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Modulation of Maturation and Ribosomal Protein S6 Phosphorylation in *Xenopus* Oocytes by Microinjection of Oncogenic *ras* Protein and Protein Kinase C

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Using *Xenopus* oocytes as a model system, we investigated the possible involvement of *ras* proteins in the pathway leading to phosphorylation of ribosomal protein S6. Our results indicate that microinjection of oncogenic T24 H-*ras* protein (which contains valine at position 12) markedly stimulated S6 phosphorylation on serine residues in oocytes, whereas normal *ras* protein (which contains glycine at position 12) was without effect. The S6 phosphorylation activity in the cell extract from T24 *ras* protein-injected oocytes was increased significantly. In addition, injection of protein kinase C potentiated the induction of maturation and S6 phosphorylation by the oncogenic *ras* protein. A similar potentiation was detected when T24 *ras* protein-injected oocytes were incubated with active phorbol ester. These findings suggest that *ras* proteins activate the pathway linked to S6 phosphorylation and that protein kinase C has a synergistic effect on the *ras*-mediated pathway.

ras p21 proteins localize on the cytoplasmic side of plasma membrane and have GDP-GTP-binding and GTPase activity (2). Point mutations can convert the normal *ras* proteins into the oncogenic proteins with reduced intrinsic GTPase activity. This biochemical property could be responsible for the ability of oncogenic *ras* proteins to induce cellular DNA synthesis and cell transformation. Given the similarity in amino acid sequences with G proteins, it is possible that *ras* proteins play a regulatory role in membrane signal transduction involved in cell profileration (10, 13, 17, 18, 27, 31). However, the biochemical pathway influenced by *ras* proteins has not been fully elucidated. In our study, we focused on the functional relationship between *ras* protein and protein kinase pathways. So far, there have been few studies on this subject, although considerable efforts have been made to investigate the links between *ras* proteins and growth factor receptor-mediated phospholipid turnover involving phospholipase C and protein kinase C (11, 19, 20, 23, 36, 37).

A chain of phosphorylation reactions is regarded as a key regulatory system in cell growth control. Ribosomal protein S6 is one of the growth-associated phosphoproteins, and its phosphorylation has been implicated in the initiation of protein synthesis, possibly exerting a role in the binding of specific mRNAs to ribosomes (6, 14). Phosphorylation of S6 is stimulated in cultured cells treated with serum or growth factors including insulin or epidermal growth factor (33) and is augmented in cells transformed with the Rous sarcoma virus oncogene ($p60^{src}$) (4) or the Abelson murine leukemia virus oncogene (*abl*) (12). These observations imply that the phosphorylation event is an important mediator for cell proliferation and cell transformation.

Increased S6 phosphorylation has also been found in viral Harvey *ras* oncogene-transformed cultured cells (3). This raises the possibility that *ras* proteins are involved in the pathway leading to S6 phosphorylation. To analyze the mechanism of induction of S6 phosphorylation by expression of oncogenic *ras* proteins, we microinjected *ras* proteins into *Xenopus* oocytes because their large size allows biochemical study on injected cells. In *Xenopus* oocytes, ribosomal protein S6 is phosphorylated during oocyte meiotic maturation (G_2-to-M transition) caused by progesterone or insulin (8, 25) and in response to microinjection of $p60^{src}$ or *abl* protein (26, 30). For *ras* proteins, microinjection of the oncogenic form of *ras* protein (containing valine at position 12) into *Xenopus* oocytes induces oocyte maturation (5), and at least one of the *ras* protein functions might be related to insulin-induced oocyte maturation (7, 21). Our data indicate that microinjection of the oncogenic H-*ras* protein into oocytes induces the phosphorylation of S6 protein and causes the activation of S6 kinase. Furthermore, protein kinase C appears to potentiate the induction of maturation and S6 phosphorylation by the oncogenic *ras* proteins.

MATERIALS AND METHODS

Protein purification. Normal and oncogenic human T24 H-*ras* proteins (containing Gly-12 and Val-12, respectively) were expressed in an *Escherichia coli* expression system (provided by R. Sweet, Smith Kline & French Laboratories) and purified to homogeneity, as described previously (18). Protein kinase C was purified from rat brain, as described previously (20). The enzyme was essentially >99% pure, and its specific activity was 230 nmol/min per mg.

