9794). We have identified activities in developing cotton fibers that resemble in specificity the geranylgeranyl- and farnesyltransferases of animals and yeast. In addition, using prenyltransferases from rabbit reticulocytes, we show that *Rac13*, having a C-terminal sequence of CAFL, can serve as an in vitro substrate for geranylgeranylation but not farnesylation. However, the presence of the uncommon penultimate F residue appears to slow the rate of prenylation considerably compared with other acceptors.

A number of genes that encode homologs of the *Ras* superfamily of small, signal-transducing GTPases have been identified in plants (Terryn et al., 1993; Verma et al., 1994; Haizel et al., 1995; Loraine et al., 1996). Genes encoding homologs of the *Rho* subfamily have recently been identified in pea (Yang and Watson, 1993; Lin et al., 1996) and in cotton (*Gossypium hirsutum*; Delmer et al., 1995). Rac, Rho, and CDC42 members of this *Rho* subfamily have been implicated in playing several diverse roles, including regulation of actin organization in animals and yeast (Hall, 1994; Nobes and Hall, 1995; Takai et al., 1995; Johnson and Pringle, 1996), regulation of the NADPH oxidase, which is involved in the oxidative burst of leukocytes (Diekmann et al., 1994), or activation of the yeast 1,3-β-glucan synthase (Diaz et al., 1993; Mol et al., 1994; Drgonova et al., 1996; Qadota et al., 1996).

Of the two genes, *Rac9* and *Rac13*, encoding homologs of Rac that we have recently identified in cotton, *Rac13* shows very high and relatively specific expression in the fibers of cotton at the stage of transition from primary to secondary wall synthesis (Delmer et al., 1995). This transition to secondary wall synthesis is characterized by several features: (a) a reorganization of the cytoskeleton and a concomitant shift in the pattern of cellulose deposition (Seagull, 1990); (b) a transient deposition of callose (1,3-β-glucan; Maltby et al., 1979; Basra and Malik, 1984) that is presumably initiated by a transient change in the level of free Ca^{2+} similar to that observed during the onset of secondary wall synthesis in differentiating tracheary elements (Fukuda, 1991, 1996); (c) a greater than 100-fold increase in the rate of cellulose deposition (Meinert and Delmer, 1977); and (d) initiation of synthesis of a unique hemicellulose, as well as lignin, in most secondary walls (cotton being a notable exception). Many of these events (e.g. elevation of cytoplasmic Ca^{2+}, induction of callose, and lignin synthesis) suggest the operation of a signal transduction pathway that bears some similarity to that elicited during pathogenesis, a pathway that shows involvement of an oxidative burst (Levine et al., 1995).

Thus, by analogy with other Rac/CDC42 GTPases, and also based on its unique pattern of gene expression, it would seem likely that the *Rac13* of cotton fibers could play a role in any of the following processes related to fiber development: (a) reorganization of the cytoskeleton, (b) regulation of an oxidative burst, and/or (c) stimulation of callose (and/or cellulose?) synthase activity. For this reason, we are initiating studies that are designed to further characterize *Rac13* structure and function. Because little is known about posttranslational processing of GTPases in plants, which is very important for their localization and function, we have directed our initial attention to this area.

It is well known that most small, signal-transducing GTPases undergo posttranslational modifications that are necessary both for their interaction with effector proteins and for their relocation from the cytoplasm to cellular membranes (for reviews of these processes, see Clarke, 1992; Shafer and Rine, 1992; Glomset and Farnsworth, 1994). For the Ras type of GTPases, a carboxy-terminal consensus sequence Ca_1a_2X (where a_1 and a_2 = any aliphatic amino acid, and X may be S, C, M, Q, or A) directs these modifications. These include the farnesylation of the C residue, followed by a proteolytic cleavage of the three distal amino acids, and a subsequent methylation of the new carboxy terminus. Members of the *Rho* subfamily undergo similar modifications and contain a similar consensus sequence, but X in this case is L or occasionally F; this sequence leads to geranylgeranylation as opposed to farnesylation (Finegold et al., 1991; Kinsella et al., 1991). How-

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Abbreviations: DPA, days postanthesis; FPP, farnesyl PPi; FTase, farnesyltransferase; GGTase, geranylgeranyltransferase; GGPP, geranylgeranylpyrophosphate; GST, glutathione *S*-transferase.

