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Phosphorylation of RAS1 and RAS2 proteins in *Saccharomyces cerevisiae*

(guanine nucleotide-binding proteins/adenylate cyclase/phospho amino acid/subcellular localization/tryptic phosphopeptides)

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ABSTRACT **RAS1 and RAS2 proteins of *Saccharomyces cerevisiae* are guanine nucleotide-binding proteins involved in the regulation of adenylate cyclase. In this paper, we report that these proteins are phosphorylated. The phosphorylation of RAS1 protein is demonstrated by treating with alkaline phosphatase as well as by labeling with [^{32}P]orthophosphate. The phosphorylation occurs exclusively on serine residues and phosphorylated RAS1 protein is predominantly membrane localized. The phosphorylation of RAS2 protein is demonstrated by similar ^{32}P-labeling experiments. The phosphorylation occurs exclusively on serine residues and phosphopeptide analyses suggest that only two major phosphorylated tryptic peptides are generated from the RAS2 protein. These results provide evidence for the phosphorylation of RAS proteins *in vivo*. Furthermore, our demonstration that the phosphorylation occurs exclusively on serine residues and that the RAS2 protein contains only two major phosphorylated tryptic peptides argues that the phosphorylation may be physiologically significant.**

RAS1 and *RAS2* genes of Saccharomyces cerevisiae were initially discovered due to their extensive homologies with *ras* oncogenes (1, 2). Yeast cells lacking both these genes are not viable (3, 4). Expression of the mammalian Ha-*ras* gene, however, enables these cells to grow (5, 6). Conversely, the *RAS1* gene can transform NIH 3T3 cells, provided that the region encoding the C-terminal portion is replaced with that of the corresponding region of the Ha-*ras* gene and an amino acid alteration is introduced into the 66th or 68th residue of the gene product (6). Like their mammalian counterpart, the RAS1 and RAS2 proteins exhibit guanine nucleotide-binding as well as GTPase activities (7–10). These activities apparently reside in the N-terminal portion of the protein, since RAS1 proteins having only the N-terminal portion exhibit these activities (9). The GTPase activity is reduced in mutant RAS2 proteins altered at the 19th, 66th, or 68th amino acid residue as well as in mutant RAS1 proteins having single amino acid changes in the 66th or 68th residues (8, 9).

Genetic as well as biochemical studies have established that these RAS proteins are required for the stimulation of yeast adenylate cyclase (11, 12). Yeast membranes lacking both the RAS1 and RAS2 proteins are defective in adenylate cyclase. However, addition of purified RAS2 protein restores GTP-dependent adenylate cyclase activity (12). The partially purified catalytic subunit of adenylate cyclase can still be stimulated by the RAS2 protein, raising the possibility that a direct interaction between the RAS2 protein and adenylate cyclase exists (13). RAS1 protein is also capable of stimulating adenylate cyclase, although the apparent stimulation is less than that of the RAS2 protein (12). The RAS1 protein has also been proposed to play a regulatory role in inositol phospholipid turnover (14).

The yeast RAS proteins undergo post-translational modification that includes fatty acid acylation (15). This paper demonstrates that the RAS proteins undergo yet another type of post-translational modification, phosphorylation. Our findings were made in the course of characterizing RAS proteins purified from yeast cells. The heterogeneity of the purified RAS1 protein led us toward the phosphorylation discovery. Treatment of the proteins with alkaline phosphatase and labeling with [^{32}P]orthophosphate demonstrated that the RAS1 proteins are phosphorylated and that the phosphorylation occurs exclusively on serine residues. Similar labeling experiments further show that the RAS2 protein is also phosphorylated. The phosphorylated proteins are predominantly localized in the membranes, the site of action of these proteins. Preliminary observations on the phosphorylation of yeast RAS proteins have been discussed (15).

