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Antibodies to *Xenopus* Egg S6 Kinase II Recognize S6 Kinase from Progesterone- and Insulin-Stimulated *Xenopus* Oocytes and from Proliferating Chicken Embryo Fibroblasts

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Ribosomal protein S6 becomes highly phosphorylated during progesterone- or insulin-induced maturation of *Xenopus laevis* oocytes. We have previously purified an M_r 92,000 protein as one of the major S6 kinases from *Xenopus* unfertilized eggs. In this paper we confirm by renaturation of activity from a sodium dodecyl sulfate-polyacrylamide gel that this protein is an S6 kinase. This enzyme, termed S6 kinase II (S6 K II), was used for the preparation of polyclonal antiserum. Immunocomplexes formed with the antiserum and purified S6 K II were able to express kinase activity with the same substrate specificity as that of the purified enzyme, including autophosphorylation of S6 K II itself. The antiserum did not react with S6 kinase I, another major S6 kinase present in *Xenopus* eggs, which is chromatographically distinct from S6 K II. The administration of progesterone to oocytes resulted in a 20- to 25-fold increase in S6 kinase activity in extracts of these cells. Immunocomplex kinase assays done on extracts revealed that anti-S6 K II serum reacted with S6 kinase from progesterone-treated oocytes. This antiserum also reacted with the activated S6 kinase from insulin-stimulated oocytes. In addition, anti-S6 K II serum reacted with activated S6 kinase from chicken embryo fibroblasts stimulated with serum or transformed by Rous sarcoma virus. These results indicate that S6 K II or an antigenically related S6 kinase(s) is subject to regulation by mitogenic stimuli in various cell types.

The phosphorylation of ribosomal protein S6 on serine residues is a common response to growth-promoting stimuli in diverse cell systems. In quiescent cultured cells, S6 is primarily unphosphorylated, whereas in stimulated cells it rapidly becomes highly phosphorylated, incorporating up to 4 to 5 mol of phosphate per mol of S6. Stimuli that elicit this response include serum, various growth factors and hormones, tumor-promoting phorbol esters, and the products of many oncogenes (1, 3, 4, 13, 22, 30, 35, 49–52). In many cases, the increase in S6 phosphorylation has been correlated with enhanced protein kinase activity specific for S6 in extracts of stimulated cells (2, 3, 9, 33, 36, 37, 40, 41, 48).

Meiotic maturation of oocytes is another example in which phosphorylation of S6 is correlated with response to hormonal stimuli. Fully grown *Xenopus laevis* oocytes are physiologically arrested in prophase of the first meiotic division. Oocytes treated with progesterone or insulin progress through meiotic cell division and arrest at metaphase II as unfertilized eggs, a process termed oocyte maturation. Studies in several laboratories have shown that in *Xenopus* oocytes S6 undergoes maximal phosphorylation upon maturation induced by treatment with progesterone or insulin or by microinjection of maturation-promoting factor (21, 24, 34, 47). In addition, S6 phosphorylation on serine residues is increased in oocytes after microinjection of the oncogene products of Rous sarcoma virus or Abelson murine leukemia virus or of purified insulin receptor kinase (30, 31, 45). In the oocyte system also, this increase is attributable at least in part to enhanced S6 kinase activity (15, 32, 46).

To begin characterization of ribosomal protein S6 kinase(s), we undertook purification of the enzyme(s) from *Xenopus* unfertilized eggs, which have maximally phosphorylated S6 and a high level of S6 kinase activity. DEAE-Sephacel chromatography revealed two peaks of S6 kinase activity, designated I and II in order of their elution from the column. The S6 kinase from peak II, purified to homogeneity, yields a protein that migrates with an M_r of 92,000 upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). This enzyme is referred to here as S6 kinase II (S6 K II).

We undertook the generation of antibody against S6 K II to aid in studying the regulation of this enzyme and to gain insight into the relationship of *Xenopus* egg S6 K II to S6 kinases from other sources. Results of immunocomplex kinase assays indicate that anti-S6 K II serum reacts with S6 kinase from both progesterone- and insulin-stimulated oocytes. Furthermore, the antibody also recognized an activated S6 kinase from chicken embryo fibroblasts (CEF) stimulated with serum and from Rous sarcoma virus-transformed CEF.

MATERIALS AND METHODS

Female *X. laevis* were obtained from either *Xenopus* I (Ann Arbor, Mich.) or the South African Snake Farm (Fish Hoek, South Africa). For production of eggs, the animals were injected with 70 IU of pregnant mare's serum gonadotropin (CalBiochem-Behring, La Jolla, Calif.) and 36 h later with 700 IU of human chorionic gonadotropin (Sigma

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Chemical Co., St. Louis, Mo.). Eggs were collected in tap water containing 0.1 M NaCl and were dejellied with 2% cysteine, pH 8. For experiments with oocytes, the animals were injected 3 days before use with 35 IU of pregnant mare's serum gonadotropin, and oocytes were isolated by manual dissection (28). 40S ribosomal subunits for use as a substrate were isolated from *X. laevis* ovaries as described previously (15). [γ-^{32}P]ATP was prepared by the method of Johnson and Walseth (23); the heat-stable inhibitor of protein kinase A, the cyclic AMP-dependent protein kinase, was purified by the procedure of Whitehouse and Walsh (54); and *Staphylococcus aureus* Cowan I was cultured and processed as described by Kessler (25). Protein concentration was determined by the method of Bradford (6).