ever, it should be emphasized that these specificities are not absolute, and substrates for geranylgeranylation can undergo farnesylation and vice versa, provided that the concentration of protein substrate and/or time of incubation is long enough. This is presumably because the GGTase and FTase are related enzymes and share a common subunit, and the difference in specificity results from different affinities for the various C-terminal motifs (Moores et al., 1991; Clarke, 1992; Boguski and McCormick, 1993). Geranylgeranylation also occurs with members of the *Rab/YPT* subfamily that have a rather different consensus sequence (CC, CXC, or CCXX), but this reaction utilizes a GGTase II that is distinct from the GGTase I that recognizes the Ca_1a_2L (or F) motif (Moores et al., 1991). Recently, an enzyme with specificity and characteristics similar to the mammalian GGTase II was reported in plants (Loraine et al., 1996).

Some recent studies suggest that both GGTase and FTase activities exist and function both in vitro and in vivo in plants (Randall et al., 1993; Yang et al., 1993; Zhu et al., 1993; Biermann et al., 1994; Morehead et al., 1995; Lin et al., 1996; Loraine et al., 1996), but none of these reactions has been characterized in detail. As part of our initial studies to elucidate the role of Rac proteins in plants, we have taken advantage of the ability to produce a nonprenylated recombinant form of Rac13 in *Escherichia coli* to test its effectiveness as a substrate for prenylation. The deduced amino acid sequences encoded by the *Rac9* and *Rac13* genes identified in cotton indicate that both have a C-terminal sequence CAFL (Delmer et al., 1995). This sequence resembles the Ca_1a_2L motif and suggests that the protein should undergo geranylgeranylation. However, an F residue in the a_2 position is very uncommon, being reported only one other time in the literature for the γ_5 subunit of brain trimeric G-proteins (Fukada, 1995).

A study of rat brain FTase has shown that peptides containing a bulky aromatic residue (F, Y, or W) at the a_2 position bind and act as competitive inhibitors but are not themselves prenylated (Goldstein et al., 1991). By analogy, the possibility exists that the penultimate F residue in Rac might affect its ability to undergo prenylation. However, the brain γ_5 subunit that has CSFL at the C terminus does apparently undergo geranylgeranylation in vivo (Yamane et al., 1990), but studies comparing the rate of prenylation of such a protein by GGTase I with other substrates lacking a penultimate F residue have not been reported. The studies presented here have identified GGTase and FTase activities in cotton fibers that show specificities similar to their animal and yeast counterparts. In addition, we show that Rac13 can be a substrate for geranylgeranylation by rabbit reticulocyte GGTase, but the F residue in position a_2 of Rac13 does, indeed, affect its rate of prenylation in vitro.

MATERIALS AND METHODS

Construction of *GST-Rac13* Gene Fusion; Expression and Purification of Fusion Proteins

Details of the cloning of the *Rac13* gene from cotton (*Gossypium hirsutum*) fibers were presented by Delmer et al. (1995). The full-length *Rac13* cDNA was excised from Bluescript S/K (Stratagene) by digestion with *Eco*RI that cleaved the insert in the polylinker upstream of the 5′ initiation site and downstream in the 3′ untranslated region of the gene. The insert was isolated, subjected to phosphatase treatment, re-ligated in-frame as a fusion with the GST gene in the pGEX-3T vector (Pharmacia), and transformed into *Escherichia coli* strain SURE. Correct orientation of the insert was determined by restriction digestion. Plasmids containing the genes encoding GST-CIIL and GST-CIIS in pGEX-1/RCT (Finegold et al., 1991) were obtained from Prof. F. Tamanoi (University of California, Los Angeles) and were also transformed into *E. coli* SURE.

