Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1266–1270, March 1986
Biochemistry

Processing and fatty acid acylation of RAS1 and RAS2 proteins in *Saccharomyces cerevisiae*

(modification/subcellular localization)

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Communicated by Donald F. Steiner, October 21, 1985

ABSTRACT **We demonstrate the pathway for the biosynthesis of *RAS1* and *RAS2* gene products of *Saccharomyces cerevisiae* leading to their localization in membranes. The primary translation products of these genes are detected in a soluble fraction. Shortly after synthesis, these precursor molecules are converted to forms that migrate slightly faster than the precursor forms on a $NaDodSO_4$/polyacrylamide gel. These processed proteins are further modified by fatty acid acylation, which is detected by [^{3}H]palmitic acid labeling. The acylated derivatives are found exclusively in cell membranes, indicating the translocation of the RAS proteins from cytosol to membranes during maturation process. The attached fatty acids can be released by mild alkaline hydrolysis, suggesting that the linkage between the fatty acid and the protein is an ester bond. The site of the modification by fatty acid is presumably localized to the COOH-terminal portion of the RAS proteins. Fractionation of the membranes by sucrose gradient demonstrates that a majority of the fatty-acylated RAS proteins are localized in plasma membrane.**

The *RAS1* and *RAS2* genes of *Saccharomyces cerevisiae* were first identified as homologues of mammalian *ras* genes and were assigned to chromosomes XV and XIV, respectively (1–3). Amino acid sequences predicted from the DNA sequences of cloned genes have demonstrated a high level of homology with other members of the ras protein family (1, 2, 4, 5). Genetic analyses have shown that haploid cells that lack one of the *RAS* genes grow normally; however, mutants that are defective in both genes are incapable of vegetative growth (3, 6). Like their mammalian counterparts, the yeast RAS proteins have been shown to bind GTP and GDP as well as to function as GTPase (7–10). Recent studies have shown that the RAS proteins are involved in the regulatory mechanisms of adenylate cyclase activity (11, 12). The level of cyclic AMP is very low in mutant cells that have *ras1*$^-$ and *ras2*$^-$ mutations. Membranes prepared from the mutant cells have a comparable level of adenylate cyclase activity to wild-type membranes when assayed in the presence of manganese but have low activity when assayed with magnesium and GTP. Addition of the isolated RAS proteins can restore the magnesium/GTP-dependent adenylate cyclase activity of the mutant membranes (12).

Compared with the biochemical functions, little is known about the structural properties of the RAS proteins and how these relate to protein function. Since, as described above, the action of the RAS proteins appears to take place in membranes, it is important to elucidate subcellular localization and biosynthetic pathway of the RAS proteins. It is also of interest to investigate whether any modifications occur on the RAS proteins and which form regulates adenylate cyclase in the membranes. Many membraneous proteins are known to undergo various modifications. In the case of $p21^{ras}$ protein of Harvey murine sarcoma virus, which is localized at the inner surface of the plasma membrane, detection of a soluble precursor pro-p21, conversion of pro-p21 to p21, and phosphorylation of p21 have been reported (13–15). Furthermore, the $p21^{ras}$ protein is modified by fatty acid acylation (13–17), which appears to be important for the transforming activity of the protein (14, 15). However, it is unclear whether the fatty acylation is responsible for the conversion of pro-p21 to p21 or whether a modification distinct from the fatty acylation is taking place. Recent studies using mutant viruses have suggested that the fatty acylation involves the addition of palmitic acid at the cysteine residue that lies near the COOH terminus of the protein (14, 15). In contrast to the $p21^{ras}$ protein, $p60^{src}$, which is also localized at the inner surface of plasma membranes, has myristic acid attached via an amide bond at the NH_2 terminus (17–19).

In this paper, we describe the biosynthesis of the yeast RAS1 and RAS2 proteins. The primary translation products are found in a soluble fraction and are subsequently converted to faster migrating forms on a $NaDodSO_4$/polyacrylamide gel. The processed molecules are further modified by fatty acid acylation, and the processed, fatty-acylated forms are localized predominantly in the plasma membrane. Based on these results, we present a model for the biosynthetic pathway of the RAS proteins.