MATERIALS AND METHODS

Chemicals, Plasmid DNAs, and Strains. [^{35}S]Methionine (1199 Ci/mmol; 1 Ci = 37 GBq) and [^{32}P]orthophosphate were purchased from ICN and Amersham, respectively. Plasmids YEp51-RAS1 and YEp51-RAS2 have been described (15). UC101 (*MATa leu2 trp1 ura3 prb pep4::URA3*) was provided by K. Matsumoto (DNAX Research Institute). KP1 (*MATa leu2 ura3 trp1 his3 ade8 can1 ras2::URA3*) was provided by M. Wigler (Cold Spring Harbor Laboratory).

Labeling Conditions. UC101 carrying YEp51-RAS1 or YEp51-RAS2 were grown in synthetic medium (7) containing 2% (wt/vol) galactose and lacking leucine, uracil, and methionine. The cells were collected in late logarithmic phase and resuspended in the same volume of Burkholder medium (16) lacking the appropriate amino acids and containing 2% galactose and potassium phosphate at 30 mg/liter. KP1 cells were dealt with in a similar manner except that the medium contained glucose and all amino acids. After brief incubation in this medium, [^{32}P]orthophosphate was added at the concentration of 150 μCi/ml and labeled for about 5 hr. ^{35}S-labeling was carried out by adding [^{35}S]methionine at 20 μCi/ml. Cells were broken with glass beads and the RAS proteins were immunoprecipitated using the monoclonal antibody Y13-259 as described (15). Franctionation into the soluble and membrane fractions were carried out as described (15). Two-dimensional gel electrophoresis was carried out according to O'Farrell (17).

Purification and Characterization of RAS Proteins. 259-Sepharose was prepared by coupling 56 mg of the monoclonal antibody Y13-259 (purified by DEAE-Sepharose) to 3.2 g of CNBr-activated Sepharose (Pharmacia) in a solution of 0.2 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl. After blocking unreacted

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groups with glycine, the gel was extensively washed and stored in a solution of 0.1 M Tris·HCl (pH 7.4) and 0.2 M NaCl.

RAS1 and RAS2 proteins were purified from UC101 cells carrying either the plasmid YEp51-RAS1 or YEp51-RAS2. Cells [3 g (wet weight)] were resuspended in 3 ml of sorbitol buffer (0.3 M sorbitol/10 mM Tris·HCl, pH 7.4/100 mM NaCl/5 mM $MgCl_2$) containing 10 μM leupeptin, 10 μM pepstatin, 10 μM bestatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 0.1 mM 7-amino-1-chloro-3-tosylamido-2-heptanone, 1 mM benzamidine, 10 mM sodium bisulfite, and 2 mM EGTA. After breaking the cells with glass beads, Triton X-100 was added to a final concentration of 1% and incubated on ice for 10 min. The extracts were centrifuged at 3000 rpm in a Sorvall HB4 rotor for 10 min and the supernatant was centrifuged in a 70 Ti rotor at 50,000 rpm for 1 hr. The supernatant was diluted 1:10 with PBS [50 mM potassium phosphate, pH 7.5/150 mM NaCl] containing 1% Triton X-100 and the protease inhibitors listed above. 259-Sepharose (1.2 ml) was added to the sample and mixed by rotating overnight at 4°C. RAS1 and RAS2 proteins were eluted with 5 ml of 0.1 M glycine hydrochloride (pH 3.0) containing 1 mM phenylmethylsulfonyl fluoride after extensive washes with PBS containing 1% Triton X-100 and the protease inhibitors. The eluate was immediately neutralized by the addition of 1 M Tris·HCl (pH 8.0). The RAS1 protein could also be eluted with 5 ml of PBS containing 1% Triton X-100 after extensive washes with 0.1 M glycine hydrochloride (pH 4.0) containing the protease inhibitors followed by 0.1 M glycine hydrochloride (pH 4.0) containing 1 M NaCl and the protease inhibitors. The recovery of RAS1 and RAS2 proteins as determined by their GDP-binding activities was between 40 and 90%, and an ≈250-fold purification was achieved. Western blotting (immunoblotting) was carried out essentially according to Turner (18) using alkaline phosphatase-conjugated anti-rat IgG (Sigma).

