Tyrosine Protein Kinase Activity during Embryogenesis*  

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The activity of tyrosine-specific protein kinase was estimated during early embryonic development of the sea urchin, Strongylocentrotus purpuratus, using the synthetic peptide Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Gly-Gly as a probe. The peptide was not phosphorylated by the purified cyclic AMP-dependent protein kinase, phosphorylase kinase, glycogen synthase kinase 3, or casein kinase 2 but was phosphorylated by the purified epidermal growth factor-receptor kinase from A-431 cells. The sea urchin tyrosine protein kinase activity was determined in a particulate fraction (17,000 × g pellet) and in a membrane fraction obtained after discontinuous sucrose gradient centrifugation; these fractions contained higher specific activities of the kinase than the cytosolic or nuclear fractions. Unfertilized eggs had very low tyrosine protein kinase activity (0.22 pmol of peptide phosphorylated per mg of protein/min) in the particulate fraction, but the activity increased 2.5-fold by 1 h after fertilization and almost 20-fold by the gastrula stage. The enzyme activity of the membrane fraction increased similarly during this time period. The tyrosine protein kinase had an apparent $K_{m}$ of 8.9 mM for the peptide, and showed one-half-maximal velocity at about 35 $\mu$M MgATP.

The phosphorylation of membrane fractions in vitro at different stages of embryonic development resulted in nine endogenous protein-staining bands ($M_{r} = 28,000$ to $>200,000$; $10\%$ Na dodecyl SO4 gels) which contained phosphorytrosine; some of the bands incorporated $^{32}$P differentially as a function of development. In the unfertilized egg membrane fraction, none of the protein-staining bands were shown to contain detectable $^{32}$P-tyrosine.

Thus, fertilization results in increases in tyrosine-specific protein kinase(s) activity which continuously increase during early embryonic development; it is suggested that these protein kinases have a functional role during early development and differentiation.

Recently, increased tyrosine-specific protein kinase activity has been associated with cell transformation induced by avian sarcoma viruses (1–3) and other viruses (4) and with the action of certain hormones or growth factors, such as EGF (5, 6), platelet-derived growth factor (7), and insulin (8). Cells responding to the viruses, EGF, or platelet-derived growth factor multiply faster (7, 9); these agents also can induce ruffling activity of plasma membranes (10–12). Embryonic cells resemble these stimulated cells with respect to the rapid multiplication and ruffled surface membranes (13). Some embryonic antigens are expressed in cancer cells (14, 15), and recently cellular genes homologous to the onc genes of Abelson murine leukemia virus, Finkel-Biskis-Jinkins murine osteosarcoma virus, and Harvey sarcoma virus were found to be expressed in the mouse embryo (16). The presence of EGF-like transforming growth factors in embryos which can mimic the transformed phenotype (17, 18) also suggests that tyrosine protein kinase activity may be associated with embryonic development.

Many currently used substrates for the tyrosine protein kinases (endogenous proteins, histones, γ-immunoglobulin, and tubulin) have the disadvantage of being variable in quantity (endogenous proteins) or phosphorylated at serine and threonine in addition to tyrosine (endogenous proteins, histones, etc.). We, therefore, chemically synthesized the peptide Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Gly (L6p), a peptide structurally similar to the tyrosine phosphorylation site of pp60$^{*}$c and to peptides synthesized by Casnellie et al. (19), and used it to estimate tyrosine protein kinase activity during early embryonic development of the sea urchin, Strongylocentrotus purpuratus. We show that the tyrosine protein kinase activity increases dramatically during early development, and that there are at least nine protein-staining bands on 10$\%$ Na dodecyl SO4-polyacrylamide gels which contain $^{32}$P-tyrosine at various times during development. Some of these $^{32}$P-labeled bands are present at all the stages from 1 h after fertilization to the late gastrula stage, while others are apparent at specific stages of development.

EXPERIMENTAL PROCEDURES

Materials—Sea urchins were obtained from Pacific Bio-Marine Laboratories, Inc., Venice, CA. Nondet P-40 and unlabeled nucleotides were purchased from Sigma. N-tert-Butyloxycarbonyl-l-aminoc acids were from Peninsula Laboratories Inc., San Carlos, CA. All other commercial chemicals of highest grade were obtained from Fisher or Sigma. [$\gamma^{32}$P]ATP was prepared according to the procedure published by Walseth and Johnson (20).

