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Expression of Normal and Activated Human Ha-*ras* cDNAs in *Saccharomyces cerevisiae*

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We expressed normal and activated human cellular Ha-*ras* cDNAs which encode 21,000-dalton polypeptides (p21s) in *Saccharomyces cerevisiae* by their insertion into a 2μm-based replicating plasmid vector under 3-phosphoglycerate kinase promoter control. We found that newly synthesized p21 in *S. cerevisiae* was produced as a soluble precursor (pro-p21) which matured into a form electrophoretically indistinguishable from the processed form (p21) observed in mammalian cells. Coincident with the processing event was translocation to a membrane component, suggesting a coupling of the two events. Using vectors that direct the synthesis of p21 variants possessing the ability to autophosphorylate in vitro, we found that processing of p21 did not significantly affect this autophosphorylation reaction. In contrast to *Escherichia coli*, marked phenotypic changes were observed in *S. cerevisiae* as a consequence of the synthesis of p21, including reduction in growth rate and induction of flocculation. Accompanying these phenotypic alterations was a significant elevation of adenylate cyclase activity.

DNA from a variety of human tumors contains altered *ras* genes capable of morphological and tumorigenic transformation of NIH 3T3 murine fibroblasts (see references 21 and 42a for recent reviews). These genes, first detected as cellular counterparts of viral (Harvey and Kirsten murine sarcoma virus) oncogenes, encode related proteins of M_r 21,000, termed p21s, whose activation typically involves a point mutation altering amino acid residue 12 or 61 (42a). Both normal and activated versions of p21 have been localized to the inner surface of the plasma membrane (10, 43). The polypeptides undergo a posttranslational modification consisting of the covalent attachment of lipid, detected by palmitic acid labeling (35), which accompanies the translocation of the nascent polypeptide from a soluble form present in the cytoplasm to the membrane-bound form (26, 35, 38). The two known biochemical activities associated with p21 are guanine nucleotide-binding ability (33, 36) and GTPase activity (12, 24, 39). Although the precise function of p21 is unclear at present, it has been demonstrated that a mutation which activates the transforming potential of the polypeptide results in impairment of GTPase activity (18, 30, 48). In addition to its guanine nucleotide-binding and GTPase activities, v-Ha-*ras* p21 displays autophosphorylating activity in vitro (11, 36), which is thought to account for the population of viral p21 phosphorylated at threonine 59 (37). Reflecting the absence of the phosphoacceptor site (3, 24, 30), cellular p21 is not phosphorylated (26).

Cellular *ras* homologs of the Harvey and Kirsten murine sarcoma virus oncogenes display a striking evolutionary conservation, having been found in *Drosophila* sp. (25) and *Dictyostelium* sp. (31). Recently, two *ras*-related genes were detected in *Saccharomyces cerevisiae* (9, 20, 27, 29, 42); genetic experiments have indicated that function of either is necessary for cell viability (20, 42). There are grounds to suspect that the function of the polypeptides encoded by yeast and mammalian *ras* genes may be similarly conserved. The polypeptides share regions of extensive homology, both proteins exhibit guanine nucleotide-binding activities (33, 36, 41), and a mutation at codon 12 which affects the transforming potential of mammalian p21 profoundly affects the physiology of yeast cells when present in the analogous region of the *RAS2* gene (20). More recently, it has been found that the human c-Ha-*ras*1 gene can complement *ras1*$^-$ *ras2*$^-$ spores, permitting vegetative growth (19). To examine the fate of human p21 in *S. cerevisiae*, as well as to obtain sufficient quantities of the polypeptide for detailed biochemical analysis, we efficiently expressed normal and activated Ha-*ras* genes in *S. cerevisiae* and report results of efforts to characterize metabolic and physiological changes resulting from the expression of this human gene.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. A tryptophan auxotroph strain of *S. cerevisiae*, 20B-12 (*MATα trp1 pep4-3*) (18), was used for transformations by standard methods (14). *S. cerevisiae* were grown in nonselective yeast extract-peptone-glucose medium or selective yeast-nitrogen base medium plus Casamino Acids (Difco Laboratories). To express p21, we utilized a derivative of plasmid YEp9T (8) which contains the yeast 2μm origin of replication, the yeast *TRP1* gene for genetic selection, a 1,538 base pair *Hin*dIII-*Eco*RI restriction fragment containing the promoter for the highly expressed yeast 3-phosphoglycerate kinase gene and the *TRP1* transcriptional terminator (8). Human c-Ha-*ras1* cDNAs encoding p21 with Gly12-Ala59, Val12-Ala59, Gly12-Thr59, or Val12-Thr59 residues were obtained from vectors previously described (24). A derivative plasmid (pYGT-111*) encoding a truncated p21 containing the first 111 amino acids of p21 but lacking the 79 carboxy-terminal residue acids was prepared by digestion of pYGT with *Nco*I and ligation of the linearized DNA after repair of the single-stranded 3′ termini with DNA polymerase I (Klenow fragment) in the presence of the four deoxynucleoside triphosphates. The encoded p21 derivative was expected to have a size of 14,300 daltons (3).