Phospho Amino Acid and Tryptic Phosphopeptide Analyses. ^{32}P-labeled RAS proteins were isolated by immunoprecipitation with the monoclonal antibody Y13-259 followed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Phospho amino acid analyses were carried out as described by Palfrey and Mobley (19). Briefly, ^{32}P-labeled proteins were eluted from a $NaDodSO_4$/polyacrylamide gel and hydrolyzed with 6 M HCl for 3 hr at 110°C, and the hydrolyzate was analyzed by thin layer electrophoresis in 2.5% formic acid/8.7% acetic acid/88.5% H_2O, pH 1.9 (vol/vol). For tryptic phosphopeptide analyses (19), ^{32}P-labeled RAS2 protein bands were excised and the gel pieces were digested with 1 ml of 0.05 M ammonium bicarbonate containing 50 μg of trypsin (Cooper Biomedical) at 37°C for 24 hr. The supernatant was removed and lyophilized, and the peptides were analyzed by thin layer electrophoresis in pyridine/acetic acid/H_2O [1:10:89 (vol/vol), pH 3.5] followed by thin layer chromatography using 1-butanol/acetic acid/H_2O/pyridine [15:3:10:12 (vol/vol)] as solvent.

RESULTS

Phosphorylation of RAS1 Protein. An indication that the yeast RAS proteins are phosphorylated was obtained when these proteins were purified by using monoclonal antibody Y13-259. Briefly, extracts of yeast cells overproducing either RAS1 or RAS2 protein were mixed with Sepharose beads covalently bound to monoclonal antibody Y13-259 (259-Sepharose). After extensive washing, the RAS1 or RAS2 protein was eluted from the 259-Sepharose. Fig. 1 shows a Coomassie stained gel of the purified RAS1 and RAS2 proteins. The purified RAS2 protein appears as a single band having the expected molecular mass of 40 kDa (Fig. 1*A*, lane 2). In contrast, multiple bands are observed with RAS1 protein including a band of the expected molecular mass, 36 kDa, and two or possibly more higher molecular mass bands (Fig. 1*A*, lane 1). The two prominent higher bands have apparent molecular masses of 42 and 40 kDa. These high molecular mass bands represent RAS1 protein and are not contaminants, as demonstrated by the Western blot shown in Fig. 1*B*. Higher molecular mass bands as well as the 36-kDa band are detected by the Y13-259 antibody (Fig. 1*B*, lane 1). In contrast, five control proteins do not bind the antibody (Fig. 1*B*, lane 2), though much larger quantities of these proteins than the RAS1 protein were present (Fig. 1*B*, lanes 3 and 4). Thus, multiple species are present.

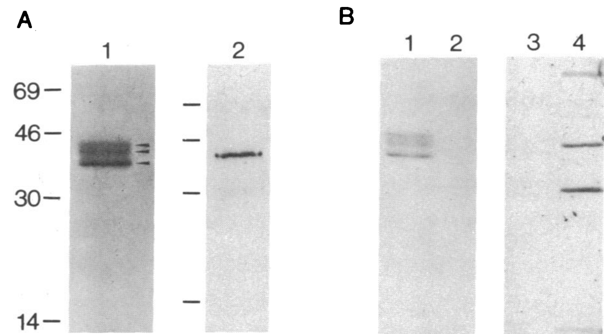

FIG. 1. (*A*) $NaDodSO_4$/polyacrylamide gel of purified RAS1 and RAS2 proteins. RAS1 and RAS2 proteins were purified by 259-Sepharose and analyzed on a $NaDodSO_4$/15% polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Lanes 1 and 2 contain 2 μg of RAS1 and RAS2 proteins, respectively. Marker proteins are albumin (M_r, 69,000), ovalbumin (M_r, 46,000), carbonic anhydrase (M_r, 30,000), and lysozyme (M_r, 14,000). (*B*) Western blot of the purified RAS1 protein. RAS1 protein and control proteins were electrophoresed on a $NaDodSO_4$/15% polyacrylamide gel. The proteins were transferred to a nitrocellulose filter and hybridized with the antibody Y13-259 followed by the alkaline phosphatase-conjugated anti-rat IgG. RAS bands were detected by the incubation with 5-bromo-4-chloro-3-indolylphosphate. Lanes: 1 and 3, 0.5 μg of the purified RAS1 protein; 2 and 4, control proteins [1.6 μg of each marker protein: albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase (M_r, 36,000), carbonic anhydrase, and lysozyme]. For lanes 3 and 4, proteins were electrophoresed on a $NaDodSO_4$/15% polyacrylamide gel and stained with Coomassie brilliant blue. The intensity of ovalbumin band is less, presumably due to breakdown.

