Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5514–5518, July 1990
Cell Biology

Microinjection of a protein-tyrosine-phosphatase inhibits insulin action in *Xenopus* oocytes

(signal transduction/S6 kinase/phosphorylation/ribosomal protein S6)

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Contributed by Edwin G. Krebs, May 7, 1990

ABSTRACT **A protein-tyrosine-phosphatase (PTPase 1B; protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48), specific for phosphotyrosyl residues, was microinjected into *Xenopus* oocytes. This resulted in a 3- to 5-fold increase in PTPase activity over endogenous levels. The PTPase blocked the insulin-stimulated phosphorylation of tyrosyl residues on endogenous proteins, including a protein having a molecular mass in the same range as the β subunit of the insulin or insulin-like growth factor I receptor. PTPase 1B also blocked the activation of an S6 peptide kinase—i.e., an enzyme recognizing a peptide having the sequence RRLSSLRA found in a segment of ribosomal protein S6 and known to be activated early in response to insulin. On the other hand, the insulin stimulation of an S6 kinase, detected by using 40S ribosomes as substrate, was unaffected even though PTPase 1B partially prevented the phosphorylation of ribosomal protein S6 *in vivo*. Mono Q chromatography of insulin-treated oocyte extracts revealed two main peaks of S6 kinase activity. Fractions from the first peak displayed S6 peptide kinase activity that was essentially abolished in profiles from PTPase 1B-injected oocytes. Material from the second peak, which was best revealed by using 40S ribosomes as substrate and had comparatively little S6 peptide kinase activity, was minimally affected by PTPase 1B. These observations suggest that at least two distinct "S6 kinases" are involved in ribosomal protein S6 phosphorylation *in vivo* and that the activation pathways for these enzymes differ in their sensitivity to PTPase 1B.**

Since the finding that insulin can induce meiotic cell division in *Xenopus* oocytes (1, 2), several additional responses to the hormone have been observed. These fall into two categories. One set of responses occurs after stimulation of oocytes with either progesterone or insulin and includes increases in internal pH (3, 4), protein synthesis (5, 6), protein kinase activities (7, 8), and protein phosphorylation (3, 9, 10). These changes occur typically 4–6 hr after insulin addition—i.e., just before germinal vesicle breakdown. In contrast, other responses are observed much sooner after hormone addition (<1 hr) and are specific for insulin. These include early increases in S6 peptide kinase (11) and ribosomal protein S6 kinase activities measured *in vitro* (7) as well as phosphorylation of S6 protein *in vivo* (7) and an increase in glucose uptake (12). Studies involving the microinjection of the insulin receptor β subunit (13) and antibodies to the insulin receptor (14) into oocytes support the notion that the early and late events are both dependent on tyrosine phosphorylation by the insulin receptor. In other systems, studies involving mutagenesis of the receptor (15–19) and insulinomimetic or inhibitory antibodies (20, 21) also suggest that insulin-stimulated events depend on the activation of the receptor kinase. On the other hand, there are indications that this kinase activity may not be required for all insulin-induced events (22–27).

Recently, a 35-kDa protein-tyrosine-phosphatase (PTPase 1B; protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) was purified to homogeneity from human placenta (28). This enzyme was shown to dephosphorylate the insulin receptor *in vitro* (29) and, therefore, was regarded as a potential tool for studying the role of tyrosine phosphorylation in insulin action *in vivo*. In a separate report, we examined the effect of PTPase 1B on late events occurring during insulin-induced meiotic cell division such as germinal vesicle breakdown (30). The present investigation addresses the question of whether microinjection of PTPase 1B will antagonize early events triggered by insulin, such as the activation of ribosomal protein S6 kinase(s) and the phosphorylation of ribosomal protein S6 *in vivo*.

MATERIALS AND METHODS

Procurement of Oocytes. Large adult *Xenopus laevis* females were purchased from Xenopus I (Ann Arbor, MI) and stage VI oocytes were obtained as described (8). The oocytes were incubated in modified OR-2 medium (lacking potassium) to ensure their response to insulin (11).

