Biochimica et Biophysica Acta, 971 (1988) 170–178
Elsevier

BBA 12307

Separation of the protein-tyrosine kinase and phosphatidylinositol kinase activities of the human placental insulin receptor §

José M. Carrascosa [a,*], Erwin Schleicher [b], Richard Maier [b], Christa Hackenberg [a] and Otto H. Wieland [a]

[a] *Institut für Diabetesforschung and* [b] *Institut für Klinische Chemie, Krankenhaus München-Schwabing, München (F.R.G.)*

(Received 19 February, 1988)
(Revised manuscript received 17 May, 1988)

Key words: Protein-tyrosine kinase; Antiphosphotyrosine antibody; Immunoprecipitation; Insulin receptor; (Human placenta)

On immunoprecipitation using a specific antiphosphotyrosine antibody, phosphatidylinositol kinase (EC 2.7.1.67) activity was separated from the protein-tyrosine kinase (EC 2.7.1.112) activity of the wheat germ agglutinin (WGA) -purified insulin receptor from human placenta. This clearly indicates that protein-tyrosine kinase and phosphatidylinositol kinase activity do not reside on the same polypeptide chain as previously has been suggested. Quantitatively, the fraction of phosphatidylinositol kinase that was bound to WGA sepharose and eluted together with the insulin receptor amounted to 2% of the Triton X-100 soluble phosphatidylinositol kinase. The apparent K_m values of the bound and unbound phosphatidylinositol kinase with respect to PI and ATP were very similar (0.4 and 0.3 mmol/l and 8 and 7 μmol/l, respectively) suggesting that the WGA-bound phosphatidylinositol kinase is not a different enzyme, but rather represents a small portion of the bulk Triton X-100-soluble phosphatidylinositol kinase that is bound to the lectin tightly associated with the insulin receptor. The synthetic polymer $(Glu^{80}Tyr^{20})_n$, a model substrate of the insulin receptor tyrosine kinase, at 0.5 mmol/l, inhibited phosphatidylinositol kinase of WGA-purified insulin receptor by 70–90%. This inhibition was not overcome by increasing the concentrations of ATP or PI as one would expect if a functional interrelationship of the protein-tyrosine kinase and the phosphatidylinositol kinase would exist.

Introduction

Several protein-tyrosine kinases, such as the viral oncogene products $p60^{v\text{-}src}$ [1] and $p68^{v\text{-}ros}$ [2], a v-*src*-related protein-tyrosine kinase from the electric ray *Narke japonica* [3], the epidermal growth factor (EGF) receptor kinase [4] and the insulin receptor kinase [5,6] have been shown to be associated with lipid kinase activities even after extensive purification. Since viral transformation [1] or exposure of cells to EGF [7] or insulin [8] leads to an increase of polyphosphoinositides and PI turnover in various tissues (cf. however in liver

§ This work is dedicated to Professor Hellmut Mehnert, Chief of the Department of Internal Medicine and Metabolism of the Schwabing City Hospital, Munich, on the occasion of his 60th birthday.

* Present address: Departamento Biologia Molecular, Facultad Ciencias, Universidad Autónoma 28049 Madrid, Spain

Abbreviations: DTT, dithiothreitol; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethylsulfonyl fluoride; IBA, insulin-binding activity; WGA, wheat germ agglutinin; EGF, epidermal growth factor.

Correspondence: O.H. Wieland, Institut für Diabetesforschung, Krankenhaus München-Schwabing, D-800 München (F.R.G.)

[9]) the firm association of phosphatidylinositol kinase activity with the protein-tyrosine kinases appeared to be of special interest. In many cases it was possible to separate lipid kinase and protein-tyrosine kinase from each other [3,4,10,11] ruling out the possibility that the two enzyme activities might reside on a single polypeptide chain. Yet, in the case of the insulin receptor, all attempts to achieve such a separation in this [5] (Machicao, F., unpublished observations) and another laboratory [6] have failed so far. On reinvestigating this problem we have now found that the accompanying phosphatidylinositol kinase can be removed from the insulin receptor without affecting the function of the protein-tyrosine kinase. Part of this work has been published in preliminary form [12].

