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DIFFERENTIAL PHOSPHORYLATION OF A 57-KDa PROTEIN TYROSINE KINASE DURING EGG ACTIVATION

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SUMMARY Fertilization results in activation of many protein kinases which function during egg activation. We have used metabolic labelling and immunoprecipitation to study changes in the phosphorylation state of a 57-KDa *src*-family protein tyrosine kinase during fertilization of the sea urchin egg. The kinase was phosphorylated on serine at all periods studied but it was also phosphorylated transiently on tyrosine at 5 minutes post insemination and then on threonine at 90 minutes after fertilization. These data indicate that the 57-KDa PTK may be under complex regulatory control during the first cell cycle.

Fertilization results in stimulation of one or more protein tyrosine kinases (PTK's) (1 - 3) that are required for successful egg activation and initiation of cell division (4). Identification of the PTK's that are activated in response to fertilization is essential to understanding the signal transduction mechanism that functions during egg activation. Eggs of D. melanogaster , sea urchins, and mammals have been shown to contain the *c-abl* encoded PTK (5, 6, 7). Oocytes of X. laevis are known to contain the insulin receptor PTK which apparently functions in oocyte maturation (8). In addition, *src*-family PTK's including *c-src* and *c-fyn* have been reported in several eggs and early embryos (9, 10, 11). Our laboratory has used an antibody against a *src*-family consensus peptide (peptide G3) to detect a 57 KDa PTK which is localized to the egg plasma membrane (12). This protein kinase appears to be a member of the *src*-family of PTK's based on the specificity of the antibody and on its apparent molecular weight, but is not recognized by many antibodies specific for *v-src* or *c-src* encoded proteins.

Since the activity of many *src*-family PTK's is regulated by phosphorylation of regulatory sites by other protein kinases, we wanted to determine whether this 57 KDa PTK was phosphorylated during the period of egg activation and whether these changes were reflected by changes in protein kinase activity.

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MATERIALS AND METHODS

Eggs and embryos Eggs from the sea urchin S. purpuratus, were collected and suspended in phosphate-free artificial sea water (1) buffered with 5mM TAPS at pH 8.3 to a final concentration of 10% (vol/vol) and maintained with constant stirring at 15°C. Eggs were prelabelled with [^{32}P]-orthophosphate (0.2 mCi/ml)(Dupont-NEN) for 18hrs, then 5µl of a concentrated suspension of sperm were added to induce fertilization.

Antibody preparation and immunoprecipitation The G3 peptide sequence (TYTAQAGAKNPIKW) was based on the cDNA sequence of a clone encoding the sea urchin homolog of the *c-abl* gene product. This G3 sequence contains 71% homology with the corresponding sequences from *c-src*, *c-lck*, or *c-lyn* and the resulting anti-G3 antibodies actually exhibit a higher affinity for the 57KDa PTK than they do for the 190KDa *c-abl* homolog in sea urchin eggs. Preparation and affinity purification of the antibody have been described elsewhere (12). Samples of radiolabelled eggs (20 - 25 mg protein) were washed by low speed centrifugation (100 X g) and resuspension in ice cold 0025 buffer (0.5 M NaCl, 0.01 M KCl,0.025 M EDTA, pH 8.0 (13)) to remove divalent cations and unincorporated isotope. The washed eggs were suspended in 0.25 ml of 2% SDS, then heated at 90°C for 5 min to inactivate phosphatases. 5ml of RIPA buffer containing 150mM NaCl, 20mM Tris pH 7.5, 10mM EDTA, 40mM NaFl, 0.1mM NaVO4, 1% NP40, 0.5% deoxycholate, and 20µg/ml aprotinin was added and the suspension was centrifuged in a Beckman SW 50.1 rotor at 35,000 rpm for 30 minutes at 4°C. The soluble material was recovered and aliquots were incubated with affinity purified anti-peptide G3 antibody at 0.2 µg/ml for 12 hrs at 4°C. Immune complexes were absorbed with 10µl of Protein A - Sepharose (Pharmacia) and washed twice with RIPA buffer. The pellet was centrifuged through a 0.5ml pad of 40% sucrose and then washed in 50mM Tris pH 8. The final pellet was suspended in SDS-PAGE sample buffer and heated at 95°C for 5 min.

