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QUANTITATION OF A SRC-LIKE TYROSINE PROTEIN KINASE DURING FERTILIZATION OF THE SEA URCHIN EGG

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Fertilization of the sea urchin egg is known to involve an increase in overall protein tyrosine kinase activity which preceeds the first cell division. In order to determine the types of tyrosine kinases that are involved in fertilization, we have used immunological and other criteria to identify a c-src related protein kinase in eggs of the sea urchin L. variegatus. Using an immune complex assay, we have measured the level of this c-src related protein kinase during fertilization and early embryonic development. Fertilization results in a decrease in the c-src kinase detectable by this technique suggesting that c-src does not contribute to the fertilization induced increase in protein tyrosine kinase activity.

Fertilization results in transformation of the quiescent, unfertilized egg into a rapidly dividing embryo capable of undergoing morphogenesis and differentiation. It is now apparent that egg activation involves an increase in protein tyrosine kinase activity (1,2,3) and in the phosphotyrosine content of egg proteins (4). In order to understand the role that egg tyrosine kinases play at fertilization, it is important to identify the kinases that exist in the unfertilized egg and measure their activity during early development. The sea urchin fertilization system is an excellent model for the study of egg activation since large quantities of eggs can be fertilized synchronously and begin the first cleavage division within 1 hr. The pp60$^{c\text{-}src}$ kinase has been identified in animals as primitive as the marine sponge (5), and exists at low levels in eggs of the

Abbreviations: TAPS, tris(hydroxynethyl)methylaminopropane sulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2ethane sulfonic acid; EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid.

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fish, amphibian, and chicken (6). The c-src enzyme can be recognized and form an immune complex with IgG from rabbits bearing Rous sarcoma virus-induced tumors (TBR serum). The kinase can then phosphorylate a tyrosine residue on the IgG heavy chain enabling quantitation of the level of active enzyme (5,7). In the present report, we have used this immune complex assay to monitor the level of active c-src kinase during egg activation and early development of the sea urchin embryo.

MATERIALS AND METHODS

Preparation of egg and embryo extracts: Unfertilized eggs were obtained from L. variegatus by intracoelomic injection of KCl, then dejellied at pH 5.5 (4). One group was inseminated and allowed to develop in sterile sea water buffered with 5mM TAPS at pH 8.0. Detergent extracts were prepared from eggs or embryos (50ul packed volume) by homogenization in 0.5ml of extraction buffer (6) containing 10mM sodium phosphate, 40mM NaF, 10mM EDTA, 1% Triton X-100, 5% Aprotinin (Sigma), pH 7.0,and 0°C. The homogenates were then centrifuged at 100,000 X G, and the soluble detergent extract was used immediately. Aliquots of the extract were precipitated with 20% TCA at 0°C for measurement of protein content (8).

Immune complex assay: Aliquots of the detergent extracts (25ul) were incubated with 5ul of TBR or control rabbit serum for 1hr at 0°C. 50ul of protein A-Sepharose (Pharmacia) washed and suspended in extraction buffer (50% vol/vol) was added and incubated on ice with agitation for 30min. The immune complexes were collected by centrifugation, washed twice in extraction buffer containing 1M NaCl, then centrifuged through a 0.5ml pad of 60% sucrose. They were washed once more in extraction buffer with 1M NaCl and once in distilled water. The kinase reaction was run by suspending the complexes in 50ul of 20mM HEPES, 50mM MgCl2, 10uM NaVO4, and 1% aprotinin, pH 7.2. The reaction was started by addition of γ-[32P]-ATP (0.9-1.0 Ci/umol) to a final concentration of 2uM, and carried out at 20°C for 2-5 min. The reaction was stopped by addition of 1ml of ice cold quench buffer (5) and the phosphorylated immune complexes were centrifuged, suspended in SDS gel sample buffer and heated at 90°C for 5min. Samples were then electrophoresed on a 10% SDS polyacrylamide gel (9) and stained briefly with coomassie blue. The radioactivity in the IgG heavy chains was detected by radioautography of the dried gels and quantitated by counting the excised bands (bleached in H_2O_2) in a scintillation counter.