Purification of Recombinant Proteins

Overexpression of GST-Rac13, GST-CIIL, and GST-CIIS was induced by treatment of early log-phase cultures with 0.1 mM isopropyl 1-β-D-thio-galactoside for 4 h at 37°C. Cells were harvested by centrifugation, resuspended in lysis buffer I containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 50 mM NaCl, 10 μg/mL leupeptin (Sigma), and 200 μM Pefabloc (Boehringer Mannheim), and lysed using a French press at 10,000 p.s.i. After centrifugation at 15,000 rpm for 10 min, the fusion proteins were allowed to bind for 90 min at 4°C to glutathione agarose beads (Sigma; 6 mL of a 50% suspension were used per liter of original culture). The beads were collected and washed 10 times with lysis buffer I. Fusion proteins were eluted from the beads by treatment with 20 mM reduced glutathione in the same buffer. Cleavage of GST-Rac13 bound to beads with thrombin (Sigma, 0.5 units/mL) in 50 mM Tris-HCl, pH 7.5, 2.5 mM $CaCl_2$, 5 mM MgC_2, and 100 mM NaCl was done for 1 to 2 h at 25°C, followed by a 16-h incubation at 4°C. Samples were concentrated using filters (Microcon, Amicon, 10-kD cutoff) and stored at −20°C in buffer containing leupeptin, Pefabloc, and 50% glycerol. Protein concentrations in solution were determined using the Bio-Rad protein assay reagent. Based on Coomassie blue staining of SDS-PAGE gels of these proteins, the degree of purity was assessed and calculation of the amount of pure protein relative to total protein was estimated. These values were used to calculate the molar concentrations of pure recombinant protein supplied to the in vitro prenylation reactions described below.

Preparation of Cotton Fiber High-Speed Supernatant and Membrane Fractions

Cotton (*G. hirsutum* Acala SJ-2) was grown in fields in Israel in the summer of 1994. Bolls of the ages indicated were harvested, and the locules were removed, rapidly frozen in liquid N_2, and stored at −80°C until use. For detecting prenyltransferase activities, locules of 17 DPA were used. Fibers were removed from seeds under liquid N_2 and ground to a fine powder. The powder was extracted at 4°C with lysis buffer II containing 50 mM Hepes-KOH, pH 7.5, 1 mM DTT, 5 μM leupeptin, and 20 μM Pefabloc using 1 to 2 mL buffer g^{-1} fresh weight of fibers. The extract was filtered through three layers of

Miracloth (Calbiochem) and centrifuged at 5,000*g* for 1 min, and the resulting supernatant was again centrifuged at 100,000*g* for 1 h at 4°C. To obtain good activity for prenyltransferases, it was necessary to use fresh, high-speed supernatant.

Prenylation Reactions

All reactions were carried out in a final volume of 50 μL. One microcurie of either [^{3}H]GGPP (Amersham, 16 Ci/mmol) or [^{3}H]FPP (NEN, 19.3 Ci/mmol) was evaporated under N_2 into each 1.5-mL microfuge reaction tube to remove the ethanol that was present as solvent. Untreated rabbit reticulocyte lysate (35 μL; Promega) was added along with recombinant protein in lysis buffer I at concentrations indicated in the figure legends. When a cotton high-speed supernatant was used in place of reticulocyte lysate, the reactions also contained 50 mM Hepes-KOH, pH 7.5, 5 mM DTT, and 20 mM $MgCl_2$. Reactions were incubated at 30°C for the times indicated and terminated by addition of 1 mL of ethanol:HCl (9:1, v/v). After incubation for 30 min at 25°C, the reactions were microfuged at high speed for 5 min. The pellets were washed twice in 100% ethanol, resuspended in 100 μL of SDS-PAGE sample buffer containing 0.5 M Tris-HCl, pH 6.8, 8 M urea, 2% SDS, 10% glycerol, and 5% mercaptoethanol, and heated for 5 min at 100°C. (For reactions that contained $MgCl_2$, 5 mM EDTA was also included in the sample buffer.) Samples (10 μg of protein) were subjected to SDS-PAGE as described by Laemmli (1970) using a minigel apparatus with gels of 0.75-mm thickness and acrylamide concentrations of 12.5 and 4.5% in the separating and stacking gels, respectively.

Following electrophoresis, the gels were fixed for 30 min in 50% methanol/12.5% acetic acid and then incubated for 20 min in 20% methanol/10% acetic acid, followed by 20 min in Amplify (Amersham). Gels were then dried and exposed for a minimum of 1 week to Curix RP2 film (Agfa-Gevaert, Leverkusen, Germany) to detect the pattern of prenylated proteins. Autoradiograms were either photographed directly or subjected to scanning and figure preparation using the Photoshop program (Adobe Photosystems, Mountain View, CA) with a MacIntosh computer. Replicate lanes were also run for staining with 0.02% Coomassie blue R-350 (Sigma) in 10% acetic acid and destaining in 10% acetic acid. We also attempted to develop a more quantitative assay for prenyltransferases, based on filtration and scintillation counting of precipitated labeled proteins; however, because of the low activity of the cotton fiber prenyltransferases and high zero time backgrounds using [^{3}H]GGPP as the substrate (a problem reported by others; Randall et al., 1993), this assay proved not to be sufficiently reproducible.