SDS-PAGE & phosphoamino acid analysis Immunoprecipitated proteins were separated by electrophoresis on a 7.5% SDS - polyacrylamide gel, then electroblotted to Immobilon P (Millipore). After localization of the radiolabelled 57 KDa protein by autoradiography, phosphoamino acid analysis of this band was performed as previously described (6).

Protein tyrosine kinase assay PTK activity was quantitated by phosphorylation of a synthetic peptide substrate (Peptide 5 = YGEVYEGVFKK in a reaction mixture containing 425 mM KCl, 50 mM PIPES, 10 mM $MgCl_2$, 10 mM EGTA, 1% NP-40, 10 µM Na_3VO_4, 1 mM peptide 5, 0.1 mg/ml soy bean trypsin inhibitor, 0.1 mg/ml aprotinin and 60 µM [γ-^{32}P]ATP (37 mCi/µmol) pH 7.3. After incubation for 10 minutes at 25°C, the reaction was stopped by the addition of 0.5 ml of 7% TCA. Reaction products were analyzed by reverse phase HPLC as described (6).

RESULTS AND DISCUSSION

In order to determine whether the 57 KDa PTK was phosphorylated in the intact egg, we used the anti-peptide G3 antibody to immunoprecipitate the 57 KDa PTK from eggs which had been labelled metabolically with [^{32}P]-orthophosphate. Unfertilized eggs were preincubated with the isotope for 18 hrs to allow labelling of the endogenous phosphoproteins. This long prelabelling step was necessary to equilibrate the ATP pools (14) because the phosphate transport system in the unfertilized sea urchin egg is largely inactive until 2 - 5 minutes post insemination (15).

Immunoprecipitation of the 57 KDa PTK from detergent extracts prepared from labelled eggs at different times after fertilization revealed that the protein was phosphorylated in both unfertilized and fertilized eggs (Figure 1). While it is apparent that

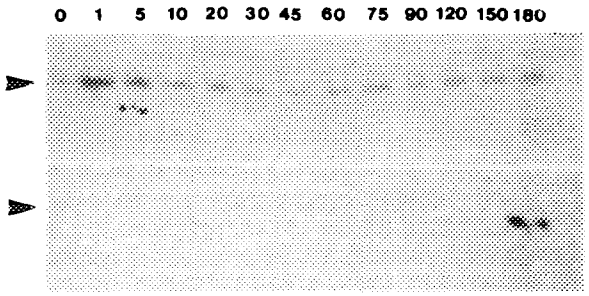

Figure 1. Immunoprecipitation of the 57-KDa protein from eggs at different times after fertilization.

Eggs preincubated with [^{32}P]-orthophosphate (0.2 mCi/ml) for 18 hr were fertilized and allowed to develop for 15 minutes after which they were washed in 0025 buffer and solubilized in RIPA buffer as described in "Methods". Samples containing 20 mg protein were recovered at different times after fertilization, then solubilized and identical aliquots of the soluble material were immunoprecipitated with the anti-peptide G3 antibody (upper panel) or with control rabbit IgG (lower panel). Immunoprecipitates were analyzed on a 7.5% SDS gel and radiolabelled proteins were detected by autoradiography. The time (minutes) after insemination is indicated at the top of the figure and the position of the 57-KDa band is indicated by the arrows.

fertilization results in some increase in the incorporation of $^{32}PO_4$ into the 57 KDa protein, it is important to remember that the 57 KDa protein was not necessarily in equilibrium with the ATP pool. Given these limitations, we conclude only that the 57 KDa enzyme is phosphorylated both before and after fertilization under physiological conditions and next addressed the question of which amino acids were phosphorylated after fertilization.