Protein kinase assays. Reaction mixtures were incubated for 15 min at 30°C in a final volume of 30 μl containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 100 μM ATP, 2×10^6 to 10×10^6 cpm of [γ-^{32}P]ATP, 3 μg of bovine serum albumin, 20 μg of 40S ribosomal subunits, and 0.2 μg of heat-stable inhibitor protein. This amount of inhibitor is sufficient to inhibit incorporation of 16 pmol of phosphate into S6 by the purified catalytic subunit of protein kinase A. The heat-stable inhibitor was omitted from the reaction when S6 kinase activity of purified catalytic subunit was assayed.

When crude extracts were assayed, 1 to 15 μg of protein was added per assay. Reactions were terminated by the addition of 0.25 volume of fivefold-concentrated electrophoresis sample buffer (sample buffer: 0.07 M Tris hydrochloride [pH 6.8], 11% glycerol, 3% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol) followed by incubation at 95°C for 1 min. The samples were electrophoresed through 12.5% SDS-polyacrylamide gels, the S6 bands were identified by staining and autoradiography, and the incorporation of radiolabel into S6 was quantified by liquid scintillation spectrometry, as described previously (15, 16). Values shown are corrected for radiolabel in the S6 region from reactions done in the absence of exogenous 40S subunits. One unit of activity is defined as the amount transferring into S6 1 pmol of phosphate per min at 30°C in the presence of 10 mM Mg^{2+}, 100 μM ATP, and 500 nM 40S subunits. For assay of the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide; Sigma), the specific activity of the ATP was reduced 10-fold, and the products of the reaction were resolved on polyethyleneimine-cellulose sheets (Machery-Nagel, Duren, Federal Republic of Germany) by ascending chromatography in deionized water (18). Autophosphorylation reactions were done in the presence of 10 μM ATP containing 1×10^8 to 2×10^8 cpm of [γ-^{32}P]ATP.

Preparation of anti-S6 K II serum. S6 K II was prepared from unfertilized *X. laevis* eggs as detailed previously (16), with the following modifications. Ammonium sulfate fractions from several preparations were stored at −70°C before further processing. Approximately 500 mg of protein was applied to a DEAE-Sephacel column (2.5 by 27 cm), which was eluted with a 900-ml gradient to 700 mM NaCl. Because of the limited capacity of the Mono S column, the preparation was subjected to Sephacryl S-200 chromatography before rather than after application to the Mono S column. DEAE peak II was concentrated by ammonium sulfate precipitation, dissolved in 1 ml of buffer, applied to the Sephacryl S-200 column, and eluted in the presence of 100 mM NaCl and 0.05% Brij 35. These modifications did not result in any significant difference in the chromatographic properties of the enzyme, or, as shown below, in the final preparation as analyzed by SDS-PAGE and silver staining, although a few minor bands did become detectable. Several preparations were pooled and concentrated, and the S6 K II to be used as the antigen was further purified by electrophoresis through an SDS-7.5% polyacrylamide gel. For the primary immunization, a sample of the enzyme was autophosphorylated and mixed with the remainder of the preparation before electrophoresis. The position of S6 K II was localized by autoradiography, and the enzyme was electroeluted from the gel with an Isco electrophoretic concentrator. To quantify the amount of antigen used for immunization, a sample of the eluted antigen was again electrophoresed through another gel with bovine serum albumin standards. As estimated by comparison with the Coomassie blue staining of the standards, there was 20 to 30 μg of S6 K II. The enzyme was emulsified with Freund complete adjuvant, a portion was injected into the popliteal lymph nodes of two rabbits (44), and the remainder was injected subcutaneously. Subsequent injections of about 15 to 20 μg each were administered at 3-week intervals. For these injections, the S6 K II was localized in the gel by Coomassie blue staining, and the band was excised, equilibrated with H_2O, and homogenized in phosphate-buffered saline. This mixture was then homogenized with Freund incomplete adjuvant and injected intramuscularly and subcutaneously in the flank and neck, respectively. Sera were tested for their ability to immunoprecipitate autophosphorylated S6 K II, and the serum used in the studies presented here was obtained 2 weeks after the second boost.

Immunoprecipitations. Radiolabeled antigen for use in testing the antisera was prepared by incubation of S6 K II in vitro in the presence of $MgCl_2$ and [γ-^{32}P]ATP. The reaction was stopped by the addition of EDTA and SDS to final concentrations of 30 mM and 0.1%, respectively. This preparation was then diluted with buffer A (100 mM NaCl, 10 mM Tris hydrochloride [pH 6.5], 1 mm EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing 500 μM unlabeled ATP. Samples were incubated at 0°C for 40 min with 5 μl of serum in a total volume of 100 μl, protein A-containing *S. aureus* was added, and incubation was continued for 20 min. The bacteria-immunocomplexes were washed twice with 1 ml of buffer A, twice with buffer B (1 M NaCl, 10 mm Tris hydrochloride [pH 7.2], 0.1% Nonidet P-40), transferred to microcentrifuge tubes, and centrifuged at 11,000 × *g* for 4 min. The immunoprecipitated proteins were solubilized by boiling for 2 min in electrophoresis sample buffer and resolved by SDS-PAGE.