RESULTS

Substrates for Prenylation Reactions

We used four different GST-fusion proteins as substrates for studying patterns of prenylation. Figure 1A, lanes 1–4, show these proteins as they were purified following expression in *E. coli*. Lanes 1 and 2 show the cotton Rac13 protein, having a CAFL C-terminal sequence, purified as the 49-kD GST-fusion protein (lane 2), and the same protein after cleavage from GST by thrombin (lane 1). This latter protein has the expected molecular mass of about 23 kD, resulting from the 21.8-kD Rac13 sequence predicted by the full-length cDNA plus 10 extra amino acids resulting from the construct in the pGEX-3T vector. The Rac13 sequence was confirmed to be produced in-frame by sequencing the thrombin-cleaved Rac13 from the N terminus and by the reaction of both GST-Rac13 and thrombin-cleaved Rac13 with antibodies against Rac proteins (not shown). Furthermore, both GST-Rac13 and thrombin-cleaved Rac13, but not GST, bind [α-^{32}P]GTP (Fig. 1B). Two other substrates of about 27 kD were derived from modifications of the GST gene itself; in these constructs the sequences encoding the GST protein were modified by addition of sequences that would encode the additional four amino acids CIIL or CIIS at the C terminus (Fig. 1A, lanes 3 and 4, respectively). The GST-CIIL protein is known to be a preferred substrate for geranylgeranylation by GGTase I, whereas the GST-CIIS protein is a preferred substrate for farnesylation by FTase (Finegold et al., 1991).

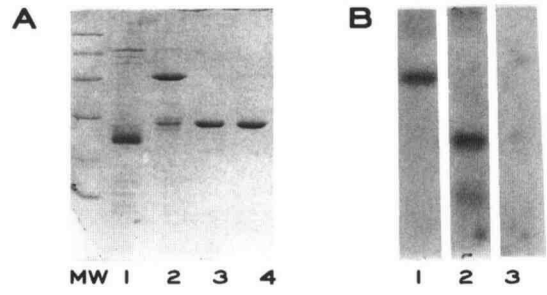

Figure 1. Recombinant proteins used as substrates for prenylation reactions. Purified proteins were separated by SDS-PAGE and stained with Coomassie blue (A, lanes 1–4) or photolabeled with [α-^{32}P]GTP and subjected to autoradiography (B) as described by Delmer et al. (1995). A, Molecular mass standards (MW) of 97, 66, 45, 31, 21, and 14 kD; lane 1, 4.5 μg of thrombin-cleaved Rac13; lane 2, 2.2 μg of GST-Rac13; lane 3, 1.5 μg of GST-CIIL; and lane 4, 1.5 μg of GST-CIIS. B, Lane 1, 1 μg of GST-Rac13; lane 2, 1 μg of thrombin-cleaved Rac13; and lane 3, 1 μg of GST.

Prenyltransferase Activities in Developing Cotton Fibers

A high-speed supernatant was prepared from cotton fibers harvested at 17 DPA, the age when the *Rac13* gene is maximally expressed (Delmer et al., 1995). This preparation was used as the source of enzyme either for geranylgeranylation, using [^{3}H]GGPP as a donor, or for farnesylation, using [^{3}H]FPP as a donor. Although the reaction rates we observed were quite low, it was nevertheless possible to show that, of the four substrates described above, GST-CIIL served as a good substrate for geranylgeranylation (Fig. 2A), whereas GST-CIIS was the only substrate that was prenylated with [^{3}H]FPP as a substrate (Fig. 2B). These results suggest that cotton fibers contain prenyltransferases with specificities similar to those of their yeast and animal counterparts (Finegold et