Microinjection of PTPase 1B. For microinjection, PTPase 1B was prepared (28) and concentrated (30). Oocytes were routinely injected with 20 nl of PTPase 1B per oocyte (0.6 mg/ml), or with buffer M [15 mM Mops, pH 7/0.38 mM EDTA/0.075% 2-mercaptoethanol/0.25 M NaCl/25% (vol/vol) glycerol] as a control, and preincubated in modified OR-2 for 2 hr to allow time for the PTPase to diffuse throughout the cell (30).

Measurement of PTPase Activity. Five PTPase 1B-injected or noninjected oocytes were homogenized in 200 μl of ice-cold buffer H (20 mM imidazole, pH 7.2/0.5 mM EDTA/0.1% 2-mercaptoethanol/0.002% phenylmethylsulfonyl fluoride/1 mM benzamidine). The homogenates were assayed as described (28), using phosphorylated, reduced carboxamidomethylated, and maleylated (RCM) lysozyme ([^{32}P]tyrosine; 5 μM) as substrate.

To measure PTPase activity *in vivo*, one of two groups of oocytes received an injection of PTPase 1B. After 2 hr, sets of five oocytes (in triplicate) from both groups were injected with 20 nl of ^{32}P-labeled reduced carboxamidomethylated and maleylated lysozyme (1.53 pmol of [^{32}P]phosphotyrosine; 0.26 mol of ^{32}P per mol of protein) per oocyte. This amount of substrate when diluted in the oocyte cytosol is still well above the K_m value (29). At various time points, oocytes were

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Abbreviations: PTPase, protein-tyrosine-phosphatase; IGF-I, insulin-like growth factor I.

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homogenized in 0.5 ml of 5% trichloroacetic acid (10 s was the earliest that oocytes could be processed after injection). The samples were then centrifuged at 12,000 × *g* for 5 min and the supernatants were assayed for ^{32}P released.

Chromatography of Oocyte Extracts on FPLC Mono Q Columns. Groups of 250 oocytes were homogenized on ice in 0.5 ml of buffer E {80 mM β-glycerophosphate/20 mM EGTA/15 mM $MgCl_2$/1 mM dithiothreitol/50 μM adenosine 5'-[γ-thio]triphosphate/lima bean trypsin inhibitor (100 μg/ml)/1 mM benzamidine, pH 7.3} and centrifuged for 10 min at 140,000 × *g* (2°C). The centrifuge tubes were sliced to remove the top (lipid) layer and the clear supernatant layer was then removed and diluted 1:10 with 10% buffer E immediately before loading onto a FPLC Mono Q column equilibrated in 10% buffer E. Processing of each group of oocytes, starting with defolliculation, was staggered by ≈2 hr to allow time for the chromatography step. The diluted sample (5 ml) was loaded on the Mono Q column (HR 5/5, 5 × 50 mm; Pharmacia); the column was washed with 10 ml of 10% buffer E and eluted with a 40-ml linear salt gradient from 0 to 0.5 M NaCl in 10% buffer E. Eighty 0.5-ml fractions were collected, divided into aliquots, and stored at −70°C until assay. The column was washed with 7.5 ml of 1 M NaCl and reequilibrated in 10% buffer E for the next run. The frozen column fractions were thawed and immediately assayed for S6 peptide and S6 kinase activities as described (8).

