Dephosphorylation of human insulin-like growth factor I (IGF-I) receptors by membrane-associated tyrosine phosphatases

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The insulin-like growth factor-I (IGF-I) receptor exhibits structural and functional similarities to the insulin receptor. Although the regulation of the insulin-receptor tyrosine kinase has been extensively investigated, the mechanisms involved in phosphorylation/dephosphorylation of the IGF-I receptor have received only little attention. To obtain a better understanding of the mode of IGF-I action, we have investigated the effects of protein phosphotyrosine phosphatases (PTPases) on the phosphorylation status of the IGF-I receptor. The dephosphorylation of the human IGF-I receptor by membrane-associated tyrosine phosphatases was studied by an immuno-enzymic assay based on the recognition of phosphotyrosine residues by anti-phosphotyrosine antibodies. Using intact IGF-I receptors as substrates, we show that they could be completely dephosphorylated by different cellular PTPases. Three pieces of evidence indicate that receptor dephosphorylation takes place on phosphotyrosine, i.e. the inhibition profile of phosphatase activity by zinc and vanadate, its absolute requirement for thiol compounds and the diminution of [³²P]phosphotyrosine labelling of the β subunit assessed by SDS/PAGE and phosphoamino acid analysis. Tyrosine kinase activity and autophosphorylation of the IGF-I receptor were decreased in a dose-dependent manner by PTPases, indicating that partial dephosphorylation of the receptor was associated with a decrease in its intrinsic activity. The sensitivity of the activated human IGF-I receptor to dephosphorylation on tyrosine leads to the speculation that IGF-I receptor activity might be regulated by mechanisms such as those described for the insulin receptor. Further investigation of the pathways of IGF-I receptor dephosphorylation will contribute to define the role(s) of PTPases in the overall mechanism of IGF-I signalling.

INTRODUCTION

The insulin-like growth-factor I (IGF-I) and the insulin receptors share a high degree of structural and functional similarity [1,2]. They are heterotetramers composed of two α and two β chains associated to form the minimal active unit, which has an $\alpha_2\beta_2$ structure. They possess an intracellular catalytic domain with a tyrosine kinase activity located on the intracellular part of the β subunit [3–5]. Comparison of the tryptic maps of the β subunits of insulin receptors [6] and IGF-I receptors [7] indicates that the closely spaced tyrosine residues 1146, 1150 and 1151 are similarly located in the so-called ‘regulatory domain’. In light of these data, it is conceivable that both receptors might be subject to similar regulatory mechanisms.

The first step in insulin-receptor activation is binding of the ligand that induces conformational changes [8] and activates the intrinsic kinase (reviewed in [9,10]). This activation leads to the autophosphorylation of the receptor on at least three tyrosine residues in the catalytic domain [11,12], and this event is followed by the transduction of the hormonal signal, likely through a cascade of phosphorylation/dephosphorylation reactions [13,14]. It has been shown that the tyrosine-phosphorylated insulin receptor is the active enzymic form and that the kinase activation is maintained as long as the receptor remains phosphorylated, even in the absence of insulin [15,16]. These data suggest a requirement for a step of dephosphorylation to deactivate the receptor. Thus, in order to regulate phosphorylation-mediated biological functions, the process of receptor tyrosine phosphorylation needs to be reversible or at least attenuated by specific dephosphorylation reactions. Although the mechanisms of tyro-

sine phosphorylation of several membrane receptors (including the insulin receptor) and other cellular proteins have been extensively investigated, the precise pathways leading to signal transmission are not yet elucidated [2]. Recently, evidence has been accumulated that most cells possess enzymes able to dephosphorylate specifically on tyrosine residues a variety of cellular proteins, including receptor tyrosine kinases (review in [17]). Protein phosphotyrosine phosphatase (PTPase) activities that can be recovered in both cytosolic and membrane fractions have been identified in cultured cells and several mammalian tissues [18–21]. Several PTPases have been purified to homogeneity [21,22], cloned and sequenced [23,24], but little information is available on their mechanism(s) of action.