Materials and Methods

Materials. All chemicals used for preparing buffers were of analytical grade and were purchased from Merck (Darmstadt) or Serva (Heidelberg). The following reagents were purchased from Sigma (München): Anti-rabbit IgG conjugated with peroxidase, bacitracin, leupeptin, $(Glu^{80}Tyr^{20})_n$, histone H_{2B}, keyhole limpet hemocyanin, *N*-acetylglucosamine, tyramine, phosphoserine, phosphotyrosine, *p*-nitrophenylphosphate, protein A, phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, wheat germ agglutinin. Silica gel 60 F_{254} thin-layer plates came from Merck (Darmstadt), CNBr-activated sepharose 4B was from Pharmacia (Freiburg) and [γ-^{32}P]ATP was from New England Nuclear (Dreieich).

Placental membrane preparation. Placental membranes were prepared essentially as described by Siegel et al. [13]. All procedures were carried out at 4°C. The freshly washed tissue (500–1200 g) was homogenized in a Waring Blendor using 5 vol. of a solution consisting of 0.25 M sucrose, 2 mM EDTA, 10 mM Tris-HCl (pH 7.4) and 0.1 mM PMSF (buffer A). The homogenate was filtered through gauze and centrifuged 30 min at $10000 \times g$. The supernatants were collected and made up to 100 mM NaCl and 0.2 mM $MgCl_2$ prior to centrifugation for 45 min at $40000 \times g$. The pellets were resuspended with a solution of 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.1 mM PMSF and 0.1 mM leupeptin (buffer B) using a glass homogenizer, and washed three times with this solution. The washed pellets were resuspended in 30–40 ml of buffer B. The membranes could be stored frozen for several weeks.

Insulin receptor purification. Placental membranes (20 ml) were thawed, made up to 10% glycerol, 0.01% bacitracin and 0.1 mM PMSF and extracted with 2% Triton X-100 by stirring at room temperature for 1 h. Thereafter, 3 vol. of buffer B was added and the extract was centrifuged 50 min at $100000 \times g$. The pellet containing the Triton-insoluble proteins was resuspended in buffer B, and phosphatidylinositol kinase activity was determined immediately. The supernatant obtained after centrifugation of the extracted membranes was mixed with WGA Sepharose prepared according to Porath et al. [14] and rotated end-over-end for 1 h at 4°C. The slurry was then poured into a column (2×8 cm), the proteins not bound to the Sepharose were collected and their phosphatidylinositol kinase activity was assayed. The Sepharose was then washed with 300 ml of a solution of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 10% glycerol and 0.05% Triton X-100. The receptor was eluted from the column using the same buffer fortified with 300 mM *N*-acetylglucosamine. 3 ml fractions were collected, assayed for IBA [15] and those with the highest activity were pooled, concentrated by pressure dialysis, and applied to a Sepharose 6B-Cl column (2×170 cm) equilibrated with 50 mM Tris-HCl (pH 7.4) 0.05% Triton X-100 and 0.1 mM PMSF. Fractions of 6 ml were collected and assayed for protein and IBA. According to the protein elution profile and the distribution of the IBA, two fractions were pooled as indicated in Fig. 1. Fraction I contains as much as possible of the first protein peak with a minimum of IBA contamination. Fraction II includes only the fractions with the highest IBA content. Additional fractions, not shown in Fig. 1, corresponding to the other protein peaks were also pooled. All fractions were assayed for phosphatidylinositol kinase activity.

Affinity purified insulin receptor was obtained by chromatography on insulin-Sepharose prepared by a modification of the procedure described by Fujita-Yamaguchi et al. [16].