Phosphoamino acid analysis was performed on the 57 KDa protein immunoprecipitated from eggs at each time point after fertilization. The results presented in Figure 2 reveal the presence of ^{32}P-Ser at all time points after fertilization. A small amount of ^{32}P-Tyr was detected in the 57 KDa protein as early as 1 minute after fertilization, and was easily detected at 5 minutes after fertilization (Fig 2C). Phosphorylation on Tyr was transient however, and by 30 minutes after fertilization, ^{32}P-Tyr was no longer detectible (Fig. 2E). Immunoprecipitates prepared between 30 and 60 minutes post-insemination contained only ^{32}P-Ser, however at 90 minutes post-insemination ^{32}P-Thr was detected indicating that different sites must be phosphorylated as the egg goes through this period of development (Fig. 2H). Phosphorylation on Thr was also transient and ^{32}P-Thr was no longer detectible by 150 minutes by which time the eggs had divided. Similar results were obtained with three different batches of eggs.

To determine whether the changes in phosphorylation state of the 57 KDa protein correlated with changes in enzyme activity, we measured the kinase activity present in immunoprecipitates prepared at different times after fertilization. Since the anti-G3 antibody is directed against peptide sequence near the catalytic site of the enzyme and

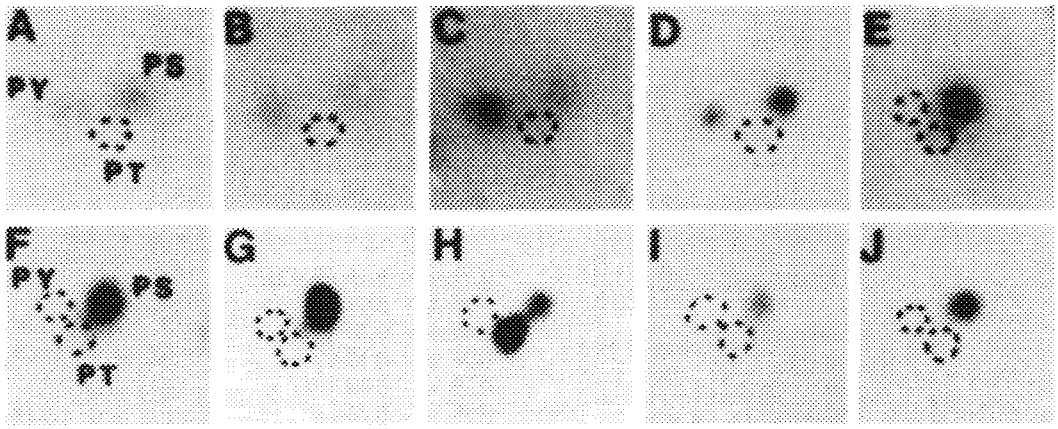

Figure 2. Phosphoamino acid analysis of the $^{32}PO_4$-labelled 57-KDa protein.

Immunoprecipitates were prepared from eggs at different points after fertilization as in Figure 1, resolved by SDS-PAGE and blotted onto an Immobilon membrane. The radioactive 57-KDa bands were localized by autoradiography, then cut out and hydrolyzed in 6N HCl. The phosphoamino acids were resolved by 2D thin layer electrophoresis at pH 1.9 (bottom to top) and pH 3.5 (left to right) and radiolabelled phosphoamino acids were detected by autoradiography. The position of phosphoamino acid standards included in each of the hydrolysis mixtures was determined by ninhydrin staining and is indicated in some cases by dotted lines (PS= P-Ser, PT= P-Thr, PY= P-Tyr). Samples were obtained at the following times after fertilization: 0 min (panel A), 1min (B), 5 min (C), 10 min (D), 20 min (E), 30 min (F), 60 min (G), 90 min (H), 120 min (I), 150 min (J).

inhibits PTK activity (12), it was necessary to elute the kinase from the immunoprecipitates under acid conditions, then measure kinase activity in the material eluted from the column. Anti-peptide G3 antibody bound to cyanogen bromide activated Sepharose (Pharmacia) was used to immunoprecipitate the 57KDa protein from detergent extracts prepared at different points after fertilization. The affinity resins were washed in RIPA buffer then washed in 3 volumes of 0.1M glycine pH 3 which was immediately neutralized by addition of 1M tris buffer at pH 8, then assayed for kinase activity (12). Attempts to quantitate the recovery of the 57 KDa PTK by Western blot were not successful due to interference by IgG heavy chain (53 KDa) leaching from the resin along with the kinase. As seen in Table I, kinase activity was detected in the unfertilized egg as well as at all times after fertilization but was highest at 5 min. and at 60 min. post-insemination.