The relationship between dephosphorylation and deactivation of the insulin receptor tyrosine kinase has recently been examined [25,26]. Studies of modulation of receptor kinase activity by transition between tris- and bis-phosphorylated receptor states have pointed out that the tris-phosphorylated form is more rapidly deactivated than the bis form. However, given the large number of phosphatases present in the cytosolic and membrane-associated cellular compartments, the specific PTPases responsible for insulin-receptor regulation remained undefined. Characterization of the regulation of IGF-I receptor dephosphorylation by specific PTPases has received little attention so far, but is essential for obtaining a more complete understanding of IGF-I and insulin actions.

The present work was aimed at approaching the dephosphorylation of the IGF-I receptor by membrane-associated PTPases. To measure the rate of tyrosine dephosphorylation of partially purified human IGF-I receptors, we have developed a

Abbreviations used: WGA, wheat-germ agglutinin; PTPase, protein phosphotyrosine phosphatase; IGF-I, insulin-like growth factor I; pNPP, *p*-nitrophenyl phosphate.

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sensitive immuno-enzymic assay. This technique, based on the e.l.i.s.a. method, allows direct and rapid quantification of the extent of dephosphorylation of phosphorylated tyrosine kinase receptors without the need for radiolabelled compounds. We were able to demonstrate that the human IGF-I receptor can be completely dephosphorylated on tyrosine residues by PTPases prepared from different tissues and cell lines. The effect of the dephosphorylation of the IGF-I receptor on its intrinsic tyrosine kinase activity was assessed *in vitro*, and the process of deactivation of the receptor was shown to be fully reversible.

MATERIALS AND METHODS

Materials

Hepes, ATP, Triton X-100, *N*-acetyl-D-glucosamine, dithiothreitol, BSA, proteinase inhibitors and poly(Glu-Tyr)(4:1) were from Sigma (St. Louis, MO, U.S.A.). Wheat-germ agglutinin (WGA) agarose (Glycaminsylex) was from Biomakor (Rehovot, Israel). Human insulin was purchased from Novo (Copenhagen, Denmark). Human recombinant IGF-I was a gift from Eli Lilly company (Indianapolis, IN, U.S.A.). Reagents for SDS/PAGE and the Bradford protein assay were from Bio-Rad (Richmond, CA, U.S.A.) or Serva (Heidelberg, Germany). All chemical reagents were of the highest purity available. [γ - 32 P]ATP was purchased from Amersham International (Amersham, Bucks., U.K.). Na 125 I was from CEA (Saclay, France). IGF-I was labelled to a specific radioactivity of 600 Ci/mmol with Na 125 I by the chloramine- τ method [27]. Briefly, 1.4 μ g of IGF-I was incubated for 40 s with 1 mCi of Na 125 I (sp. radioactivity 17 mCi/ μ g) and 4 μ g of chloramine- τ in 0.2 M-phosphate buffer. Na $_2$ S $_2$ O $_5$ (13 μ g) and BSA (0.6%) were added, and IGF-I was purified on a Sephadex G-50 column. Sheep anti-phosphotyrosine antibody was produced in the laboratory, and γ -globulins (4 μ g of protein/ml) purified by affinity chromatography were used in all experiments [28]. Peroxidase-conjugated rabbit anti-(sheep γ -globulins) antibody was purchased from DAKO (Glostrup, Denmark). Plastic ware for tissue culture and the immunoassay technique was from Nunc (Copenhagen, Denmark) and Poly Labo (Strasbourg, France). Culture media and fetal-calf serum were from Gibco (Grand Island, NY, U.S.A.). α IR-3 is a murine-specific monoclonal antibody directed against the α -subunit of the IGF-I receptor, and was generously provided to us by Dr. S. Jacobs (Wellcome, Research Triangle Park, NC, U.S.A.).

Animals

Female Wistar rats were purchased at a weight of 180 g from IFFA CREDO (L'Arbresle, France), housed and fed *ad libitum* in the laboratory. They were mated in the laboratory, and the day of mating was taken as day 1 of gestation. On the day of the experiment, the rats were killed by cervical dislocation, and the livers and placentas (20 days of gestation) were rapidly removed after maternal laparotomy.