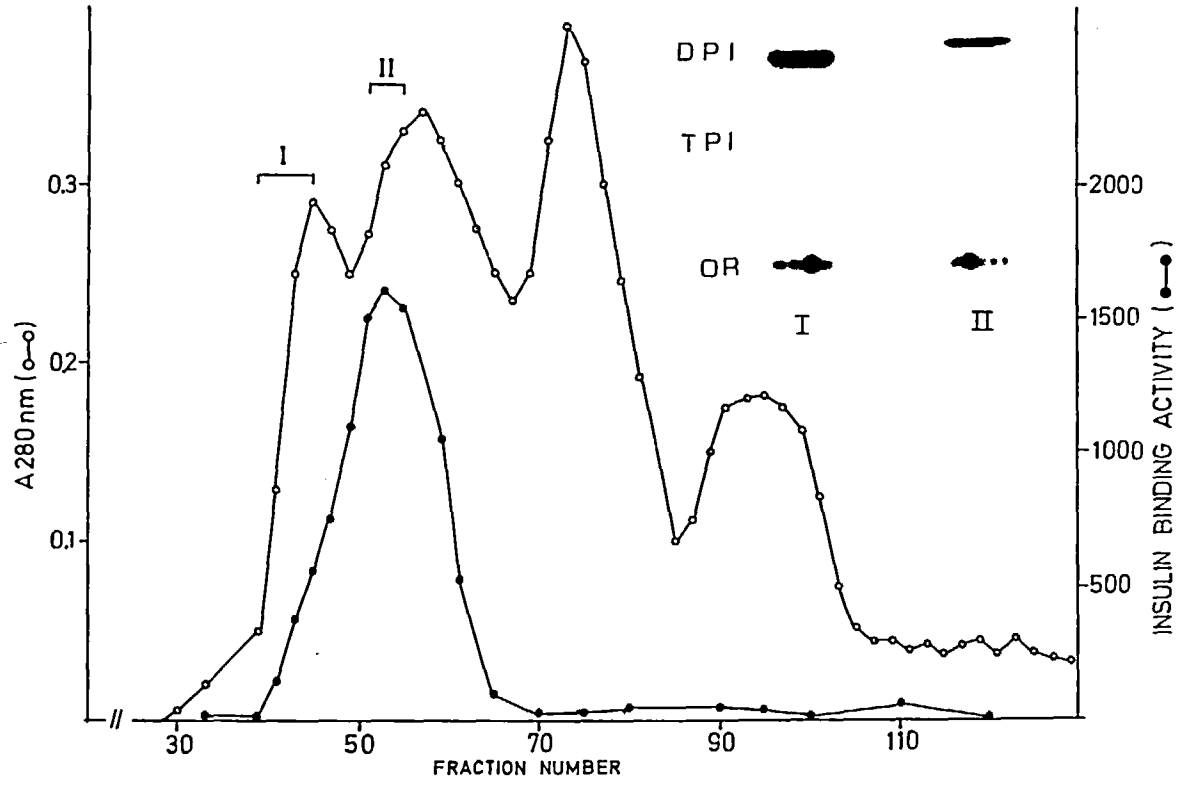

Fig. 1. Partial separation of phosphatidylinositol kinase and IBA by chromatography on Sepharose 6B-Cl. Separation was performed as indicated in Materials and Methods. IBA, phosphatidylinositol kinase and protein-tyrosine kinase activities were tested as described. Inset shows the ^{32}P-incorporation into phosphatidylinositol catalyzed by equal amounts of protein of fractions I and II. Results are representative of a single separation and were reproduced four times. DPI, phosphatidylinositol 4-phosphate, TPI, phosphatidylinositol 4,5,-bisphosphate; OR, origin.

Phosphorylation assays. The 'standard phosphorylation medium' consisted of 50 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, 6 mM $MnCl_2$, 1 mM vanadate, 0.1% Triton X-100 and 50 μM [γ-^{32}P] ATP (3600 cpm/pmol). Samples (20–30 μg of protein) were preincubated 30 min without or with $5 \cdot 10^{-7}$ M insulin, before the reaction was started by addition of [γ-^{32}P]ATP.

Phosphatidylinositol kinase assay. Phosphatidylinositol kinase activity was assessed by the rate of ^{32}P incorporation from [γ-^{32}P]ATP using the phosphorylation medium as described in Ref. 5 with 0.5 mg PI per ml as substrate. Incubation was for 5 min at 23°C, well within the linear range. Lipids were separated on silica gel thin-layer plates. Chromatograms were developed with chloroform/methanol/4 M NH_4OH (9 : 7 : 2 v/v). Lipids were detected with iodine vapor and the ^{32}P-labelled spots by autoradiography. The spots corresponding to labelled PIP were scraped out and counted for ^{32}P radioactivity by liquid scintillation. Phosphoinositides were used as standard in each chromatogram.

Protein-tyrosine kinase assays

With $(Glu^{80}, Tyr^{20})_n$ as substrate. Assays were carried out in 50 μl of 'standard phosphorylation medium' at 23°C. The peptide concentration was 1 mM. After 5 min of reaction, samples were taken and applied on square (2 × 2 cm) filter papers (Whatman 3 MM) soaked with 10% trichloroacetic acid and 20 mM sodium-pyrophosphate and filters were submerged in the same solution. After washing four times with fresh 10% trichloroacetic acid/20 mM sodium pyrophosphate, filters were counted for radioactivity. Phos-

phorylation in the absence of peptide was always subtracted to determine the net peptide phosphorylation.