For immunocomplex kinase assays, the immunoprecipitations were done in either buffer A and B or in buffer C (150 mM NaCl, 50 mM Tris hydrochloride [pH 7.2], 1 mM EDTA, 0.05% Nonidet P-40). In addition, all buffers contained 2 mM dithiothreitol (DTT) and 100 μM phenylmethylsulfonyl fluoride (PMSF), and the buffer used for the final transfer comprised 150 mM NaCl and 50 mM Tris hydrochloride, pH 7.2. Immunocomplex kinase reactions were done essentially as developed by Collett and Erikson (10) for the immunoglobulin G kinase activity of pp60$^{v\text{-}src}$, the product of the Rous sarcoma virus oncogene. The pellets were suspended in kinase reaction buffer, substrates were added, and the samples were incubated at 30°C for 30 min. The reactions were terminated by the addition of an equal volume of 2× sample buffer and incubated at 95°C for 2 min. The bacteria were pelleted, and the products of the reaction in the supernatant were analyzed as described above.

Incubation of cells and preparation of extracts. Oocytes were incubated at ambient temperature (18 to 22°C) in either

OR-2 or Barth medium (19, 28). Maturation in vitro was induced by the addition of either progesterone or insulin to a final concentration of 35 or 1 μM, respectively. At the times indicated in the figure legends, the oocytes were homogenized in 3 volumes of buffer containing 55 mM β-glycerophosphate (pH 6.8), 5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 100 mM $Na_2P_4O_7$, 5 mM phosphotyrosine, 2 mM DTT, 100 μM PMSF, 100 μM sodium orthovanadate, and 10 μg of leupeptin per ml. The homogenates were centrifuged at 37,000 × *g* for 30 min, the lipid layers were removed, and the supernatants were dialyzed against 55 mM β-glycerophosphate–5 mM EGTA–5 mM $MgCl_2$–2 mM DTT–100 μM PMSF.

Uninfected CEF and CEF infected with NY 72-4, a mutant of Rous sarcoma virus temperature sensitive for transformation (42) (ts-CEF), were grown at 41.5°C in Richter improved modified Eagle medium with insulin (Irvine Scientific, Santa Ana, Calif.) 5% calf serum and 10% tryptose phosphate broth in 100-mm culture dishes until they were nearly confluent. Then the cells were rendered quiescent by incubation for 20 h in Dulbecco modified Eagle medium without serum. One culture from each set was left untreated, one was stimulated by the addition of serum to 10%, and one culture of ts-CEF was transferred to 35°C. After 1 h, each monolayer was washed gently, and the cells were scraped from the dish and then homogenized in 750 μl of buffer containing 55 mM β-glycerophosphate (pH 6.8), 5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 2 mM DTT, 100 μM PMSF, 1 mM sodium orthovanadate, and 10 μg of leupeptin per ml. The homogenates were centrifuged at 100,000 × *g* for 30 min before further analysis of the supernatants.

RESULTS

Verification that the M_r 92,000 protein possesses S6 kinase activity. Previous data had shown that highly purified S6 K II yields a protein that migrates with an M_r of 92,000 upon SDS-PAGE (16). The chromatographic and sedimentation profiles of this protein coincided exactly with the S6 kinase activity in all procedures (15, 16); nevertheless, the possibility still existed that the kinase activity resided in another protein of different M_r that was not detectable by our procedures. To resolve the question of whether S6 kinase activity is inherent to the M_r 92,000 protein, a sample of S6 K II was subjected to SDS-PAGE and slices of the gel were examined for S6 kinase activity as described in the legend to Fig. 1. S6 kinase activity could be recovered from the gel slice that contained the M_r 92,000 protein (Fig. 1). This result provides strong evidence that this protein is indeed an S6 kinase.

Immunoprecipitation of S6 K II. To generate anti-S6 K II serum, we injected the purified protein into rabbits as described in Materials and Methods. The production of antibody was monitored by immunoprecipitation of autophosphorylated S6 K II followed by SDS-PAGE and autoradiography. Sera from both rabbits showed a positive response after the first boost, and serum from one of the rabbits showed an increased titer after the second boost. A third boost did not cause any further increase. The antibody was able to immunoprecipitate radiolabeled antigen, and the amount immunoprecipitated was proportional to the concentration of antigen, implying that the antibody was in excess. However, the amount of antigen immunoprecipitated could be increased by increasing the quantity of antibody (data not shown). Therefore, this antibody probably has a low avidity for S6 K II. This would make it difficult to do experiments requiring saturation of the antibody or the complete removal of antigen from a preparation.