Cell culture

NIH-3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). 3T3-NHIR cells, mouse embryo fibroblasts transfected with an expression plasmid encoding the human insulin receptor (6×10^6 receptors/cell) were obtained from Dr. J. Whittaker (Stony Brook, NY, U.S.A.). NIH-3T3 cells transfected with an expression plasmid encoding the human IGF-I receptor (2×10^5 receptors/cell) were a gift from Dr. P. De Meyts (Hagedorn Institute, Copenhagen,

Denmark). The human glioma cell lines U343 and U259 were kindly provided by Dr. S. Gammeltoft (Copenhagen, Denmark). Fibroblasts were grown in Dulbecco's modified Eagle medium, and glioma cells in RPMI-1640 medium. All media were supplemented with 10% (v/v) fetal-calf serum and antibiotics. Cells were fed by changing medium every other day and were used 3–6 days after plating just before reaching confluency.

Partial purification of receptors and binding assay

IGF-I and insulin receptors were partially purified by affinity chromatography on WGA as previously described for the insulin receptor [29]. IGF-I-receptor-binding assays were performed with 20 μ l of partially purified receptor preparation, incubated for 16 h at 4 °C in the presence of 125 I-labelled IGF-I (sp. radioactivity 600 Ci/mmol). Bound iodinated hormone was separated from the free ligand by precipitation with 25% poly(ethylene glycol) 6000 and 0.5% γ -globulins as a carrier [30]. Under the same experimental conditions 125 I-insulin binding to 20 μ l of the WGA preparation was negligible (results not shown).

Preparation of membrane-associated phosphatase fractions

Extraction from rat liver and placenta. The technique is a modification of the procedure described for placental tissue by Roome *et al.* [31]. Briefly, rats were killed by cervical dislocation. The livers and placentas were rapidly removed, cut and washed extensively in an ice-cold buffer containing 250 mM-sucrose, 25 mM-Hepes and 15 mM- β -mercaptoethanol, pH 7.2, then homogenized for 45 s at medium setting, with a Polytron (Proscience, Paris, France). All subsequent steps of tissue extraction were performed at 4 °C in the presence of the proteinase inhibitors phenylmethanesulphonyl fluoride (1 mM) and Trasylol (100 units/ml). Homogenates were centrifuged at 600 g for 20 min. NaCl (0.1 M) and MgCl $_2$ (0.2 mM) were added to the supernatants, which were then centrifuged for 30 min at 180 000 g. At the end of this step, the supernatant was considered as the cytosolic fraction, and the remaining pellet, corresponding to the microsomal fraction, was solubilized by stirring for 60 min at 4 °C in 25 mM-Hepes/1% Triton X-100 in the presence of proteinase inhibitors. After solubilization and centrifugation for 30 min at 100 000 g, the final supernatant was designated the membrane-associated fraction. After protein determination, all preparations were stored at –80 °C, and kept for 1 week without any detectable decrease in phosphatase activity.

Extraction from cell lines. Confluent cells were washed twice in PBS (phosphate-buffered saline: 137 mM-NaCl, 2.6 mM-KCl, 6.4 mM-Na $_2$ HPO $_4$, 1.5 mM-KH $_2$ PO $_4$, pH 7.4) and once in 50 mM-Hepes/150 mM-NaCl containing 1 mM-EDTA, 15 mM- β -mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride and 100 units of Trasylol/ml. Cells were scraped directly in the same buffer and centrifuged for 3 min at 600 g. The pellet was sonicated (Vibra Cell; Sonics and Materials Inc., Danbury, CT, U.S.A.) for 15 s at the lowest setting in 2 vol. of buffer and centrifuged for 30 min at 100 000 g. After this step, the pellet was solubilized in 50 mM-Hepes/150 mM-NaCl/1% Triton X-100 supplemented with proteinase inhibitors for 60 min at 4 °C, and centrifuged for 40 min at 180 000 g. The final supernatant was designated the membrane-associated fraction and stored at –80 °C.