With histone H_{2B} as substrate. Assays were performed in 50 μl of 'standard phosphorylation medium' containing 1 mg histone H_{2B} per ml. After 7 min at 23°C, the reaction was stopped with 6 μl of a solution containing 20% SDS, 10% glycerol, 250 mM DTT and 50 mM Tris-HCl (pH 7.4) (sample buffer) and the solution was heated for 15 min at 95°C. Proteins were analyzed on SDS-PAGE and the radioactive bands were identified by autoradiography.

Preparation of the phosphotyrosine-directed antibody

Antibody was essentially prepared as described by Pang et al. [17]. The purity of the synthesized *O*-phosphotyramine and *N*-bromoacetyl-*O*-phosphotyramine was assessed by reverse-phase HPLC. The modified keyhole limpet hemocyanin used for immunization contained approx. ten *O*-phosphotyramine residues per 100 aminoacyl residues of hemocyanin. After immune absorption of the antiserum on an *O*-phosphotyramine-Sepharose column, no phosphotyrosine-directed antibody could be eluted with 0.15 mol/l phosphoserine. Upon elution with 0.2 mol/l *p*-nitrophenylphosphate, a protein solution (approx. 1 mg/ml) consisting of more than 90% γ-globulin was obtained. Antibody to the hemocyanin-phosphotyramine conjugate was tested against a BSA-phosphotyramine conjugate adsorbed on microtitre plate wells and the bound antibodies were determined by an antirabbit IgG antibody conjugated with peroxidase.

Antibody solution obtained from the *O*-phosphotyramine column had titers ranging from $1:10^4$ to $1:10^5$. Specificity was tested by addition of increasing amounts of phosphotyramine, phosphoserine and phosphotyrosine to a $1:10^5$ dilution of the antibody. With 2, 10 and 50 mmol/l phosphotyramine and phosphotyrosine, respectively, an inhibition of 12, 38, 70 and 23, 60, 89% was obtained, whereas phosphoserine did not significantly interfere with antibody antigen reaction.

Immunoprecipitation procedures

WGA-Sepharose-purified insulin receptor, preincubated without or with $5 \cdot 10^{-7}$ M insulin, was phosphorylated in 50 μl of 'standard phosphorylation medium' and immunoprecipitated with the anti-phosphotyrosine specific antibodies. Phosphorylated receptor was incubated overnight at 4°C with a 1:20 dilution of the immunoabsorbed antibody. Receptor antibody complexes were immunoprecipitated with 50 μl of a suspension of protein A in 25 mM Hepes (pH 7.4) (100 mg/ml) for 2 h at 4°C. Following centrifugation aliquots of the supernatant were taken and assayed for histone and phosphatidylinositol kinase activity. Pellets were washed three times with 50 mM Tris-HCl (pH 7.4) 1% Triton X-100 and 0.15 M NaCl, resuspended in 50 μl of 'standard phosphorylation medium' and assayed for histone and phosphatidylinositol kinase activities.

Results

Distribution of phosphatidylinositol kinase activity of placenta membrane

Previous studies from this laboratory [5], (Machicao, F., unpublished results) confirmed by others [6] have shown that phosphatidylinositol kinase activity accompanies the insulin receptor up to extensive purification on insulin-Sepharose chromatography and immunoprecipitation. In order to obtain more quantitative information, we examined the contribution of receptor-associated phosphatidylinositol kinase to the total phosphatidylinositol kinase activity present in placental membranes. Table I shows the distribution of phosphatidylinositol kinase activity during partial purification of the insulin receptor. As can be seen, about 50% of total membranal phosphatidylinositol kinase was Triton-soluble, the other 50% remaining insoluble. The bulk of the Triton-soluble phosphatidylinositol kinase activity is not bound to WGA-Sepharose, and only 2% of this activity is recovered in the WGA-Sepharose eluate.

Table I further shows that while IBA was purified about 20-fold by Sepharose chromatography the specific phosphatidylinositol kinase activity decreases notably. These results clearly indicate that the phosphatidylinositol kinase activity apparently associated with the insulin receptor represents only a small part of the total phosphatidylinositol kinase present in placental membranes.

TABLE I

DISTRIBUTION OF PHOSPHATIDYLINOSITOL KINASE ACTIVITY OF HUMAN PLACENTAL MEMBRANES DURING INSULIN RECEPTOR PURIFICATION

Phosphatidylinositol kinase and IBA was determined as indicated in Materials and Methods. Total phosphatidylinositol kinase activity present in 20 ml of membrane suspension corresponds to 45 nmol of ^{32}P incorporated into PI/min. Mean values ± S.E. of six different experiments are given.