Isotopic labeling. Cells were grown at 30°C in yeast-nitrogen base medium plus Casamino Acids to an optical density at 660 nm of 1.0, pelleted by low-speed centrifuga-

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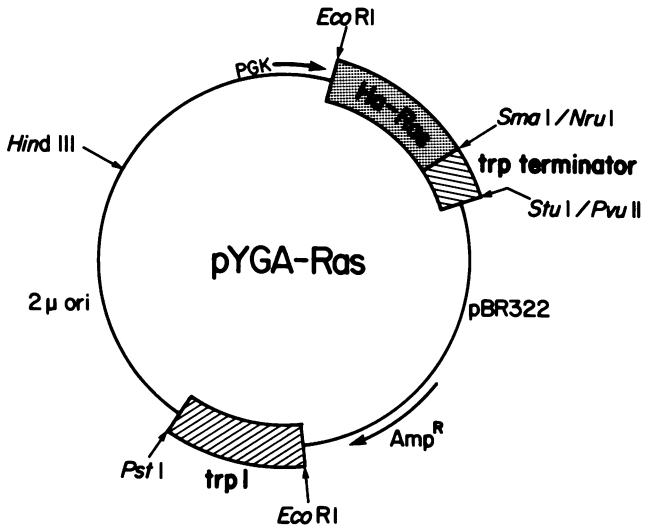

FIG. 1. Construction of p21-*ras* expression vectors. Human c-Ha-*ras*1 cDNAs encoding p21(Gly12-Ala59), p21(Val12-Ala59), p21(Gly12-Thr59), and p21(Val12-Thr59) were isolated as previously described (24) and introduced into plasmid YEp9T (8) containing the 3-phosphoglycerate kinase promoter as described in the text to generate plasmids pYGA, pYVA, pYGT, and pYVT, respectively. 2μ ori, 2μm origin of replication.

tion, and suspended in 1/5 of a volume of yeast-nitrogen base medium containing 2% glucose and amino acids (minus methionine). [^{35}S]methionine was added to a final concentration of 300 μCi/ml, and the cultures were incubated at 30°C for the times indicated. For chase reactions, unlabeled methionine was added to 400 μg/ml after labeling, and incubation was continued to appropriate endpoints. Labelings were terminated by chilling and centrifugation.

Subcellular fractionation and immunoprecipitations. Cell pellets were suspended in 0.2 ml of HNE (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.5], 50 mM NaCl, 1 mM EDTA) and disrupted by vigorous mixing on a Vortex mixer with glass beads. Nonidet P-40 was added to a final concentration of 1% to solubilize membrane components, and the extract was clarified by centrifugation. Immunoprecipitations were performed with rabbit preimmune sera or sera directed against bacterially derived p21 (24), as previously described (34), and analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (22). For analysis of soluble and membrane-bound polypeptides, cultures were lysed after radiolabeling by vigorous agitation with glass beads. Membrane-bound material (P15) was separated from soluble components (S15) by centrifugation at 15,000 × *g* for 10 min. Soluble polypeptides were directly immunoprecipitated, whereas membrane-bound polypeptides associated with the P15 fraction were released with 1% Nonidet P-40, removed from the remaining insoluble components by centrifugation, and analyzed by immunoprecipitation.