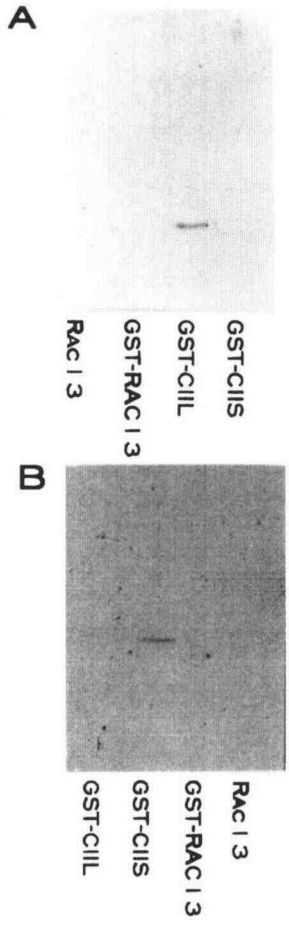

Figure 2. Fluorograms showing prenylated products resulting from incubation of the indicated recombinant proteins with a high-speed supernatant derived from cotton fibers as a source of prenyltransferases. Radioactive substrate used was either [^{3}H]GGPP (A) or [^{3}H]FPP (B) with acceptor proteins as indicated. All reactions were incubated for 1 h at 30°C and contained 87 μg of cotton protein extract. Concentrations of recombinant proteins were: GST-CIIL and GST-CIIS, 6.7 μM for both A and B; GST-Rac13, 6.7 μM for A and 4.6 μM for B; Rac13, 3.1 μM for A and 2.0 μM for B.

al., 1991; Glomset and Farnsworth, 1994). We noted, however, that neither GST-Rac13 nor Rac13 could be demonstrated to serve as a substrate with either prenyl donor (Fig. 2). Because the reaction rates were so low, it was not possible at this point to distinguish whether this lack of reaction was due to an inability to serve as a substrate or whether these proteins were simply much poorer substrates than GST-CIIL or GST-CIIS.

Rac13 as a Substrate for Reticulocyte GGTase

Because of the low activity and relative instability of the cotton prenyltransferases, we chose to study the prenylation of Rac13 using a commercial preparation derived from rabbit reticulocytes as a source of enzyme. This preparation is primarily known for its ability to carry out in vitro translation of proteins; however, it is known to contain good activities for the three prenyltransferases GGTase I, GGTase II, and FTase I. However, it does not contain the protease or methyltransferase that catalyzes the posttranslational modifications following prenylation and, because of this, little or no change in mobility of the prenylated product compared with unprenylated substrate is observed upon SDS-PAGE electrophoresis (Wilson and Maltese, 1995). Figure 3A shows an experiment using this system with [^{3}H]GGPP as a substrate. In this case, as expected, the GST-CIIL served most efficiently as an acceptor, and GST-CIIS was geranylgeranylated less efficiently than GST-CIIL. In this more active sytem, observations of a very weak labeling of the bands of GST-Rac13 and Rac13 indicate that these proteins could also serve as acceptors, although both were far less effective than either GST-CIIL or GST-CIIS. The GST protein that lacks a consensus sequence for prenylation was not prenylated.

A more detailed study of this activity as a function of time comparing GST-Rac13 and GST-CIIL as acceptors is shown in Figure 3B. These studies show that geranylgeranylation of GST-CIIL occurs very rapidly, with the reaction being maximal at the earliest time tested (1 h). In contrast, GST-Rac13 at a similar molar substrate concentration to that used for GST-CIIL, although clearly serving as an