Egg Preparation—Spawning was induced by the injection of 0.5 M KCl. Eggs were collected and washed in normal seawater, and then suspended in pH 5.0 seawater for 15 min to remove the jelly coat. These eggs were pelleted, washed, and fertilized as described by Hinegardner (21). The embryos were raised at 4–5 °C in normal seawater with 0.4% Gibco penicillin/streptomycin solution.

Peptide Synthesis—The peptide was synthesized by previously described methods (22). It was then purified by ion exchange chromatography on CM-Sepharose CL-6B followed by high pressure liquid chromatography on a C$_{18}$ column. The expected structure was confirmed by amino acid analysis. Two other peptides, E$_{a}$G$_{1}$ (Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Gly) and E$_{a}$P-G$_{1}$ (Glu-Asp-Ala-Glu-Phe-Ala-Ala-Arg-Gly), were custom synthesized by Peninsula Laboratories Inc.

Protein Estimation—Protein concentration was determined by the method reported by Bradford (23), $\gamma$-globulin was used as the standard.
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Peptide Phosphorylation Assay—The 100 \( \mu l \) of reaction mixture (30°C) contained 50 mM Tris-HCl (pH 7.8), 50 mM magnesium chloride, 10 \( \mu M \) Na orthovanadate, 0.05% Nonidet P-40, and 11 \( \mu M \) [\( \gamma ^{32}P \)]ATP. Blank values for each incubation were determined in the absence of peptide. The reactions were stopped at the times indicated by the addition of 100 \( \mu l \) of 5% trichloroacetic acid; the samples were then kept on ice for 15 min. The precipitated proteins were pelleted by centrifugation at 1500 \( \times g \) for 30 min. Phosphorylated peptide was estimated by the application of 50 \( \mu l \) of the supernatant fluid onto Whatman P-81 phosphocellulose paper and washing the paper in acetic acid (25). To determine that the peptide being phosphorylated, the supernatant fluid was chromatographed by reverse-phase, high pressure liquid chromatography (C18 column). The amount of \( ^{32}P \) obtained in the peptide fraction from the column was quantitatively the same as that estimated in the acid supernatant fluid after application to phosphocellulose paper. The nature of the phosphorylated amino acid was also determined (see below).

Membrane Phosphorylation—Endogenous phosphorylation of membrane fractions was performed in 50 \( \mu l \) of a reaction mixture containing membranes (50 \( \mu g \) of protein), 50 mM Tris-HCl (pH 7.8), 50 mM magnesium chloride, 10 \( \mu M \) Na orthovanadate, 0.05% Nonidet P-40, and 11 \( \mu M \) [\( \gamma ^{32}P \)]ATP (70,000 cpm/pmol). The reaction at 30°C was initiated by the addition of radiolabeled ATP and after 2 min was terminated by the addition of 50 \( \mu l \) of a solution containing 40 mM Tris-Cl (pH 7.5), 200 mM dithiothreitol, 6% Na dodecyl SO4, and 5% glycerol.

Identification of Peptide Bands Containing Phosphorysine—Samples were prepared for 2–3 min at 100°C, and electrophoresis was conducted in 10% acrylamide, 0.27% bisacrylamide slab gels in 0.1% Na dodecyl SO4 with the buffers described by Laemmli and Favre (26). These gels were stained with 0.2% Coomassie blue in a solution containing 30% methanol and 10% acetic acid and were destained in 30% methanol and 10% acetic acid solution in water. Parallel gels were incubated for 2 h in 1 M KOH at 55°C (6) and then dried. The radioactive peptides were subsequently detected by exposing Kodak X-ray films to the alkali- as well as nonalkali-treated gels for 1–3 days.

Identification of the Phosphoamino Acids—Bands were excised from the dried nonalkali- and alkali-treated gels, and proteins were precipitated according to the procedure reported by Beemon and Hunter (27). The pellets were washed with acetone at 0°C. The dried proteins were partially hydrolyzed in 0.5 ml of 6 N HCl at 110°C for 2 h, and the hydrolysates were then analyzed on Whatman No. 3MM paper by electrophoresis (2.5 kV, 45 min) at pH 3.5 (pyridine/acetic acid/H2O, 1:10:189). The positions of standards (phosphotyrosine, phosphothreonine, and phosphoserine) were located by staining with 0.2% ninhydrin solution in acetone. The Kodak X-ray film was exposed to the alkali- as well as nonalkali-treated gels, and proteins were identified.