Adenylate cyclase assays. Membrane extracts were prepared, and cyclase assays were performed as described previously (7), except that cells were lysed with a Dounce homogenizer rather than a cavitation bomb. Production of cAMP was monitored by the method of Salomon (32).

RESULTS

Vector constructions. Vectors directing the synthesis of normal (Gly12-Ala59) and activated (Val12-Ala59) human c-Ha-*ras* p21 in *S. cerevisiae* were derived from plasmid YEp9T, which can replicate autonomously in *S. cerevisiae* by virtue of the presence of the origin of replication from the yeast 2μm plasmid. Plasmids pYGA and pYVA were generated by inserting c-Ha-*ras* cDNAs, obtained from COS cells transfected with c-Ha-*ras*1 genes positioned behind the simian virus 40 early promoter (24), under the transcriptional control of the yeast 3-phosphoglycerate kinase promoter (Fig. 1) (8, 15, 16). The p21 translational initiation codon is located five nucleotides 3′ of the *Eco*RI restriction site used to isolate each cDNA fragment (24). Vectors directing the synthesis of normal and activated p21 with a threonine phosphoacceptor residue at position 59 (pYGT and pYVT, respectively) were produced by in vitro recombination with appropriately mutated cDNAs (24). We assured transcription termination by including the *TRP1* terminator sequences 3′ of the *ras* cDNAs. In addition, the vector contained the intact *TRP1* gene for genetic selection in a haploid auxotroph. The structure of the plasmids is illustrated in Fig. 1.

Expression of human p21 in *S. cerevisiae*. To express human Ha-*ras* p21 in *S. cerevisiae*, we transformed a *trp1* strain of *S. cerevisiae* with plasmids pYGT and pYVT and isolated Trp$^+$ colonies. To demonstrate the presence of the polypeptides, we labeled logarithmically growing cultures with [^{35}S]methionine for 60 min. After lysis and solubilization with detergent, extracts were treated with either preimmune sera or immune sera raised against bacterially derived human p21 (24). The immune complexes were collected, and the proteins were resolved by electrophoresis on SDS-polyacrylamide gels. Two polypeptides of about 23,000 daltons were specifically precipitated by anti-p21 immune sera (Fig. 2, lanes b) from cultures transformed with plasmids pYGT (panel C) and pYVT (panel D) with electrophoretic mobilities similar to those of pro-p21 (upper band) and p21 (lower band) found in mammalian cells. These proteins

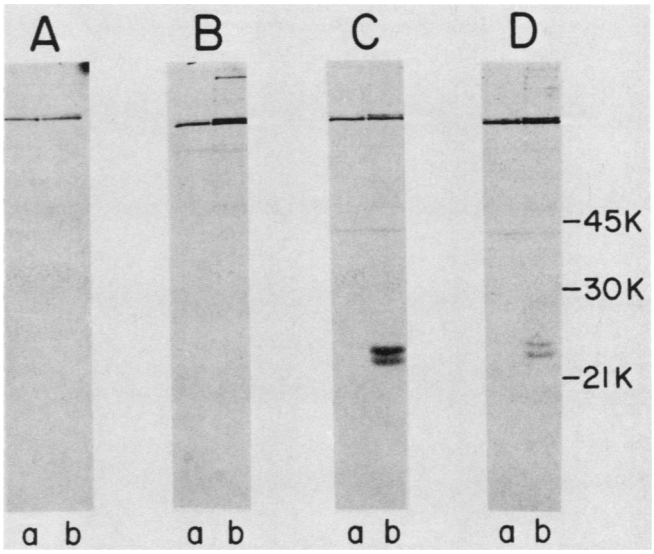

FIG. 2. Immunoprecipitation of p21-*ras* from pYGT- and pYVT-transformed yeast cells. Cultures transformed with YEp9T (panel A), pYeHBsd (panel B), pYGT (panel C), and pYVT (panel D) were labeled with [^{35}S]methionine for 60 min. The cells were lysed, and soluble extracts were treated with preimmune sera (lanes a) or anti-p21 sera (lanes b). Immune complexes were isolated with Staph A cells and analyzed by electrophoresis on SDS-polyacrylamide gels. Molecular weight designations are on the right.

were not precipitated by preimmune sera (lanes a), nor were they precipitated by either sera from extracts of YEp9T-transformed *trp*$^-$-carrying yeast cells (panel A) or yeast cells transformed with a related expression vector (pYeHBsd) directing the synthesis of the surface antigen encoded by human hepatitis B virus (21) (panel B). Immunological specificity and apparent molecular weight correspondence to authentic p21s establish these proteins as translational products of Ha-*ras* cDNAs, a conclusion supported by the observation that the electrophoretic mobility of the Gly12-Thr59 form of p21 is distinct from that of the Val12-Thr59 form (Fig. 2) (24).