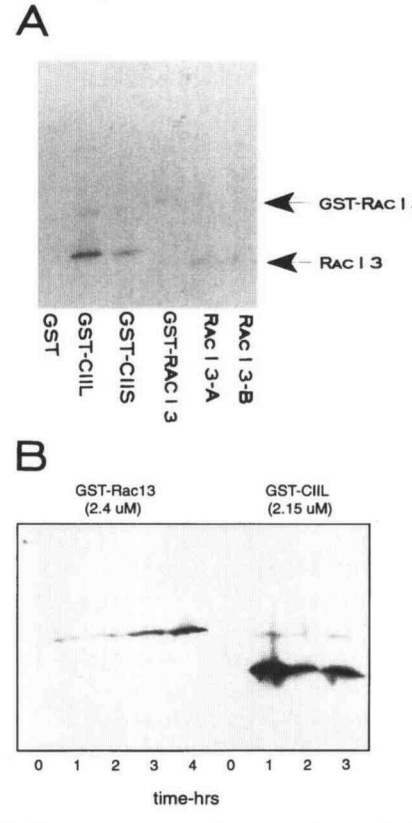

Figure 3. A, Fluorogram comparing geranylgeranylation of various recombinant proteins using rabbit reticulocyte lysate as a source of prenyltransferases. Incubations were for 4 h at 30°C as described in "Materials and Methods." The concentrations of recombinant proteins were: GST-CIIL and GST-CIIS, 1 μM; GST-Rac13, 2.6 μM; Rac13-A, 1.3 μM; Rac13-B, 0.9 μM; and GST, 2.2 μM. B, Fluorogram comparing the rate of geranylgeranylation of GST-Rac13 and GST-CIIL by rabbit reticulocyte lysates. The concentration of GST-CIIL was 2.15 μM and that of GST-Rac13 was 2.4 μM.

acceptor, is a much less efficient one. Preloading of the recombinant GST-Rac, with either GTP-γ-S or GDP-β-S, did not affect the rate of prenylation (not shown).

Long-term incubations of 4 h with high acceptor protein concentrations were used in another experiment to compare prenylation of GST-Rac13 using either [^{3}H]GGPP or [^{3}H]FPP as a substrate (Fig. 4). Again, it is clear that GST-Rac13 can undergo geranylgeranylation, but no reaction was observed when [^{3}H]FPP was used as a substrate. Under such long incubation conditions with high protein acceptor concentrations, the specificity of GGTase I and FTase I for GST-CIIL versus GST-CIIS is not apparent because the reactions have gone virtually to completion. (The nature of the much weaker prenylated bands of higher molecular weight that were observed in these long-term exposures in preparations of GST-CIIL and GST-CIIS is not known.) However, the experiment does show clearly that GST-Rac13 is an even poorer substrate for geranylgeranlyation than GST-CIIS and shows no evidence of undergoing farnesylation. Such results strongly support the notion that the CAFL sequence in Rac13 does allow geranylgeranylation in vitro but that the bulky F residue in position a_2 does hinder the reaction.

DISCUSSION

Although the activities were low and unstable, GGTase and FTase activities can be demonstrated in extracts of developing cotton fibers (Fig. 2). The specificities of these enzymes, with respect to the C-terminal motif recognized, appear to be similar to those reported for other eukaryotic prenyltransferases (Clarke, 1992; Glomset and Farnsworth, 1994). Studies with suspension-cultured tobacco cells (Randall et al., 1993; Morehead et al., 1995) and *Atriplex nummularia* (Zhu et al., 1993; Lin et al., 1996) also demonstrated in vitro activities for GGTase and FTase, and these enzymes also showed specificities similar to those we observed for cotton. Many of the Rab proteins identified in plants have recently been shown to be membrane-associated in vivo and geranylgeranylated in vitro, providing another piece of evidence that prenylation of these proteins occurs (Haizel et al., 1995; Loraine et al., 1996). Finally, a pea cDNA clone for a close homolog of the β subunit of a protein FTase has recently been identified (Yang et al., 1993). Although none of these prenyltransferases has been purified and studied in detail, the limited data accumulated to date suggest that the mechanisms of protein prenylation in plants will be similar to those of other eukaryotes.

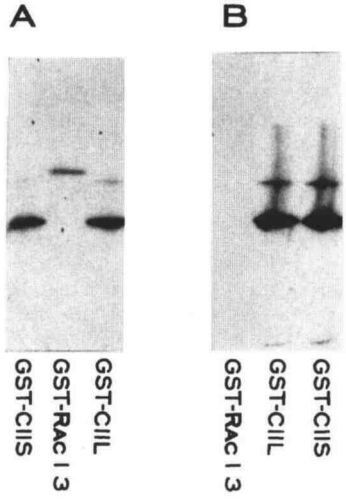

Figure 4. Fluorograms showing a comparison of the ability of various recombinant proteins to undergo geranylgeranylation (A) versus farnesylation (B) using rabbit reticulocyte lysates as sources of prenyltransferases. Concentrations of GST-CIIL and GST-CIIS were 2.2 μM; GST-Rac 13 was present at 2.4 μM. Time of incubation was 4 h at 30°C.

