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Insulin and progesterone activate a common synthetic ribosomal protein S6 peptide kinase in *Xenopus* oocytes

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A synthetic peptide Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala, the structure of which is based on that of a phosphorylated sequence in ribosomal protein S6, was employed as a probe for stimulated kinase activity in *Xenopus laevis* oocytes induced to mature with insulin or progesterone. Insulin elicited an early (20–30 min) 3-fold stimulation of S6 peptide phosphorylating activity that was not evident with progesterone. However, both hormones produced a delayed 7–12-fold stimulation of S6 peptide phosphorylating activity at the time of germinal vesicle breakdown. The results of DEAE-Sephacel, Sephacryl S-200, TSK-400, and heparin-Sepharose chromatographic fractionation experiments imply that a common S6 peptide kinase is activated as a consequence of short and long term insulin exposure, as well as in long term progesterone treatment of oocytes. Omission of potassium from the oocyte culture medium greatly facilitated insulin-induced meiotic maturation.

S6 kinase; Insulin; Progesterone; Oocyte maturation

1. INTRODUCTION

Recent studies conducted in several laboratories including this one [1–8] have indicated that the increased phosphorylation of ribosomal protein S6 that occurs when mammalian cells are exposed to insulin, other serum growth factors, or the tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is catalyzed by a novel protein-serine kinase. Similarly, an avian S6 kinase is stimulated when chick embryo fibroblasts are treated with calf serum or TPA, or upon retroviral transformation [8,9]. Typically, these stimulations of S6 kinase activity occur within the first 30 min of treatment [6,7,9]. Insulin treatment of *Xenopus* oocytes also causes the rapid stimulation of an S6 kinase [10], and a rapid increase in S6 phosphorylation (in vivo) was noted when oocytes were microinjected with purified human placental insulin receptor [11]. In addition to this 'immediate response', S6 phosphorylation in oocytes has been found to be stimulated several hours later in response to insulin [12] as well as to progesterone [13–16]. Although not examined in response to insulin, an S6 kinase is activated several hours after stimulation with progesterone, just prior to germinal vesicle breakdown (GVBD) [17] and again as maturing oocytes approach second metaphase arrest [17–20]. Based on its elution characteristics from DEAE-Sephacel, the S6 kinase that becomes activated under these latter conditions would appear to be the enzyme referred to as S6 kinase II [18,19], which has been purified from *Xenopus* eggs. While purified S6 kinase II from *Xenopus* eggs has many similarities to the insulin-activated S6 kinase in partially purified preparations from mammalian cells [2,4–6,17,21], it is not settled whether the short term insulin-activated kinase and the long term progesterone-activated S6 kinase (S6 kinase II) in *Xenopus* oocytes are the same enzyme. Suggesting that they might be different are the reports that they elute from DEAE-Sephacel at 0.3 M NaCl [10] and 0.16–0.17 M NaCl [17,19], respectively. The present study was undertaken to

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clarify this point and also to determine characteristics of a TPA-stimulated S6 peptide kinase in *Xenopus* oocytes.

2. MATERIALS AND METHODS

Bovine (sodium) insulin was purchased from Calbiochem. Unless stated otherwise, the sources of all other materials, including the *Xenopus laevis* females, and the procurement of stage VI oocytes are as described [17]. Oocytes were incubated either in OR-2 [22] or in the modified OR-2 of Maller and Koontz [23] at 20–24°C in the absence or presence of 10 μM insulin or 1 μg/ml progesterone. Oocytes were scored for GVBD as described [17].

For the preparation of extracts, oocytes were transferred into a 2-ml Potter-Elvehjem tissue grinder on ice, and ice cold buffer A (75 mM β-glycerophosphate, 20 mM Mops, pH 7.2, 15 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM Na_3VO_4) was added. The oocytes were rapidly homogenized (5 strokes) and centrifuged (5°C) for 15 min at $150000\times g$ in a Beckman airfuge or for 20 min at $100000\times g$ in a Beckman TL-100 tabletop ultracentrifuge. The clear supernatant layer was removed, aliquoted, and frozen at −70°C until column chromatography or assay. The details concerning all column chromatographies are provided in the figure legends.