Determination of total phosphatase activity

This was done by measuring their ability to dephosphorylate *p*-nitrophenyl phosphate (*p*NPP) [32]. Samples (20 μ l) of different phosphatase preparations were incubated for 10 min at 30 °C with 10 mM-*p*NPP in Hepes buffer, and the reaction was stopped with 0.2 M-NaOH. With alkaline phosphatase as a standard, total activity was calculated from A_{405} readings. Protein was

measured in the same samples by the method of Bradford [33], with IgG as a standard.

Measurement of PTPase activity by immuno-enzymic assay

The test is based on the recognition by anti-phosphotyrosine antibodies of phosphotyrosine residues remaining on intact IGF-I receptors after incubation in the absence or presence of phosphatases. WGA-purified human IGF-I receptors (250 ng of protein/well) were stimulated with IGF-I (0.1 μ M) for 45 min at 22 °C. The phosphorylation reaction was initiated by addition of 30 μ M-ATP/8 mM-MgCl₂/4 mM-MnCl₂ in 30 mM-Hepes/30 mM-NaCl/0.1 % Triton X-100, pH 7.4, continued for 30 min at 4 °C, and terminated by addition of coating buffer (15 mM-Na₂CO₃/35 mM-NaHCO₃, pH 9.6). A 50 μ l portion of autophosphorylated receptor solution was loaded in each well of a microtitre plate and allowed to coat overnight at 4 °C. The plates were then washed twice with PBS/0.05 % Tween 20 and saturated for 2 h at 37 °C with 2 % BSA in PBS. Three washes were performed with PBS/Tween, and the dephosphorylation reaction was initiated by addition of 50 μ l of phosphatase preparation or buffer (25 mM-Hepes/5 mM-EDTA/0.1 % BSA, pH 7.4). After a 30 min incubation at 22 °C and five washes with PBS/0.05 % Tween 20, 50 μ l of antiphosphotyrosine antibody (1.8 μ g/well in PBS 1 % BSA) was added to the solution, which was then incubated for 45 min at 37 °C. The plates were washed five times, and the second antibody [50 μ l of peroxidase-conjugated rabbit anti-(sheep γ -globulins); 0.25 μ g/well] was added for 90 min at 22 °C. After five further washes with PBS/0.05 % Tween 20, 50 μ l of *o*-phenylenediamine dihydrochloride (3 mg/ml) in 0.1 M-citric acid/0.1 M-Na₂HPO₄/0.02 % H₂O₂, pH 5.5, was added as a chromogen. *A*₄₀₅ readings were performed on an e.l.i.s.a. mini-reader (MR700; Dynatex, Saint Cloud, France). Percentage receptor dephosphorylation was calculated as the ratio of *A*₄₀₅ of dephosphorylated receptors (incubated in the presence of phosphatases) over that of control receptors (incubated in the absence of phosphatases).

Autophosphorylation *in vitro* and kinase activity of the WGA-purified IGF-I receptor

Partially purified IGF-I receptors were preincubated for 90 min at 4 °C in the absence or presence of 0.1 μ M-IGF-I in 50 mM-Hepes/0.1 % Triton X-100, pH 7.4. The phosphorylation reaction was initiated by addition of 15 μ M-[γ -³²P]ATP, 4 mM-MgCl₂ and 8 mM-MnCl₂ and continued for 30 min at 22 °C. The reaction was stopped by addition of 60 μ l of 1 × Laemmli sample buffer containing 100 mM-dithiothreitol, and all samples were boiled for 3 min [34]. For immunoprecipitation experiments, 500 μ l of stopping solution (50 mM-Hepes/0.1 % Triton containing 100 mM-NaF, 10 mM-Na₄P₂O₇, 5 mM-EDTA, phenylmethanesulphonyl fluoride and aprotinin) were added to the autophosphorylated receptor and incubated with antibody α IR3 or with an anti-phosphotyrosine antibody coupled to Protein A-Sepharose for 2 h at 4 °C. After three washes, 60 μ l of 1 × Laemmli buffer was added and SDS/PAGE was performed. The proteins were separated and analysed by one-dimensional SDS/PAGE under reducing conditions as described previously [3]. The intensity of the signals shown on the autoradiograms was quantified by scanning densitometry (Hoefer Scanner; Hoefer Scientific Instruments, San Francisco, CA, U.S.A.).