In summary, immunoprecipitation of the 57 KDa protein from eggs labelled metabolically with [^{32}P]-orthophosphate allowed us to detect the incorporation of radioisotope into the 57 KDa PTK at different times after fertilization. The extensive changes in $^{32}PO_4$ transport and metabolism at fertilization made it difficult to achieve equilibrium labelling of the protein and therefore quantitation of phosphorylation stoichiometry would not be reliable. However, qualitative changes in phosphorylation of

TABLE I

Protein tyrosine kinase activity immunoprecipitated by the anti - peptide G3 antibody

Min Post-insemination	activity (fmol/min)
Unfertilized	0.35 +/- 0.15 (n=3)
1	0.28 +/- 0.09 "
5	0.60 +/- 0.20 "
10	0.24 +/- 0.13 "
20	0.29 +/- 0.08 "
30	0.35 +/- 0.24 "
60	0.53 +/- 0.36 "
90	0.30 +/- 0.24 "
120	0.27 +/- 0.11 "
150	0.28 +/- 0.04 "

Samples of eggs containing 10 mg protein were washed in 0025 buffer, solubilized in RIPA buffer, and the 57-KDa kinase was immunoprecipitated with anti-G3 antibody bound to Sepharose beads or with control rabbit IgG bound to beads. Enzyme bound to 25µl of resin was eluted with 50 µl of 0.1 M glycine buffer,pH 3.0,and immediately neutralized with 1 M tris,pH 8. The activity eluted from each sample was assayed using a peptide substrate as described in Methods. Values represent the activity eluted from the anti-peptide G3 resin - the activity eluted from the control resin and are the average of three experiments +/- S.D.

Ser, Thr, and Tyr residues would still be detectible. The results demonstrate that the 57 KDa protein is phosphorylated on Ser at all times examined. In addition, two transient phosphorylation events occurred after fertilization. First was a Tyr phosphorylation which occurred during the first few minutes post-insemination. Secondly, the 57 KDa PTK was phosphorylated on Thr about 90 minutes post-insemination.

The fact that many *src*-family PTK's are themselves substrates for other protein kinases has been documented in many cases. The *c-abl*- encoded PTK, for example, undergoes phosphorylation on up to ten different sites at different points during the cell cycle (16). This is thought to be due to autophosphorylation as well as the action of the cdc2 kinase, although kinase C has been shown to phosphorylate $p150^{c\text{-}abl}$ in other systems (17). To date, the changes in phosphorylation of *c-abl* kinase have not been correlated with changes in catalytic activity. In contrast, the phosphorylation of other *src*-family members has a dramatic effect on kinase activity. The $pp60^{c\text{-}src}$ kinase activity is negatively regulated by phosphorylation of tyr 527 by the CSK kinase (18), while phosphorylation of Ser and Thr in the N-terminal region of this protein is thought to function in subcellular localization (19, 20). Another example is *c-lck* where, phosphorylation on Tyr activates kinase activity (21).

The fertilization - dependent changes in phosphorylation state of the 57 KDa kinase indicates that this enzyme might be under complex regulatory control. The highest levels of enzyme activity correlated with Tyr phosphorylation of the 57KDa protein at 5 min. post-insemination, however elevated kinase activity was also present at 60 min. post-insemination when P-Tyr was not detected in the 57KDa protein. In contrast, phosphorylation on Thr appears to have no effect on kinase activity and may be involved aspects of enzyme function other than catalytic activity, such as subcellular localization, interactions with other proteins, or possibly substrate specificity. Future in vitro studies to examine the effect of phosphorylation of different sites on kinase activity will help explain how the changes in phosphorylation state observed in the fertilized egg could function during egg activation.

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