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The Posttranslational Processing of *ras* p21 Is Critical for Its Stimulation of Yeast Adenylate Cyclase

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Mammalian *ras* genes substitute for the yeast *RAS* gene, and their products activate adenylate cyclase in yeast cells, although the direct target protein of mammalian *ras* p21s remains to be identified. *ras* p21s undergo posttranslational processing, including prenylation, proteolysis, methylation, and palmitoylation, at their C-terminal regions. We have previously reported that the posttranslational processing of Ki-*ras* p21 is essential for its interaction with one of its GDP/GTP exchange proteins named *smg* GDS. In this investigation, we have studied whether the posttranslational processing of Ki- and Ha-*ras* p21s is critical for their stimulation of yeast adenylate cyclase in a cell-free system. We show that the posttranslationally fully processed Ki- and Ha-*ras* p21s activate yeast adenylate cyclase far more effectively than do the unprocessed proteins. The previous and present results suggest that the posttranslational processing of *ras* p21s is important for their interaction not only with *smg* GDS but also with the target protein.

The *ras* genes have been identified as potent oncogenes in the mutant forms (reviewed in reference 1). They comprise a family of at least three members (Ki-, Ha-, and N-*ras*) which share structural and functional properties. Their products, *ras* p21s, have GDP/GTP-binding and GTPase activities. Recent evidence indicates that c-*ras* p21 is a downstream molecule of the receptor-type tyrosine kinases, such as the platelet-derived growth factor and epidermal growth factor receptors, which convey their signals to an intracellular effector pathway (6, 14, 19, 38, 39). However, the direct target protein of *ras* p21s remains to be clarified. The *ras* genes are highly conserved in evolution. Homologs of the mammalian *ras* genes are found in simple organisms such as the budding yeast *Saccharomyces cerevisiae* (12, 36). In *S. cerevisiae*, the *RAS1* and *RAS2* proteins have been shown to regulate adenylate cyclase, although the precise mechanism of the interaction remains unclear (4, 18, 44). Mammalian *ras* genes substitute for yeast *RAS* genes, and their products activate yeast adenylate cyclase (4, 13, 29).

ras p21s have the distinctive C-terminal structure CAAX (A, aliphatic amino acid; X, any amino acid). This C-terminal region is posttranslationally processed; farnesylation of the cysteine residue is followed by removal of the AAX portion and subsequent carboxyl methylation of the exposed C-terminal cysteine residue (8, 21, 22, 26). Ha- and N-*ras* p21s are, in addition, palmitoylated at a second cysteine residue (21). Ki(2B)-*ras* p21 lacks this second cysteine residue and is not palmitoylated, but it has a polybasic region just upstream of the farnesylated cysteine residue (8, 22, 26). It has been shown that the posttranslationally processed C-terminal region of *ras* p21s and the polybasic region are essential for their transforming and membrane-binding activities (8, 21, 22, 26, 40).

ras p21s have GDP-bound inactive and GTP-bound active forms which are interconvertible by GDP/GTP exchange and GTPase reactions (1, 43). The GTPase reaction is regulated by the GTPase-activating protein (GAP) (45). The GDP/GTP exchange reaction is regulated by the GDP/GTP exchange protein (GEP) (1, 43). Several groups have previously detected GEP activity for *ras* p21 in mammalian tissues, but the existence of GEP activity for *ras* p21 has not been substantiated (15, 25, 46). In *S. cerevisiae*, the *CDC25* gene product has been genetically and biochemically identified as the GEP for the *RAS* protein (5, 10, 27, 37). The homolog of the *CDC25* gene, which complements the *CDC25* mutation, has recently been isolated from a mouse cDNA library (31). We have previously purified a stimulatory GEP for Ki-*ras* p21, named *smg* GDS, and determined its primary structure (28, 33, 47). This *smg* GDS is also active on *smg* p21A/*rap*1A p21/*Krev*-1 p21, *smg* p21B/*rap*1B p21, *rho*A p21, *rho*B p21, *rac*1 p21, and *rac*2 p21, which are members of the *ras* p21-related small GTP-binding protein superfamily, but is inactive on Ha-*ras* p21 (23, 32, 33). We have reported that the posttranslational processing of Ki-*ras* p21 as well as that of other substrate small GTP-binding proteins is essential for their interaction with *smg* GDS (24, 33).