Receptor tyrosine kinase activity was measured with the artificial substrate poly(Glu-Tyr) (4:1). IGF-I receptors were first incubated for 90 min at 4 °C in the absence or presence of 0.1 μ M-IGF-I. Phosphorylation of 0.2 mg of poly(Glu-Tyr) (4:1)/ml was carried out for 30 min at 22 °C in 50 mM-Hepes buffer containing 0.1 % Triton X-100 and 30 μ M-[γ -³²P]ATP in

the presence of 10 mM-magnesium acetate. Incorporation of radioactive phosphate into the substrate was measured by a filter-paper assay [35].

Identification of phosphoamino acids

Phosphoamino acid analysis was performed by the method of Hunter [36]. ³²P-labelled bands were localized by autoradiography of fixed and dried gels, and the appropriate portions were excised. After incubation for 12 h in 10 % (v/v) methanol, the gel fragments were dried, rehydrated, and further processed as previously described [37].

RESULTS

Quantification of IGF-I receptor dephosphorylation on tyrosine residues by an immuno-enzymic assay

The time course of phosphatase action is shown in Fig. 1. The half-time of receptor dephosphorylation was achieved after a 5 min incubation in the presence of phosphatases, and the maximal dephosphorylation was reached at 30 min. Similar experiments performed in the presence of increasing concentrations (25–1000 ng of protein/well) of IGF-I receptor-substrate allowed us to set the optimal assay conditions (results not shown). Subsequent studies were carried out with a receptor concentration of 250 ng of protein/well and a dephosphorylation time of 30 min.

Dephosphorylation of the IGF-I receptor

Effect of membrane-associated phosphatases and inhibitors. We investigated the ability of phosphatases from different cell types and tissues to dephosphorylate the IGF-I receptor. A shown in Table 1, the ratio of membrane-associated to cytosolic phosphatase activity calculated for each preparation varied from 2 to 5, and reached the highest level in brain glioma cells. These phosphatase activity ratios were totally independent of the number of IGF-I-binding sites present in the different tissues and cells. In general, the extent of dephosphorylation of the IGF-I receptor was higher in cell extracts than in tissue extracts. At low phosphatase concentrations, i.e. 10 m-units, the phosphatase activity from glioma cells was significantly elevated compared with other cell lines (results not shown). At high phosphatase concentrations, i.e. 100 m-units, the activity was also significantly

Fig. 1. Dephosphorylation of the human IGF-I receptor by rat liver PTPases

The time course of IGF-I-receptor dephosphorylation in the presence of phosphatases is shown; 250 ng of protein/well was coated overnight in the microtitre plate and incubated for 0 to 60 min in the presence of 100 m-units of rat liver phosphatases. Results are expressed as percentage receptor phosphorylation calculated relative to basal. Values are means $\pm$ S.E.M. of three experiments performed in quadruplicate.

Table 1. Ability of phosphatases from various origins to dephosphorylate the human IGF-I receptor

Phosphatase activities of each preparation were calculated in m-units/mg of protein (measured with alkaline phosphatase to establish the standard curve and *p*NPP as a substrate) and are expressed as activity ratio of particulate to cytosolic fractions. IGF-I binding activities were determined on 20 μ l samples of each preparation. Specific binding was calculated by subtracting the radioactivity (c.p.m.) obtained in the presence of 1 μ M unlabelled IGF-I and expressed per mg of protein. Values are means of two or three determinations (ND, not determined). Total counts per assay were 100 000 c.p.m. Percentage dephosphorylation of the IGF-I receptor was measured with the immuno-enzymic assay with auto-phosphorylated human IGF-I receptors (250 ng protein/well) as substrate in the presence of 100 m-units of phosphatases prepared from the particulate fractions of the indicated cells and tissues. Results were compared with control values obtained in the absence of phosphatases and are expressed as means $\pm$ S.E.M.