Kinase activitiy was measured at 30°C for 15–30 min in the presence of 0.25 mM Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (RRLSSLRA or S6 peptide); 25 μM (crude extracts) or 50 μM (column fractions) [γ-^{32}P]-ATP; 400 nM cAMP-dependent protein kinase inhibitor peptide [24,25]; and buffer B (30 mM β-glycerophosphate, 20 mM Mops, pH 7.2, 20 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM Na_3VO_4) in a final volume of 25 μl. The reactions were terminated and the radioactivity incorporated into S6 peptide was quantitated as described [2,17]. Protein was estimated by the method of Bradford [26] with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

3.1. *Insulin induction of oocyte maturation*

While insulin has been reported to induce meiotic cell division in *Xenopus* and *Rana* oocytes [23,27–30], other workers [31–34] have found little or no insulin induced oocyte maturation. In our own initial experiments with insulin, it was also extremely difficult to trigger maturation of oocytes. For example, with oocytes from 6 out of 8 females, there was no evidence of GVBD after 10–18 h of incubation with 4–400 μg/ml insulin in OR-2 medium. With oocytes from the remaining 2 females, only a small percentage of the oocytes underwent GVBD (10–30%). However, when the medium of Maller and Koontz [23] was used, a higher and more consistent response to insulin was observed. Using populations of oocytes from 21 females, 15 showed 100% GVBD 8–22 h after insulin addition, 1 underwent 70% GVBD by 22 h, and the remaining 6 responded poorly or not at all. As reported [12,27], insulin-induced GVBD lagged behind progesterone-induced GVBD, although the lag varied from less than 1 h to as much as 4–5 h depending on the female from which the oocytes were obtained.

The levels of components present in cell culture, media, such as sodium, bicarbonate, and phosphate ions, are known to greatly affect the responsiveness of cells to insulin [35,36]. Since the Maller and Koontz medium is essentially a modified OR-2 medium that lacks KCl and $NaHPO_4$, the influence of these components was tested. As evident from table 1, the omission of potassium appeared to facilitate insulin-induced oocyte maturation. Potassium-free medium has previously been found to lower the threshold concentration for the induction of maturation by a number of divalent cations [37]. Significant induction of maturation by insulin-like growth factor 1 (IGF-1) also appeared to require the absence of potassium (not shown). All of the subsequent experiments were performed using the modified OR-2 medium of Maller and Koontz [23].

3.2. *Activation of S6 peptide kinases in maturing* Xenopus *oocytes*

An S6 peptide (RRLSSLRA), used in the presence of an inhibitor of the cAMP-dependent protein kinase, has served as a convenient probe for activated 40 S ribosomal S6 kinases in progesterone-treated *Xenopus* oocytes [17] and other model systems [1,2,38], and therefore its phosphorylation was regarded as a probable indicator of insulin-activated S6 kinases in the present study. In addition, it is known that purified *Xenopus* S6 kinase II will phosphorylate this peptide (personal communication from Dr J.L. Maller). As previously reported for S6 kinase activity [10], insulin treatment of *Xenopus* oocytes caused a rapid 3-fold stimulation of soluble S6 peptide kinase activity (fig.1). In our hands, this activation could be detected without a 6 h dialysis step before assay [10] and was also seen with 100 nM IGF-1. The phosphorylating activity transiently declined to control levels and underwent an even greater increase (12-fold) by the time of 50% GVBD. This

Table 1

Effect of potassium on insulin induction of *Xenopus* oocyte maturation

Defolliculation/ recovery medium	Addition/ subtraction	% GVBD (9 h)	% GVBD (21 h)
OR-2	none	0	0
OR-2	−2.5 mM KCl	63	97
OR-2	−0.5 mM Na_2HPO_4	0	0
OR-2	− both components	68	100
mod. OR-2 [Maller and Koontz (16)]	none	100	100
mod. OR-2 [Maller and Koontz (16)]	+2.5 mM KCl	35	53
mod. OR-2 [Maller and Koontz (16)]	+0.5 mM Na_2HPO_4	96	100
mod. OR-2 [Maller and Koontz (16)]	+ both components	11	82