Analysis of Phosphorylated Amino Acids. Excised gel samples were swollen and homogenized in 1 ml of 50 mM NH_4HCO_3, 200 μl of trypsin (1 mg/ml) was added, and the samples were incubated overnight at 37°C. After an additional 8 hr of incubation with 5 ml of 50 mM NH_4HCO_3, the samples were centrifuged at 2000 × *g*, the gel pellets were washed overnight with an additional 3 ml of NH_4HCO_3 and centrifuged, and the combined supernatants (85% recovery of radioactivity) were evaporated repeatedly after readdition of H_2O to remove residual NH_4HCO_3. Acid hydrolysis and TLC were performed as described (31). TLC plates (10 × 10 cm, precoated cellulose from EM reagents) were subjected to electrophoresis in the first dimension (pH 1.9) at 1000 V for 50 min. After thorough drying, electrophoresis was performed in the second dimension (pH 3.5) at 1000 V for 20 min. The plates were dried, sprayed with 0.2% ninhydrin in acetone, heated at 60°C for 5 min, and exposed to Kodak X-Omat AR film for 60 hr with DuPont Cronex Lightning Plus intensifying screens at −70°C.

RESULTS

Augmentation of Endogenous PTPase Activity. Homogenates from *Xenopus* oocytes displayed an endogenous PTPase activity of 20 ± 7 (SD; $n = 7$) pmol of phosphate released per min per oocyte. After microinjection of PTPase 1B, the average level increased to 86 ± 36 (SD; $n = 7$) pmol per min per oocyte, representing a 3- to 5-fold increase over the endogenous levels. Since 70 pmol of activity per min was introduced per oocyte, the activity measured in homogenates appeared to reflect accurately the additional amount of enzyme provided. The enhanced level of activity remained stable for >18 hr. Microinjection of buffer alone had no effect on endogenous PTPase activity.

Because the activity measured in a homogenate might not reflect that present in the cell, attempts were made to obtain an indication of PTPase activity *in vivo*. To do this, the substrate, ^{32}P-labeled reduced carboxamidomethylated and maleylated lysozyme, was microinjected into oocytes. In control cells, the substrate was dephosphorylated at a rate of 1.3 pmol per min per oocyte with no evidence for proteolysis as assessed by SDS/polyacrylamide gel electrophoresis. This rate is low because of the limited amount of time (10 s) allowed for diffusion of the substrate during the linear phase of the reaction. Thus, only a fraction (≈1/20th) of the total oocyte cytoplasm was assayed. In oocytes that were preinjected with PTPase 1B, the activity increased 3-fold to at least 4.3 pmol per min per oocyte, representing approximately the same relative increase as seen in homogenates.

PTPase 1B Blocks Protein Tyrosine Phosphorylation Induced by Insulin. To determine whether the injected PTPase could affect the level of endogenous protein tyrosine phosphorylation in response to insulin, extracts from ^{32}P-labeled cells were subjected to SDS/polyacrylamide gel electrophoresis. Fig. 1*A* shows the insulin-dependent phosphorylation of a 100-kDa protein. No stimulated phosphorylation was detected in insulin-treated oocytes injected with PTPase 1B. When the gels were treated with alkali to enhance detection of proteins phosphorylated on tyrosyl residues (Fig. 1*B*), the 100-kDa band became particularly prominent and exhibited a 2- to 3-fold increase in phosphorylation in response to insulin (quan-