RESULTS AND DISCUSSION

We first tested the ability of different TBR sera to immunoprecipitate pp60$^{c\text{-}src}$ from sea urchin egg plasma membranes which were phosphorylated in vitro. Several antisera were found to precipitate a prominant 60 KDa phosphoprotein as well as a 54KDa protein which may represent a proteolytic fragment (Fig. 1). When these TBR sera were tested in an immune complex kinase assay, phosphorylation of the IgG heavy chain was readily detected

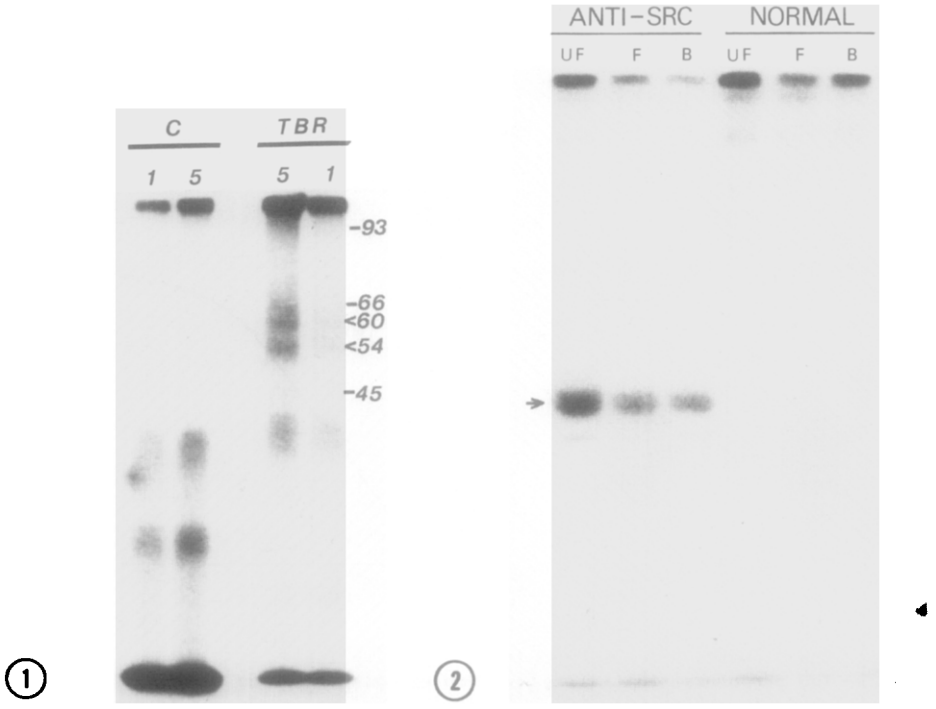
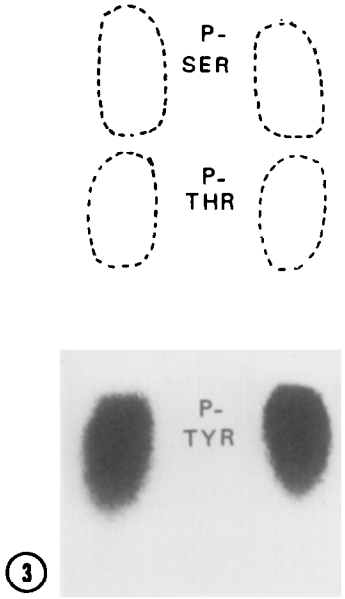


Fig. 1. Immunoprecipitation of [32P]-labelled plasma membrane proteins with TBR antisera.
Plasma membranes (10ug protein) prepared from fertilized eggs (4) were autophosphorylated in 15ul of a buffer containing HEPES, 25mM; MgCl2, 50mM; NP40, 0.5%; VaVO4, 10uM; pH 7.2 containing 1.7uM γ-[32P]-ATP (3 Ci/mmol) at 20C for 10min. The reaction was stopped by addition of excess ice cold EDTA (0.1M), and the phosphoproteins were immunoprecipitated with 1 or 5ul of normal (C) or TBR (TBR) serum followed by protein A sepharose. The phosphoproteins were analyzed on a 10% SDS polyacrylamide gel and autoradiographed for 24 hrs with an intensifying screen. The above autoradiograph demonstrates that the TBR antisera precipitated proteins of 60 and 54 KDa which were not precipitated by the control serum.