Analysis of maturation and S6 phosphorylation in *ras*-injected oocytes. Oocytes at stage VI were manually dissected from the ovarian follicle of sexually mature female *Xenopus laevis* (*Xenopus* One, Wis.) and prelabeled for 3 h at 19°C in MBS-H buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$) containing $^{32}P_i$ (0.2 mCi/ml). The labeled oocytes were refed with fresh MBS-H buffer.

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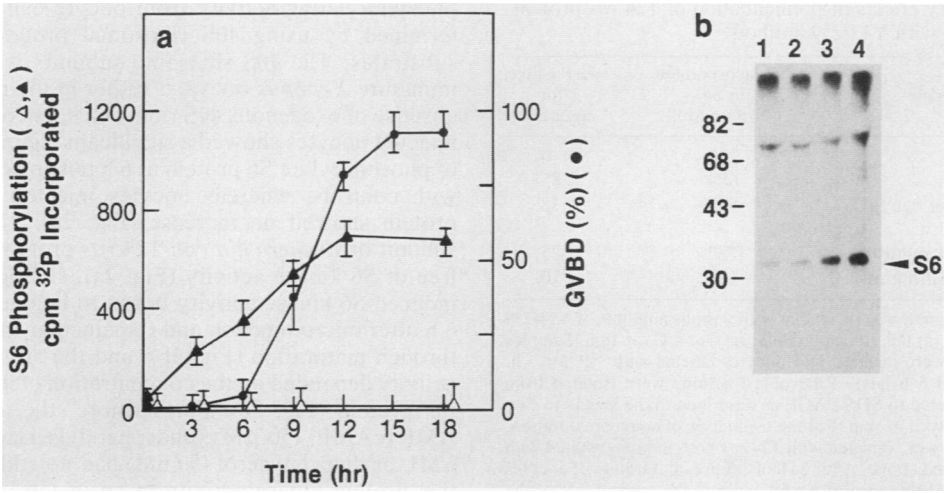

FIG. 1. Phosphorylation of S6 protein in oocytes after microinjection of *ras* proteins. (a) ^{32}P-labeled oocytes at stage VI were injected with normal (△) or oncogenic (▲) T24 *ras* protein. Injected oocytes were harvested at the indicated times after injection, and phosphorylated S6 proteins in ribosomes were analyzed by SDS-PAGE followed by autoradiography. Maturation of oocytes induced by T24 *ras* proteins (●) was determined for the same set of oocytes. Data represent means ± standard errors of three separate experiments. GVBD, Germinal vesicle breakdown. (b) Lanes 1 to 4 show S6 phosphorylation induced by 0, 12, 25, and 50 ng, respectively, of T24 *ras* protein at 6 h postinjection. The radioactivity in the S6 band in lanes 1 to 4 was 95, 101, 251, and 480 cpm, respectively.

After microinjection of either purified *ras* proteins (50 nl; 1 mg/ml), bovine serum albumin (BSA; 50 nl, 1 mg/ml), or protein kinase C (50 nl, 0.5 mg/ml), injected oocytes (15 to 20) were chronologically harvested and homogenized in 2 ml of lysing buffer A [50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.5), 5 mM $MgCl_2$, 5 mM KCl, 50 mM NaF, 4 μM EDTA, 1% (wt/vol) deoxycholate, 1% (wt/vol) Triton X-100]. Extracts were separated by high-speed centrifugation (10,000 × *g* for 10 min at 4°C) and then layered onto 2 ml of 1.6 M sucrose–50 mM PIPES–5 mM $MgCl_2$–0.5 M KCl–4 μM EDTA. The ribosome fractions were pelleted by centrifugation (100,000 × *g* for 16 h at 4°C). Pelleted samples were suspended in Laemmli sample buffer (24) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) followed by autoradiography. To determine oocyte maturation, we examined the appearance of a white spot in the animal pole of the oocyte or the breakdown of the germinal vesicle (5).

Determination of S6 kinase and phosphatase activity. The S6 kinase assay was performed by a modification of the published method (9). After microinjection of *ras* proteins (50 ng), ca. 20 injected oocytes were harvested at various times and homogenized in 500 μl of homogenization buffer [55 mM β-glycerophosphate (pH 6.8), 5 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 50 mM NaF, 100 mM $Na_4P_2O_7$, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml] with a glass homogenizer (10 strokes). The homogenates were centrifuged at 5,000 × *g* for 10 min, and the yolk part was removed. The extracts were further centrifuged at 100,000 × *g* for 2 h at 42°C. Phosphorylation was carried out for 15 min at 30°C in a volume of 80 μl containing 20 mM HEPES (pH 7.0), 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 100 μM ATP, 2 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), 10 μg of 40S ribosomal protein, and an appropriate amount of extracts. The 40S ribosomes were isolated by a previously published procedure (38). The reaction was terminated by the addition of Laemmli sample buffer. The samples were boiled for 2 min and analyzed by SDS-PAGE (10% acrylamide) followed by autoradiography.