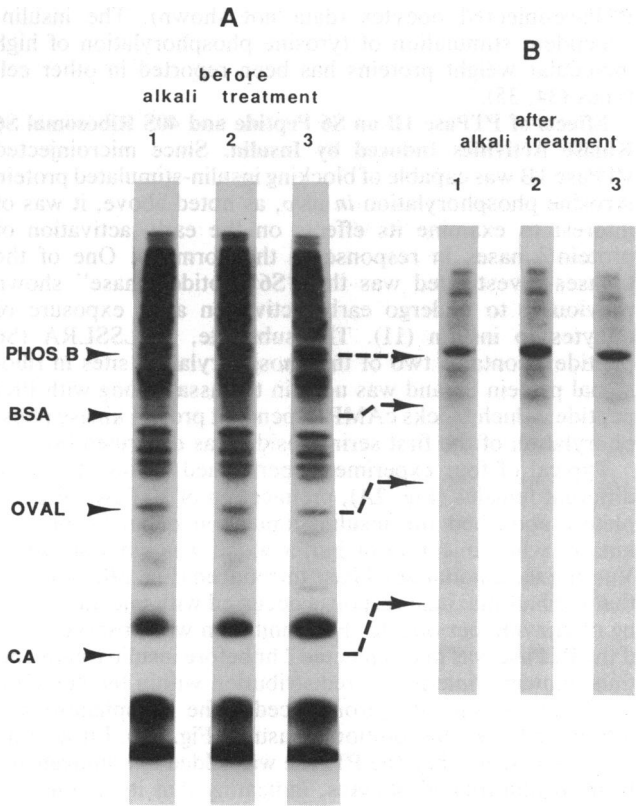

FIG. 1. PTPase blocks insulin-stimulated protein tyrosine phosphorylation. (*A*) Before alkali treatment. Oocytes were injected with buffer M (lanes 1 and 2) or with PTPase 1B (lane 3) and then with $^{32}P_i$ as in Fig. 3*B*. After exposure to 10 μM insulin (lanes 2 and 3) for 40 min, batches of 20 oocytes per lane were homogenized in 0.4 ml of buffer E on ice and centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was removed (350 μl) and added to 90 μl of 5× concentrated SDS sample buffer (32), the samples were placed in a boiling water bath for 5 min, and 70 μl (equivalent to 1.8 oocytes) per lane was loaded in triplicate on a 10% polyacrylamide gel. After electrophoresis, proteins were stained with Coomassie blue and then destained, and the dried gel was exposed to Kodak X-Omat AR film for 2 hr with DuPont Cronex Lightning Plus intensifying screens at room temperature. Molecular size standards were as follows: phosphorylase b (PHOS B) (97 kDa), bovine serum albumin (BSA) (66 kDa), ovalbumin (OVAL) (43 kDa), and carbonic anhydrase (CA) (31 kDa) (Bio-Rad). Lanes: 1, control (buffer injected); 2, insulin treated (buffer injected); 3, insulin treated (PTPase injected). For simplicity, only one of each triplicate gel lane is shown. Similar results were observed 10, 20, and 60 min after insulin treatment. (*B*) After alkali treatment. After autoradiography, the gel in *A* was treated with KOH as described (33), dried, and exposed to x-ray film as described above, for 16 hr at room temperature.

titated by Cerenkov counting); this increase was blocked by injection of the PTPase. Control experiments involving microinjection of the PTPase into the homogenization buffer rather than oocytes indicated that PTPase 1B acted *in vivo*, not after the cells had been disrupted (data not shown). Analysis of the material in the 100-kDa band excised from the alkali-treated gels demonstrated that phosphorylation was on tyrosyl residues (data not shown). As indicated in the *Discussion*, we suggest that this band may represent the oocyte insulin or insulin-like growth factor I (IGF-I) receptor β subunit.

In a slightly different experiment from that illustrated in Fig. 1, a microsomal fraction obtained by subjecting the 12,000 × *g* supernatant from the oocyte homogenate to centrifugation at 100,000 × *g* for 1 hr was examined. The pellet, in addition to revealing material migrating at a size expected for the insulin or IGF-I receptor, showed the presence of a labeled band at 160 kDa, the insulin-stimulated tyrosine phosphorylation of which was blocked in the PTPase-injected oocytes (data not shown). The insulin-dependent stimulation of tyrosine phosphorylation of high molecular weight proteins has been reported in other cell types (34, 35).

Effects of PTPase 1B on S6 Peptide and 40S Ribosomal S6 Kinase Activities Induced by Insulin. Since microinjected PTPase 1B was capable of blocking insulin-stimulated protein tyrosine phosphorylation *in vivo*, as noted above, it was of interest to examine its effects on the early activation of protein kinases in response to the hormone. One of the kinases investigated was the "S6 peptide kinase" shown previously to undergo early activation after exposure of oocytes to insulin (11). The substrate, RRLSSLRA (S6 peptide), contains two of the phosphorylation sites in ribosomal protein S6 and was used in the assay along with PKI peptide, which blocks cAMP-dependent protein kinase phosphorylation of the first serine residue [as described (8)].