Origin of phosphatases	Activity ratio particulate/cytosol	IGF-I binding activity (% of total)	IGF-I-receptor dephosphorylation (%)
Cell lines			
Human glioma U343	5	16.5	100 $\pm$ 0.5
Human glioma U251	ND	10.9	98 $\pm$ 0.8
3T3 fibroblasts	5.5	46.5	90 $\pm$ 2.2
3T3 fibroblasts transfected with IGF-I receptors			
3T3 fibroblasts	3.8	14.6	80 $\pm$ 1.6
Rat tissues			
Placenta	2	10.3	70 $\pm$ 3.2
Liver	1.7	3.8	80 $\pm$ 3.1

Table 2. Effect of cations and EDTA on PTPase activity

Phosphatase activity was measured with an immunoassay using human IGF-I receptors as substrate (250 ng of protein/well). Phosphatases from rat liver (25 μ g of protein) or from glioma cells (10 μ g of protein) were added to the incubation buffer for 20 min at room temperature to dephosphorylate 80 % of the auto-phosphorylated receptor under control conditions. The different compounds were added together with the phosphatases in the assay buffer at the concentrations indicated. Results are expressed as percentage inhibition of phosphatase activity measured in the absence of any additional compounds. Data are means of two to three experiments performed with different phosphatase preparations and carried out in quadruplicate.

Compound	Concn. (μ M)	Inhibition of phosphatase activity (%)	
		Rat liver	Glioma
Zinc	10	15	55
	100	100	100
	500	100	100
Vanadate	25	20	75
	50	50	95
	100	100	100
	200	100	100
EDTA	5 $\times$ 10 ³	0	0
	25 $\times$ 10 ³	15	0
	100 $\times$ 10 ³	75	5

increased ($P < 0.01$) in the transformed cells derived from human glioma brain tumours as compared with fibroblasts.

As previously reported for several purified PTPases, a series of compounds modulate their intrinsic activity in a way which

Fig. 2. Effect of glioma and liver PTPases on the level of phosphorylation of the human IGF-I receptor

(a) Immuno-enzymic assay. IGF-I receptor (250 ng of protein/well) was autophosphorylated with 30 μ M unlabelled ATP, 4 mM-MnCl₂ and 8 mM-MgCl₂ for 30 min at 22 °C. The autophosphorylated receptors were coated overnight in a microtitre plate and incubated with increasing concentrations of phosphatases prepared from human glioma cells or normal rat liver. Results are expressed as percentage phosphorylation relative to basal, i.e. autophosphorylated receptor incubated in the absence of phosphatases. Values are means of two to three experiments carried out in quadruplicate. (b) SDS/PAGE analysis of IGF-I receptor after immunoprecipitation with anti-phosphotyrosine antibody. WGA-purified receptors (3 μ g of protein/lane) were incubated for 90 min at 4 °C in the absence (No) or presence (With) of 0.1 μ M unlabelled IGF-I. The receptors were autophosphorylated with 25 μ M-[γ -³²P]ATP, 4 mM-MnCl₂ and 8 mM-MgCl₂ for 30 min at 22 °C; then increasing concentrations of phosphatases (P-ases) were added to the receptor solutions and allowed to incubate for another 30 min at 22 °C. The reaction was terminated by addition of 500 μ l of stopping solution containing 100 mM-EDTA and 5 mM-NaF, and anti-phosphotyrosine antibody (1.7 μ g/lane) coupled to Protein A-Sepharose was added for 2 h at 4 °C. The immunoprecipitated proteins were separated under reducing conditions on 7.5%-acrylamide resolving gels and autoradiographed.

appears to be specific for PTPases [17,21]. Therefore, we examined the effect of such agents on the activity of membrane-associated phosphatase fractions prepared from rat liver and glioma cells. As shown in Table 2, the PTPase activities tested for their ability to dephosphorylate the IGF-I receptor were totally inhibited by micromolar concentrations of zinc and vanadate. By contrast, the effect of EDTA to inhibit the PTPase activity varied according to the origin of phosphatases. The residual phosphorylation of the IGF-I receptor in the presence of 100 mM-EDTA was 25 % with rat liver and 95 % with glioma-cell phosphatase preparations. The known absolute dependency on thiol compounds for full activity of PTPases was analysed by studying the effects of β -mercaptoethanol in the phosphatase assay buffer. We observed that this compound could be omitted from the phos-