MATERIALS AND METHODS

Chemicals and Plasmid DNAs. [^{35}S]Methionine (1064 Ci/mmol; 1 Ci = 37 GBq), [^{35}S]cysteine (1008 Ci/mmol), and [9,10-^{3}H(N)]palmitic acid (27.5 Ci/mmol) were purchased from New England Nuclear. Plasmids YEp51-RAS1 and YEp51-RAS2 were designed to produce intact RAS1 and RAS2 proteins in yeast cells under the control of *GAL10* promotor (7). Plasmids pKS43 and pKH502-RAS2 were used to produce intact RAS1 and RAS2 proteins in *Escherichia coli*, respectively. Briefly, complete *RAS1* and *RAS2* coding sequences were placed downstream of the *recA* promotor, which is induced by the addition of nalidixic acid (20). pKS43 has a synthetic ribosome binding sequence to produce the protein more efficiently than does pKH502-RAS2. Details of the construction of the plasmids will be described elsewhere.

Labeling Conditions. *S. cerevisiae* JR25-2A (*leu2, ura3, trp1, his3, can1*) carrying an expression plasmid was grown at 31° C to 0.7–1.1 × 10^7 cells per ml in a synthetic medium containing 4% (wt/vol) galactose (7). Cells were labeled with [^{35}S]methionine or cysteine at 20 μCi/ml or tritiated fatty acid at 15 μCi/ml. Extracts from the labeled cells were prepared as described (9). To express RAS proteins in *E. coli*, C600 cells were grown at 37° C in SOB medium (21) to 6 × 10^8 cells per ml, and the *recA* promotor was induced by nalidixic acid at 50

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μg/ml. One to 2 hr after the induction, the cells were labeled with [^{35}S]cysteine at 25 μCi/ml. Labeled cells were disrupted by sonication in 10 mM Tris·HCl, pH 7.4/50 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/1% Triton X-100. The conditions for immunoprecipitation with the monoclonal antibody Y13-259 (22) and $NaDodSO_4$/PAGE have been described (7).

Preparation of Crude Membrane and Soluble Fractions. Labeled yeast cells from 20–50 ml of culture were washed with sorbitol buffer (0.3 M sorbitol/0.1 M NaCl/5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4) (23) and were mixed with glass beads in the sorbitol buffer containing 1 mg of aprotinin per ml and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by agitation on a Vortex mixer for 6 min with 1-min bursts, and undisrupted cells and large cell debris were removed by centrifugation at 1500 × *g* for 10 min. After centrifugation at 15,000 × *g* for 10 min (omitted in some experiments), crude membrane (P100) and soluble (S100) fractions were separated by further centrifugation at 105,000 × *g* for 120 min. Each pellet was washed by suspension in the sorbitol buffer (0.5 ml) and recentrifugation. The membranes were resuspended in 0.8 ml of phosphate-buffered saline (50 mM potassium phosphate/150 mM NaCl, pH 7.5)/1% Triton X-100/0.5% deoxycholate/1 mM phenylmethylsulfonyl fluoride and incubated on ice for 1 hr to extract the RAS proteins. Remaining insoluble material was removed by centrifugation at 15,000 × *g* for 10 min.

Characterization of Fatty-Acylated RAS Proteins. Yeast cells carrying YEp51-RAS2 were labeled with [^{3}H]palmitic acid for 60 min, and membrane extracts were prepared as described above. Labeled RAS2 proteins were isolated from the extract by immunoprecipitation. After gel electrophoresis, the recovered proteins were treated with diethyl ether to remove free fatty acids. For the analysis of the bound fatty acids, labeled proteins were hydrolyzed in 0.1 M KOH/methanol at 25° C for 60 min. The incubation mixture was extracted with $CHCl_3/H_2O$, 8:3 (vol/vol), as described by Schmidt *et al.* (24). The released materials were methylated and analyzed by reversed-phase TLC (Si-C_{18}, J. T. Baker Chemical) with 95% acetonitrile/5% H_2O as a developing solvent.

Fractionation by Sucrose Density Gradient. The crude membranes from 300 ml of culture were further fractionated by centrifugation in a gradient of sucrose from 25% (wt/wt) to 60%. GDP binding activity of the RAS protein was measured by using monoclonal antibody (7). Other marker enzymes were assayed as described (23).