Fig. 2. Phosphorylation of pp60v-src specific IgG by sea urchin egg extracts. Detergent extracts prepared from eggs or embryos and aliquots (150ug protein) were incubated with anti-src (TBR) or normal rabbit serum as described in materials and methods. The immune complexes were adsorbed to protein A-Sepharose, washed, suspended in phosphorylation buffer and incubated with γ-[32P]-ATP for 5min as described in materials and methods. The phosphoproteins were then analyzed on a 10% SDS polyacrylamide gel. The above autoradiograph demonstrates the specific phosphorylation of the IgG heavy chain (arrow) from TBR serum but not from control serum. Unfertilized eggs (UF), fertilized eggs extracted 30min post insemination (F), and blastula stage embryos (B).

(Fig. 2). No phosphorylation was observed with control rabbit sera indicating that the reaction depended on the presence of anti-src antibodies. Phosphoamino acid analysis of the [^{32}P]-labelled IgG produced in this reaction revealed that only tyrosine residues were phosphorylated (Fig. 3). This indicated that no contaminating serine/threonine protein



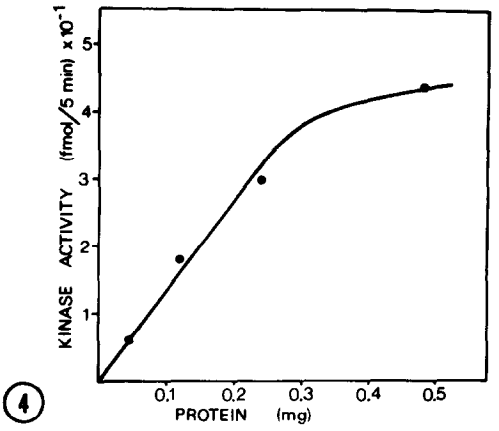


Fig. 3. Phosphoamino acid analysis of 32P-labelled IgG.
TBR-IgG phosphorylated in immune complex assays such as in Fig. 2 was electroeluted from polyacrylamide gels, pooled, freeze dried, and subjected to partial acid hydrolysis in 6N HCl at 110 C for 3hr. The phosphoamino acids were resolved by paper electrophoresis in 0.5% pyridine/ 5.0% acetic acid, pH 3.5 at 2,400 V for 1hr. Phosphoamino acid standards included in the samples were detected with ninhydrin and their positions are indicated by dashed lines. The above autoradiograph demonstrates [32P]-phosphotyrosine in TBR IgG phosphorylated in immune complexes from unfertilized eggs (left) and blastula stage embryos (right).

Fig. 4. Protein dependence of the TBR immune complex assay.
Aliquots containing different amounts of unfertilized egg extract were incubated with 5ul of TBR or normal rabbit serum, the immune complexes were collected on protein A-Sepharose and phosphorylated as in Fig 2. The [32P]-IgGwas cut from polyacrylamide gels and counted in a scintillation counter. Src-specific activity was obtained by subtracting the nonspecific (normal serum) incorporation from that in the TBR IgG.

kinases were present. IgG phosphorylation was linearly dependent on the amount of egg extract (up to 250ug protein) added to the reaction (Fig. 4).