Typical of four experiments performed on oocytes from different females (Fig. 2*A*), preinjection of PTPase 1B completely abolished the insulin-stimulation of an S6 peptide kinase, while injection of buffer alone was without effect. Varying the amount of PTPase introduced (Fig. 2*B*) indicated that the half-maximal response occurred with injection of ≈3 ng of enzyme per oocyte. Full inhibition was observed only if the PTPase was microinjected 2 hr before insulin treatment, thus allowing time for its redistribution within the cell (30); inhibition became less pronounced if the phosphatase was introduced after the addition of insulin (Fig. 2*C*). Little or no effect was seen when the PTPase was added to homogenates from insulin-treated oocytes, indicating that its action was intracellular.

In addition to insulin-stimulated enhancement of protein kinase activity directed toward S6 peptide, it is known that the treatment of oocytes with insulin also increases the S6 kinase activity that is seen when 40S ribosomes are used as substrate (7). It was of interest to determine whether this latter activity was also affected by microinjection of PTPase 1B. Surprisingly, as exemplified in Fig. 3*A*, this was not the case. At the highest concentration of PTPase 1B available, there was little or no effect on the activation of S6 kinase activity.

Insulin is known to stimulate the early phosphorylation of ribosomal protein S6 in oocytes (7, 13) and, as anticipated, this also occurred in the present study (Fig. 3*B*). In cells microinjected with PTPase 1B partial inhibition of this effect was seen (Fig. 3*B*), but it was not possible to completely block S6 phosphorylation *in vivo* with the PTPase.

Chromatography of Oocyte Extracts on FPLC Mono Q Columns. One possible explanation for the results presented above would be that two or more S6 kinases are involved and that the pathways leading to the regulation of these kinases differ in their sensitivity to PTPase 1B. Furthermore, it could be assumed that at least one of the insulin-stimulated S6