In this study, we examined whether the posttranslational processing of Ki- and Ha-*ras* p21s was critical for their stimulation of yeast adenylate cyclase in a cell-free system. We report that the posttranslationally fully processed Ki- and Ha-*ras* p21s activate yeast adenylate cyclase far more effectively than do the unprocessed proteins.

MATERIALS AND METHODS

Materials and chemicals. The cDNAs of Ki(2B)- and Ha-*ras* p21s were kindly provided by R. A. Weinberg (Massachusetts Institute of Technology, Boston) and F. Tamanoi (The University of Chicago, Chicago, Ill.), respectively. *Spodoptera frugiperda* cells overexpressing Ki- or Ha-*ras* p21 were made as described previously (33). The posttranslationally unprocessed and fully processed Ki- and Ha-*ras* p21s were purified from the soluble and membrane fractions, respectively, of the *Spodoptera frugiperda* cells as described previously (33). Their C-terminal structures were confirmed to be posttranslationally unprocessed and fully

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processed, respectively (33). *Escherichia coli* overexpressing Ha-*ras* p21 was kindly provided by M. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) (20). Bacterial Ha-*ras* p21 was purified from Ha-*ras* p21-overexpressing *E. coli* (20). [α-^{32}P]ATP and [^{3}H]cyclic AMP (cAMP) were obtained from Amersham Corp. Guanosine 5'-(3-*O*-thio)triphosphate (GTPγS) and GDP were from Boehringer Mannheim and Yamasa Shoyu Co. (Chiba, Japan), respectively.

Preparation of yeast adenylate cyclase. Plasmid *YEP24-ADC1-CYR1*, which carried the complete *S. cerevisiae* adenylate cyclase gene (*CYR1*) under the control of the yeast alcohol dehydrogenase I (*ADC1*) promoter, was transformed into *S. cerevisiae* TK35-1 (*MAT*α *leu2 his3 trp1 ura3 cyr1-2 ras2::LEU2*) as described previously (42). The crude membrane fraction was obtained from about 10^{10} cells and resuspended in 4 ml of buffer A {50 mM 2-*N*-morpholinoethanesulfonic acid (pH 6.2), 0.1 mM $MgCl_2$, 0.1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride, 1 μM leupeptin, 100 μM benzamidine}. The suspended crude membrane fraction (12.0 mg/ml of protein) was used as the membrane-associated adenylate cyclase sample. The rest of the suspended crude membrane fraction was resuspended in buffer A containing 0.5 M NaCl and 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, rotated at 4°C for 20 min, and centrifuged at 100,000 × *g* for 60 min. The supernatant (1.7 mg/ml of protein) was used as the solubilized yeast adenylate cyclase sample.