Oocytes were defolliculated and allowed to recover for approx. 2 h in the various media (± components). Sets of 20 oocytes were then treated with 10 μM insulin and scored for GVBD at 9 h and 21 h as described previously [13]

coincided with the increase in S6 kinase activity seen after progesterone addition [17,39] and the major burst in total protein phosphorylation that precedes the first meiotic division in *Xenopus* oocytes [40]. Both insulin and progesterone treatments cause increases in internal pH [12,41] and the rates of protein synthesis [35,42] at the time of the burst in protein phosphorylation and therefore appear to trigger a common set of late events that are closely associated with GVBD and the mechanics of cell division. The early transient stimulation of S6 peptide kinase activity following insulin treatment did not necessarily correlate with insulin induction of GVBD, since this increase was detected when OR-2 medium was used and GVBD was not induced (see above). Conversely, there were a few cases using modified OR-2 in which GVBD was induced, but the early increase in S6 peptide kinase activity was not found. The early increase in S6 peptide kinase activity, as seen with insulin, is relatively small compared to the later rise and is not observed when progesterone is used to induce maturation. Hence, it follows that this action of insulin may be unrelated to the induction of normal maturation events and may involve a second track of insulin action. Interestingly, the early insulin-stimulated increase in S6 peptide kinase activity was not found to be accompanied by a significant increase in total protein phosphorylation (not shown) as measured for progesterone-treated oocytes at the time of GVBD [40].

3.3. *Chromatographic fractionation of insulin- and progresterone-activated S6 kinases*

To address whether short term insulin (20–30 min) and long term progesterone (to ~50% GVBD) treatments of oocytes produced the activation of common or distinct S6 peptide kinases, soluble extracts from treated oocytes were fractionated on DEAE-Sephacel (fig.2). With extracts from progesterone-treated oocytes, three discernable peaks of stimulated S6 peptide kinase activity were consistently eluted from DEAE-Sephacel with ~100, ~140, and ~180 mM NaCl, respectively (fig.2A and B). The relative sizes of the activity peaks varied considerably between experiments performed with oocytes from different females, although the ~180 mM NaCl peak was generally the major peak of increased S6 peptide kinase activity. This peak was seen earlier in progesterone-treated oocyte extracts in which it was found to phosphorylate ribosomal protein S6 [17], and is likely to correspond to the 'S6 kinase II' identified in *Xenopus* egg cytosol [18,19]. Long term treatment of oocytes with insulin also resulted in the activation of the same three peaks, with the 180 mM peak predominating (not illustrated). With short term insulin treatment, a major peak of stimulated activity eluted from DEAE-Sephacel at 160–170 mM NaCl (fig.2A and C) and several minor stimulated peaks were observed, although not all in any one experiment. The minor peak detected at ~300 mM NaCl might correspond to the S6 kinase

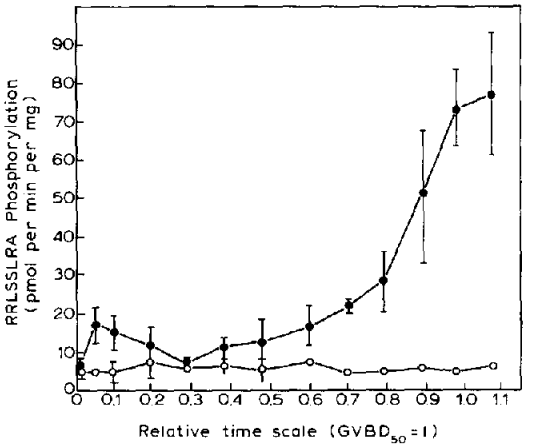

Fig.1. Time course of insulin stimulation of *Xenopus* oocyte RRLSSLRA phosphorylating activity. The mean ± SD ($n=3$) RRLSSLRA phosphorylating activity was determined with soluble extracts from control (○) and 10 μM insulin-treated (●) oocytes. $GVBD_{50}$ = 1 = 10.5 ± 0.5 h.

described by Stafanovic et al. [10]. When the elution profiles from many experiments were normalized and averaged (fig.2B and C), it was found that the major short term insulin stimulated kinase activity peak (160–170 mM NaCl) was almost the same as the major progesterone-stimulated kinase activity (180 mM NaCl), although it appeared to elute slightly ahead of the progesterone-stimulated kinase activity.