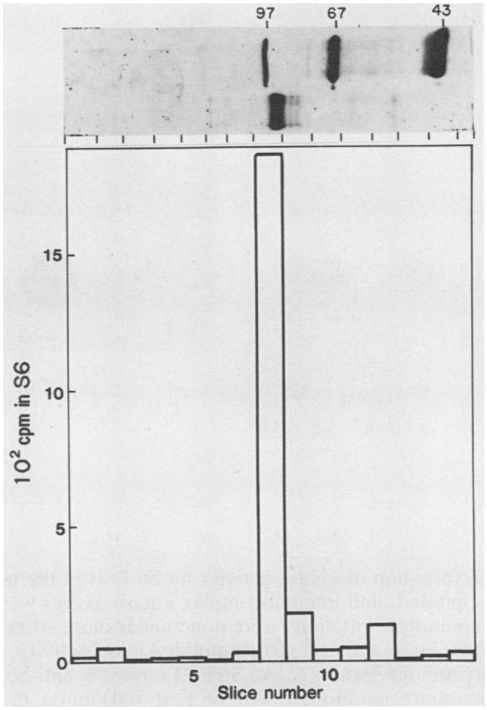

FIG. 1. Renaturation of S6 kinase activity in the M_r 92,000 protein after SDS-PAGE. Two samples of S6 K II (27 units each) were diluted into electrophoresis sample buffer, heated at 95°C for 1 min, and electrophoresed through an SDS-7.5% polyacrylamide gel. One track was silver stained (38), and the other track was cut into 15 sections. Elution, precipitation, and renaturation of enzyme activity were done by the procedure of Hager and Burgess (20), except that after being dissolved in 6 M guanidine the samples were not diluted, but rather were microdialyzed against kinase reaction buffer for 4 h at 22°C. Then 40S ribosomal subunits, $MgCl_2$, and [γ-^{32}P]ATP were added, and the reaction mixtures were incubated at 30°C for 30 min. Incorporation of radiolabel into S6 was quantified as detailed in Materials and Methods. Recovery of activity was approximately 1% of that applied to the gel. Top panel: Silver-stained portion of the gel. Upper lane, Molecular weight standards. The M_rs of the standards (×10^3) are indicated: phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), all from Pharmacia Fine Chemicals (Piscataway, N.J.); lower lane, S6 K II preparation. Migration was from left to right. Bottom panel: Incorporation of radiolabel into S6 by the eluates of the indicated slices.

The ability of some enzymes to retain activity while sequestered in an immunocomplex has greatly facilitated investigation into the expression and regulation of their activity. One notable example of this is the work on $pp60^{c\text{-}src}$, the normal cellular homolog of $pp60^{v\text{-}src}$. Immunoprecipitation of radiolabeled $pp60^{c\text{-}src}$ and immunocomplex kinase assays have enabled investigators to correlate the phosphorylation of certain tyrosine residues on this molecule with changes in its activity and have led to insight regarding the control of the activity of this enzyme under different conditions in various tissues (5, 8, 11, 12, 27). To ascertain whether immunocomplex assays would be possible with S6 K II, the bacteria-immunocomplexes were resuspended, 40S subunits, $MgCl_2$, and [γ-^{32}P]ATP were added, and the incorporation of radiolabel into S6 was determined. Immunocomplexes formed with S6 K II and immune serum

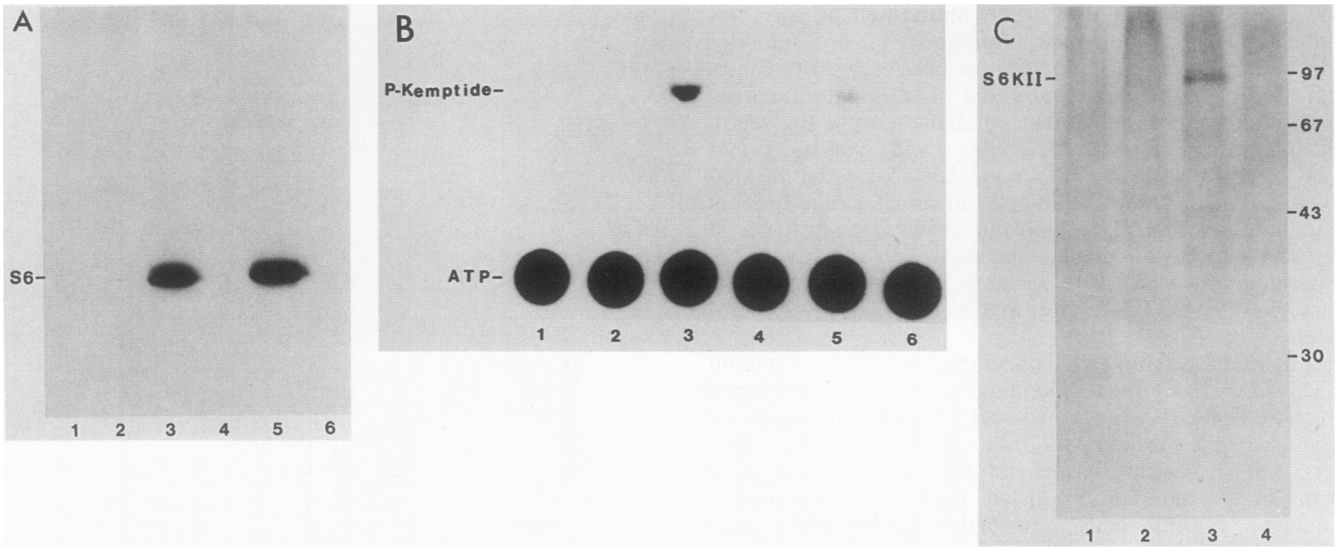

FIG. 2. Expression of kinase activity by S6 K II in the immunocomplex. A sample of S6 K II (1.5 units) was diluted into buffer C and immunoprecipitated, and immunocomplex kinase assays were done as described in Materials and Methods. Similar results were obtained when the immunoprecipitations were done under more stringent conditions, with buffers A and B, but the recovery of activity was slightly lower. (A) S6 kinase activity; (B) Kemptide kinase activity; (C) autophosphorylation. Lanes 1 to 4, Immunocomplex kinase assays: 1, no serum; 2, preimmune serum; 3, anti-S6 K II serum; 4, anti-S6 K II serum without the addition of S6 K II. Lanes 5 and 6, Substrate incubated without immunoprecipitation: 5, with S6 K II (0.03 units); 6, without S6 K II. Exposures were for 2 h (A), 6 h (B), or 7 h (C). The positions of the molecular weight standards ($\times 10^3$) are indicated on the right side of panel C.