Assay for adenylate cyclase. To make the GTPγS- and GDP-bound forms of *ras* p21, *ras* p21 (maximally 80 pmol) was first incubated with 10 μM GTPγS or GDP for 30 min at 30°C in a mixture (20 μl) containing 10 mM Tris-HCl (pH 8.0), 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol, 1 mM L-α-dimyristoylphosphatidylcholine, and 0.125% sodium cholate. After this incubation, 4 μl of $MgCl_2$ was added to give a final concentration of 21 mM, and the mixture was immediately cooled on ice. The membrane-associated yeast adenylate cyclase (48 μg of protein) or the solubilized yeast adenylate cyclase (17 μg of protein) and 250 μM [α-^{32}P]ATP (100 to 600 cpm/pmol) were added to this mixture. For the assay for membrane-associated yeast adenylate cyclase, the second incubation was performed for 30 min at 32°C in a mixture (100 μl) containing 27 mM Tris-HCl (pH 8.0), 2 mM Tris-HCl (pH 7.5), 5 mM 2-*N*-morpholinoethanesulfonic acid (pH 6.2), 5 mM $MgCl_2$, 2 mM EDTA, 0.01 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 0.2 mM dithiothreitol, 1 mM cAMP, 10 mM theophilline, 20 mM creatine phosphate, 20 U of creatine phosphokinase per ml, 1 mM L-α-dimyristoylphosphatidylcholine, 0.025% sodium cholate, and 2 μM GTPγS or GDP. For the assay for solubilized yeast adenylate cyclase, the second incubation was performed in the same way except that the reaction mixture additionally contained 50 mM NaCl and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid. The reaction was stopped, and the [^{32}P]cAMP produced was measured as described previously (4, 42, 44). The adenylate cyclase activity was expressed as picomoles of cAMP produced per milligram of enzyme protein.

Other procedures. The [^{35}S]GTPγS-binding activity was assayed by the filtration method, using nitrocellulose filters as described previously (47). Protein concentrations were determined with bovine serum albumin as a standard (3).

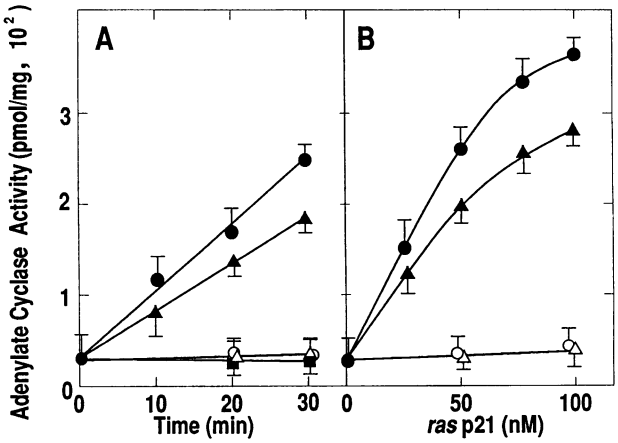

FIG. 1. Effects of the GTPγS- and GDP-bound forms of posttranslationally processed Ki- and Ha-*ras* p21s on the activation of solubilized yeast adenylate cyclase. (A) Time course. The activity of solubilized yeast adenylate cyclase was measured for various periods of time with the GTPγS- or GDP-bound form of the posttranslationally processed Ki- and Ha-*ras* p21s at a final concentration of 50 nM. (B) Dose response. The activity of solubilized yeast adenylate cyclase was measured with various concentrations of the GTPγS- or GDP-bound form of the posttranslationally processed Ki- and Ha-*ras* p21s. Symbols: ●, with the GTPγS-bound form of Ki-*ras* p21; ○, with the GDP-bound form of Ki-*ras* p21; ▲, with the GTPγS-bound form of Ha-*ras* p21; △, with the GDP-bound form of Ha-*ras* p21; ■, without *ras* p21. The results are means ± standard errors of three independent experiments.

RESULTS

First, the effects of the posttranslationally processed and unprocessed Ki- and Ha-*ras* p21s on the activation of the solubilized yeast adenylate cyclase were examined in a cell-free system. The GTPγS- and GDP-bound forms of the posttranslationally processed Ki- and Ha-*ras* p21s were prepared by incubating each protein with GTPγS and GDP, respectively. The GTPγS-bound form of both *ras* p21s stimulated the solubilized yeast adenylate cyclase in time- and dose-dependent manners, but the GDP-bound form was almost inactive (Fig. 1). The doses of Ki- and Ha-*ras* p21s necessary for this effect were similar. The GTPγS-bound form of both *ras* p21s also markedly stimulated the membrane-associated yeast adenylate cyclase, but in this case the GDP-bound form of both *ras* p21s was slightly active (Fig. 2). This weak activation of the membrane-associated yeast adenylate cyclase by the GDP-bound form of Ki- and Ha-*ras* p21s may be due to rebinding of GTP regenerated from GDP as described previously (17). The degree of activation of the membrane-associated yeast adenylate cyclase by both *ras* p21s was much greater than that obtained with the solubilized yeast adenylate cyclase.