Table 3. Effect of PTPases on human IGF-I receptor kinase activity

The tyrosine kinase activity and the autophosphorylation of the WGA-purified IGF-I receptor were compared in the absence or presence of increasing phosphatase concentrations from rat liver. For poly(Glu-Tyr) phosphorylation, the phosphorylated receptors were incubated for 30 min in buffer alone or in the presence of phosphatases to allow dephosphorylation. The phosphorylation of the exogenous substrate poly(Glu-Tyr) was carried out for 30 min at 22 °C. Data are expressed as ³²P incorporated into the substrate and represent means of two separate experiments performed in triplicate. For autophosphorylation, the receptors were stimulated for 90 min at 4 °C with 0.1 μM-IGF-I. Phosphatases were added and incubated for 30 min at 22 °C. After immunoprecipitation with anti-phosphotyrosine antibody, the phosphorylated proteins were separated by SDS/PAGE under reducing conditions followed by autoradiography. The labelled bands corresponding to the receptor β subunit were cut out of the dried gel and counted for radioactivity. Data, presented as ³²P c.p.m. incorporated into the β subunit, represent values obtained in a typical experiment reproduced at least three times.

IGF-I receptor incubation condition	Kinase activity (c.p.m. incorporated)	
	Into poly(Glu-Tyr) (4:1)	Into β subunit
No IGF-I	62000	6500
With IGF-I		
No PTPases	100000	16000
With PTPases		
10 m-units	45000	7400
50 m-units	27000	3300
100 m-units	15000	1500

phatase assay buffer provided that it was present during the purification procedure. In this regard, we found that extracts of phosphatases prepared in the absence of dithiothreitol or β-mercaptoethanol displayed a 85% decrease in their ability to dephosphorylate the IGF-I receptor (results not shown).

Assessment of receptor dephosphorylation by use of unlabelled or ³²P-labelled ATP. The pattern of IGF-I-receptor dephosphorylation studied with the immuno-enzymic assay was compared with autophosphorylated receptors analysed by SDS/PAGE (Fig. 2). Partially purified receptors were stimulated by IGF-I and phosphorylated either with unlabelled ATP or with [γ-³²P]ATP. They were then incubated with the anti-phosphotyrosine antibody and processed for analysis by the immuno-enzymic assay or SDS/PAGE (see the Materials and methods section). Maximal extent of receptor dephosphorylation was reached with 100 m-units of phosphatase from rat liver and 25 m-units from human glioma cells. With the immuno-enzymic assay, the ED₅₀ for dephosphorylation was obtained with 20 m-units of rat liver membrane-associated phosphatase and only 10 m-units of the glioma-cell preparation.

Effect of PTPases on IGF-I-receptor kinase activity

We next investigated the effects of PTPases on IGF-I receptor activity assessed by phosphorylation/dephosphorylation reactions. The receptor kinase activity was evaluated by the extent of phosphorylation of the exogenous substrate poly(Glu-Tyr) (4:1) and receptor autophosphorylation (Table 3). We observed a diminution of the kinase activity, evidenced by a dose-dependent relationship of the incorporation of ³²P into poly(Glu-Tyr) (4:1). The ED₅₀ was achieved with 10 m-units of phosphatase prepared from glioma cells. The same phosphatase preparation was tested for its ability to modulate IGF-I-receptor autophosphorylation. IGF-I receptors stimulated *in vitro* were autophosphorylated and immunoprecipitated with anti-phos-