Studies presented here using the reticulocyte lysate as the source of prenyltransferases clearly show that GST-Rac13 and Rac13 can undergo geranylgeranylation but not farnesylation (Figs. 2–4). This result is predicted based on the Ca_1a_2L rule developed in other eukaryotes as a site for geranylgeranylation (Clarke, 1992; Glomset and Farnsworth, 1994). However, our further speculation that the bulky F residue at the a_2 position might interfere with prenylation, as found for rat or bovine brain FTases (Goldstein et al., 1991; Moores et al., 1991), also appears to be true, since GST-Rac13 and Rac13 were geranylgeranylated at rates far slower than GST-CIIL or even GST-CIIS. Since proteins with CAIL motifs are excellent substrates for geranylgeranylation, we do not consider the A residue in the second position to be a limiting factor for prenylation (Clarke, 1992). Furthermore, since GGTase I specificities are determined only by the C-terminal motif and appear to be insensitive to the nature of the protein outside the motif sequence (Moores et al., 1991; Glomset and Farnsworth, 1994), it is unlikely that other portions of the Rac13 sequence contributed to the slower rate of prenylation. There may be an analogy to these results in the studies of Zhu et al. (1993) with the plant chaperone ANJ1. As predicted, this protein that has CAQQ as a C-terminal motif is a good substrate for farnesylation and a weak substrate for geranylgeranylation. However, it was surprising that changing the CAQQ motif to CAQL did not enhance geranylgeranylation and, in fact, prevented it. This may be another indication that the nature of the a_2 residue is important for determining the rate of prenylation (Moores et al., 1991).

The low rate of prenylation of Rac13 in vitro raises the question as to whether this protein is prenylated in vivo in the developing cotton fiber. Previous studies provide a hint that the protein may well be prenylated in vivo; thus, we identified a 19-kD cotton fiber membrane polypeptide that shows a pattern of developmental expression analogous to the mRNA expression for Rac13, and this protein can be photolabeled with [α-^{32}P]GTP (Delmer et al., 1995). Since prenylation, proteolytic cleavage, and methylation modifications are known to enhance migrations and reduce the apparent molecular size of small GTPases by about 1 to 2 kD (Zhu et al., 1993), the observed 19-kD molecular mass observed for this putative native Rac is reduced by the amount predicted by prenylation and further posttranslational processing. Also, some of this

protein was found in the membranes, which is also indicative of processing (Ziman et al., 1993; Glomset and Farnsworth, 1994). Thus, the relatively slow rate of prenylation observed in vitro may not accurately reflect the situation in vivo, but more experiments will clearly be necessary to confirm this point. From the relative rates of prenylation observed with the reticulocyte compared with the cotton extracts, we strongly suspect that the reason we did not see geranylgeranylation of Rac13 with cotton fiber extracts is simply a matter of the very low activity and instability of the GGTase detected and not to any difference in specificity. We have estimated that, in assays using the amounts of enzymes described herein, the rate for the cotton GGTase I preparation is at least 50 times slower than the reticulocyte system using GST-CIIL as a substrate. At such low rates, it would certainly have been impossible to detect in vitro prenylation of the less-favored Rac13 acceptor using the cotton system.

It is interesting to speculate why the CAFL sequence, a less than ideal prenylation motif, evolved for the Rac9 and Rac13 proteins. One possibility is that it relates to tissue specificity; both Rac9 and Rac13 are expressed in a limited number of tissues in cotton, the fiber being the most notable. In extensive screening of our cotton fiber cDNA library prepared using mRNA from 21 DPA fibers, we never discovered any other genes for Rac-related proteins other than Rac13 or Rac9. Yet, our studies with three different anti-Rac antibodies suggest that other tissues and the fibers at an earlier stage of development do contain other related Rac proteins (T. Trainin and D.P. Delmer, unpublished observations). The studies of expression of the pea Rac homolog, which has the highly preferred geranylgeranylation motif CSIL at its C terminus, indicate that this protein is probably widely expressed in a variety of tissues (Yang and Watson, 1993). One way of assuring that even a low level of Rac13 or Rac9 would not be functional in some cell types might be to endow it with a C-terminal sequence that makes it a much less preferred substrate for prenylation than other GTPases that are more highly expressed in those cell types. Clearly, further studies will be needed to clarify these issues.

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