RESULTS

Immunoprecipitation of the RAS Proteins. An indication that the RAS proteins in yeast cells were modified was first obtained when they were compared with the proteins produced in *E. coli*. Bacterial cells carrying pKS43 or pKH502-RAS2 were induced by nalidixic acid to produce intact RAS proteins, which were labeled with [^{35}S]cysteine. The labeled RAS proteins were precipitated from the crude extracts by using a monoclonal antibody Y13-259 and then were separated by $NaDodSO_4$/PAGE and compared with products similarly prepared from yeast cells. Both RAS1 and RAS2 proteins synthesized in *E. coli* cells (Fig. 1, lanes 2 and 5) migrated more slowly than those of yeast cells (Fig. 1, lanes 3 and 6). The apparent molecular weight of the RAS1 protein produced in *E. coli* was M_r 37,000, whereas the product of yeast was M_r 36,000. In the case of RAS2 protein, the protein from *E. coli* had an apparent M_r of 41,000, and the protein synthesized in yeast cells had an apparent M_r of 40,000. In addition, RAS1 and RAS2 proteins synthesized in a rabbit reticulocyte cell-free translation system showed the same mobility with the product from *E. coli* (data not shown). Since

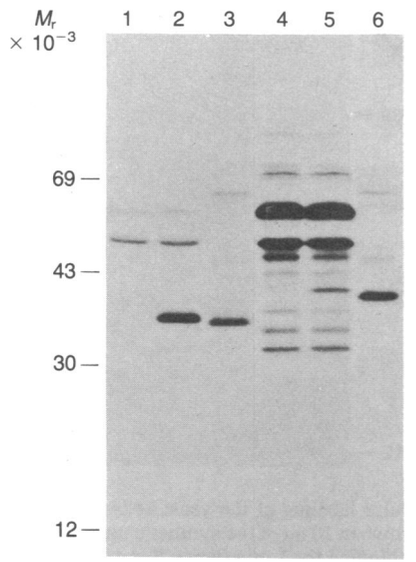

FIG. 1. $NaDodSO_4$/polyacrylamide gel analysis of RAS1 (lanes 1–3) and RAS2 (lanes 4–6) proteins. Yeast cells carrying YEp51-RAS1 or YEp51-RAS2 were labeled with [^{35}S]cysteine for 20 min at 31° C (lanes 3 and 6). *E. coli* C600 carrying pKS43 or pKH502-RAS2 were induced by nalidixic acid and labeled with [^{35}S]cysteine as described (lanes 1, 2, 4, and 5). RAS proteins were precipitated from the crude extracts with the monoclonal antibody Y13-259 and separated on a 15% $NaDodSO_4$/polyacrylamide gel. Lanes 1 and 4 show controls without the monoclonal antibody. Bands of the radioactive proteins were visualized by fluorography with Amplify (Amersham) and Kodak-XAR5 film. Size markers are albumin (M_r 69,000), ovalbumin (M_r 43,000), carbonic andydrase (M_r 30,000), and cytochrome *c* (M_r 12,000).

the yeast RAS proteins are not likely to be processed or modified in *E. coli* or in the rabbit reticulocyte lysate, these results suggest that some processing or modification of the RAS proteins occurs in yeast cells, which results in the change in their mobility on $NaDodSO_4$/polyacrylamide gels.

Detection of the Precursor Molecules. To examine whether the slowly migrating form exists in yeast cells and whether there is a precursor–product relationship between the two forms, we carried out a pulse–chase experiment. Yeast cells carrying YEp51-RAS2 were labeled with [^{35}S]methionine and fractionated into crude membrane and soluble fractions. M_r 41,000 molecules (p41) were detected in the soluble fraction after 1 min of labeling (Fig. 2, lane 2) but not in the membrane fraction (Fig. 2, lane 1). The label in the p40 molecules accumulated rapidly in the soluble fraction after 2 min of labeling (Fig. 2, lanes 3 and 4). When cells labeled for 1 min were chased with an excess amount of cold methionine for 30 min, almost all of the label was found in the p40 molecule (Fig. 2, lanes 5 and 6). These results indicate that the proteins are first synthesized as p41 and then converted to p40. Since a significant amount of p40 was detected in cells labeled for 1 min, the conversion of p41 to p40 appears to be rapid. Similar results were obtained with RAS1; a precursor form of RAS1 was found in the soluble fraction, and a rapid conversion from the M_r 37,000 form (p37) to the M_r 36,000 form (p36) of RAS1 was also observed (data not shown).