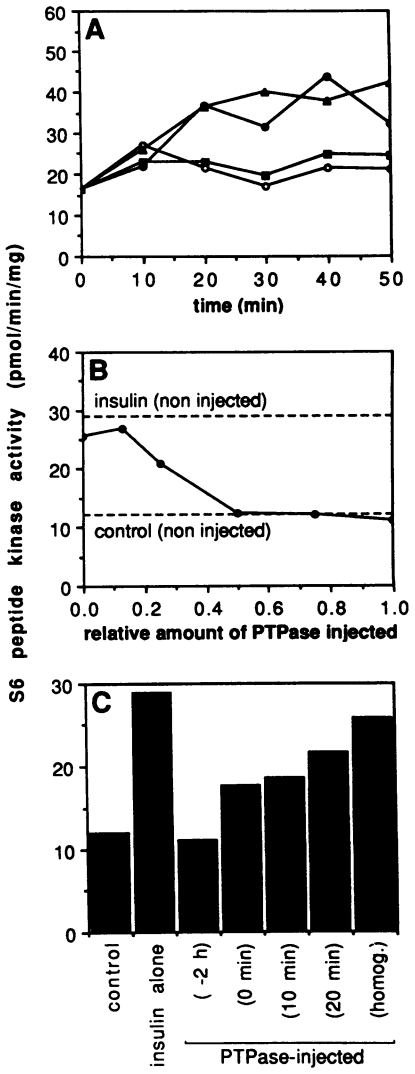

FIG. 2. PTPase effect on insulin-stimulated S6 peptide kinase. (*A*) Time course. PTPase-injected, buffer-injected, and noninjected oocytes (see *Materials and Methods*) were incubated for 2 hr and then exposed to 10 μM insulin or a control solution lacking insulin for various lengths of time. Ten oocytes per time point were homogenized in 200 μL of assay buffer (50 mM β-glycerophosphate, pH 7.3/7 mM NaF/0.3 mM EDTA/15 mM $MgCl_2$/2 mM dithiothreitol) on ice and centrifuged at 150,000 × *g* for 20 min (4°C); the clear supernatant was stored in aliquots at −70°C until assay. The extracts were assayed as described with S6 peptide (RRLSSLRA) used as substrate in the presence of PKI peptide (8). ○, Control (noninjected); ●, insulin-treated (noninjected); ▲, insulin treated (buffer injected); ■, insulin treated (PTPase injected). (*B*) Dose–response. Various dilutions of PTPase 1B in buffer M were injected. After 2 hr, 10 μM insulin was added for 30 min, and the oocytes were homogenized, processed, and assayed for S6 peptide kinase activity as in *A*. (*C*) Sensitive period. PTPase 1B was microinjected at various times relative to stimulation by 10 μM insulin (0 min). Thirty minutes after insulin addition, the oocytes were homogenized, processed, and assayed as in *A*. In the last group, PTPase was added directly to the homogenate 30 min after insulin addition.

kinases is active on the S6 peptide and that its activity is abolished by PTPase 1B. To test this hypothesis, extracts from control and PTPase-injected oocytes, after insulin treatment, were applied to an FPLC Mono Q column. As shown in Fig. 4*A*, the major peak of insulin-stimulated S6 peptide kinase eluted at ≈0.23 M salt. This stimulated activity was not seen in the profile from the cells microinjected with PTPase 1B (Fig. 4*A*). When fractions from the same elution profile shown in Fig. 4*A* were analyzed by using rat 40S

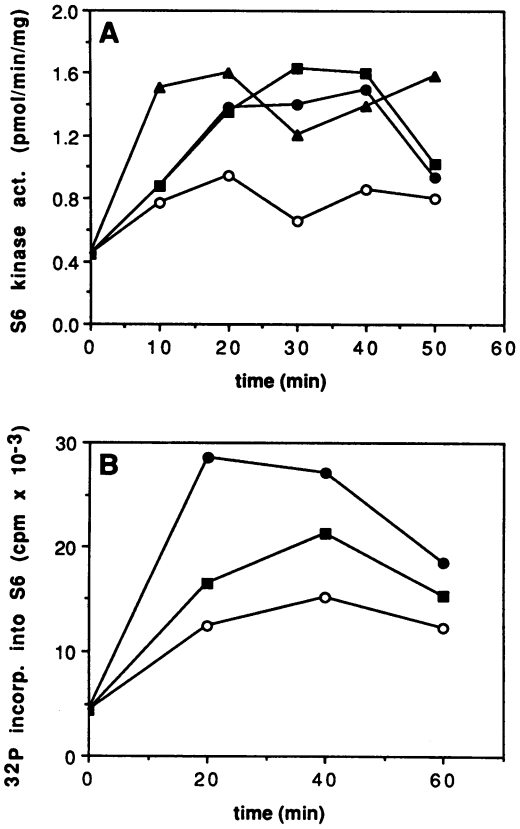

FIG. 3. (*A*) PTPase effect on insulin-induced S6 kinase activity. Extracts as described in Fig. 2 were assayed for S6 kinase activity by using rat 40S ribosomal subunits as substrate (8). The kinase reactions were terminated by the addition of 5× SDS sample buffer (32) and subjected to electrophoresis on 12.5% polyacrylamide gels. The Coomassie blue-stained bands representing ribosomal protein S6 were excised and counted. The data are representative of two such experiments performed. ○, Control (noninjected); ●, insulin treated (noninjected); ▲, insulin treated (buffer injected); ■, insulin treated (PTPase injected). (*B*) PTPase inhibition of S6 phosphorylation *in vivo*. Oocytes were either not injected or were microinjected with PTPase 1B and then microinjected with 20 nl of $^{32}P_i$ (600 mCi per ml of H_2O; 1 Ci = 37 GBq; ICN) per oocyte. After 2 hr allowed for equilibration (30, 36), the oocytes were exposed to 10 μM insulin. At each time point after insulin addition, ribosomal protein S6 was isolated from 20 oocytes and counted as described (37). The data are representative of three such experiments performed. ○, Control (noninjected); ●, insulin treated (noninjected); ■, insulin treated (PTPase injected).