Fraction	% of total phosphatidyl-inositol kinase activity [a]	Specific phosphatidyl-inositol kinase activity (pmol per min per mg protein)	IBA (μU/mg protein)
Membranes	100	54.4 ± 7.4	–
Triton X-100-insoluble	47 ± 0.5	59.2 ± 17.4	–
Triton X-100-soluble	53 ± 0.5	113.6 ± 28.5	22 ± 2
WGA-Sepharose, not bound	98 ± 1.0 [b]	178.4 ± 45.9	–
WGA-Sepharose, eluate	2 ± 0.2	23.4 ± 4.6	400 ± 30

[a] Refers only to membrane bound phosphatidylinositol kinase.
[b] Triton X-100 soluble fraction = 100%.

If the WGA-eluate was further purified through insulin-Sepharose affinity chromatography, a similar degree of purification was reached for the histone and the phosphatidylinositol kinase activities (not shown). Thus, it seemed possible that the minor component of cellular phosphatidylinositol kinase activity that copurifies with the insulin receptor might be either intrinsic to the receptor itself, or tightly associated with it.

Partial separation of phosphatidylinositol kinase from IBA

Partial separation of phosphatidylinositol kinase from IBA was achieved by Sepharose 6B-Cl chromatography (Fig. 1). Thus, in fraction II the specific activity of phosphatidylinositol kinase was 2.5-times lower than in fraction I (see also inset of Fig. 1) while the specific activity of IBA was about 5-times higher in fraction II than in fraction I (Table II).

There was no detectable phosphatidylinositol kinase activity in the other protein peaks eluted from the Sepharose 6B-Cl column. These results suggest that phosphatidylinositol kinase activity and IBA are part of different proteins which can be partially separated on Sepharose 6B-Cl. The loss of phosphatidylinositol kinase-specific activity of the WGA eluate after Sepharose chromatog-

TABLE II

PARTIAL SEPARATION OF PHOSPHATIDYLINOSITOL KINASE AND IBA BY CHROMATOGRAPHY ON SEPHAROSE 6B-Cl

For experimental conditions see legend to Fig. 1.

Fraction	A	B	A/B	Insulin effect $(Glu^{80}Tyr^{20})_n$ phosphorylation (fold)
	phosphatidylinositol kinase activity (pmol per min per mg protein^{-1})	IBA (mU/mg protein)		
WGA eluate	23.5	0.400	59	1.8
Sepharose 6B-Cl fraction I [a]	20.1	0.164	123	1.6
Sepharose 6B-Cl fraction II [a]	8.0	0.800	10	5.4

[a] Fractions I and II shown in Fig. 1.

raphy is probably due to the fact that the separation was carried out overnight and the fractions were pooled and tested later on the following day.

In several instances insulin produced some (1.5-fold) stimulation of phosphatidylinositol kinase activities using WGA eluate preparations. With fractions I and II, after Sepharose-6B-Cl chromatography (Fig. 1), no clear stimulation of phosphatidylinositol kinase activity by insulin could be observed, in contrast to the stimulation of tyrosine phosphorylation of the synthetic peptide (Table II).

Separation of phosphatidylinositol kinase from insulin receptor protein-tyrosine kinase activity by immunoprecipitation

In some cases, including the insulin receptor, specific antibodies were observed to precipitate protein-tyrosine kinase and phosphatidylinositol kinase activities together suggesting that the two enzymes may be identical [1,2,6]. In the present work we used a specific antibody against phosphotyrosine which precipitates the phosphorylated insulin receptor from human placenta. Phosphorylated WGA-purified insulin receptor was incubated with this antibody, and the immunoprecipitate and supernatant were tested for phosphatidylinositol kinase and protein kinase activities. Confirming our previous findings [5], WGA-purified insulin receptor preparations catalyze the incorporation of ^{32}P into PI to form PIP (Fig. 2, lanes A and B). After immunoprecipitation the major part of phosphatidylinositol kinase activity (95%) remains in the supernatant (lanes G and H), whereas the small precipitated activity (lanes E and F) is comparable to that obtained with unspecific rabbit serum (lanes C and D). In this experiment, insulin had no effect on phosphatidylinositol kinase activity.