Analysis of S6 phosphorylation by two-dimensional electrophoresis. The ribosomal fractions were isolated as described above. The procedure of Thomas et al. (33) was used to extract the ribosomal proteins, precipitate them, and subject them to two-dimensional gel electrophoresis with carrier ribosomal proteins from *Xenopus* oocytes and unfertilized eggs. The ribosomal proteins were stained with Coomassie blue, and the labeled S6 phosphoproteins were analyzed by autoradiography.

RESULTS

Induction of ribosomal protein S6 phosphorylation by microinjection of oncogenic H-*ras* protein. Microinjection of the oncogenic *ras* protein into *Xenopus* oocytes induces oocyte maturation in the absence of hormone (5). To analyze the effect of *ras* proteins on the phosphorylation of S6 protein, we have purified bacterially made normal (Gly-12) or oncogenic (Val-12) human T24 H-*ras* proteins and injected them (50 ng) into immature *Xenopus* oocytes which were prelabeled with $^{32}P_i$ for 3 h. These labeling conditions were sufficient to equilibrate the intracellular [^{32}P]ATP pool. Ribosomal fractions were then fractionated, and phosphoproteins were analyzed by SDS-PAGE. As reported previously (5), about 90% of oocytes were mature 15 h after microinjection of the oncogenic *ras* protein. In contrast, the same amount of normal *ras* protein had no detectable effect on oocyte meiotic maturation. In the time course experiment under the same conditions, oncogenic *ras* protein was found to markedly increase the phosphorylation of S6 protein, whereas normal *ras* protein had little or no effect (Fig. 1a). S6 phosphorylation induced by the oncogenic *ras* protein reached a maximal level by 12 h postinjection (Fig. 1a) and increased in a dose-dependent manner (Fig. 1b). To examine whether the stimulation of S6 phosphorylation by microinjected T24 *ras* protein is dependent upon de novo protein synthesis, we pretreated oocytes with cycloheximide (100

TABLE 1. Inhibitory effects of preincubation of T24 *ras* protein with Y13-259 antibody[a]

Microinjected sample	^{32}P incorporated in S6 (% of control)	% Positive for maturation
BSA	100	0
T24 *ras*	689	85
T24 *ras* + nonimmune rat IgG (10 mg/ml)	593	78
T24 *ras* + Y13-259 (4 mg/ml)	182	25
T24 *ras* + Y13-259 (10 mg/ml)	129	10

[a] T24 *ras* protein (1 mg/ml) was incubated with various amounts of Y13-259 antibody or nonimmune rat immunoglobulin G (IgG) at 4°C for 18 h. Samples (50 nl) of the mixtures were injected into oocytes labeled with $^{32}P_i$ for 3 h. Oocytes were harvested 6 h later. Ribosome fractions were isolated from lysed oocytes and subjected to SDS-PAGE as described in the legend to Fig. 1. The control value shows 110 cpm. For the evaluation of oocyte maturation, ca. 20 immature oocytes were injected with T24 *ras* protein preincubated with the antibodies as described above. After 24 h of culture, maturation of oocytes was determined by the appearance of a white spot in the animal pole of the oocytes or by the breakdown of the germinal vesicle. Data show the mean values of duplicate experiments, and similar results were obtained in separate experiments.