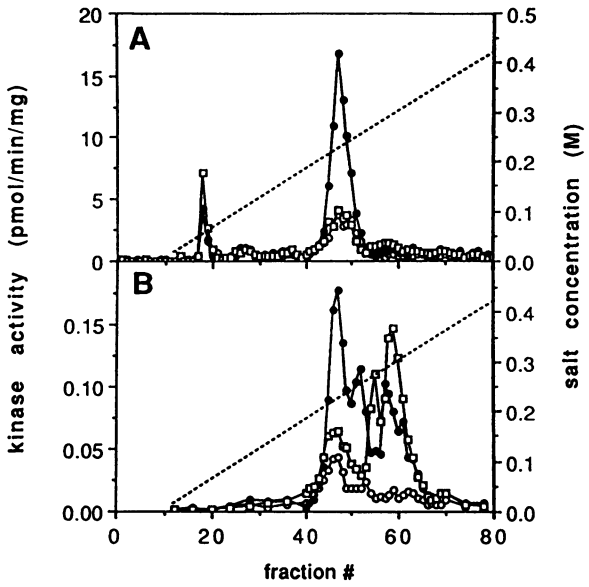

FIG. 4. Mono Q column profiles of S6 and S6 peptide kinase activities. Oocytes were microinjected with buffer M or PTPase 1B, as in Fig. 3, and then exposed to 10 μM insulin for 40 min. Extracts were chromatographed on an FPLC Mono Q column and assayed for S6 peptide (*A*) and 40S ribosomal protein S6 kinase (*B*) activities as described (8). Since the salt gradients for each column profile were essentially the same, a dotted line representing the average salt gradient for each experiment is shown. ○, Control (buffer injected); ●, insulin treated (buffer injected); □, insulin treated (PTPase injected).

ribosomal subunits as substrate (Fig. 4*B*), a stimulated peak of activity again eluted at ≈0.23 M salt, but, in addition, a few peaks of insulin-stimulated kinase activity were found to elute at higher salt concentrations. While the stimulated peak of activity eluting at 0.23 M salt was again essentially abolished by the PTPase, the peak(s) eluting at higher salt concentrations was not reduced to control levels in PTPase 1B-injected oocytes. Similar effects were also observed after 20 min of insulin stimulation (data not shown).

DISCUSSION

Microinjection of PTPase 1B into *Xenopus* oocytes elevated PTPase activity to a level that accurately reflected the sum of the endogenous and the injected phosphatase. The enhancement of PTPase activity *in vivo* was sufficient to counteract the insulin-stimulated phosphorylation of an oocyte protein of 100 kDa. It is likely that this protein represents the β subunit of the frog insulin or IGF-I receptor (2) for the following reasons: (*i*) it is the major protein phosphorylated on tyrosyl residues; (*ii*) its phosphorylation is stimulated by insulin; (*iii*) its apparent size is approximately that of the mammalian receptor; and (*iv*) it is a substrate of PTPase 1B as shown for the insulin receptor *in vitro* (29). It has been suggested that insulin acts through IGF-I receptors in the oocyte (2, 12) and, interestingly, the β subunit of the IGF-I receptor from NRK cells has been tentatively identified as a 100-kDa protein (34). Attempts to provide more definitive identification of the 100-kDa band via immunoprecipitation and immunoblotting with anti-insulin or anti-IGF-I receptor antibodies were unsuccessful.