The high molecular mass RAS1 protein bands contain phosphorylated proteins as shown by treatment with calf intestinal alkaline phosphatase (Fig. 2). The RAS1 protein purified by 259-Sepharose was treated with calf intestinal alkaline phosphatase and analyzed on a $NaDodSO_4$/polyacrylamide gel. The high molecular mass bands were no longer seen after the treatment (lanes 2 and 3). This phosphatase reaction was inhibited by the addition of 10 mM potassium phosphate (pH 7.5) (data not shown). The 69-kDa band is alkaline phosphatase.

Phosphorylation of RAS1 protein was further confirmed by labeling with radioactive phosphate. Yeast cells overproducing RAS1 protein were labeled with [^{32}P]orthophosphate and the RAS1 proteins were isolated by immunoprecipitation with Y13-259. Fig. 3*A* shows the results of the analysis of the ^{32}P-labeled RAS1 proteins on a $NaDodSO_4$/polyacrylamide gel. Multiple bands of phosphorylated RAS1 protein are detected and migrate slower than the 36-kDa band (lane 4). The position of the 36-kDa band was identified by a brief labeling with [^{35}S]methionine (lane 2).

Phosphorylated RAS1 proteins can be detected without RAS1 overproduction. We have employed KP1 cells for this experiment. In this mutant, the *RAS2* gene is deleted; only RAS1 protein is present. ^{32}P-labeling of these cells followed by immunoprecipitation with the monoclonal antibody Y13-

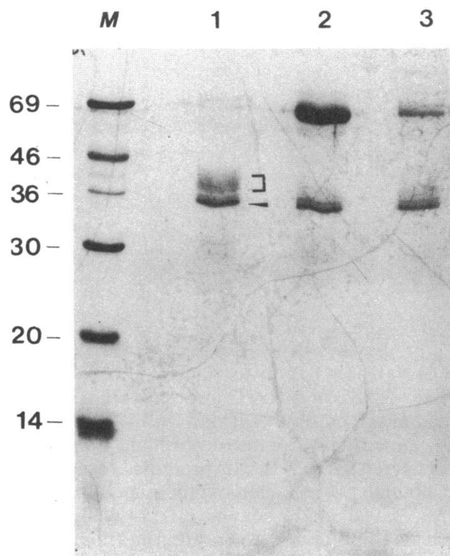

FIG. 2. Alkaline phosphatase treatment of the purified RAS1 protein. RAS1 protein (0.15 μg) purified by 259-Sepharose was treated with 0.48 unit (lane 3) or 4.8 units (lane 2) of calf intestinal alkaline phosphatase (Boehringer Mannheim) in 30 μl containing 5 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 0.05% Triton X-100, and 25 mM KCl. After the incubation at 37°C for 1 hr, the reaction mixtures were electrophoresed on a $NaDodSO_4$/15% polyacrylamide gel and the proteins were stained with Coomassie brilliant blue. Lane 1 shows untreated RAS1 protein. Marker proteins at 36 and 20 kDa are glyceraldehyde-3-phosphate dehydrogenase and trypsin inhibitor, respectively.

259 revealed the presence of ^{32}P-labeled bands (Fig. 3B, lane 2). Multiple bands are present at a position similar to that found with the UC101 cells overproducing RAS1 (compare lane 1 and lane 2 in Fig. 3B).

To determine which amino acid contained phosphate, the radioactive protein bands were eluted from the gel and acid-hydrolyzed. The resulting amino acids were analyzed by thin layer electrophoresis. As shown in Fig. 3C, the ^{32}P radioactivity was found exclusively on serine residues.