Processing of human p21. Cellular p21 found in mammalian cells exists in two electrophoretically distinct forms, a slower migrating species representing the primary translational product and a more rapidly migrating form thought to result from the covalent attachment of lipid to the polypeptide (26, 35). This modification apparently accompanies the localization of the protein to the plasma membrane (26, 35, 38). Although this particular posttranslational modification does not occur when the protein is synthesized in *Escherichia coli* (24), it could account for the characteristic doublet seen in yeast cells (Fig. 2). Alternatively, it was possible that the slower migrating species resulted from a fortuitous posttranslational modification (e.g., glycosylation) of the more rapidly migrating one. To address this possibility, we pulse-labeled yeast strains transformed with pYGT and pYVT with [^{35}S]methionine for 20 min and subsequently chased them with an excess of unlabeled methionine for 0, 20, 60, 120, or 180 min. The cells were lysed, and p21 was immunoprecipitated and analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. It was evident that p21 was synthesized in a precursor form (pro-21) which matured to a more rapidly migrating species (p21) similar to that observed in mammalian cells (Fig. 3) (38). The half-life of this processing event was approximately 20 min for both

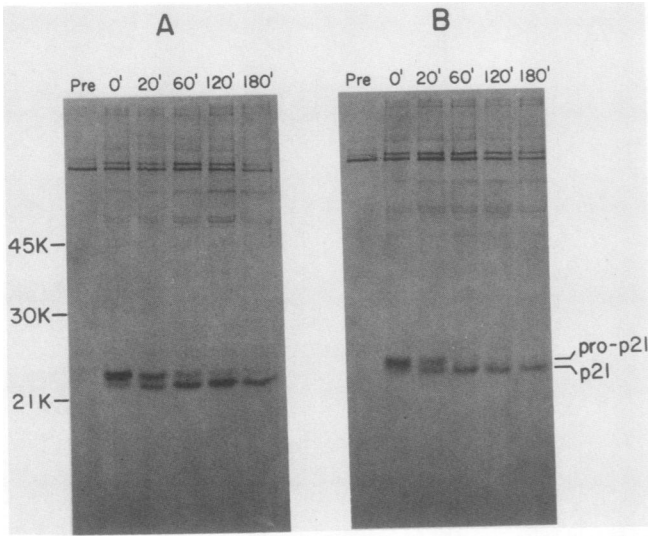

FIG. 3. Analysis of p21-*ras* processing in yeast cells. Yeast cultures transformed with pYGT (panel A) or pYVT (panel B) were pulse-labeled with [^{35}S]methionine for 20 min and chased with an excess of unlabeled methionine for 0, 20, 60, 120, or 180 min. p21 was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. The length of the chase period is indicated. Pre, Extracts treated with preimmune sera. Molecular weight designations are on the left.