In order to determine the effect of fertilization on the level of c-src kinase, we prepared egg extracts at different times during development and assayed them for the ability to phosphorylate TBR IgG. As seen in Fig. 5, the amount of kinase detected by this technique declined rapidly after fertilization and did not increase significantly during cleavage, blastula, or gastrula stages. This result was surprizing because previous experiments by our laboratory (2) as well as others (3) had demonstrated

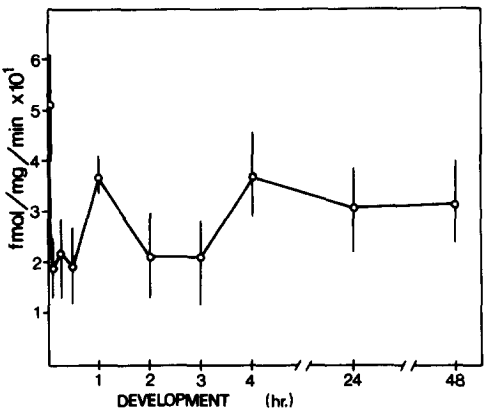


Fig. 5. SRC-Kinase levels during sea urchin development.
Extracts were prepared from eggs and embryos at different times during development. Aliquots of these extracts were assayed for src-kinase levels by the immune complex assay described in materials and methods. Results obtained from five different batches of eggs are presented above with the range indicated by the vertical bars. Time points taken during the first hour of development are: unfertilized (0hr), 5min, 15min, and 30min post insemination.

that the overall protein tyrosine kinase activity increased in response to fertilization. We measured the total protein tyrosine kinase activity in the detergent extracts used for one of the immune complex assays to confirm that the total tyrosine protein kinase activity did increase after fertilization. As expected, total tyrosine protein kinase activity increased about 50% by 30min post fertilization and over 100% by 12 hrs (early blastula) (Table 1) demonstrating that the conditions used in the immune complex experiments did not differ radically from those used in our previous studies.

TABLE 1

Time Post-Fertilization	Pmol/mg/min
Unfertilized	0.251
30min	0.376
24hr	0.621

Tyrosine protein kinase activity during development. Aliquots of detergent extracts used in one set of experiments from Figure 4 were assayed in duplicate for protein tyrosine kinase activity using neurotensin as a substrate as described before (2). The specific activity is expressed as pmoles of [32P]-neurotensin produced/mg protein/minute at 20°C.

Previous work has shown that the level of protein tyrosine kinase activity increases in response to fertilization and continues to increase through early embryonic development (1). Our findings demonstrate that, while the c-src kinase exists in the unfertilized egg, the amount detectable by immune complex assay actually decreases after fertilization. While the results of an immune complex assay cannot be directly compared to enzyme activity measured with a soluble peptide substrate, our findings indicate that it is unlikely that c-src contributes to the post-fertilization increase in protein tyrosine kinase activity. Through clearing experiments, we have found that the TBR antisera immunoprecipitate up to 30% of the total protein tyrosine kinase activity present in the egg. Apparently one or more additional tyrosine kinases are activated at fertilization to levels sufficient to overshadow the decline in the relatively small amount of c-src.

There are several possible explanations for the relatively abrubt decrease the the level of c-src kinase following fertilization. One possiblility is that a population of c-src became tightly associated with insoluble cytoskeletal components and thus would have been removed by centrifugation prior to immunoprecipitation. Alternatively, active kinase may have been destroyed during the active recycling of the egg plasma membrane which occurs during the first few minutes after fertilization (10). Since we have been unable to radiolabel c-src metabolically in the unfertilized eggs, we are unable to distinguish between the above possibilities.

The level of c-src kinase in the sea urchin egg (per mg protein) is comparable to that reported for eggs of the fish and frog (6). As in these vertebrate embryos, the level of c-src kinase in the sea urchin embryo did not increase significantly during cleavage and blastula formation (6). Studies in vertebrate and insect developmental systems have indicated that action of c-src kinase may relate to functions performed by differentiated (principally neuronal) tissues which are formed well after gastrulation (6, 11-14). The relatively low level of c-src present in the egg may serve some

maintenance function or represent a role earlier in oogenesis. The identity of the other protein tyrosine kinases present in eggs and their role in development remains a question for future research.

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