μg/ml) for 2 h prior to microinjection of the *ras* protein and cultured the injected oocytes in the presence of cycloheximide until they were harvested. There was no difference in the level of S6 phosphorylation between cycloheximide-treated and untreated oocytes, indicating that the stimulatory effect of T24 *ras* protein on S6 phosphorylation does not require de novo protein synthesis (data not shown). When the total nucleotides extracted from oocytes were analyzed in a Dowex AG1X-8 column with a linear ammonium formate gradient (20 mM to 1 M in 8 M formic acid), there was no significant difference (less than 10%) in the specific activity of ATP (cpm per A_{254} unit) between BSA-injected and *ras* protein-injected oocytes. This indicates that the specific activity of ATP was not changed by the administration of *ras* proteins. The specificity of the effects of *ras* was examined by using the monoclonal anti-*ras* antibody Y13-259, which specifically blocks the *ras* guanine nucleotide exchange activity (15) and serum-induced DNA synthesis in NIH 3T3 cells (27). Experiments demonstrated that preincubation of T24 *ras* protein with the *ras* neutralizing antibody Y13-259 blocked the induction of both S6 phosphorylation (80% inhibition) and oocyte maturation (89% inhibition) by T24 *ras* protein (Table 1). In contrast, nonimmune-rat immunoglobulin G did not have inhibitory effects (Table 1). Thus, the different potencies of the oncogenic and normal *ras* proteins in inducing S6 phosphorylation correlate with their different abilities to induce maturation in oocytes. This reflects the previous observation that oncogenic mutant *ras* proteins have less GTPase activity and are more potent in inducing cellular DNA synthesis than their normal counterparts (2, 10, 31). So far, only serine residues were identified as a phosphate acceptor in phosphorylated S6 protein in oocytes treated with various reagents. Two-dimensional phosphoamino acid analysis of phosphorylated S6 in oocytes injected with T24 *ras* protein showed no phosphotyrosine or phosphothreonine, but phosphoserine was detected exclusively (data not shown). Therefore, as in the case of progesterone- or insulin-treated (25) and *abl*- or $p60^{src}$-injected (26, 30) oocytes, a certain type of serine kinase could be activated or a serine phosphatase could be suppressed under these conditions.

Activation of S6 kinase activity by oncogenic H-*ras* protein. To further explore the above possibility, the S6 protein phosphorylating activity from oocyte cell extracts was determined by using 40S ribosomal proteins as exogenous substrates. The 40S ribosome subunits were isolated from immature *Xenopus* oocytes. Either in the presence or in the absence of exogenous 40S ribosomes, oncogenic *ras* protein-injected oocytes showed a significant increase in their ability to phosphorylate S6 protein at 6 h postinjection as compared with controls, whereas oocytes injected with normal *ras* protein showed no increase (Fig. 2a). The 40S ribosome subunit or *Escherichia coli* T24 *ras* protein preparation was free of S6 kinase activity (Fig. 2a). Oncogenic *ras* protein-induced S6 kinase activity began to increase between 3 and 6 h after microinjection and remained at the maximum level through maturation (Fig. 2b), and the stimulation of kinase activity depended on the concentration of injected oncogenic *ras* protein (Fig. 2c). Furthermore, the addition of cyclic AMP (cAMP) (50 μM), phosphatidylserine (5 μg)-Ca^{2+} (50 μM), or diacylglycerol (50 nM) had no stimulatory effect on this protein kinase activity in an in vitro assay, indicating that the activated kinase is different from cAMP-dependent protein kinase or protein kinase C (data not shown).

Synergistic effects of protein kinase C on oncogenic H-*ras* action. *ras* p21 proteins may be important elements of the signal transduction pathway involved in cell growth. One of the pathways would be coupled with calcium and phospholipid-dependent protein kinase C. Recent studies have shown that the level of diacylglycerol, an endogenous activator of protein kinase C, is rapidly increased following the introduction of the oncogenic *ras* protein into *Xenopus* oocytes (23). Therefore, it was of interest to investigate whether protein kinase C affects oncogenic *ras* protein-induced biological responses in oocytes. For this purpose, ^{32}P-labeled oocytes were injected with protein kinase C (25 ng) purified from rat brain either alone or in combination with T24 *ras* protein (50 ng). Our data indicate that T24 *ras* protein alone induced about 35% of oocytes to mature at 10 h postinjection, whereas 74% of oocytes matured in 10 h when T24 *ras* protein and protein kinase C were coinjected (Table 2). At 24 h after injection, the level of maturation was 80% for oocytes coinjected with the two proteins and 85% for oocytes injected with T24 *ras* protein alone. Although normal *ras* protein is much less potent than T24 *ras* protein in the induction of maturation, a high dose of normal *ras* protein (200 ng per oocyte) can induce maturation in 10 to 20% of oocytes at 24 h postinjection (Table 2) (5). In contrast, coinjection of normal *ras* protein and protein kinase C resulted in a twofold increase in maturation (Table 2). This suggests that protein kinase C synergistically acts with both T24 *ras* protein and high concentrations of normal *ras* protein. Microinjection of 25 ng of protein kinase C alone did not cause a significant increase in oocyte maturation. Analysis of the phosphorylation level of S6 protein revealed that the degree of S6 phosphorylation was higher in oocytes coinjected with T24 *ras* protein and protein kinase C than that in oocytes injected with T24 *ras* protein alone. However, there was no effect on S6 protein phosphorylation 25 min, 6 h, 10 h, and 24 h after injection of protein kinase C alone (Fig. 3a). When the specific activity of ATP was examined as described for Fig. 1, there was no significant change (less than 10%) between oocytes injected with T24 *ras* protein and oocytes injected with T24 *ras* protein plus protein kinase C. Heat inactivation (at 100°C for 5 min) of the protein kinase C preparation abolished its stimulatory effects. Serine residues of S6 protein were shown to be phosphorylated in these phosphorylation reactions (data not shown). A similar enhancement of maturation (Table 2) and

