Activation of a ribosomal protein S6 protein kinase in *Xenopus* oocytes by insulin and insulin-receptor kinase

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Communicated by P. Cohen (Dundee)

Previous studies in this laboratory have shown that insulin treatment of *Xenopus* oocytes leads to an increase in phosphorylation of ribosomal protein S6. To investigate the mechanism of this increase, S6 kinase activity was measured in lysates of oocytes exposed to insulin. Insulin caused a rapid 4- to 6-fold increase in S6 kinase activity, which was maximal by 20 min and which could be reversed by removal of insulin prior to homogenization. Dose-response curves showed a detectable increase in specific activity at 1 nM insulin with a maximal effect at 100 nM. Treatment of oocytes with puromycin did not prevent this increase in S6 kinase activity, suggesting activation rather than synthesis of the enzyme. DEAE-Sephacel chromatography of extracts from insulin-treated oocytes revealed two peaks of S6 kinase activity, and the specific activity of the peak eluting at 300 nM NaCl was increased 3-fold in oocytes treated with insulin. The same peak of S6 kinase activity was increased 40% within 10 min in oocytes injected with highly purified insulin-receptor kinase. These results indicate that the insulin-dependent increase in S6 phosphorylation is due, at least in part, to activation of an S6 protein kinase, and this activation may result from the action of the insulin receptor at an intracellular location. **Key words:** insulin/S6 phosphorylation/*Xenopus* oocytes/protein kinase

Introduction

Despite many years of investigation, the molecular basis of how insulin exerts its effects on cells is not known. It seems clear that binding of insulin to its receptor on the plasma membrane results in transduction of a signal across the membrane to the interior of the cell. This signal initiates a variety of metabolic processes that occur on a time scale ranging from a few minutes to as long as a few days (for review, see Kahn *et al.*, 1981). Shortly after insulin is bound, the insulin receptor complex is internalized by endocytosis and in various cell systems such internalized receptors are either degraded, reshtutled back to the plasma membrane or maintained in an intracellular pool (Marshall *et al.*, 1981; Wang *et al.*, 1983; Heidenreich and Olefsky, 1985). In 1982 Kahn and co-workers demonstrated that the insulin receptor possesses a protein-tyrosine kinase activity that is stimulated upon insulin binding (Kasuga *et al.*, 1982). This finding has been confirmed in numerous laboratories, and it has been shown that autophosphorylation of the receptor activates its protein kinase activity towards exogenous substrates (Petrzelli

et al., 1982; Avruch *et al.*, 1982; Rosen *et al.*, 1983; Yu and Czech, 1984). These studies have suggested the possibility that increased protein-tyrosine kinase activity of the insulin receptor is, in fact, the signal produced by insulin. It has been thought for some time that protein phosphorylation might underlie some of the effects of insulin. Insulin is known to change the phosphorylation state of several proteins and in some cases the relevant proteins and phosphorylation sites have been identified (Avruch *et al.*, 1985; Brownsey and Denton, 1985; Cohen *et al.*, 1985). In all cases, however, these phosphorylation events have been on serine residues indicating that there must be intermediates between the tyrosine-specific insulin receptor kinase and the protein-serine kinases involved in serine phosphorylation.

One of the best-studied examples of phosphorylation stimulated by insulin is ribosomal protein S6. In several lines of cultured cells responsive to insulin, a rapid increase in S6 phosphorylation occurs following insulin administration (Smith *et al.*, 1979; Lastick and McConkey, 1981; Thomas *et al.*, 1982). An increase in S6 phosphorylation also occurs in *Xenopus* oocytes treated with insulin (Stith and Maller, 1984). The exact function of S6 phosphorylation is not yet known, but it has been associated with an increase in protein synthesis and may aid in recruitment of specific mRNA species (Scott and Pardue, 1981; Thomas *et al.*, 1982; Duncan and McConkey, 1982; Burkhard and Traugh, 1983). Although S6 phosphorylation has been studied in many systems, the mechanism responsible for increased phosphorylation has not yet been elucidated. In this paper insulin is shown to activate rapidly and reversibly an S6 protein kinase activity in *Xenopus* oocytes. In addition, S6 kinase activity is increased following microinjection of highly purified insulin-receptor kinase into oocytes. The activated form of the S6 kinase has been partially purified by anion exchange chromatography.