To test further the possibility that the major short term insulin-stimulated kinase activity might be a different kinase than the progesterone-stimulated enzyme, pooled DEAE-Sephacel fractions from the short term insulin peak and the long term progesterone-stimulated peak were concentrated by ammonium sulfate precipitation and loaded onto a Sephacryl S-200 gel filtration column. As shown in fig.3A, the two peaks coeluted at an approximate molecular mass of 110 kDa, indicating that they were not grossly different in apparent size. The peak fractions from the Sephacryl S-200 gel filtration column were pooled separately and applied to a heparin-Sepharose column (fig. 3B). Again, the two peaks of stimulated activity cofractionated, eluting in the range of 0.36–0.38 M NaCl.

One additional fractionation approach was performed using crude extracts applied to two different types of gel filtration columns. In fig.4A,

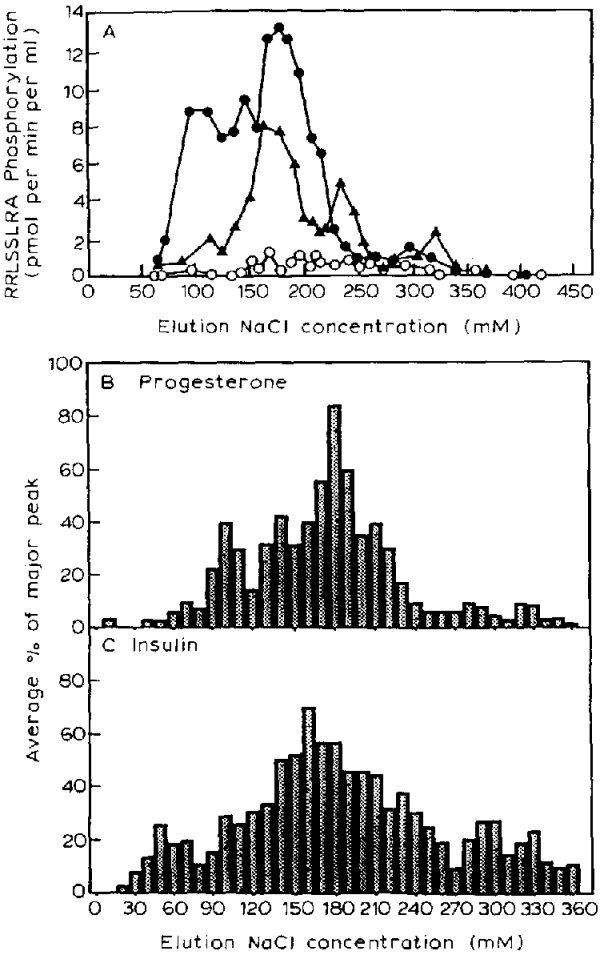

Fig.2. DEAE-Sephacel chromatography of RRLSSLRA kinases from insulin- and progesterone-treated *Xenopus* oocytes. Extracts were prepared in buffer C (30 mM β-glycerophosphate, 10 mM Mops, pH 7.2, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol and 0.5 mM vanadate), as described in section 2, from oocytes that were exposed to 10 μM insulin for 20 min (▲), 1 μg/ml progesterone for 6.5 h (●) or left untreated (○). Each extract (2.5 mg cytosolic protein) was loaded onto a 2.5 ml DEAE-Sephacel column (Sigma) equilibrated in buffer C. The column was washed with 5 ml of buffer C, and proteins were eluted with a 40 ml linear gradient of 0–450 mM NaCl in buffer C with a flow rate of 0.4 ml/min. The 1.0 ml column fractions were assayed for RRLSSLRA phosphorylating activity and conductivity. The conductivity values were converted to salt concentrations with a standard curve of pure NaCl in deionized H_2O. No RRLSSLRA kinase activity was detected in the wash through fractions. (A) Results from a typical experiment; (B) GVBD extracts after progesterone treatment; (C) 20 min insulin treatment, 4 averaged results; 100% corresponded to the RRLSSLRA phosphorylating activity detectable in the highest peak fraction of a column run.