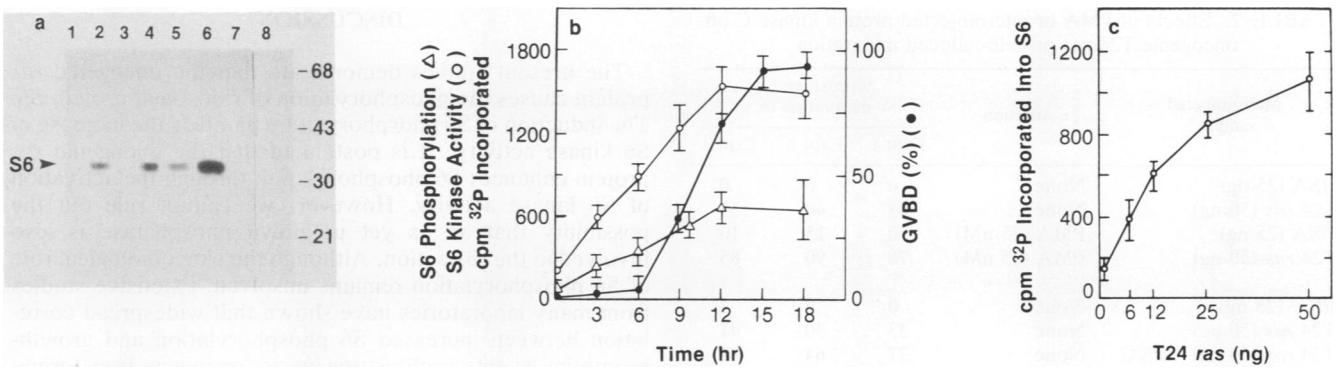

FIG. 2. S6 kinase activity following microinjection of *ras* proteins. The extracts were prepared from oocytes microinjected with *ras* proteins (50 ng) and were incubated with [γ-^{32}P]ATP in the presence or absence of exogenous 40S ribosomes (10 μg) as described in Materials and Methods. Then phosphorylated samples were analyzed by SDS-PAGE. (a) S6 phosphorylation with oocyte extracts (10 μg) at 6 h postinjection. Samples are BSA-injected oocyte extracts incubated with (lane 2) or without (lane 1) 40S ribosomes, normal *ras* protein-injected oocyte extracts incubated with (lane 4) or without (lane 3) 40S ribosomes, T24 *ras* protein-injected oocyte extracts incubated with (lane 6) or without (lane 5) 40S ribosomes, 40S ribosomes alone (lane 7), and 40S ribosomes plus T24 *ras* protein (lane 8). The radioactivity (cpm) in S6 was as follows: lane 1, 86; lane 2, 214; lane 3, 88; lane 4, 213; lane 5, 221; lane 6, 757; lane 7, 10; lane 8, 20. (b) Time course of induction of S6-kinase activity by T24 *ras* protein (50 ng) (○). Extracts (10 μg) were prepared from T24 *ras* protein-injected oocytes at the indicated times after injection and were reacted with 40S ribosomes (10 μg). The level of S6 phosphorylation (△) and percent germinal vesicle breakdown (GVBD) (●) were examined in the same set of oocytes as those used for Fig. 1 and Table 1. Data represent means ± standard errors of three separate experiments. (c) Effects of concentration of injected T24 *ras* protein on S6 kinase activity. The indicated amounts of T24 *ras* protein were injected into oocytes. Oocytes were harvested 6 h later, and 10 μg of cell extracts was reacted with 40S ribosomes (10 μg) in the S6 kinase assays described above. In panels b and c, the radioactivity in the S6 band in the reactions without the addition of 40S ribosomes (maximum values were 210 cpm for panel b and 290 cpm for panel c) has been subtracted for the value shown. Data represent means ± standard errors of three separate experiments.