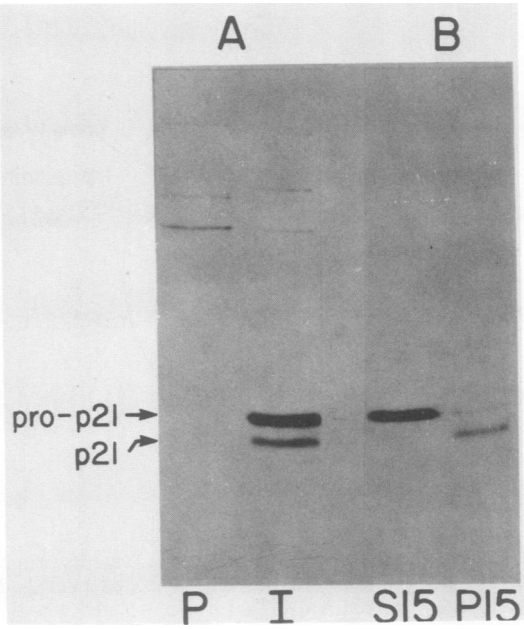

FIG. 4. Subcellular localization of pro-p21 and p21 expressed in yeast cells. Cultures transformed with pYGA or pYVA were labeled for 60 min with [^{35}S]methionine and lysed by vigorous agitation with glass beads. Membrane material was separated from soluble components by centrifugation at 15,000 × *g* for 10 min. The presence of p21 was determined by immunoprecipitation and SDS-polyacrylamide gels as described in the text. Panel A depicts an unfractionated extract treated with either preimmune (P) or anti-p21 (I) sera. Panel B shows the supernatant (S15) and pellet (P15) fractions treated with p21-specific immune sera.

the Gly12-Thr59 and Val12-Thr59 forms of p21 and for the Ala59 analogs (Gly12-Ala59 and Val12-Ala59) as well (data not shown).

Subcellular localization of p21. In mammalian cells, viral and cellular p21s are synthesized as soluble cytoplasmic proteins which subsequently associate with the inner surface of the plasma membrane (26, 35, 38). Concomitant with the change in intracellular localization is acylation of p21 (26, 35, 38), which is believed to affect the electrophoretic mobility of the protein. To provide further evidence that the processed version of p21 in yeast cells reflects a related posttranslational modification, we asked whether p21 similarly locates to a membranous structure in yeast cells after synthesis as a soluble polypeptide. We assessed the subcellular location of the two electrophoretically distinct forms of p21 by labeling yeast cultures transformed with pYGA with [^{35}S]methionine for 60 min, fractionating whole-cell lysates into soluble and membrane-bound components, and determining the presence of p21 in each fraction by immunoprecipitation. Both pro-p21 and mature p21 were present in unfractionated whole-cell extracts, whereas only pro-p21 was observed in the soluble fraction (Fig. 4). In contrast, all of the mature p21 was found in the membranous fraction in a form subject to release by nonionic detergent. These data establish that nascent p21 is present primarily as a soluble component of the cytoplasm, whereas processed p21 is affiliated with sedimentable membrane components through interactions presumably hydrophobic in nature. It remains to be established whether this association of p21 with a sedimentable structure reflects affiliation with the plasma membrane or another sedimentable component.

Phosphorylation of pro-p21 and p21. It has recently been

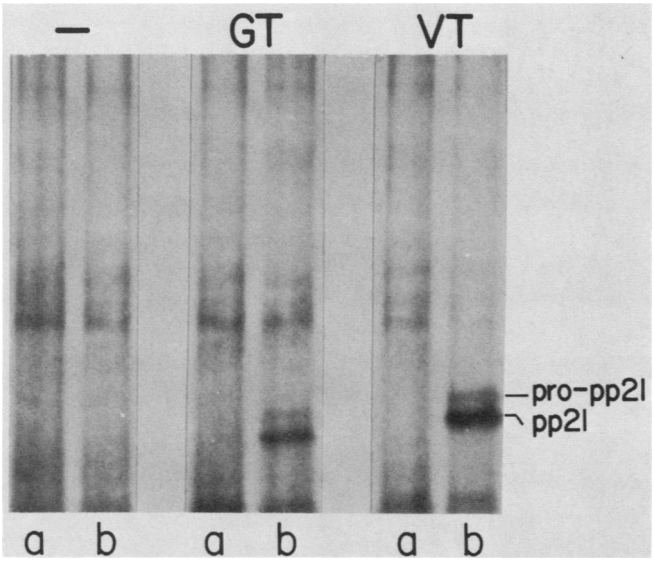

FIG. 5. Autophosphorylating activities of pro-p21 and p21. Unlabeled extracts of yeast cultures transformed with YEp9T (−), pYGT (GT) and pYVT (VT) were subjected to immunoprecipitation with preimmune sera (lanes a) and anti-p21 sera (lanes b) as described in the legend to Fig. 2. The immunoprecipitates were assayed for phosphotransferase activity in a reaction mixture (100 μl) containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 1 μCi of [γ-^{32}P]GTP for 15 min at 37°C. We washed the immunoprecipitate to remove unbound phosphate and analyzed it by SDS-polyacrylamide gel electrophoresis.