That our antibody did precipitate the insulin-stimulatable receptor-associated 95 kDa protein-tyrosine kinase that phosphorylates also histone as an exogenous substrate is shown in Fig. 3, lanes C and D. Controls with unspecific serum yielded no [^{32}P]phosphoprotein containing precipitates (Fig. 3, lanes A and B). In Fig. 3, lanes E and F, it appears that immunoprecipitation was not complete, 20–25% of the insulin receptor

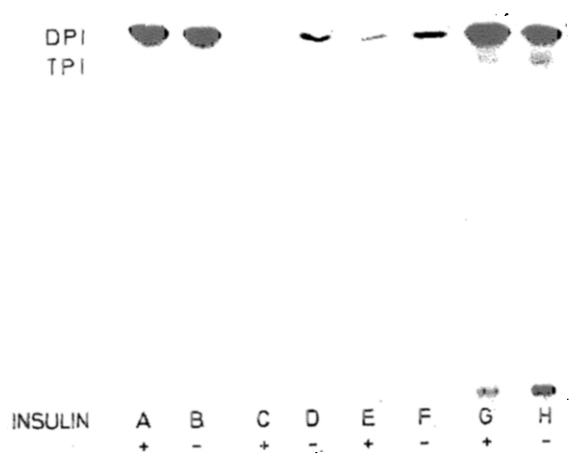

Fig. 2. Distribution of phosphatidylinositol kinase activity of the WGA eluate after insulin receptor immunoprecipitation. For determination of phosphatidylinositol kinase activity, a sample of WGA eluate (20 μl) was preincubated in 0.1 ml of standard phosphorylation medium containing 50 μg of PI without (lane A) or with $5 \cdot 10^{-7}$ M insulin (lane B) for 30 min. [γ-^{32}P]ATP was then added and after 5 min lipids were extracted and separated as indicated in Materials and Methods. For immunoprecipitation, another sample 20 μl of the WGA eluate was preincubated for 30 min in 50 μl of standard phosphorylation medium (with no PI added) without (lanes C, E and G) or with $5 \cdot 10^{-7}$ M insulin (lanes D, F and H). [γ-^{32}P]ATP was then added and phosphorylation was stopped after 5 min with 30 μl of a solution containing 20 mM EDTA, 20 mM sodium pyrophosphate, 1 mM ATP, 2 mM vanadate and 1 mM DTT. Thereafter the samples were mixed with the anti-phosphotyrosine antibody (lanes E–H) or unspecific rabbit serum (lanes C and D), kept overnight at 4°C, supplemented with 50 μl of protein A suspension and, after a further 2 h incubation at 4°C, the immunoprecipitates were centrifuged for 5 min. Phosphatidylinositol kinase activities were determined using 60 μl portions of the supernatants (lanes G and H) or the washed and resuspended precipitates (lanes C–F) as described in Materials and Methods Abbreviations as in Fig. 1.

kinase remaining in the supernatant. Nevertheless, it seems clear from these experiments that by immunoprecipitation, the bulk of phosphatidylinositol kinase activity can be separated from the protein-tyrosine kinase indicating that they are different proteins.

The question still remains of whether the WGA bound phosphatidylinositol kinase may differ from the enzyme that is not bound to the lectin, or whether it is just a small fraction of the Triton X-100-soluble phosphatidylinositol kinase which, in tight association with the insulin receptor and

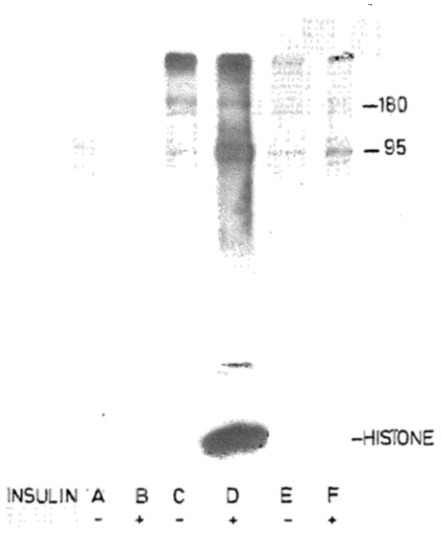

Fig. 3. Distribution of insulin receptor kinase activity after immunoprecipitation of WGA eluate. Incubations were performed as described in Fig. 2 except that histone H_{2B} (1 mg/ml) was used as substrate instead of PI. Proteins were separated on SDS-PAGE and the radioactive bands were detected by autoradiography. Lanes A and B, precipitate with unspecific rabbit serum; lanes C and D, precipitate with specific anti-phosphotyrosine antibody; lanes E and F, supernatant after immunoprecipitation with specific anti-phosphotyrosine antibody.