Reduction of insulin-stimulated endogenous protein tyrosine phosphorylation correlated with the abolition of the insulin-induced S6 peptide kinase activity. When subjected to Mono Q chromatography, S6 peptide kinase eluted at the same salt concentration (0.23 M) as reported for S6 kinase II (38). Since antibodies to ribosomal S6 kinase II, a member of the *rsk* gene family (39), immunoprecipitate the oocyte S6 kinase that is stimulated within 10 min by insulin (40) and this enzyme is known to phosphorylate the S6 peptide, RRLSSLRA (41), it would appear that the PTPase 1B-sensitive S6 peptide kinase represents S6 kinase II. When fractions were assayed with 40S ribosomes as substrate, not only was this activity detected, but a second peak(s) of activity eluting at ≈0.3 M salt was also observed; yet this material was insensitive to PTPase 1B. It therefore appears that PTPase 1B-sensitive and insensitive pathways of S6 kinase activation exist in oocytes and may account for the partial effect of the microinjected enzyme on the phosphorylation state of endogenous S6 protein.

While it is possible that a single kinase, such as the 90-kDa rsk with its dual catalytic domains (42), may act as a point of convergence of multiple signals and behave, in essence, as two separate kinases, genetic (39), biochemical (38, 43, 44), and immunological evidence (39, 40) indicates that more than one S6 kinase exists. It should be noted that a distinct S6 kinase of 70 kDa has been isolated in homogeneous form from

a number of sources, including 3T3 cells (45), and does not appear to be a proteolytic fragment of the 90-kDa rsk molecule. Whether this enzyme corresponds to the material in the second peak of activity eluting from the Mono Q column remains to be established.

It should be noted that a PTPase 1B-insensitive reaction does not necessarily imply independence of tyrosine phosphorylation; it may simply reflect the substrate specificity of the enzyme or its localization in the cell after microinjection. Our previous data indicated that the injected enzyme is targeted to a location predominantly in the animal hemisphere (30). Upon sucrose density gradient centrifugation, it appeared to be associated with a band of heterogeneous membrane vesicles; it did not localize in nuclear or plasma membranes. This implies that PTPase 1B could become associated with certain internal membrane fractions where its concentrated activity could block specific physiological responses to insulin.

It has been suggested that not all pathways of insulin action are initiated entirely at the level of the plasma membrane but that some might be triggered by internalized receptors (46–48). For example, isolated nuclei have been shown to respond directly to insulin, resulting in an increase in RNA synthesis (49), protein phosphorylation (50), and transport of macromolecules (51, 52). In addition, microinjection of an insulin receptor complex was found to stimulate S6 phosphorylation *in vivo* without recycling to the plasma membrane (13). These studies indicate a number of potential sites other than the plasma membrane at which PTPase 1B could exert its effect. As discussed previously (30), localization of PTPase 1B to an internal membrane site may explain why PTPase 1B does not block insulin-induced germinal vesicle breakdown yet delays maturation by acting on an event downstream of the receptor. Recent evidence has shown that the PTPase represents a truncated form of the 50-kDa human placenta phosphatase having an extension of ≈12 kDa at the C terminus (53, 54). Expression of a close homolog of the placental enzyme (55) in BHK cells indicates that it localizes in a particulate fraction requiring detergents for extraction, while expression of its truncated form, analogous to PTPase 1B, shows that this enzyme can be readily extracted with aqueous buffers of low ionic strength (D. E. Cool, N.K.T., E.H.F., and E.G.K., unpublished data). Thus, full-length PTPases or truncated forms, or receptor-linked PTPases as typified by CD45 (56), may localize to different compartments of the cell, possess different substrate specificities, and block different tyrosine phosphorylation events. Consequently, these enzymes represent potentially a whole spectrum of tools for examining the role of protein tyrosine phosphorylation in cells.

We wish to thank Dr. Ora M. Rosen and Dr. Richard A. Roth for providing us with anti-insulin receptor antibodies. This work was supported, in part, by Public Health Service Grant DK07902 from the National Institutes of Health, National Institute of General Medical Sciences Grant GM42508, and the Muscular Dystrophy Association of America.

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