To determine the amino acid residue phosphorylated in the synthetic peptide, the high pressure liquid chromatography fraction containing phosphopeptide was lyophilized. Hydrolysis of the phosphopeptide was performed in 8 N HCl at 100°C for 2 h, followed by electrophoresis to detect the \( ^{32}P \)-labeled amino acids. In all cases, \( ^{32}P \)-tyrosine was the only phosphorylated amino acid detected.

RESULTS

Tyrosine Protein Kinase Activity in the Developing Embyro—S. purpuratus embryos raised at 4–5°C in normal seawater progress through development at about one-half the rate observed at 15°C. Unfertilized eggs and embryos at different developmental stages were washed with seawater and then suspended in a buffer (pH 7.5) containing 50 mM Tris, 2 mM MgCl2, and 1 mM EDTA. These eggs were homogenized with a Polytron probe at a setting of 6–7 for 3 min; the homogenate was then centrifuged at 1,100 \( \times g \) for 5–6 min to pellet unbroken eggs, cells, and nuclei. The resultant supernatant fluid was then centrifuged at 17,000 \( \times g \) for 20 min. The pellet obtained had a 5–10-fold higher specific activity of tyrosine-specific protein kinase than the 1,100 \( \times g \) nuclear pellet or the 17,000 \( \times g \) supernatant fluid. For further subfractionation, the high speed pellet (particulate fraction) was suspended in the above mentioned buffer and centrifuged on a discontinuous sucrose gradient in a Beckman SW 27 rotor at 26,000 rpm for 4 h. The gradient consisted of steps of 30, 40, and 45% (w/v) sucrose solution in buffer (50 mM Tris, 2 mM MgCl2, 1 mM EDTA [pH 7.5]). The 30/40% interface (particulate fraction) had the maximum (2–5-fold higher) specific activity of tyrosine-specific protein kinase. Both the particulate and membrane fractions were used in subsequent experiments to estimate tyrosine-specific protein kinase activity at different stages of development.

Before fertilization, the specific activity of the kinase was 0.22 pmol of peptide phosphorylated per mg of protein/min and 0.36 pmol of peptide phosphorylated per mg of protein/min in the particulate and membrane fractions, respectively. Within 1 h after fertilization, the specific activity of the protein kinase in the particulate fraction had increased to 0.56 pmol of peptide phosphorylated per mg of protein/min; it further increased to 4.5 pmol of peptide phosphorylated per mg of protein/min at the gastrula stage (Fig. 1). There was a corresponding 7-fold increase in the specific activity in the membrane fraction between the 1 h and gastrula stage.

Phosphorylation of Peptide and Identification of Phosphotyrosine—To establish that the peptide was phosphorylated at the tyrosine residue and to determine the specificity of the peptide as a substrate in the following experiments were done. The peptide EoG (Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) was phosphorylated using the 48-h membrane. The reaction was stopped with trichloroacetic acid and the proteins were precipitated. The high pressure liquid chromatography profile of the supernatant fluid radioactivity was obtained. Supernatant fluid without peptide was also chromatographed on the same chromatography column. Two profiles show the difference between the two profiles. The amount of \( ^{32}P \) obtained in the peptide fraction from the column was quantitatively the same as that estimated in the acid supernatant fluid after application to phosphocellulose paper. The high pressure liquid chromatography fraction containing phosphopeptide was processed for phosphoamino acid analy-
To determine the specificity of the peptide as a substrate, it was incubated with purified cyclic AMP-dependent protein kinase, phosphorylase kinase, glycogen synthase kinase 3, and casein kinase 2, or the EGF-receptor protein kinase of A-431 cells. Except for the purified EGF-receptor kinase (28), none of the other kinases phosphorylated the peptide (not shown).

Effect of Time, Protein, Peptide, and MgATP on the Kinase Activity—The amount of $^{32}$P incorporated into the peptide $\text{L}_{12}\text{G}_1$ increased linearly up to 15 min (Fig. 3a). The incorporation also remained linear over a restricted range as a function of increasing protein concentration (Fig. 3b). The apparent maximal velocity was 61 pmol of peptide phosphorylated per mg of protein/min using the 48-h embryo membranes. The protein kinase was most active at pH 8.0 and showed approximately one-half-maximal velocity at 35 µM MgATP (not shown).