were able to phosphorylate S6 (Fig. 2A). The phosphorylation was dependent on the presence of both S6 K II and immune serum; assays with preimmune serum, without serum, or with immune serum incubated without enzyme were all negative. The S6 kinase activity expressed by the immunocomplex was approximately 1 to 2% of the activity subjected to the immunoprecipitation procedure (Fig. 2A, lanes 3 and 5). There could be several reasons for this low level: the antibody most likely does not precipitate all of the enzyme; certain immunoglobulin G molecules may inactivate the enzyme; accessibility of the substrate to the enzyme may be restricted because of the large size of the bacteria-immunocomplexes and the 40S subunits.

The fact that immunoprecipitated S6 K II is still capable of expressing S6 kinase activity enabled us to address two other questions regarding its enzymatic properties. We previously reported that purified S6 K II was capable of phosphorylating Kemptide (16) and that this activity coeluted with S6 kinase activity upon various chromatographic procedures. Kemptide kinase activity was also expressed by immunocomplexes formed with S6 K II and anti-S6 K II serum (Fig. 2B). In this case the level of activity expressed by the immunocomplex was approximately 10 to 20% of the activity subjected to immunoprecipitation (Fig. 2B, lanes 3 and 5). This higher level is consistent with greater accessibility of the peptide substrate. We had also shown that highly purified S6 K II became phosphorylated upon incubation with Mg^{2+} and [γ-^{32}P]ATP and suggested that this enzyme could undergo autophosphorylation (15), a feature common to most protein kinases (17). When immunocomplexes containing S6 K II were suspended in $MgCl_2$–[γ-^{32}P]ATP, radiolabel was incorporated into an M_r 92,000 protein (Fig. 2C). The phosphorylated M_r 92,000 protein was also detectable in Fig. 2A upon longer exposure of the gel. Both of these results are consistent with the idea that the phosphorylation of Kemptide and of S6 K II itself are properties of S6 K II and are not due to the activity of another kinase(s).

Incorporation of radiolabel into S6 in the immunocomplex assay was linear with enzyme concentration for amounts up to 5 or 6 units per immunoprecipitation (data not shown). Preliminary experiments similar to those depicted in Fig. 2A were done on extracts of unfertilized eggs. S6 kinase activity was expressed by immunocomplexes containing anti-S6 K II serum, whereas those formed with preimmune serum were negative, and as shown below, incorporation increased with increasing amounts of extract. This demonstrates that the antibody is capable of recognizing S6 K II in a crude extract and, thus, that the immunocomplex kinase assay should provide a valid means of monitoring changes in the level or activity of this enzyme or antigenically related S6 kinases during oocyte maturation.

Specificity of anti-S6 K II serum. For immunocomplex assays to be useful for analyses of crude extracts, it was important to ascertain whether anti-S6 K II serum reacted only with S6 K II-like enzymes or with other kinases as well. Consequently, several additional immunoprecipitations were done to address this issue. There was no specific immunoprecipitation of casein kinase activity or of Ca^{2+}- and phospholipid-dependent histone H1 kinase activity from crude extracts. Moreover, there was no detectable phosphorylation of S6 by the immunocomplex even when a large amount (20 units of S6 kinase activity) of purified catalytic subunit of protein kinase A was subjected to immunoprecipitation (data not shown). Therefore, anti-S6 K II serum does not appear to react nonspecifically with kinases in general, nor does it react with protein kinase A or C, both of which are able to phosphorylate S6 in vitro (14, 26, 39, 53).

As mentioned above, two peaks of S6 kinase activity are evident upon DEAE-chromatography of extracts of unfertilized eggs (16). Further purification and biochemical characterization of the activity from peak I (S6 K I) have not yet led

to a definitive conclusion about whether this enzyme is related to S6 K II. Partially purified S6 K I has a restricted substrate specificity similar to that of S6 K II; however, the two enzyme activities are separable by several chromatographic procedures, and the M_r 92,000 protein is not detectable in highly purified S6 K I (unpublished data). To obtain additional information about this question and about the specificity of the antibody, we performed immunocomplex kinase assays on a sample from each peak. Only peak II yielded immunoprecipitable activity (Fig. 3). This suggests that the polyclonal antibody does not recognize S6 K I or alternatively that it reacts with S6 K I in a manner that prevents expression of activity. It also cannot be ruled out that S6 K I was inactivated during the immunoprecipitation procedure, but activity was stable when the enzyme was kept in buffer C at 0°C for 2 h. Regardless of the explanation, Fig. 3 demonstrates that S6 K I is silent in the immunocomplex assay and therefore that S6 kinase activity detected by this procedure reflects the presence of S6 K II or antigenically related S6 kinases.

Immunoprecipitation of S6 kinase activity from maturing oocytes. Several studies have demonstrated that S6 undergoes multiple phosphorylation during meiotic maturation of *Xenopus* oocytes induced by either progesterone or insulin (21, 24, 34, 47). With insulin treatment, increased S6 kinase activity is detectable within 10 min, and by 30 min a four- to fivefold stimulation is evident (46). There have been no studies reported to date following S6 kinase activity after progesterone treatment. Accordingly, oocytes were homogenized at various times after the addition of progesterone, and S6 kinase activity was measured. Increased activity was detectable 1 h after the administration of hormone, culminating in a 20- to 25-fold increase by 3 h, and activity remained high for several hours after germinal vesicle breakdown (GVBD) (Fig. 4). This time course is consistent with the time course of the increase in S6 phosphorylation during oocyte maturation (34).