Fractionation of the ^{32}P-labeled cell extracts suggests that the ^{32}P-labeled RAS1 proteins are predominantly localized in the membrane. Yeast cells carrying YEp51-RAS1 were labeled with [^{32}P]orthophosphate. After breaking the cells, soluble and membrane fractions were separated and the RAS1 proteins in each fraction were immunoprecipitated using the monoclonal antibody Y13-259. As shown in Fig. 4, the ^{32}P radioactivity was predominantly detected in the membrane fraction (lane 6), with only a minute amount detected in the soluble fraction (lane 4). Minor radioactivity was detected in the soluble fraction at a position corresponding to proteins smaller than RAS1 and may represent degraded protein released from the membrane. Labeling of the identical cells with [^{35}S]methionine followed by subcellular fractionation demonstrated that RAS1 proteins were present in the soluble fraction at a level similar to that found in the membrane fraction (data not shown). In fact, in yeast cells overproducing RAS proteins, the RAS protein concentration in the soluble fraction is similar to that in the membrane fraction (15, 20). Therefore, only the RAS1 proteins in the membrane are phosphorylated.

Phosphorylation of RAS2 Protein. Similar analyses utilizing [^{32}P]orthophosphate were carried out with the RAS2 protein, revealing that the RAS2 protein is also phosphorylated. Yeast cells overproducing the RAS2 protein were labeled with [^{32}P]orthophosphate and the radioactive incorporation into the RAS2 protein was examined. ^{32}P radioactivity corresponds to the position of the RAS2 protein (Fig. 5A, lane 3).

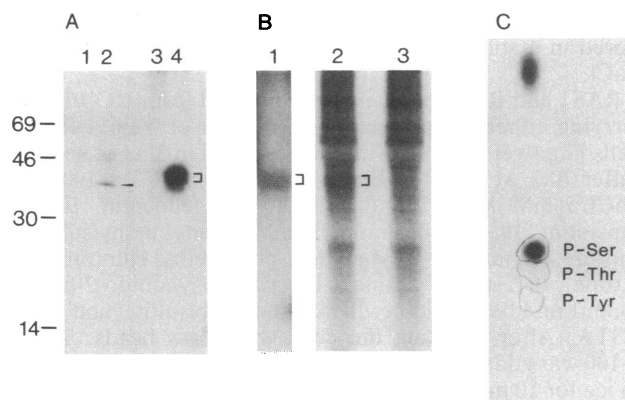

FIG. 3. (A) [^{32}P]Orthophosphate labeling of RAS1 protein. UC101 cells carrying YEp51-RAS1 were grown to late logarithmic phase at 30°C. The cells were collected, resuspended in Burkholder medium, and labeled with [^{32}P]orthophosphate. After breaking the cells, ^{32}P-labeled proteins (2×10^6 cpm) were immunoprecipitated with (lane 4) or without (lane 3) the antibody Y13-259 and the immunoprecipitates were analyzed on a $NaDodSO_4$/15% polyacrylamide gel. The cells were also labeled with [^{35}S]methionine for 5 min and the labeled proteins were immunoprecipitated with (lane 2) or without (lane 1) Y13-259. (B) ^{32}P-labeling of RAS1 proteins in KP1 cells. KP1 cells were grown to late logarithmic phase and labeled with [^{32}P]orthophosphate. After breaking the cells, ^{32}P-labeled proteins (5×10^7 cpm) were immunoprecipitated with (lane 2) or without (lane 3) Y13-259 and the immunoprecipitates were washed 10 times before analyzing on a $NaDodSO_4$/polyacrylamide gel. Lane 1 shows [^{32}P]RAS1 protein immunoprecipiated from extracts of UC101 cells carrying YEp51-RAS1. Radioactivity used here was less than that used in lane 4 of A. (C) Phospho amino acid analyses. ^{32}P-labeled RAS1 proteins isolated from UC101 carrying YEp51-RAS1 and separated on a $NaDodSO_4$/polyacrylamide gel were eluted from the gel, acid-hydrolyzed, and analyzed by thin layer electrophoresis as described by Palfrey and Mobley (19). Marker phospho amino acids were electrophoresed with the sample as well as electrophoresed separately, and their spots were identified by ninhydrin staining.