In contrast to the posttranslationally processed Ki- and Ha-*ras* p21s, the GTPγS-bound form of the posttranslationally unprocessed Ki- or Ha-*ras* p21 was far less effective on the solubilized yeast adenylate cyclase (Fig. 3). Similar results were obtained when bacterial Ha-*ras* p21 was used instead of the posttranslationally unprocessed Ki- and Ha-*ras* p21s. Although the GTPγS-bound form of the posttranslationally unprocessed Ki- and Ha-*ras* p21s and bacterial Ha-*ras* p21 slightly activated the membrane-associated yeast adenylate cyclase, the processed Ki- and Ha-*ras* p21s were far more effective (Fig. 4; Table 1). Kinetic analysis of the

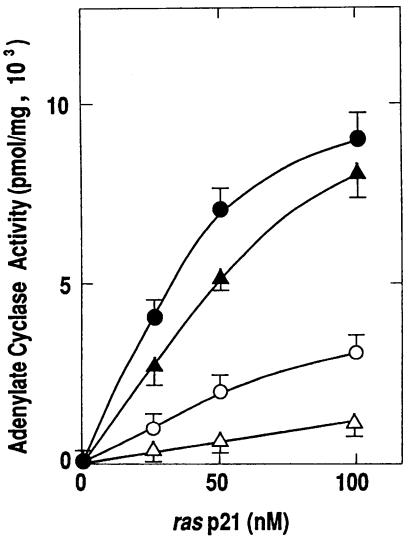

FIG. 2. Effects of the GTPγS- and GDP-bound forms of posttranslationally processed Ki- and Ha-*ras* p21s on the activation of membrane-associated yeast adenylate cyclase. The activity of membrane-associated yeast adenylate cyclase was measured with various concentrations of the GTPγS- or GDP-bound form of the posttranslationally processed Ki- and Ha-*ras* p21s. Symbols: ●, with the GTPγS-bound form of Ki-*ras* p21; ○, with the GDP-bound form of Ki-*ras* p21; ▲, with the GTPγS-bound form of Ha-*ras* p21; △, with the GDP-bound form of Ha-*ras* p21. The results are means ± standard errors of three independent experiments.

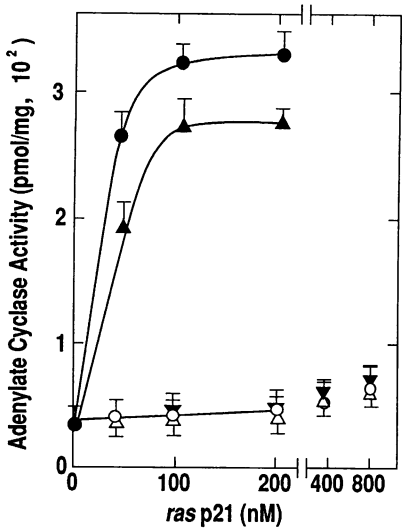

FIG. 3. Effects of the GTPγS-bound form of posttranslationally processed or unprocessed Ki-*ras* and Ha-*ras* p21s on the activation of solubilized yeast adenylate cyclase. The activity of solubilized yeast adenylate cyclase was measured with various concentrations of the GTPγS-bound form of posttranslationally processed or unprocessed Ki- and Ha-*ras* p21s and bacterial Ha-*ras* p21. Symbols: ●, with posttranslationally processed Ki-*ras* p21; ○, with posttranslationally unprocessed Ki-*ras* p21; ▲, with posttranslationally processed Ha-*ras* p21; △, with posttranslationally unprocessed Ha-*ras* p21; ▼, with bacterial Ha-*ras* p21. The results are means ± standard errors of three independent experiments.

data shown in Fig. 4 revealed that the K_a values for the posttranslationally processed Ki- and Ha-*ras* p21s, the posttranslationally unprocessed Ki- and Ha-*ras* p21s, and bacterial Ha-*ras* p21 were about 50, 74, 950, 800, and 670 nM, respectively, and the V_{max} values were about 430, 420, 67, 33, and 57 pmol/min/mg, respectively. These K_a and V_{max} values for bacterial Ha-*ras* p21 are almost the same as those observed by Farnsworth and Feig (16).