perhaps other glycoproteins, is retained by the lectin. In the case of different enzymes, one might expect differences in kinetic properties. We, therefore, studied the substrate dependence of the WGA-bound and unbound phosphatidylinositol kinase. As indicated in Table III, apparent K_m values for both PI and ATP as extrapolated from Lineweaver-Burk plots were very similar, supporting the view that the phosphatidylinositol kinase activity of the two fractions is attributable to the same enzyme.

Inhibition of phosphatidylinositol kinase activity by $(Glu^{80}Tyr^{20})_n$

The effect of the polymer $(Glu^{80}Tyr^{20})_n$, a model substrate of the insulin receptor associated protein-tyrosine kinase [18], on the phosphorylation of PI by WGA-purified insulin receptor preparations was investigated. As shown in Fig. 4 $(Glu^{80}Tyr^{20})_n$ inhibited the phosphatidylinositol kinase activity by 70%, i.e., from 4.2 to 1.25 pmol per min per mg in (A), and by more than 90%, i.e., from 2.9 to 0.3 pmol per min per mg protein in (B). This inhibition was not overcome by increasing the concentrations of the substrates as indicated by the unchanged apparent K_m values for ATP (8.3 μM) and PI (150 μM). The apparent K_i of $(Glu^{80}Tyr^{20})_n$ for inhibition of phosphatidylinositol kinase measured at 20 μM ATP and 1 mM PI was about 0.4 mM.

The different V_{max} values of the phosphatidylinositol kinase activities of Fig. 4A and B and also of Table III are due to the use of different

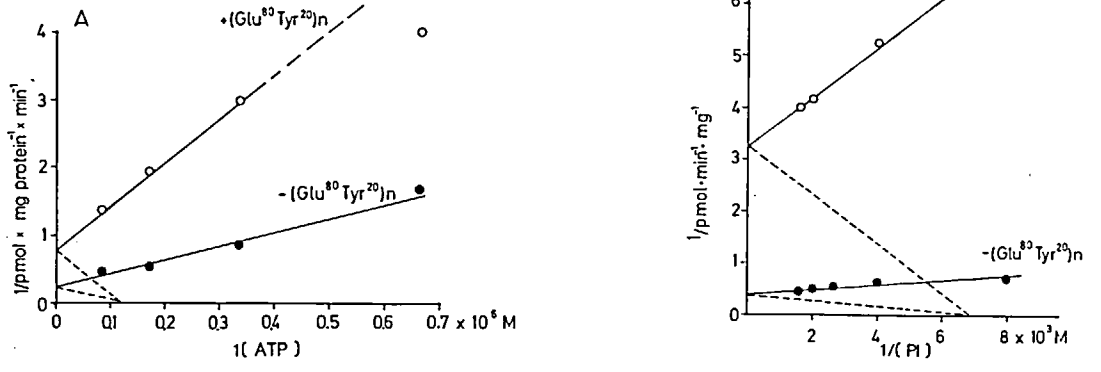

Fig. 4. Effect of $(Glu^{80}Tyr^{20})_n$ on PI phosphorylation by WGA-purified insulin receptor as a function of (A) ATP and (B) PI concentration. Phosphorylation was carried out for 5 min, essentially as described in Materials and Methods. The PI concentration in (A) was 1 mM and the ATP concentration in (B) was 20 μM. The $(Glu^{80}Tyr^{20})_n$ concentration was 0.5 mM throughout assuming an average molecular weight of 45000 [18].

TABLE III

SUBSTRATE AFFINITY DATA OF PHOSPHATIDYLINOSITOL KINASE FROM HUMAN PLACENTAL MEMBRANES

Phosphatidylinositol kinase activity of the WGA eluate and the non WGA bound fraction of the Triton X-100 soluble membrane extract was measured as indicated in Materials and Methods except that the concentrations of PI and ATP were varied over a range appropriate for establishing Michaelis-Menton kinetics.

Phosphatidylinositol kinase fraction	V_{max} [a] (pmol per mg per min)	K_{mapp}	
		PI (mmol/l)	ATP (μmol/l)
Wheat germ eluate	6	0.4	8
Not bound to WG	100	0.3	7

[a] Measured at 0.6 mM PI and 12 μM ATP, molecular weight of PI was taken to be 800.