Three other bands of about M_r 30,000 were also detected by the monoclonal antibody Y13-259 in the soluble fraction. These bands were not detected in [^{35}S]cysteine-labeled cells nor in an *in vitro* translation product (see Fig. 1 and ref. 7). Since the cysteine residues are only present close to the COOH terminus of the RAS proteins, we believe that these molecules are lacking the original COOH terminus. Results from the partial proteolysis of these proteins by V8 protease (25) also supported this idea (data not shown).

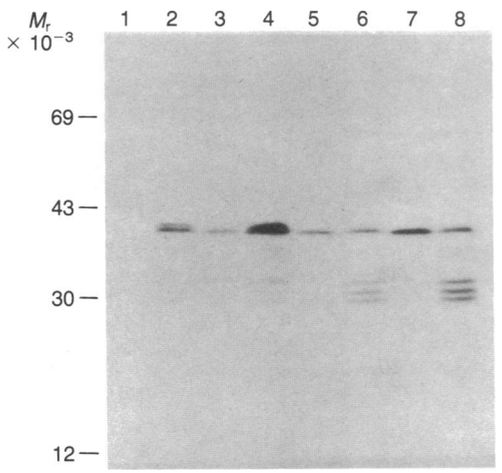

FIG. 2. Pulse labeling of the yeast cells carrying YEp51-RAS2. Cells were grown in 10 ml of the synthetic medium and pulse-labeled with [^{35}S]methionine for 1 min (lanes 1 and 2) or 2 min (lanes 3 and 4). For chase experiments, 1-min labeled cells were further incubated in the presence of cold methionine at 20 μg/ml for 30 min (lanes 5 and 6) or 90 min (lanes 7 and 8). After labeled cells were mixed with unlabeled cells (50-ml culture), membrane extracts (lanes 1, 3, 5, and 7) and soluble fractions (lanes 2, 4, 6, and 8) were prepared as described. Immunoprecipitated RAS2 proteins were separated as described in the legend to Fig. 1.

The p36 molecule of RAS1 and the p40 molecule of RAS2 were found in the membrane fraction as well as in the soluble fraction. The radioactivity in these p36 and p40 molecules in the membrane fraction accumulated after long labeling or chase, indicating the conversion of the RAS proteins from soluble to membrane-bound form (Fig. 2, lane 7). Because the p36 protein as well as the p40 protein remained in the soluble fraction after a 90-min chase but disappeared after a 120-min chase (data not shown), the transportation mechanism, which makes the RAS proteins accessible to membranes, might include some slow reaction(s).

Fatty Acid Acylation of the RAS Proteins. Since the RAS proteins are present in both soluble and membrane fractions, we tested if the p36 and p40 molecules in the membrane fraction are chemically distinct from the molecules in the soluble fraction. Many membraneous proteins including $p60^{src}$ and $p21^{ras}$ have been reported to be modified by fatty acylation (17, 26–34). Therefore, we performed labeling with fatty acids. Cells carrying the expression plasmids were labeled with [^{3}H]palmitic acid for 30 min. After fractionation, RAS proteins were precipitated with the monoclonal antibody Y13-259. Results in Fig. 3 clearly demonstrate that only the RAS proteins in the membrane fraction have ^{3}H label associated with them, and no RAS proteins in the soluble fraction were labeled. Parallel experiments using [^{35}S]methionine showed the distribution of RAS proteins in both fractions. Thus, fatty-acylated p36 and p40 are exclusively localized in the membranes. This indicates that the RAS proteins in soluble and membrane-bound forms are chemically different because of the fatty acylation, although both of them have the same mobility on NaDodSO$_4$/PAGE. RAS proteins could be labeled with [^{3}H]myristic acid with nearly the same efficiency as labeling with [^{3}H]palmitic acid (data not shown). Since virtually no ^{3}H labeling of the proteins in the soluble fraction was detected, conversion of the ^{3}H label from fatty acid to amino acids appears to be negligible under the conditions used.