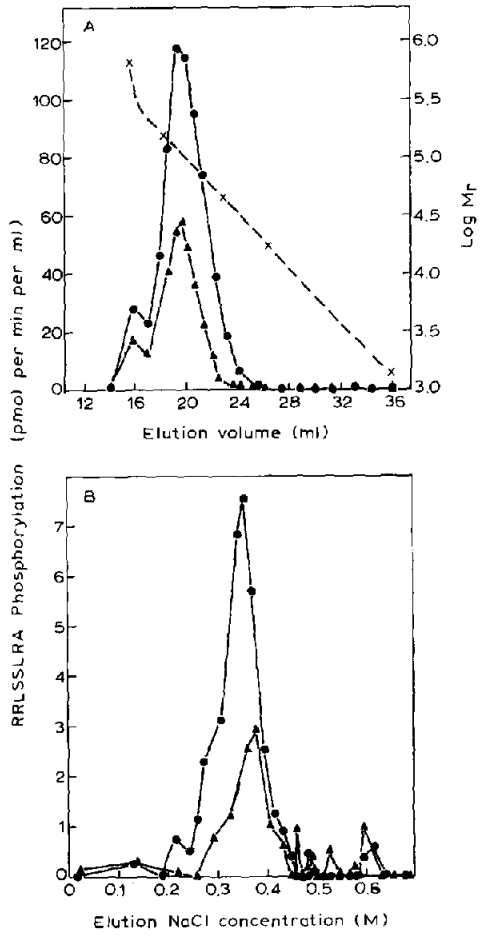

Fig.3. Successive Sephacryl S-200 and heparin-Sepharose fractionation of the short term insulin- and long term progesterone-stimulated RRLSSLRA kinase activity. (A) The major peak fractions (160–190 mM NaCl) from DEAE-Sephacel chromatography of 20 min insulin-treated (▲) and 7 h progesterone-treated (●) oocyte extracts were each pooled (approx. 8 ml total), 100 mg BSA was added, protein was precipitated with 60% ammonium sulfate, redissolved in buffer C, and applied (50 mg protein; 250 μl) to a 1 × 55 cm Sephacryl S-200 (Sigma) column equilibrated with buffer C plus 100 mM NaCl and 0.1% Brij-35. The applied sample was eluted at a flow rate of 0.15 ml/min into approx. 0.67 ml fractions. Elution positions of the marker proteins, thyroglobulin, γ-immunoglobulin, ovalbumin, myoglobin and vitamin B_{12} are indicated in kDa. (B) The peak fractions (4 fractions eluting between 18.5 and 20.5 ml) of RRLSSLRA kinase activity from the Sephacryl S-200 column shown in panel A were diluted 6-fold in buffer C and applied to a 1 ml heparin-Sepharose (Sigma) column equilibrated in buffer C. The column was washed with 5 ml of buffer C and eluted with a 40 ml linear gradient of 0–1 M NaCl in buffer C with a flow rate of 0.4 ml/min into 1.0 ml fractions. Symbols are identified as in panel A. No RRLSSLRA kinase activity was detected in the wash through fractions.