The position of this ^{32}P band is identical to that of [^{35}S]methionine-labeled RAS2 protein (compare lanes 2 and 3). This

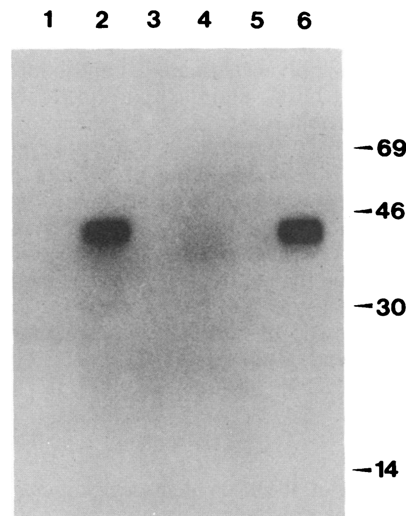

FIG. 4. Subcellular fractionation of phosphorylated RAS1 proteins. UC101 cells carrying YEp51-RAS1 were labeled with [^{32}P]orthophosphate for 5 hr in Burkholder medium. After breaking the cells, soluble and membrane fractions were fractionated as described (15). Immunoprecipitations of total cell extracts (lanes 1 and 2), soluble fraction (lanes 3 and 4), and membrane fraction (lanes 5 and 6) were carried out with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) monoclonal antibody Y13-259 and analyzed by $NaDodSO_4$/12.5% polyacrylamide gel.

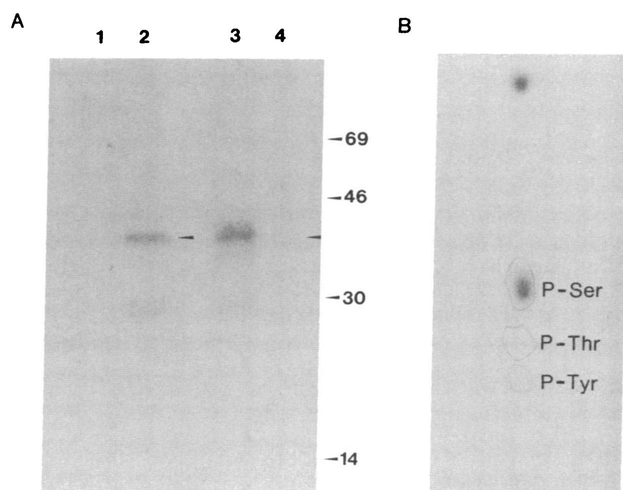

FIG. 5. (*A*) [^{32}P]Orthophosphate labeling of RAS2 protein. UC101 cells carrying YEp51-RAS2 were labeled with [^{32}P]orthophosphate in Burkholder medium for 5 hr. ^{32}P-labeled proteins were immunoprecipitated with (lane 3) or without (lane 4) the monoclonal antibody Y13-259 and the immunoprecipitates were analyzed on a $NaDodSO_4$/12.5% polyacrylamide gel. The cells were also labeled with [^{35}S]methionine for 1 hr and immunoprecipitations were carried out with (lane 2) or without (lane 1) the monoclonal antibody Y13-259. (*B*) Phospho amino acid analyses. ^{32}P-labeled RAS2 protein separated on a $NaDodSO_4$/polyacrylamide gel was eluted, acid-hydrolyzed, and analyzed by thin layer electrophoresis as described in Fig. 3. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

is in marked contrast to the RAS1 situation. Apparently, the phosphorylation of RAS2 protein does not change its mobility on a $NaDodSO_4$/polyacrylamide gel. This explains why only a single band was observed when the RAS2 protein purified by the 259-Sepharose was analyzed on a $NaDodSO_4$/polyacrylamide gel (Fig. 1*A*, lane 2).