WGA eluate preparations. As regards the different apparent K_m values for PI (Table III and Fig. 4B) one should also consider the difficulties inherent in the supply of lipid substrates.

Discussion

The fact that even after extensive purification the insulin receptor from human placenta displayed phosphatidylinositol kinase activity had led us [5] and others [6] to consider the possibility that the tyrosine-specific protein kinase and the lipid kinase may reside on the same enzyme molecule. In support of this, in several experiments, though not regularly, insulin stimulated the phosphorylation of PI concomitantly with the receptor autophosphorylation or the phosphorylation of histone and other exogenous substrates [5]. More recently, Sale et al. [6] using human serum anti-insulin receptor autoantibodies showed that most of the phosphatidylinositol kinase activity was precipitated together with the insulin receptor, further suggesting the possibility that the insulin receptor itself might bear phosphatidylinositol kinase activity. The possible interdependence of the insulin receptor-associated protein-tyrosine kinase and phosphatidylinositol kinase was also studied by Sale et al. using the synthetic polymer $(Glu^{80} Tyr^{20})_n$, which is a good substrate for the protein-tyrosine kinase and, hence, can competitively inhibit receptor autophosphorylation. The finding that this compound also inhibited PI phosphorylation [6] seemed to provide supportive evidence that protein-tyrosine kinase and phosphatidylinositol kinase and share the same active sites on the insulin receptor. If this is the case, one would expect that the suppression of phosphatidylinositol kinase by (Glu, Tyr) could be overcome by increasing the concentrations of the substrates PI and ATP. This was, however, not the case as indicated by our kinetic studies showing that the inhibition of PI phosphorylation is attributable to reduction of the V_{max} rather than to changing the affinity for PI and ATP (Fig. 4). The mechanism of this inhibition is not clear but may depend on other properties of the (Glu, Tyr) polymer rather than functioning as a phosphorylatable substrate. In a few experiments it was found that the soluble, non-receptor-bound phosphatidylinositol kinase is also inhibited by about 90% by (Glu, Tyr) (data not shown). The mechanism of this inhibition was not further investigated.

In the present study we could achieve clear separation of the protein kinase and the lipid kinase by immunoprecipitation with a specific anti-phosphotyrosine antibody prepared in this laboratory. Hence, it now seems clear that phosphatidylinositol kinase activity is not intrinsic to the insulin receptor-associated protein-tyrosine kinase, but rather seems tightly bound to the latter by physical interactions. Similarly, the assumption that the $p60^{v\text{-}src}$ kinase might also act as a lipid kinase [1] was later discarded when the activity of the two enzymes could be separated by immunoprecipitation [11].

Quantitatively, the phosphatidylinositol kinase activity associated with the insulin receptor accounts for only 2% of the total Triton X-100-soluble phosphatidylinositol kinase activity (Table I). The question then arises is whether this fraction is a separate enzyme, different from the bulk phosphatidylinositol kinase of the Triton X-100 extract. Our studies on the substrate saturation kinetics of the two phosphatidylinositol kinase fractions yielded very similar apparent K_m values for both ATP and PI (Table III). Although this does not exclude two different enzymes it seems more likely to us that the phosphatidylinositol kinase of the WGA eluate stems from the pool of

the Triton X-100 soluble phosphatidylinositol kinase, and is retained by the lectin through its tight binding to the insulin receptor and other glycoproteins.

In conclusion, the present results clearly demonstrate that the phosphatidylinositol kinase activity which is purified together with the insulin receptor from human placenta is separable by specific immunoprecipitation, and hence – unlike what was hitherto suggested – is not intrinsic to the protein (tyrosine) kinase of the receptor. However, the striking affinity of phosphatidylinositol kinase activity to the insulin receptor still raises the question of the possible functional significance in insulin action. Stimulation of phosphatidylinositol kinase, as occasionally is observed with solubilized receptor preparations [5,6], would need more conclusive evidence, perhaps under in vivo conditions, i.e., using intact cells. Other effects of insulin, such as increasing cellular phosphoinositides and PI turnover [8] and to activating phospholipase C [19–21] in adipose tissue, further point to a regulatory interrelationship between the insulin receptor and the PI cycle activity.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, F.R.G. We thank Mr. Paul Delly for preparing the figures and Mrs. Dagmar Lehmeier and Mrs. Dorothea Berberich for secretarial assistance.

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