shown that human p21 displays the autophosphorylating activity exhibited by viral p21 when its cDNA is engineered by site-directed mutagenesis to encode the appropriate phosphoacceptor site located at amino acid 59 (24). To ascertain whether the posttranslational modification of p21 associated with alteration of electrophoretic mobility affects its autophosphorylating activity, we immunoprecipitated Thr59 derivatives of p21 (Gly12-Thr59 and Val12-Thr59) from yeast strains transformed with the appropriate vectors and carried out autophosphorylation reactions in vitro with labeled GTP (36). Both pro-p21 and p21 become phosphorylated under these conditions (Fig. 5). Since the relative intensities of the two species approximated their steady-state levels (Fig. 3; unpublished results), we conclude that this posttranslational modification did not significantly affect the autophosphorylating activity of p21. As previously observed (11, 24), we noted that the Val12 form of p21 exhibited slightly greater autophosphorylating activity than the Gly12 form (12, 24, 39).

Effect of p21 on yeast phenotype. Yeast cells expressing p21 exhibited an abnormal phenotype; they displayed a diminished growth rate (data not shown) and became extensively aggregated when grown in liquid media (Fig. 6). No phenotypic differences were observed between strains expressing normal or activated versions of *ras* gene products in these experiments. Aggregation did, however, become more extensive at higher cell densities (unpublished results). It appears that this effect is mediated by p21, since the expression of a truncated p21 (lacking 79 amino acids) or other membrane-bound heterologous proteins (e.g., hepatitis B surface antigen) failed to induce this phenotype (Fig. 6). The capacity for diploid cells to sporulate did not seem to be affected by the expression of p21 (data not shown).

Effect of human p21 on yeast adenylate cyclase activity. In view of the similarities between p21 and the alpha subunit of G protein involved in mediating the activity of adenylate cyclase (13, 17) and the importance of cAMP in yeast metabolism (22), we examined whether yeast cells synthesizing human p21 exhibit altered levels of adenylate cyclase activity. Adenylate cyclase activity was measured in membranes prepared from yeast strains transformed with plasmids YEp9T, pYGA, pYGT, pYVA, pYVT, pYGT-111*, and pYeHBsd. Adenylate cyclase activity in yeast cells expressing any of the four complete forms of p21 was significantly higher than the activity in YEp9T-transformed control yeast cells or in yeast cells expressing another membrane-bound polypeptide (hepatitis B surface antigen [21]) (Table 1). Further documentation attributing this response to p21 comes from results with yeast cells bearing plasmid pYGT-111*, which encodes an enzymatically inactive *ras*-related polypeptide lacking the terminal 79 amino acid residues. Membranes derived from such yeast cells exhibited a normal level of adenylate cyclase activity. In the presence of GTP, which is known to stimulate yeast adenylate cyclase in vitro (6), an expected increase in adenylate cyclase activity in membranes prepared from YEp9T-, pYeHBsd-, and pYGT-111*-transformed cultures was observed (6) (Table 1). No evidence of stimulation by GTP was observed in membranes derived from p21-expressing strains, however.

DISCUSSION

We found that a high-copy-number vector containing the yeast 3-phosphoglycerate kinase promoter and the *TRP1* terminator sequences could be used to obtain efficient expression of human p21 in yeast cells. The polypeptide underwent a form of posttranslational processing which may closely resemble that occurring in mammalian cells; it was synthesized as a precursor which was subject to a modification that resulted in increased electrophoretic mobility. It is believed that posttranslation attachment of lipid accounts for the altered electrophoretic mobility of the processed form of p21 in mammalian cells (35); if so, the modification that occurs in yeast cells may be similar, if not identical, in nature. Although the possibility exists that proteolytic cleavage accounts for the mature form of p21 in yeast cells, we consider this less likely in view of the observation that the processed form of p21 is metabolically stable and precisely coelectrophoreses with p21 derived from *ras*-transfected rat1 cells (Fig. 2; unpublished data). Furthermore, we showed that the processed form of p21, but not pro-p21, is preferentially associated with yeast membranes. It is noteworthy that membrane-associated processed p21 was readily extractable by nonionic detergent. This observation sup-

TABLE 1. Adenylate cyclase activity of yeast membranes

Plasmid used to transform cells	Adenylate cyclase activity (nmol) in the presence of:[a]	
	Mg^{2+}	Mg^{2+} + GTP
YEp9T	8	15
pYGA	45	47
pYGT	44	39
pYVA	44	43
pYVT	42	46
pYGT-III*	10	13
pYeHBsd	11	15

[a] Expressed as nanomoles of cAMP produced/mg of protein per 30 min. Mg^{2+} 10 mM; GTP, 100 μM.