When the ^{32}P-labeled RAS2 protein was acid-hydrolyzed and phospho amino acid analysis was carried out, it was found that the phosphorylation occurred exclusively on serine residues (Fig. 5*B*). Thus, the phosphorylation is serine specific for both the RAS1 and RAS2 proteins.

Although the phosphorylated RAS2 proteins could not be separated on a one-dimensional gel, it was possible to separate the phosphorylated proteins by two-dimensional gel electrophoresis. As shown in Fig. 6*A*, two major spots with several minor spots were found when the ^{32}P-labeled RAS2 protein was analyzed by two-dimensional gel electrophoresis. The two major spots correspond to proteins with pI values of 5.9 and 5.6. Minor spots toward the lower right side have low molecular masses and are likely to be degradation products. When a similar gel was silver stained, an additional, more basic spot was detected. This spot presumably represents the unphosphorylated RAS2 protein.

To gain insight into the number of sites (regions) phosphorylated on the RAS2 protein, tryptic phosphopeptide analyses were carried out. As shown in Fig. 6*B*, two major spots and possibly three minor spots were found. According to the sequence of the *RAS2* gene, there should be 30 peptides, 19 of which should contain serine residues. Thus, the phosphorylation occurs at a relatively small number of specific sites on the RAS2 protein.

DISCUSSION

In this paper, we have presented *in vivo* evidence for the phosphorylation of RAS1 and RAS2 proteins. RAS1 protein phosphorylation was first hypothesized upon the realization that the protein consists of heterogeneous species. Phospho-

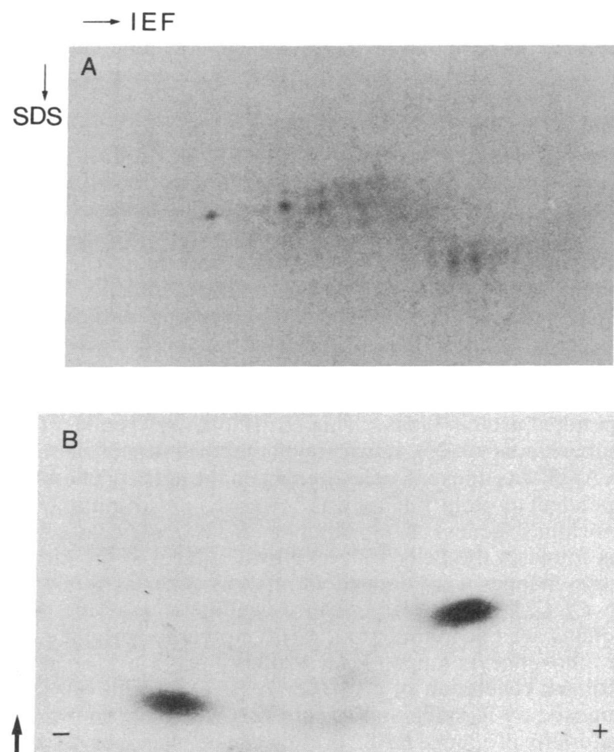

FIG. 6. (*A*) Two-dimensional gel electrophoresis of ^{32}P-labeled RAS2 protein. ^{32}P-labeled RAS2 proteins were isolated from UC101 cells carrying YEp51-RAS2 by the immunoprecipitation with Y13-259 antibody. The sample was separated by isoelectric focusing (IEF) in the first dimension and $NaDodSO_4$/12.5% polyacrylamide gel, electrophoresis in the second dimension. (*B*) Tryptic phosphopeptide analysis of ^{32}P-labeled RAS2. Tryptic peptides were applied to the lower right hand corner and subjected to thin layer electrophoresis in the first dimension and ascending chromatography in the second dimension in the direction of the arrow. We have only shown half of the thin layer plate but the other half did not have any radioactive spots.

rylation of RAS1 protein was suggested by Temeles *et al.* (21). However, no results were given in their report. To the best of our knowledge, our paper provides the first demonstration that the phosphorylation of RAS1 protein occurs in yeast cells. We have further shown that the RAS2 protein is also phosphorylated. In contrast to RAS1, phosphorylation does not alter the mobility of the RAS2 protein on a $NaDodSO_4$/polyacrylamide gel. It is surprising that the two rather homologous proteins behave so differently. Since these two proteins differ markedly in their C-terminal portions, it is possible that conformational changes due to phosphorylation may also differ between the two.