Phosphorylation of Endogenous Proteins at Tyrosine during Development—Since the above results indicated the presence of a tyrosine protein kinase(s) in the sea urchin embryo, we determined whether or not endogenous proteins in the embryo could be phosphorylated at tyrosine. For this purpose, membrane fractions from the stages shown in Fig. 1 were incubated with [$\gamma$-$^{32}$P]ATP, solubilized in Na dodecyl SO$_4$, and then electrophoresed on 10% polyacrylamide gels and stained with Coomassie blue (Fig. 5A). Parallel gels were incubated in 1 M KOH for 2 h to remove most of the $^{32}$P-serine and $^{32}$P-threonine from the proteins. Both the nontreated and KOH-treated gels were subjected to autoradiography (Fig. 5, B and C).

The alkali-treated gels had several bands containing $^{32}$P (Fig. 5C). Proteins ($M_\text{r}$ = 26,000, 42,000, 45,000, 50,000, 57,000, 74,000, 84,000, 126,000, and >200,000) prominent on the alkali-treated gels were eluted from the nonalkali- and alkali-treated gels and processed for phosphoamino acid analysis (see "Experimental Procedures"). All of the protein bands from the nonalkali-treated gels were found to contain $^{32}$P-tyrosine, although the $M_\text{r}$ = 45,000, 57,000, 74,000, and 84,000 bands had the highest amounts (Fig. 6). $^{32}$P-tyrosine was...
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FIG. 5. Na dodecyl SO₄ gel electrophoresis of the membrane proteins obtained at different stages of development. The gels were 10% polyacrylamide, and 50 μg of protein were loaded in each lane. A, Coomassie blue-stained gel. B, autoradiograph of the gel in A. Proteins were phosphorylated with [γ-³²P]ATP in vitro as described under "Experimental Procedures." C, autoradiograph of a parallel gel treated with alkali. The developmental stages (after fertilization) depicted on each gel are 1 h (a), 24 h (b), 48 h (c), 72 h (d) 96 h (e), and 0 h (f) (unfertilized egg). 200K, for example, represents Mᵦ = 200,000.

FIG. 6. Electrophoretic separation and autoradiography of labeled phosphoamino acids of ³²P-containing bands corresponding to Mᵦ = 45,000 (A), 57,000 (B), 74,000 (C), and 84,000 (D). The labeled bands were excised from nonalkali-treated polyacrylamide gels and processed as described under "Experimental Procedures." The bands were treated with 10% KOH, and the resulting amino acids were identified by peptide phosphorylation, was coincident with an increase in the number of endogenous proteins phosphorylated at tyrosine in a membrane fraction. Of the nine protein bands analyzed for ³²P-tyrosine, all were shown to contain the radiolabeled amino acid. Significantly, however, no radiolabeled bands were detected on alkali-treated gels when membranes from unfertilized eggs were analyzed.

DISCUSSION

To date, tyrosine-specific protein kinase activity has been studied in normal and neoplastic cells (1-8, 30-32), but there have been no reports of its activity in invertebrates or embryos. Here, we show that tyrosine protein kinase activity is present in the sea urchin embryo and that its activity markedly increases during early embryonic development. The increase in the enzyme activity following fertilization, estimated by peptide phosphorylation, was coincident with an increase in the number of endogenous proteins phosphorylated at tyrosine in a membrane fraction. Of the nine protein bands analyzed for ³²P-tyrosine, all were shown to contain the radiolabeled amino acid. Significantly, however, no radiolabeled bands were detected on KOH-treated gels when membranes from unfertilized eggs were analyzed.

Some of the acutely transforming retroviral onc gene products have tyrosine protein kinase activities (1-4). Although the viral onc genes appear to have originated from the vertebrate genome (16), the transcription of c-onc genes may be normally repressed or at a very low level. Recently, Muller et al. (16) have shown stage- and tissue-specific differential expression of c-onc genes during pre- and postnatal development of the mouse. The cellular genes homologous to Finkel-Biskis-Jenkins murine osteosarcoma, Abelson murine leukemia, and Harvey sarcoma viral onc genes were studied. It is noteworthy that the Abelson murine leukemia virus onc gene product is known to possess tyrosine protein kinase activity (4). It has been suggested that cellular onc genes are involved in normal development (33, 34), and the data presented here could support such a concept. Whether or not the tyrosine protein kinase activity measured during sea urchin early development is the product of what may be considered an "onc gene," however, is not known. Sea urchin embryos, because of their availability in large amounts and because of certain early developmental similarities with chordates (35), may prove to be an excellent model to study the role of tyrosine phosphorylation in processes like cell proliferation and differentiation.

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