Results

Previous studies in this laboratory have shown that meiotic maturation of *Xenopus* oocytes induced by insulin is associated with increased phosphorylation of ribosomal protein S6 (Stith and Maller, 1984). To investigate whether this increase might be a reflection of increased S6 kinase activity, groups of 30 oocytes were treated with 1 μ M insulin for various periods of time and then homogenized under conditions that have been shown previously to preserve S6 kinase activity in *Xenopus* unfertilized eggs (Erikson and Maller, 1985), as described under Materials and methods. Figure 1A shows that S6 protein kinase-specific activity was rapidly increased ~3-fold, reaching a maximum level within 20 min after insulin addition. In the continued presence of insulin, the S6 kinase specific activity remained elevated, but following removal of insulin, activity declined to near basal values within 10 min (Figure 1A). S6 kinase activity was not inhibited by inclusion of the heat-stable inhibitor of cAMP-dependent protein kinase in the assay. Dose-response curves comparing S6 protein kinase specific activity 30 min after exposure to various concentrations of insulin revealed an increase

Fig. 1. S6 kinase activity in insulin-treated oocytes. (A) Time course of activation of S6 kinase activity. Groups of 30 oocytes were treated for various times with 1 μ M insulin, then homogenized, dialysed and assayed for S6 kinase activity as described under Materials and methods (●—●). Prior to homogenization, one group exposed to insulin for 60 min was washed free of insulin for 10 min prior to homogenization (Δ). (B) Dose-dependence of insulin-stimulated S6 kinase activity. Groups of 30 oocytes were incubated with the indicated concentration of insulin for 30 min, then homogenized, dialysed, and assayed for S6 kinase activity as described under Materials and methods.

Fig. 2. Fractionation of S6 kinase activity by DEAE-Sephacel chromatography. One hundred oocytes were incubated with or without 1 μ M insulin for 30 min, followed by homogenization, dialysis, and chromatography as described under Materials and methods. (A) Control oocytes; (B) insulin-treated oocytes. ○—○, S6 kinase activity; ---, NaCl concentration; —, A₂₇₈.

at subnanomolar concentrations, with a maximal effect at 100 nM (Figure 1B). This concentration is similar to that required for insulin-induced S6 phosphorylation in the intact oocytes (Stith and Maller, 1984). An important question concerns whether the increase in S6 kinase activity is due to activation or synthesis. The rapidity of the increase (Figure 1) is most consistent with

Fig. 3. S6 kinase activity following microinjection of insulin-receptor kinase. For each time point each of 25 oocytes was injected with 50 nl of receptor kinase buffer (40 mM imidazole pH 7.2, 10% glycerol, 200 mM NaCl, 0.5% Triton-X-100) containing 100 nM insulin, 12 mM MgCl₂, 2 mM MnCl₂, 100 μ M ATP, without (control) or with 100 μ g/ml insulin receptor kinase, affinity-purified as described under Materials and methods. In other experiments control injections also included boiled receptor kinase preparations. At various times, oocytes were homogenized, dialysed and assayed for S6 kinase activity. In the data shown, activity at each time point has been corrected for the activity seen in control injected oocytes. The ordinate thus represents the increased S6 kinase specific activity due to injected insulin receptors.

an activation mechanism. To investigate this question further, oocytes treated with 0.1 mM puromycin for 50 min were exposed to insulin and S6 kinase activity was determined. As reported by several laboratories, protein synthesis inhibition by itself led to an increase in S6 phosphorylation (Gressner and Wool, 1974; Lastick and McConkey, 1980), and lysates from puromycin-treated oocytes exhibited increased S6 kinase activity (data not shown). However, this increase was additive with that of insulin, indicating an activation mechanism accounts for the ability of insulin to increase S6 kinase activity.

To determine whether the increase in S6 kinase specific activity could be accounted for by a change in one or more specific enzymes, lysates from insulin-treated oocytes were subjected to DEAE-Sephacel chromatography. As shown in Figure 2, two peaks of S6 protein kinase were resolved, and the peak eluting at 300 mM NaCl was elevated in insulin-treated oocytes. Specific activity measurements on the pooled peaks from control and hormone-treated oocytes showed an insulin-dependent increase of 3-fold in 30 min. This chromatographic behavior suggests that insulin causes a stable modification of the enzyme.

Activation by insulin-receptor kinase

As described in the Introduction, it has been suggested that the insulin-receptor kinase might activate metabolic processes by phosphorylating protein substrates on tyrosine residues. One approach towards testing this hypothesis would be to show the ability of microinjected receptor to cause an insulin-like response. As a step in this direction, human placental insulin receptors purified to near homogeneity were microinjected into oocytes, and the S6 kinase activity was determined. As shown in Figure 3, injection of insulin receptors caused an ~2-fold increase in total S6 kinase activity in oocyte lysates. The increase was maximal within 10 min and activity returned to basal levels by 30 min. Data are expressed relative to controls injected with insulin alone in the receptor kinase buffer, or to injection of boiled receptor preparations. In several experiments of this type the increase in specific activity ranged from 40 to 200%, and the rate of increase was faster than that observed following external insulin administration (Figure 1). Upon DEAE-Sephacel chrom-