The specific activity of S6 kinase from unfertilized eggs is usually higher than that from oocytes matured in vitro collected at the time of GVBD, (e.g., see the legend to Fig. 5), indicating that some additional increase occurs during further maturation to metaphase II. This difference in activity is consistent with the state of phosphorylation of S6 in oocytes at GVBD and in unfertilized eggs. At the time of GVBD S6 exists in derivatives that contain 2 to 4 mol of phosphate per mol of S6, whereas in unfertilized eggs it contains 4 to 5 mol of phosphate per mol (34). In view of the large number of ribosomes in oocytes (7), additional S6 kinase activity may be necessary after GVBD to complete maximal phosphorylation of S6. Since anti-S6 K II serum is able to distinguish between the two major S6 kinases present in extracts of unfertilized eggs (Fig. 3), experiments were undertaken to assess whether the S6 kinase that appears after progesterone treatment could be recognized by this antibody. Extracts were prepared, and several concentrations of protein were subjected to immunoprecipitation and immunocomplex assays. Extracts of progesterone-treated oocytes did yield immunoprecipitable S6 kinase activity (Fig. 5). The amount of immunoprecipitable activity was slightly lower than that expressed by corresponding immunocomplexes prepared from extracts of unfertilized eggs, and as expected, the immunoprecipitable activity from unstimulated oocytes was very low.

As mentioned above, activation of S6 kinase with insulin is detectable much earlier than the increase in activity observed with progesterone. To assess whether the insulin-stimulated enzyme is recognized by anti-S6 K II serum, experiments similar to those shown in Fig. 5 were performed on extracts of insulin-treated oocytes. The results, summarized in Fig. 6, reveal that the appearance of immunoprecipitable activity coincided with the appearance of S6 kinase activity in the extracts. Therefore, anti-S6 K II serum can

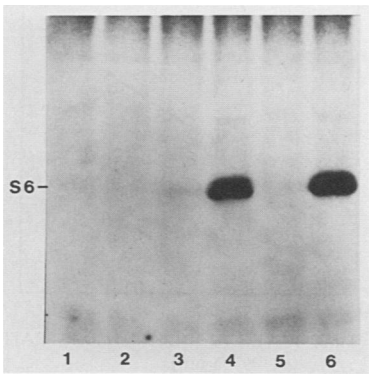

FIG. 3. Immunoprecipitation of S6 kinase activity from DEAE peaks I and II. Samples from DEAE peaks I and II were diluted into buffer C, and immunocomplex kinase activity was determined as described in Materials and Methods. Samples and sera were as follows: lane 1, peak I (1.2 units), anti-S6 K II; lane 2, peak I (2.4 units), preimmune; lane 3, peak I (2.4 units), anti-S6 K II; lane 4, peak II (1.2 units), anti-S6 K II; lane 5, peak II (2.4 units), preimmune; lane 6, peak II (2.4 units), anti-S6 K II. Exposure was for 16 h. Specific radioactivity associated with S6 was 383 cpm in lane 4 and 716 cpm in lane 6.

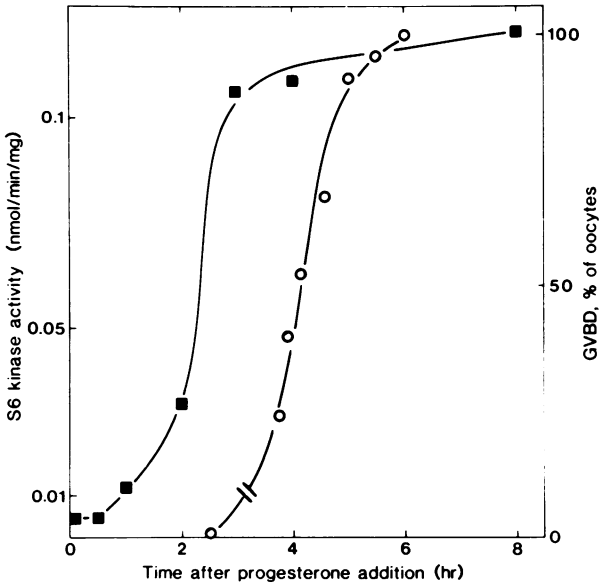

FIG. 4. S6 kinase activity in *Xenopus* oocytes after progesterone treatment. Oocytes in groups of 25 were incubated in the presence of progesterone (35 μM) for the indicated times, extracts were prepared, and protein concentration and S6 kinase activity were determined as described in Materials and Methods. The time of GVBD was established by examining oocytes for white-spot formation with a dissecting microscope. Symbols: ■, S6 kinase activity; ○, GVBD.