Although most of our analyses were carried out with yeast cells overproducing RAS proteins, phosphorylated RAS1 and RAS2 proteins are readily detected in yeast cells without overproduction. Using a mutant having the *RAS2* gene deleted, we have detected ^{32}P-labeled RAS1 proteins with high molecular masses (Fig. 3*B*). When a similar experiment was carried out with another mutant having the *RAS1* gene deleted, we detected only one band of an apparent molecular mass of 40 kDa by labeling with [^{32}P]orthophosphate (A.R.C. and F.T., unpublished results). This is as we observed with yeast cells overproducing the RAS2 protein. Thus, phosphorylation occurs in a similar manner in cells not overproducing RAS proteins.

Three observations have been made concerning the phosphorylation of RAS proteins. First, the phosphorylation of RAS1 and RAS2 proteins occurs exclusively on serine residues. Second, it appears that only a limited number of

phosphorylation sites are present on these proteins. In this paper, we have shown by tryptic phosphopeptide analyses that there are only two major phosphorylation sites (regions) on the RAS2 protein. We have also carried out a similar analysis with RAS1 protein. Preliminary results suggest that there are only a few phosphorylation sites on this protein (A.R.C. and F.T., unpublished observation). Finally, phosphorylated RAS proteins are predominantly localized in the membrane, where the action of these proteins occurs. Thus, these results suggest that the phosphorylation is not just a random event but may play a physiologically significant role.

What possible physiological roles could then be served by phosphorylation of the RAS proteins? As described earlier, RAS proteins stimulate adenylate cyclase thus controlling the level of cAMP, which in turn regulates the activity of cAMP-dependent protein kinase. This cAMP pathway is under tight feedback control (22). It was found that the intracellular level of cAMP was unusually elevated when the activity of cAMP-dependent protein kinase was lowered due to mutation of these kinase genes. On the other hand, a lower level of cAMP was found in the cells with an elevated level of the kinase. These changes were dependent on *RAS* genes as well as on the *CDC25* gene (22). Because we know that the RAS proteins are phosphorylated, it is tempting to speculate that the phosphorylation of RAS proteins plays a role in the feedback regulation of cAMP in yeast cells. This has been proposed by Resnick and Racker (23) who have shown that bacterially produced RAS2 protein can be phosphorylated by purified cAMP-dependent protein kinase and that the phosphorylated protein is not as effective as the unphosphorylated proteins in stimulating adenylate cyclase. Using RAS2 protein purified from yeast cells, we have also observed that the protein can be phosphorylated by cAMP-dependent protein kinase but not by protein kinase C (A.R.C. and F.T., unpublished results). Comparison of tryptic phosphopeptides between the *in vitro* phosphorylated protein and the *in vivo* phosphorylated protein reveals that the same set of peptides are phosphorylated but that the relative intensities are not identical (A.R.C. and F.T., unpublished results). Further experiments are needed to determine whether the phosphorylation carried out by cAMP-dependent protein kinase *in vitro* reflects the phosphorylation we observe *in vivo*.

Among *ras* gene products, phosphorylation is not limited to the yeast proteins. A low but significant level of phosphorylation has been detected on the serine residues of c-Ha-ras proteins (24, 25). A serine phosphorylation event was observed with c-Ki-ras protein when the cells were treated with a phorbol ester, suggesting that the phosphorylation in this case is catalyzed by protein kinase C (26). Thus, phosphorylation may be a general feature of ras proteins and play a role in regulating their action. Elucidating the biochemical consequences of the phosphorylation should contribute to the further understanding of the action of ras proteins in general.

Note Added in Proof. c-Ha-ras protein has been demonstrated to be phosphorylated at a single site (serine-177) (27).

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