Fig. 4. DEAE-Sephacel chromatography of S6 kinase activity in receptor kinase-injected oocytes. One hundred oocytes were injected either with or without insulin receptor kinase as described in Figure 3, and after 10 min oocytes were homogenized, dialysed and subjected to DEAE-Sephacel chromatography as described in the legend to Figure 2. (A) Control injected oocytes; (B) insulin receptor-kinase injected oocytes. $\circ$ — $\circ$, S6 kinase activity; — — —, NaCl; —, A_{278} .

atography of extracts from oocytes injected with insulin receptors, a 2-fold increase in total activity was evident in the peak of S6 kinase eluting at 300 mM NaCl (Figure 4). In several experiments of this type, the increase in specific activity of the pooled peak ranged from 40 to 150%. Although a small increase in specific activity was occasionally seen with the S6 kinase activity eluting at 200 mM NaCl, this increase was not consistent from experiment to experiment.

Discussion

The results presented in this paper show that insulin caused rapid activation of an S6 protein kinase in *Xenopus* oocytes. The activation was fully reversible and continued exposure of the oocyte to insulin was required to maintain the elevated level of S6 kinase activity. The concentration of insulin required to activate the enzyme in oocytes was similar to the concentration needed to increase S6 phosphorylation, supporting the possibility that this enzyme accounts for insulin-induced S6 phosphorylation *in vivo*. Preliminary experiments indicate that the insulin-stimulated enzyme does not phosphorylate histones, casein or phosvitin. This suggests that the enzyme is not related to protease-activated kinase II or other broad specificity protein kinases reported to phosphorylate S6 *in vitro* (Perisic and Traugh, 1983; Cobb and Rosen, 1983; Donahue and Masaracchia, 1984; Parker *et al.*, 1985). Although the mechanism of activation of the oocyte enzyme is not clear, the activated form was clearly stable through anion exchange chromatography. The same peak of S6 kinase activity on DEAE-Sephacel was activated following injection of purified insulin receptors into oocytes. This result demonstrates the activation of a specific enzyme *in vivo* in response to an exogenous protein-tyrosine kinase. This activation was more rapid than with external insulin. However, the extent of activation of the S6 kinase by injected receptor was not as great as with external insulin, and the activation was transient, with activity returning

to basal levels within 30 min (Figure 3). This could be a consequence of the inactivation of the receptor after injection, since even with external insulin, removal of the hormone leads to a rapid loss of elevated S6 kinase activity (Figure 1). Nevertheless, the fact that the insulin receptor did elicit a response after injection supports the hypothesis that transduction of the hormone signal by the insulin receptor can occur when the receptor is at an intracellular location. This suggests that internalized insulin receptor itself could act as a 'messenger' for insulin-induced S6 phosphorylation. The exact location at which the receptor kinase acts has not yet been identified, but could involve the inner surface of the plasma membrane as well as other possible locations. Under our current experimental conditions, incubation of purified insulin receptors with the partially purified S6 kinase and MgATP does not appear to lead to activation of S6 phosphorylation (data not shown), supporting the idea of one or more intermediates between insulin receptor kinase and the S6 kinase. Further work is needed to determine whether or not the protein-tyrosine kinase activity of the insulin receptor accounts for its ability to activate the S6 kinase, but injection of oocytes should facilitate analysis of how insulin causes an increase in S6 phosphorylation.

It is becoming clear that analysis of how observed increases in serine phosphorylation in insulin-treated or virally transformed cells occur is an important element in defining the mechanism of action of protein-tyrosine kinases. Recent studies demonstrate that the protein-tyrosine kinase from Rous sarcoma virus, pp60^{v-src}, phosphorylates and inactivates a protein-serine phosphatase *in vitro* (Johansen and Ingebritsen, 1986). This suggests that protein-tyrosine kinases may affect serine phosphorylation by altering both protein kinases and protein phosphatases. Other studies in this laboratory have shown that microinjection of the protein-tyrosine kinases associated with Rous sarcoma virus and Abelson murine leukemia virus also leads to increased S6 phosphorylation in oocytes (Spivack *et al.*, 1984; Maller *et al.*, 1985). It will be of interest to determine whether these protein-tyrosine kinases also act, at least in part, through activation of the protein-serine kinase shown in this report to be stimulated by insulin and by insulin-receptor kinase.