To rule out the possibility that the unprocessed Ki- and Ha-*ras* p21s purified from the soluble fraction of the insect cells and bacterial Ha-*ras* p21 were denatured, we carried out a series of experiments and obtained the following results. First, the unprocessed Ki- and Ha-*ras* p21s and bacterial Ha-*ras* p21 showed GDP/GTP-binding and GTPase activities equivalent to those of the processed Ki- and Ha-*ras* p21s (data not shown). Second, *ras* GAP stimulated the GTPase activity of the unprocessed Ki- and Ha-*ras* p21s and bacterial Ha-*ras* p21 as well as that of the processed Ki- and Ha-*ras* p21s (data not shown). Third, microinjection of the GTPγS-bound form of the unprocessed Ki- and Ha-*ras* p21s stimulated DNA synthesis in Swiss 3T3 cells and induced germinal vesicle breakdown in *Xenopus* oocytes (data not shown). The GTPγS-bound form of bacterial Ha-*ras* p21 showed the same effects. These results were consistent with earlier observations (2, 41).

DISCUSSION

We have shown that the posttranslationally processed Ki- and Ha-*ras* p21s activate the solubilized and membrane-associated yeast adenylate cyclases far more effectively than do the unprocessed Ki- and Ha-*ras* p21s and bacterial Ha-*ras* p21 in a cell-free system. The difference between the processed and unprocessed *ras* p21s involves three modifications: farnesylation, removal of three amino acids, and carboxyl methylation. It remains to be clarified which modification is important for the different effects of the processed and unprocessed *ras* p21s on the yeast adenylate cyclase. Marcus et al. have previously shown that the posttranslational processing of a-factor is critical for its biological activity (30). Our result is consistent with this observation. On the other hand, Buss et al. have reported that in the absence of normal C-terminal processing, *ras* p21 can still transform NIH 3T3 cells if *ras* p21 is targeted to the membrane by the addition of the N-terminal myristoylation signal (7). This result suggests that the sole function of the C-terminal processing of *ras* p21 is to attach the protein to the membrane and seems inconsistent with our observation that the posttranslational processing is important for the activation of yeast adenylate cyclase. However, Buss et al. used an extremely powerful promoter, and the mutated *ras* p21 was vastly overexpressed in their NIH 3T3 cells. Thus, it is possible that the myristoyl moiety at the N-terminal portion enhances the interaction of *ras* p21 with its target protein to some extent. The same group has recently shown that Ha-*ras* p21 containing a C-terminal CVLL or CAIL sequence instead of a CVLS sequence, which is geranylgeranylated instead of farnesylated at this cysteine residue, transforms NIH 3T3 cells when overexpressed but not when moderately expressed (9). Under the same conditions, the oncogenic-type Ha-*ras* p21 transforms NIH 3T3 cells when moderately expressed. These results suggest that overexpression is necessary for the C-terminal mutant of *ras* p21 to show its transforming activity and that the function of the prenyl moiety is not only membrane attachment, consistent with our observation that the posttranslational processing of *ras* p21 greatly increases its potency for activation of the