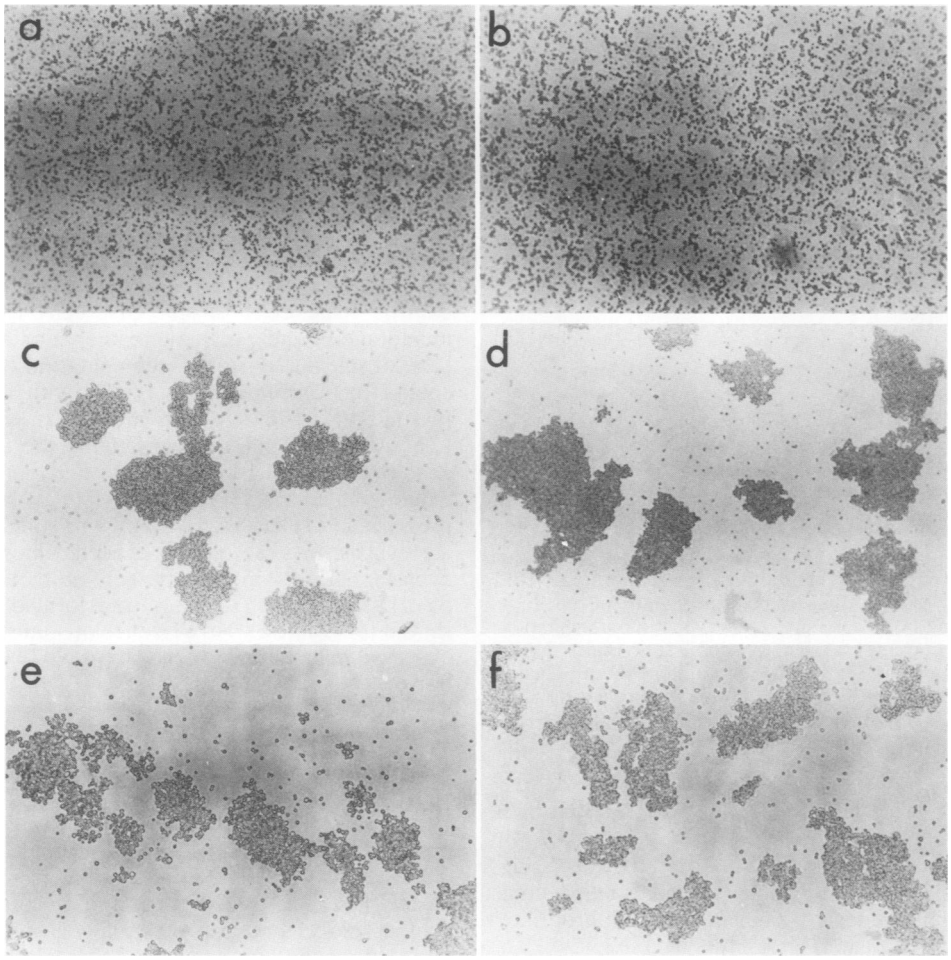

FIG. 6. Light micrographs of *S. cerevisiae* transformed with YEp9T (a), pYGT-111* (b), pYGT (c), pYVT (d), pYGA (e), and pYVA (f). Cultures were grown at 30°C in liquid medium containing yeast-nitrogen base medium and Casamino Acids.

ports the interpretation that localization indeed represents a specific cellular response to polypeptide modification and argues against the notion that the protein is merely precipitating in response to its synthesis in an inappropriate environment, as can occur when p21 is synthesized in *E. coli* (24).