Materials and methods

β -Glycerophosphate was from US Biochemical Corp. and pregnant mare's serum gonadotropin was from Calbiochem. Porcine zinc insulin was a gift of Eli Lilly Co. and DEAE-Sephacel was from Pharmacia. Female *X. laevis* were from the South African Snake Farm and were primed 3 days before use with 35 IU of pregnant mare's serum gonadotropin. Isolation of oocytes and preparation of 40S ribosomal subunits was carried out as described previously (Maller, 1983; Erikson and Maller, 1985). [γ -³²P]ATP was synthesized by the method of Johnson and Walseth (1979) and protein was measured by the Bradford procedure (1976). The heat-stable inhibitor of cAMP-dependent protein kinase was purified as described by Whitehouse and Walsh (1983).

Preparation of oocyte lysates

All operations were carried out at 4°C. 20–30 oocytes were homogenized in 3 volumes of 55 mM β -glycerophosphate (pH 6.8), 5 mM MgCl₂, 5 mM EGTA, 50 mM NaF, 100 mM Na₂P₂O₇, 5 mM phosphotyrosine, 2 mM dithiothreitol (DTT), 100 μ M phenylmethylsulfonyl fluoride (PMSF), 100 μ M Na orthovanadate and 1 μ g leupeptin per ml. The homogenate was centrifuged at 37 000 *g* for 30 min, the lipid layer removed, and the supernatant dialysed for 6 h against 55 mM β -glycerophosphate, 5 mM MgCl₂, 5 mM EGTA, 2 mM DTT and 100 μ M PMSF.

DEAE-Sephacel chromatography

The dialysed supernatant from 100 oocytes was diluted to a conductivity of 1.9 mOhm with distilled water and applied to a DEAE-Sephacel column (1 $\times$ 3 cm) equilibrated in 20 mM Tris-Cl, pH 7.0, 5 mM MgCl₂, 2 mM EGTA, 25 mM NaCl, 2 mM DTT. The column was washed with the same buffer and proteins were eluted with a 60 ml linear gradient to 600 mM NaCl in the same buffer.

S6 protein kinase activity

Reactions were incubated for 30 min at 30°C in a total volume of 30 µl containing 20 mM Hepes, pH 7.0, 5 mM 2-mercaptoethanol, 5 mM MgCl₂, 100 µM ATP containing 1×10^7 c.p.m. of [γ -³²P]ATP, 100 µg bovine serum albumin/ml, 19 µg of 40S ribosomal subunits, 3 µg of the heat-stable inhibitor of cAMP-dependent protein kinase and an appropriate amount of enzyme. Reactions were terminated by addition of 1/4 volume of 5 × electrophoresis sample buffer (1 × buffer: 70 mM Tris-Cl, pH 6.8, 11% glycerol, 3% SDS, 0.01% bromophenol blue, 5% mercaptoethanol) followed by incubation at 95°C for 3 min. The reaction mixture was electrophoresed through 12.5% gels by the Laemmli (1970) procedure. The gels were stained with Coomassie blue, destained, dried, exposed to Kodak XRP film and radioactivity in S6 was quantified by liquid scintillation spectrometry of the excised band. This protein has been demonstrated previously by this laboratory to be S6, based on migration in a two-dimensional gel system designed for basic ribosomal proteins and by reaction with a monoclonal antibody against chicken liver S6 (Nielsen et al., 1982). All data have been corrected for radioactivity in the S6 region in parallel assays conducted without addition of 40S subunits.

Purification of insulin-receptor kinase

Membranes from a fresh human placenta were prepared and detergent-solubilized proteins chromatographed on wheat germ agglutinin agarose as described previously (Pike et al., 1984). For affinity purification, the peak eluted from this column was adsorbed to and eluted from insulin-Sepharose using the procedure described by Pike et al. (1986). The purified receptor was concentrated by adsorption onto a small column of DEAE-cellulose and eluted with a small volume of 40 mM imidazole, pH 7.2, 10% glycerol, 250 mM NaCl, 0.5% (w/v) Triton X-100. The receptor had a specific activity for insulin binding of 3850 pmol/mg and for phosphorylation of 300 nmol/min/mg, using as substrate a synthetic peptide corresponding to the autophosphorylation site of pp60^{v-src} (Pike et al., 1984).

Prior to injection, the receptor was incubated at room temperature for 15 min with 100 nM insulin, 12 mM MgCl₂, 2 mM MnCl₂ and 100 µM ATP. This procedure activated the receptor kinase as judged by autophosphorylation. The activated receptor was kept on ice prior to loading of the microinjection needle.

Acknowledgements

We thank Jan Kyes for help in dissecting oocytes. This work was supported by grants from the NIH (AM 28353), the American Cancer Society (NP-517A) and by the Howard Hughes Medical Institute. JLM is an Established Investigator of the American Heart Association.

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Received on 8 October 1985; revised on 25 November 1985