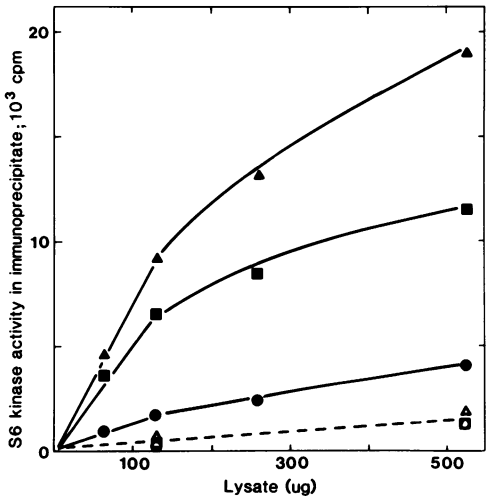

FIG. 5. Immunoprecipitation of S6 kinase activity from maturing oocytes and from unfertilized eggs. Extracts were prepared from unstimulated oocytes, from progesterone-treated oocytes collected at the time when 50% of them had undergone GVBD, and from unfertilized eggs. Protein concentration and S6 kinase activity were determined. Extracts were adjusted to 0.05% Nonidet P-40, and the indicated amounts of protein were immunoprecipitated from a volume of 100 μl. The immunocomplexes were washed with buffer C, and incorporation of radiolabel into S6 was quantified as described in Materials and Methods. The specific activities (nanomoles per minute per milligram) of S6 kinase in the extracts were: unstimulated oocytes, 0.004; progesterone-treated oocytes, 0.078; unfertilized eggs, 0.160. Symbols: ▲, unfertilized eggs; ■, progesterone-treated oocytes; ●, unstimulated oocytes; solid lines, anti-S6 K II serum; dashed line and open symbols, preimmune serum.

recognize the S6 kinase activated by insulin and also the S6 kinase that arises after progesterone treatment. Whether in these cases the antiserum recognizes the same enzyme or an antigenically related enzyme is not certain at present.

Recognition of avian S6 kinase by anti-S6 K II serum. When serum is added to quiescent CEF, S6 rapidly becomes maximally phosphorylated (1, 4, 22) and a four- to eightfold increase in S6 kinase activity is seen within 15 min (unpublished data). Immunocomplex S6 kinase assays were performed to ascertain whether anti-S6 K II serum could recognize serum-activated S6 kinase from CEF. Extracts of quiescent CEF yielded a very low level of immunoprecipitable activity, whereas immunoprecipitable activity from serum-stimulated CEF was eightfold higher (Fig. 7A).

For ts-CEF, S6 kinase activity is temporally correlated with the activity of $pp60^{v\text{-}src}$ (2). At the nonpermissive temperature of 41.5°C, ts-CEF exhibit a nontransformed phenotype because the thermolabile mutant $pp60^{v\text{-}src}$ is inactivated. In addition to reduced S6 phosphorylation and reduced S6 kinase activity, such cells also contain reduced levels of phosphotyrosine in proteins (43). Upon transfer to the permissive temperature of 35°C, $pp60^{v\text{-}src}$ is activated and S6 kinase activity attains the maximal level within 15 min. Conversely, after transfer of ts-CEF from 35 to 41.5°C, the activities of both $pp60^{v\text{-}src}$ and S6 kinase decline in parallel (2). Experiments similar to those shown in Fig. 7A were done on ts-CEF after stimulation with serum or after transfer to 35°C. Immunoprecipitable activity from ts-CEF stimulated with serum was eightfold higher than that from quiescent ts-CEF maintained at 41.5°C, and that from ts-CEF transferred to 35°C was approximately sevenfold higher (Fig. 7B). In these experiments the amount of activity expressed by the immunoprecipitates was approximately 0.5 to 1%, similar to the recoveries obtained with lysates of eggs or oocytes. These results demonstrate that antibody raised against S6 K II recognizes an S6 kinase in CEF that is activated by serum treatment or by expression of $pp60^{v\text{-}src}$.

DISCUSSION

In eucaryotic cells phosphorylation of ribosomal protein S6 on serine is one of the most common responses to growth-promoting stimuli. Therefore, much effort has been directed recently toward characterization of the relevant S6 kinase(s). Several properties of S6 kinase activity from cells stimulated by growth-promoting agents indicate that it differs from protein kinases A and C, both of which are able to phosphorylate S6 in vitro (14, 26, 39, 53). These properties include lack of inhibition by the heat-stable inhibitor protein (2, 3, 9, 15, 16, 32, 40, 46, 48) and the inability of phospholipids to affect activity (2, 9, 15, 33, 40, 48). In addition, the substrate specificity of S6 kinase activity from stimulated cells is highly restricted in vitro compared with either protein kinase A or C (9, 15, 48). It has not been rigorously established whether the same protein kinase or distinct enzymes are activated by the various stimuli used. In this regard, however, Blenis and Erikson (2) have shown that CEF stimulated by serum, tumor-promoting phorbol esters, or expression of $pp60^{v\text{-}src}$ yield S6 kinase activity that displays similar properties upon ion-exchange chromatography. Moreover, Tabarini et al. (48) reported that both the insulin- and phorbol ester-stimulated S6 kinase activities from 3T3-L1 cells sediment with an M_r of 50,000 to 60,000 upon sucrose density gradient centrifugation. To date, however, in most systems little is known about the molecular nature of the enzyme(s) responsible for S6 phosphorylation.