The significance of the posttranslational processing event is as yet unclear. Although it appears not to affect the intrinsic ability of Thr59-containing p21 molecules to autophosphorylate (Fig. 5), the specific association of the processed protein with a membrane component is consistent with observations that lipid-containing p21 is similarly associated with the plasma membrane in mammalian cells. Although there is no direct evidence that lipid is responsible for anchoring the polypeptide to the membrane, it is strongly suggested by the observation that mutants of p21 lacking the presumed lipid attachment site are unable either to affiliate with the plasma membrane or to transform mammalian cells (44). Further support for this conclusion comes from the observation that the first 14 amino acids of $pp60^{v\text{-}src}$ encompassing its lipid binding site direct cytoplasmic proteins to the membrane when fused to their N termini (28), although there are reports of other polypeptides that remain soluble after acylation (1, 5). The suggestion that yeast cells are capable of acylating human p21 is therefore consistent with both our data and various observations made in other systems; we emphasize, however, that direct demonstration of this form of posttranslational modification in yeast cells awaits additional experiments.

An intriguing resemblance between mammalian *ras*-encoded proteins and a class of proteins known as G proteins involved in the regulation of cAMP synthesis in mammalian cells has been noted (13). Since the discovery that they share common intracellular location and guanine nucleotide-binding activities (36), this resemblance was reinforced by the discovery of structural homologies (13, 17) and shared enzymatic properties (12, 24, 39). After the detection of *ras*-related genes in yeast cells (9, 20, 27, 29, 42), genetic and biochemical criteria established that the products of these genes are involved in the positive regulation of the yeast adenylate cyclase system (40). Our results indicate that adenylate cyclase activity is significantly elevated in yeast cells expressing human p21 compared with normal strains or with strains expressing a truncated *ras* protein, clearly implicating the polypeptide in this process. Although there is evidence suggesting that yeast *RAS* gene products participate directly in regulation of this enzyme system (40), we have been unable to demonstrate direct stimulation of adenylate cyclase present in wild-type yeast membrane preparations by purified *E. coli*-derived human p21, nor have we been successful in establishing that antibody to p21 inhibits stimulation of yeast adenylate cyclase activity (unpublished

data). Whether this indicates that human p21 participates indirectly in this pathway or is attributable to some other reason is unclear at present. In any case, the result is informative in the context of data indicating that the human polypeptide allows vegetative growth of otherwise nonviable adenylate cyclase-deficient *ras1*$^-$ *ras2*$^-$ yeast strains (19).

Surprisingly, although in mammalian cells these proteins ordinarily elicit drastically different cellular responses, both normal and activated p21s produce similar stimulatory effects on yeast adenylate cyclase. Furthermore, each appears to induce adenylate cyclase activity that is relatively resistant to further stimulation by GTP. It is possible that characteristic differences between the two forms (Gly12 and Val12) of p21 are obscured by the fact that the expression system used ensures a relatively high level of p21 synthesis (~0.5% of total cellular protein), as has been reported when the normal c-Ha-*ras*1 gene is expressed at high levels in murine fibroblasts (7). Although it appears that yeast *RAS* genes play a role in modulating yeast metabolism and adenylate cyclase activity and that human p21 may be able to substitute for some functions of the yeast protein (19), we stress that there is as yet no evidence that p21 directly interacts with or otherwise stimulates adenylate cyclase activity in mammalian cells. On the contrary, it has been reported that the activity of adenylate cyclase (2), as well as the steady-state concentration of cAMP in high-density cell cultures (4), is reduced in rat kidney (NRK) fibroblasts in response to transformation by v-Ki-*ras*. Although we cannot presently account for this disparity, it is possible that human *ras* genes are members of an extended family whose functions have expanded considerably during evolution for purposes in some cases unique to vertebrates. If so, early hopes that genetic and biochemical analyses of *ras* genes in yeast cells would reveal fundamental insights into the biochemical basis of *ras*-mediated transformation of mammalian cells will be frustrated. In any case, progress toward understanding mammalian *ras* function should be facilitated by access to readily obtainable quantities of human p21. Since *E. coli*-derived pro-p21 may be unsuitable in view of its extreme insolubility and failure to become posttranslationally modified (24), yeast cells may represent an attractive host from which these polypeptides can be easily isolated and characterized in a native state.

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