S6 phosphorylation (Fig. 3b) was observed when 4-β-phorbol-12-myristate 13-acetate (PMA) was added to oocytes injected with T24 *ras* protein. Treatment of the oocytes with 4-α-phorbol-12,13-didecanoide (PDD), an inactive analog of PMA, did not have the stimulatory effect. Taken together, these results provide evidence that protein kinase C potentiates the *ras* protein action by increasing the rate of induction of both oocyte maturation and S6 phosphorylation.

Two-dimensional gel electrophoresis of S6 phosphorylation. To further analyze the synergistic effect of protein kinase C on T24 *ras* protein-induced S6 phosphorylation, we examined the phosphorylated S6 derivatives by two-dimensional gel electrophoresis. In T24 *ras* protein-injected oocytes, S6 derivatives b and c were predominantly phosphorylated and some d form was phosphorylated (Fig. 4C). In contrast, only the "a" form was phosphorylated in BSA-injected oocytes (Fig. 4A). These results indicate that microinjected T24 *ras* protein increased the phosphorylation of S6 protein quantitatively as well as qualitatively. In protein kinase C-injected oocytes, the species of phosphorylated derivatives were virtually only of the "a" form and there was almost no increase in ^{32}P labeling (Fig. 4B). Moreover, in oocytes coinjected with protein kinase C and T24 *ras* protein, the species of ^{32}P-labeled S6 derivatives were quite similar to that of T24 *ras* protein-injected oocytes, although there was a slight increase in the c form. Also, ^{32}P incorporation into the S6 derivatives in the former was increased compared with that in the latter (Fig. 4D). This indicates that microinjected protein kinase C alone did not stimulate S6 phosphorylation and that protein kinase C enhanced T24 *ras* protein-induced S6 phosphorylation quantitatively but not qualitatively. Thus, the synergistic effect of protein kinase C on T24 *ras*-induced S6 phosphorylation could be due to an overall increase in phosphorylation of S6 derivatives rather than a qualitative change in the species of phosphorylated S6 derivatives.

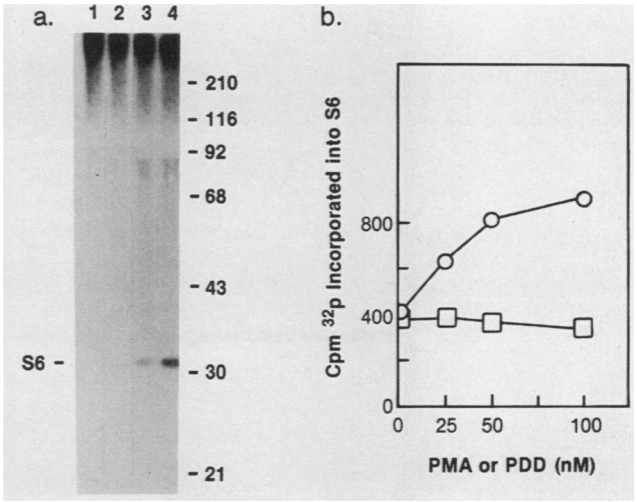

FIG. 3. Stimulation of S6 phosphorylation by microinjected protein kinase C or PMA in T24 *ras* protein-injected oocytes. (a) ^{32}P-labeled oocytes were injected with BSA (50 nl; 0.5 mg/ml) (lane 1), protein kinase C (50 nl; 0.5 mg/ml) (lane 2), T24 *ras* protein (50 nl; 1 mg/ml) (lane 3), or protein kinase C plus T24 *ras* protein (lane 4). After incubation for 6 h, ribosome fractions were extracted and subjected to SDS-PAGE (10% acrylamide) followed by autoradiography, as described in the legend to Fig. 1. The radioactivity of ^{32}P incorporated into S6 in lanes 1 to 4 was 150, 165, 383, and 685 cpm; respectively. (b) In another set of experiments, labeled oocytes were injected with T24 *ras* protein (50 nl; 0.5 mg/ml), and injected oocytes were treated with increasing amounts of PMA (○) or 4-α-phorbol-12,13-didecanoide (PDD) (□) for 6 h. Data represent the mean value of duplicate experiments.