We previously proposed that one of the major S6 kinase

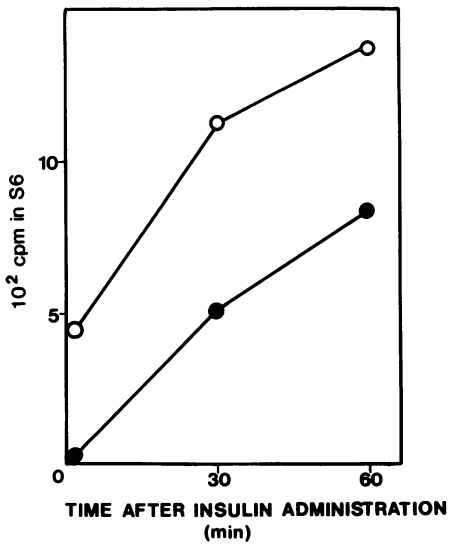

FIG. 6. Kinetics of S6 kinase activation in insulin-treated oocytes. Oocytes were treated with 1 μM insulin for the indicated times. Extracts were prepared, and S6 kinase activity and immunocomplex kinase activity were determined as described in Materials and Methods and in the legend to Fig. 5. The data have been corrected for radioactivity in the S6 region in assays conducted without exogenous 40S subunits in the case of direct assays, and for radioactivity in the S6 region in immunocomplex assays performed with nonimmune serum. Symbols: ○, S6 kinase activity in the extract; ●, immunocomplex S6 kinase activity.

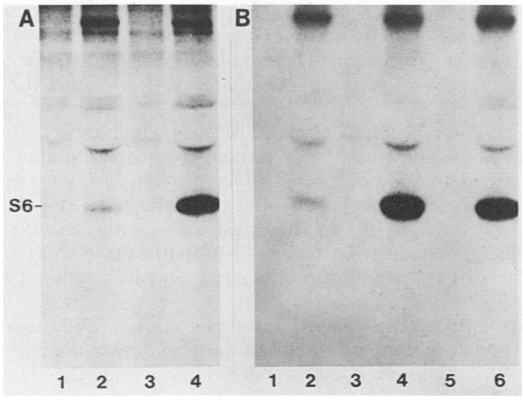

FIG. 7. Immunoprecipitation of activated S6 kinase from CEF. CEF and ts-CEF were stimulated by the addition of serum or by transfer from 41.5°C to the permissive temperature, 35°C. Extracts were prepared, and protein concentration, S6 kinase activity, and immunocomplex kinase activity were determined as described in Materials and Methods and in the legend to Fig. 5. The specific activities (nanomoles per minute per milligram) of S6 kinase in the extracts were: CEF, 0.008; serum-stimulated CEF, 0.037; ts-CEF (41.5°C), 0.007; serum-stimulated ts-CEF (41.5°C), 0.042; ts-CEF (35°C) 0.034. (A) Lanes: 1 and 2, CEF; 3 and 4, serum-stimulated CEF. (B) Lanes: 1 and 2, ts-CEF (41.5°C); 3 and 4, serum-stimulated ts-CEF (41.5°C); 5 and 6, ts-CEF (35°C). Odd-numbered lanes, Preimmune serum; even-numbered lanes, anti-S6 K II serum. The amounts of protein used were 25 μg for the CEF experiments and 70 μg for the ts-CEF experiments. Exposures were for 22 h (A) and 6 h (B). Specific radioactivity associated with S6 was as follows: panel A, lane 2, 60 cpm; lane 4, 470 cpm; panel B, lane 2, 360 cpm; lane 4, 3,000 cpm; lane 6, 2,410 cpm.

activities present in *Xenopus* unfertilized eggs resides in a protein of M_r 92,000 (16). In this communication, renaturation of S6 kinase activity from the region of an SDS-polyacrylamide gel that contained the M_r 92,000 protein provides compelling evidence that this protein, designated S6 K II, does catalyze the phosphorylation of S6. Antibody specific for this enzyme can be used to immunoprecipitate S6 kinase activity from purified enzyme preparations or from various cell extracts. The antibody appears to be specific for S6 K II-like enzyme(s) since it failed to immunoprecipitate casein kinase or protein kinase C activities from extracts, nor did it immunoprecipitate S6 kinase activity from the purified catalytic subunit of protein kinase A. In addition, the antibody did not immunoprecipitate S6 K I, the other major S6 kinase in unfertilized eggs, which is chromatographically distinct from S6 K II.

Anti-S6 K II antibody was used in immunocomplex assays to analyze the S6 kinase activity stimulated in oocytes after administration of either progesterone or insulin. The results demonstrate that S6 K II-like activity contributes to S6 phosphorylation in both progesterone- and insulin-stimulated oocytes. We also showed here that antiserum raised against S6 K II specifically immunoprecipitated activated S6 kinase from CEF stimulated to proliferate by serum or by the activation of $pp60^{v\text{-}src}$. This suggests that an avian S6 protein kinase is closely related to S6 K II, or at least shares a domain antigenically related to S6 K II.

The major issue presented by these data is that a multiplicity of diverse stimuli activate one, or at most a few, serine-specific S6 protein kinases. The initial event in the case of serum, growth factors, insulin, or $pp60^{v\text{-}src}$ stimulation most likely involves tyrosine phosphorylation, whereas tumor-promoting phorbol esters activate the serine-threonine-specific enzyme protein kinase C. In amphibian oocytes the effect of progesterone is mediated by a plasma membrane steroid receptor linked to the adenylate cyclase system (see reference 29 for a review). Thus, progesterone stimulation of S6 kinase activity adds another potentially distinct pathway of activation. Therefore, the data presented here suggest that S6 K II-like enzymes are subject to activation by several stimuli that initially probably have different mechanisms of action. We have as yet no information concerning the number of steps between the initial event and the activation of S6 K II, or whether the pathways converge at a common point before S6 K II activation.

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