To analyze the specificity of fatty acylation, we isolated [^{3}H]palmitic acid-labeled RAS2 protein from the membranes. After removal of free fatty acids, labeled proteins (42,982 cpm) were hydrolyzed by methanolic KOH; 88% of the

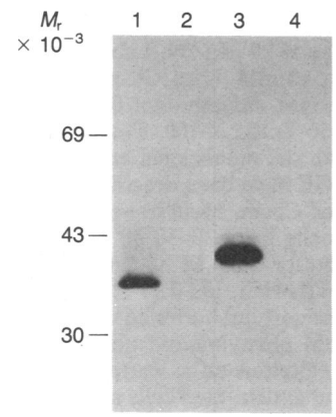

FIG. 3. Distribution of fatty-acylated RAS1 and RAS2 proteins in yeast cells. Cultures (100-ml) of yeast cells carrying YEp51-RAS1 (lanes 1 and 2) or YEp51-RAS2 (lanes 3 and 4) were labeled with [^{3}H]palmitic acid for 30 min at 31°C and fractionated as described. RAS proteins were precipitated with the monoclonal antibody Y13-259 from the extracts of pellets from centrifugation at 105,000 × *g* (lanes 1 and 3) and from the soluble fractions (lanes 2 and 4). NaDodSO$_4$/PAGE and fluorography were performed as described in the legend to Fig. 1.

radioactivity was recovered in the organic phase (37,844 cpm), indicating an alkali-labile ester bond between the fatty acid and the RAS protein. We were able to identify in the released material palmitic acid as well as myristic and lauric acids by TLC analysis. Similar results were obtained with the myristic acid-labeled proteins (data not shown).

Fractionation of the Membranes. To further analyze the distribution of RAS proteins in membranes, we fractionated the crude membranes by sucrose density gradient. A majority of the RAS-specific GDP binding activity cosedimented with the vanadate-sensitive ATPase activity, which was used as the marker for plasma membrane (35) (Fig. 4). On the other hand, NADPH-cytochrome *c* reductase, which is a marker for endoplasmic reticulum membrane (35), gave a peak at a different density. Proteins were scattered, giving peaks at positions corresponding to 38.6% sucrose and 48.8% sucrose (data not shown). Thus, a majority of the RAS proteins are localized in plasma membrane. It is possible that a minor portion of the RAS proteins are also localized in other

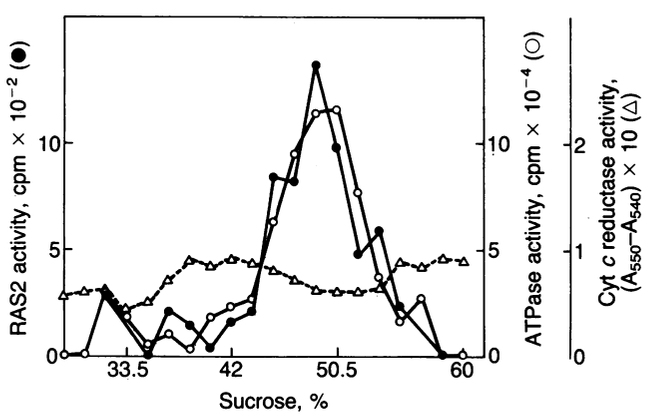

FIG. 4. Fractionation of the crude membranes by sucrose density gradient. Crude membranes (17 mg) prepared from the yeast cells carrying YEP51-RAS2 plasmid were fractionated by sucrose density gradient centrifugation for 14 hr at 30,000 rpm in a SW 41 rotor. GDP binding activity of the RAS2 protein, vanadate-sensitive ATPase (marker for plasma membrane), and NADPH-cytochrome *c* reductase (marker for endoplasmic reticulum membrane) activities are shown.

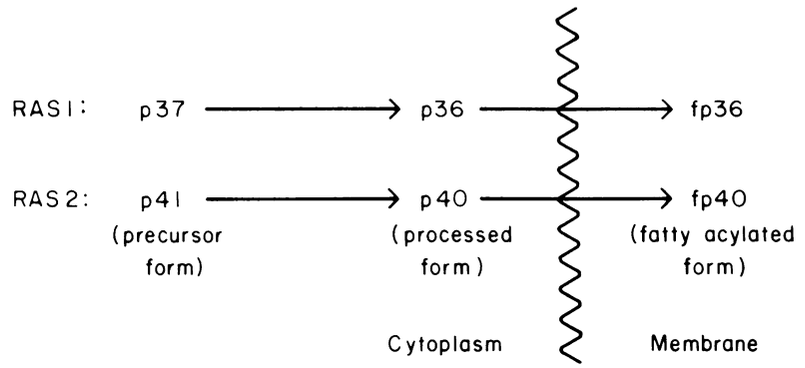

FIG. 5. Main pathway for the biosynthesis of yeast RAS proteins.

membranes, since we noted small shoulders of the GDP binding activity on the sucrose gradient.