TABLE 2. Effects of PMA or microinjected protein kinase C on oncogenic T24 *ras* protein-induced maturation

Microinjected sample[a]	Addition	% Positive for maturation in:		
		Expt 1	Expt 2	Expt 3
BSA (25 ng)	None	0	0	0
T24 *ras* (50 ng)	None	43	40	33
BSA (25 ng)	PMA (25 nM)	0	15	10
T24 *ras* (50 ng)	PMA (25 nM)	76	90	85
PKC (25 ng)	None	0	0	7
T24 *ras* (50 ng)	None	33	30	41
T24 *ras* (50 ng) +PKC (25 ng)	None	77	64	82
BSA (25 ng)	None	0	0	0
PKC (25 ng)	None	0	5	0
wt *ras* (200 ng)	None	10	17	11
wt *ras* (200 ng) +PKC (25 ng)	None	28	30	21

[a] Immature oocytes at stage VI (about 20) were injected with BSA (50 nl; 0.5 mg/ml) or T24 *ras* protein (50 nl; 1 mg/ml), and injected oocytes were incubated with PMA (25 nM) as described in the legend to Fig. 3. In another set of experiments, oocytes (about 20) were injected with purified protein kinase C (PKC) (50 nl; 0.5 mg/ml) alone, T24 *ras* protein (50 nl; 1 mg/ml) alone, normal *ras* protein (wt) alone, protein kinase C plus T24 *ras* protein, or protein kinase C plus normal *ras* protein. After incubation in MBS-H buffer at 19°C, maturation of oocytes was determined at 10 h postinjection.

DISCUSSION

The present studies demonstrate that the oncogenic *ras* protein causes the phosphorylation of ribosomal protein S6. The induction of S6 phosphorylation parallels the increase of S6 kinase activity. It is postulated that the oncogenic *ras* protein enhances S6 phosphorylation through the activation of S6 kinase activity. However, we cannot rule out the possibility that an as yet unknown phosphatase is also involved in the *ras* action. Although the exact biological role of S6 phosphorylation remains unsolved, extensive studies from many laboratories have shown that widespread correlation between increased S6 phosphorylation and growth-promoting events such as oogenesis, oncogene transformation, and growth factor-induced cell proliferation (3, 4, 8, 12, 25, 30, 33). Thus, our studies provide further evidence of the important role of S6 phosphorylation in the cell cycle, and the *ras*-transformed phenotype could be associated with the constitutive activation of S6 phosphorylation in the cell cycle, as reported previously (3).

A number of protein kinases capable of phosphorylating S6 have been characterized from various sources. Among them, S6 II kinase purified from *Xenopus* eggs appears to have a high substrate specificity for S6 (9). This protein kinase has two kinase domains which have high sequence similarity to the regions of protein kinase C and the catalytic subunit of cAMP-dependent protein kinase (16). The kinase activity cross-reactive with anti-S6 II kinase antibody has been detected in progesterone- or insulin-stimulated oocytes (16). Whether the S6 serine kinase activated by T24 *ras*