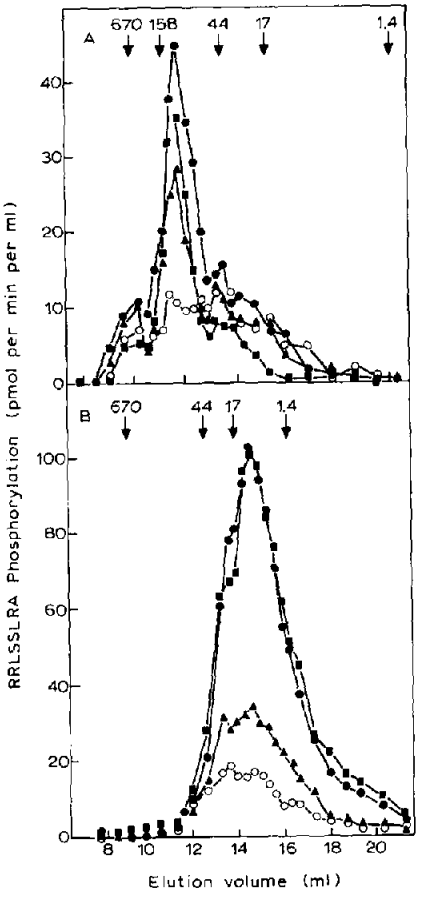

Fig.4. Gel filtration chromatography of insulin- and progesterone-treated oocyte extracts. (A) Cytosolic extracts (2.5 mg protein) from oocytes that were exposed to insulin for 20 min (▲) or 12 h (GVBD extracts) (■), progesterone for 7 h (GVBD extracts) (●) or left untreated (○) were each applied to a 1 × 55 cm Sephacryl S-200 (Sigma) column equilibrated with buffer C plus 100 mM NaCl and 0.1% Brij-35, and eluted at a flow rate of 0.15 ml/min into ~0.67 ml fractions. (B) The above extracts (2.0 mg protein) were, alternatively, applied to a 0.75 × 30 cm TSK-400 GSWP HPLC column (Bio-Rad) and eluted with buffer C plus 100 mM NaCl and 0.1% Brij-35 at a flow rate of 0.3 ml/min into 0.33 ml fractions. Marker proteins are identified in the legend of fig.3.

control, short term insulin-, long term insulin-, and long term progesterone-treated extracts were chromatographed on Sephacryl S-200. The peaks of stimulated activity all coeluted, even though on DEAE-Sephacel several insulin- and progesterone-stimulated peaks often appeared. in fig.4B, these same types of extracts were applied to a TSK-400

gel filtration column, which has previously been found to presumably interact with an S6 kinase from 3T3 cells causing it to elute at an anomalously low molecular size [1,2]. When applied to the column, all the stimulated activities eluted at the same low molecular size (~12 kDa), and, thus, were not separated.

These data would tend to support the notion that the major insulin-stimulated S6 peptide kinase and the major progesterone-stimulated S6 peptide kinase represent the same enzyme. Furthermore, since the major peak of progesterone-stimulated S6 peptide kinase activity elutes from DEAE-Sephacel at the same salt position as S6 kinase II from *Xenopus* eggs [17–19] and also shows 40 S ribosomal protein phosphorylating activity [17], the major S6 peptide kinase and the S6 kinase II are probably the same enzyme. While this work was in progress, Erikson et al. [39] reported that polyclonal antibodies prepared against the *Xenopus* egg S6 kinase II immunoprecipitate stimulated S6 kinase activity in extracts from short term insulin-treated or long term progesterone-treated *Xenopus* oocytes and serum-stimulated or Rous sarcoma virus-transformed chick embryo fibroblasts. This S6 kinase also resembles the mitogen-activated S6 kinase from mammalian sources with respect to its behaviour on DEAE-Sephacel, Sephacryl, TSK-400 and heparin-Sepharose columns, although the 85 kDa mammalian enzyme may be a slightly smaller protein [1,2,4,5,8,21].

The mammalian mitogen-activated S6 kinase and its avian counterpart both undergo activation after exposure of cultured cells to the tumor promoter TPA [1,2,4,9]. Exposure of *Xenopus* oocytes to 0.5 μM TPA for 15 min caused a modest (1.8-fold) stimulation of a cytosolic S6 peptide phosphorylating activity which was found to cofractionate with the major short term insulin- and long term progesterone-activated S6 kinase on DEAE-Sephacel, Sephacryl S-200 and TSK-400 (not shown). Although multiple S6 kinases may become activated during oocyte maturation ([17,38] and this report), the *Xenopus* S6 kinase II appears to be regulated by two different pathways, one involving MPF (Maturation Promoting Factor) appearance [17] in preparation for cell division (any inducing agent) and the other, a more direct result of cell stimulation by certain agents (insulin, IGF-1, and TPA), not involving MPF appearance.

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