DISCUSSION

We have identified various forms of RAS proteins within yeast cells. The relationship between these forms is summarized in Fig. 5. First, precursor forms are detected exclusively in a soluble fraction, which then are converted to a form that migrates slightly faster on $NaDodSO_4$/PAGE. This conversion proceeds rapidly after the termination of translation as compared with the later modification steps. The processed proteins are further modified by the addition of fatty acids. The majority of the fatty-acylated molecules are associated with the plasma membrane (Fig. 4). The overall picture of this biosynthetic pathway is similar to that of mammalian ras protein. However, in the case of yeast RAS proteins, we find the presence of processed but not fatty-acylated molecules (Figs. 2 and 3). This clearly indicates that a processing is occurring in addition to the fatty acylation. The conclusion that the two types of modification take place on the yeast RAS proteins is further supported by the difference in the sites of these modifications (see below).

To localize the site of processing, we performed [^{35}S]cysteine labeling. Since the cysteine residues exist solely close to the COOH termini of the RAS proteins, the protein molecules that have an intact COOH terminus can be labeled with [^{35}S]cysteine. The label was found in the processed forms of RAS1 and RAS2 (Fig. 1). Thus, in the case of the yeast RAS proteins, the processing does not involve cleavage of COOH termini from the precursor molecules. The processing might rather involve cleavage at the NH_2 terminus and/or modification that changes the net charge of the protein. In contrast to the processing, the fatty acylation appears to involve the COOH terminus. We found that only mature-sized p36 of RAS1 and p40 of RAS2 could be modified by fatty acylation, and the protein lacking COOH-terminal cysteine residues remained unmodified in the soluble fraction (Figs. 2 and 3). The exact site of the fatty acylation might be at the COOH-terminal cysteine, since this cysteine is a part of a sequence Cys-Aaa-Aaa-Xaa (Aaa is an aliphatic amino acid) that is also present in mammalian $p21^{ras}$, and it has been suggested that this sequence is involved in the fatty acylation of $p21^{ras}$ (14, 15). The linkage between the bound fatty acid and protein molecules appeared to involve an ester bond, based on the sensitivity to mild alkali. The presence of an ester bond has been reported for many membraneous proteins (17, 26–29).

Wen and Schlesinger (36) reported the accumulation of several fatty-acylated proteins in yeast secretory mutants (*sec1*, *sec7*, and *sec18*) at the nonpermissive temperature. They also reported that no [^{3}H]palmitic acid-labeled proteins were found in *sec53* mutant cells, a mutant defective in translocation of nascent polypeptides into the endoplasmic reticulum (23). However, we found that the fatty-acylated RAS proteins did not accumulate in the *sec7* and *sec18* mutants, and the fatty acylation of the RAS proteins was not affected by *sec53* and *sec59* mutations (unpublished data). These suggest that the RAS proteins utilize a biosynthetic pathway distinct from the secretory pathway.

In addition to the above modifications, phosphorylated RAS proteins were detected in both the soluble and membrane fractions with diverse mobility in a $NaDodSO_4$/polyacrylamide gel. The phosphorylation of RAS1 protein was also reported by Temeles *et al.* (9).

In conclusion, we have elucidated a pathway for the biosynthesis of the yeast RAS proteins. This involves processing, fatty acylation, and association with the membranes. How these modifications relate to the function of the proteins—i.e., stimulation of adenylate cyclase—will become clear by the purification and characterization of proteins produced in yeast.

We thank Drs. Bruce Stillman and Fevzi Daldal for critical reading of the manuscript and Madan Rao and Dr. Daniel Broek for stimulating discussions. We also thank Nasrollah Samiy for excellent technical assistance. This work was supported by a Cancer Center grant from the National Cancer Institute and National Institutes of Health Grant RO1 CA41996-01. A.F. is on leave from the Institute for Molecular and Cellular Biology, Osaka University, Japan.

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