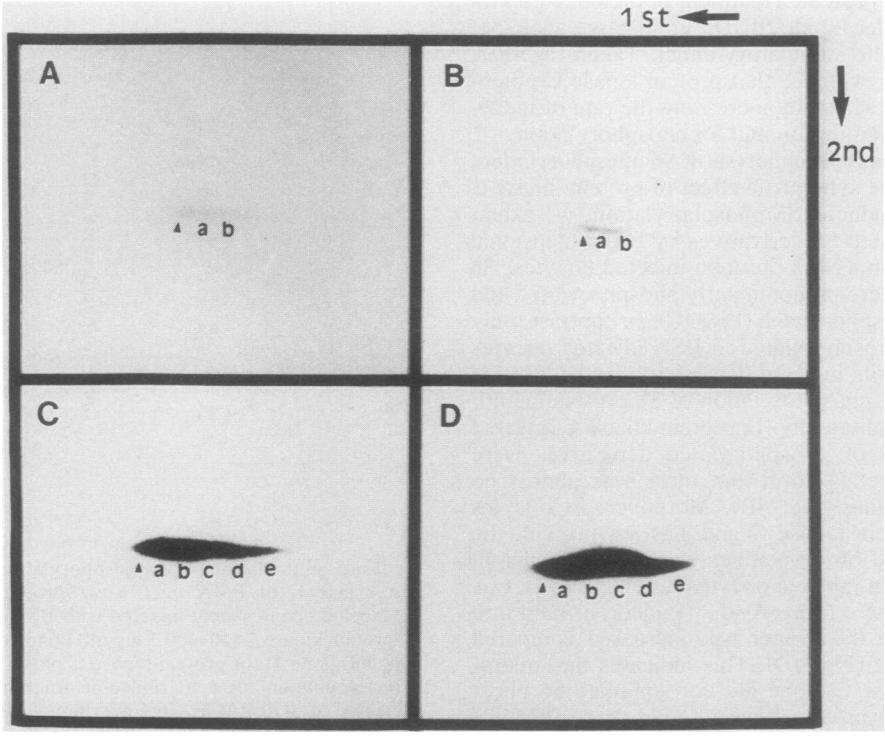

FIG. 4. Analysis of S6 phosphorylation by two-dimensional gel electrophoresis. ^{32}P-labeled oocytes were injected with BSA (A), protein kinase C (B), T24 *ras* protein (C), or T24 *ras* protein plus protein kinase C (D) as described in the legend to Fig. 3. Ribosomal proteins were prepared from injected oocytes at 6 h postinjection, and two-dimensional gel electrophoresis was performed by the method of Thomas et al. (33). ^{32}P-labeled S6 protein was analyzed by autoradiography. The arrowhead marks the position of unphosphorylated S6, and the letters a to d indicate the positions of derivatives with increasing numbers of phosphate groups. 1st and 2nd refer to the first and second dimensions of the gel electrophoresis system, respectively.

oncogenic protein is identical to S6 II kinase remains to be determined. At least, the enzyme described in our study seems to be different from protein kinase C or cAMP-dependent protein kinase.

In preliminary experiments, we found that microinjection of normal *ras* protein preincubated with GTPrS, a nonhydrolyzable GTP analog (10 μM), resulted in a remarkable increase in S6 phosphorylation in oocytes, indicating that a GTP-bound form of normal *ras* protein is in the active state (unpublished data). Since *ras* proteins convert bound GTP to GDP (2) and yet lack phosphotransferase activity, it is conceivable that S6-phosphorylating activity is regulated by *ras* guanine nucleotide exchange activity through cellular factors. There could be a number of steps between the expression of *ras* activity and the stimulation of S6 kinase activity. *Xenopus* oocytes contain a cytoplasmic factor (GAP) which can stimulate the GTPase activity of *ras* protein, and the mutated oncogenic *ras* protein is possibly kept in the active GTP-bound state because it fails to interact with the factor (34). GAP protein has been postulated to be an intermediate between *ras* proteins and the catalytic domain of some effector molecule (35). If this is the case, GAP should mediate the effect of *ras* on S6 phosphorylation. Studies are under way to determine whether microinjection of GAP into *Xenopus* oocytes affects the activation of S6 phosphorylation by GTP-*ras* protein complexes.

In the course of study to find cellular factors which influence the actions of *ras*, we have found that oncogenic H-*ras* protein and protein kinase C synergistically act on the induction of maturation and S6 phosphorylation in oocytes. Consistent with these data, microinjection of the oncogenic H-*ras* protein and protein kinase C into NIH 3T3 cells has also indicated the possible interaction of the two proteins in eliciting a mitogenic signal in NIH 3T3 cells (22). In addition, *ras* oncogene-transformed cells showed the accumulation of diacylglycerol (11, 20, 36) and profound attenuation of an 80-kilodalton endogenous substrate of protein kinase C (20, 36).

The molecular mechanism underlying the synergistic effect of protein kinase C on the action of *ras* is not clear at present. It seems that *ras* proteins act through a different pathway from the protein kinase C-regulated pathway and that the effect of protein kinase C on the action of *ras*, at least, involves the stoichiometric change of phosphorylation of S6 (Fig. 4). An alternative possible explanation for *ras*-protein kinase C synergism is that protein kinase C phosphorylates *ras* proteins or the cellular proteins mediating the *ras* effects and thereby modulates their activities. It should be noted that Harvey or Kirstein *ras* protein can be phosphorylated by protein kinase C both in vivo and in vitro, although the functional significance of the phosphorylation has not yet been clarified (1, 29). In any case, the present data further emphasize the regulatory role of *ras* proteins and protein kinase C in signal transduction in early development. As for the role of protein kinase C in *X. laevis* development, it has been shown that protein kinase C mediates neural induction (28) and enhances insulin-induced oocyte maturation (32).

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