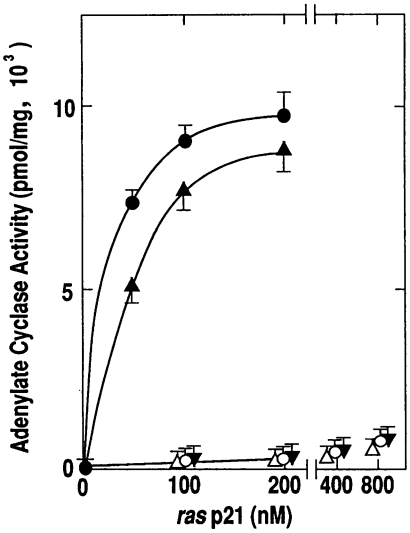

FIG. 4. Effects of the GTPγS-bound form of posttranslationally processed or unprocessed Ki-*ras* and Ha-*ras* p21s on the activation of membrane-associated yeast adenylate cyclase. The activity of membrane-associated yeast adenylate cyclase was measured with various concentrations of the GTPγS-bound form of posttranslationally processed or unprocessed Ki- and Ha-*ras* p21s and bacterial Ha-*ras* p21. Symbols: ●, with posttranslationally processed Ki-*ras* p21; ○, with posttranslationally unprocessed Ki-*ras* p21; ▲, with posttranslationally processed Ha-*ras* p21; △, with postranslationally unprocessed Ha-*ras* p21; ▼, with bacterial Ha-*ras* p21. The results are means ± standard errors of three independent experiments.

yeast adenylate cyclase in terms of the concentration required and the maximal effect. Taken together, these results suggest that the posttranslational processing of *ras* p21s is critical for their stimulation of yeast adenylate cyclases. However, it remains to be clarified whether Ki- and Ha-*ras* p21s directly or indirectly act on yeast adenylate cyclase, because the solubilized yeast adenylate cyclase used in this study was a partially purified sample.

It is essential for our understanding of the mode of action of *ras* p21s in the regulation of cell proliferation, differentiation, and transformation to identify their direct target proteins in mammalian tissues. The target protein of *ras* p21 has not yet been identified. Since *ras* GAP inhibits the K^+ channel in the presence of *ras* p21, *ras* GAP has been proposed to be one of the candidates of the target proteins (48). We have shown here that *ras* GAP is active on the unprocessed as well as the processed *ras* p21. However, recent genetic and biochemical lines of evidence indicate that *ras* GAP is a negative regulator of *ras* p21s rather than the target protein for cell proliferation or transformation (11, 34, 35, 49). The role of *ras* GAP as the target protein is still controversial. Thus, our present results together with our earlier observations (24, 33) suggest that the posttranslational processing of *ras* p21, presumably its C-terminal region, is critical for its interaction with its target protein as well as at least one of its GEPs.

We cannot rule out the possibility that the unprocessed Ki- and Ha-*ras* p21s purified from the soluble fractions of insect cells and bacterial Ha-*ras* p21 were denatured. However, we assume that this possibility is unlikely because (i) these fractions show GDP/GTP-binding and GTPase activities, (ii) *ras* GAP stimulates their GTPase activity, and (iii) microinjection of them induces DNA synthesis in Swiss 3T3 cells and germinal vesicle breakdown in *Xenopus* oocytes.

We have shown here that the GTPγS-bound form of the processed Ki- and Ha-*ras* p21s effectively activates the yeast adenylate cyclases and that the GDP-bound form is less effective. Several lines of evidence that the GTP-bound form of *ras* p21s is the active form have been presented (1, 17). Our present study has provided additional evidence for this conclusion.

TABLE 1. Effects of the GTPγS-bound form of posttranslationally processed or unprocessed Ki-*ras* and Ha-*ras* p21s on the activation of membrane-associated yeast adenylate cyclase[a]

ras p21	Concn (nM)	Adenylate cyclase activity (pmol/mg [10^3])
Control	0	0.05 ± 0.02
Ki-*ras* p21		
Processed	200	9.71 ± 0.42
Unprocessed	800	0.71 ± 0.16
Ha-*ras* p21		
Processed	200	8.73 ± 0.39
Unprocessed	800	0.50 ± 0.10
Bacterial	800	0.72 ± 0.21

[a] Data are from the experiment shown in Fig. 4.

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