Fig. 3. Analysis of the human IGF-I-receptor dephosphorylation by SDS/PAGE

WGA-purified human IGF-I receptors (3 μg of protein/lane) were stimulated for 90 min at 4 °C in the absence (lane a) or presence (lanes b and c) of 0.1 μM unlabelled IGF-I, and autophosphorylated with 25 μM-[γ-³²P]ATP, 4 mM-MnCl₂ and 8 mM-MgCl₂ for 30 min at 22 °C. The receptor solution was then incubated in the absence or presence (lane c) of 100 m-units of rat liver phosphatases (P-ases). The dephosphorylation reaction was stopped by addition of Laemmli sample buffer, and the phosphoproteins were separated under reducing conditions on 5–15%-acrylamide-gradient resolving gels. Lane d corresponds to P-ases incubated with the same phosphorylation mixture in the absence of IGF-I receptor.

photyrosine antibodies before electrophoretic separation by SDS/PAGE. A decrease in receptor autophosphorylation was observed, which was characterized by a dose-dependent relationship of ³²P incorporation into the β-subunit for concentrations of phosphatases between 10 and 100 m-units.

Amino acid analysis of the dephosphorylated IGF-I receptor

To establish the specificity of the receptor dephosphorylation on tyrosine, the capacity of liver phosphatases to dephosphorylate the human IGF-I receptor was examined by gel electrophoresis and phosphoamino acid analysis of the β subunit. In the presence of IGF-I, the autophosphorylation of a 98 kDa protein was stimulated 2–3-fold (Fig. 3, lanes a and b). This band was believed to correspond to the β chain of the IGF-I receptor, since it was also precipitated by antibody αIR3 (results not shown). The signal was decreased by 60% after the addition of phosphatases (lane c). The large number of radioactive bands appearing in lanes c and d (compared with lane b) correspond to proteins present in the phosphatase preparation phosphorylated in presence of the phosphorylation buffer containing [γ-³²P]ATP. The disappearance of these additional bands after immunoprecipitation with an anti-phosphotyrosine antibody (see Fig. 2b) indicates that these proteins were phosphorylated mainly on threonine and serine residues. Examination of the gel revealed that no additional radiolabelled bands appeared when the receptor was incubated in the presence of phosphatases (lane c) compared with phosphatases added in phosphorylation buffer

Fig. 4. Phosphoamino acid analysis of the dephosphorylated IGF-I receptor

After gel electrophoresis of WGA-purified IGF-I receptors incubated in the absence or presence of phosphatases and autoradiography, the portions of the gels corresponding to the β subunit (95–97 kDa) of the receptors were excised and subjected to phosphoamino acid analysis as described in the Materials and methods section. Lanes A and B correspond to IGF-I receptors incubated in the absence of phosphatases. Lanes C and D correspond to receptors incubated in the presence of 100 m-units of rat liver phosphatases (P-ases). Lane D was included as a control and contained phosphatase preparation phosphorylated in the absence of IGF-I receptor (IGF-IR).

Fig. 5. Re-phosphorylation of the dephosphorylated IGF-I receptor

Partially purified IGF-I receptors were stimulated for 90 min at 4 °C in the presence of 0.1 μ M-IGF-I and autophosphorylated with 25 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min at 22 °C. The autophosphorylated receptors were incubated in the absence or presence of phosphatases (P-ases; 100 m-units) for 30 min at 22 °C to allow dephosphorylation. At the end of the dephosphorylation process, vanadate (200 μ M) was added for 10 or 30 min to the whole receptor solution, and the reaction was stopped by addition of Laemmli sample buffer. The phosphorylated proteins were separated by SDS/PAGE under reducing conditions on 7.5%-acrylamide resolving gels and subjected to autoradiography.

Fig. 6. Assessment of dephosphorylation of receptor tyrosine kinases by PTPases

Dephosphorylation of the IGF-I receptors (■; IGF-I-R) by PTPases was compared with that of insulin (●; Insulin-R) and epidermal growth factor receptors (△; EGF-R); by the immuno-enzymic assay. Autophosphorylated receptors (250 ng of protein) were coated overnight on microtitre plates. They were incubated in the presence of increasing concentrations of glioma-cell phosphatases for 30 min at 22 °C. Results are expressed as percentage receptor phosphorylation compared with controls (autophosphorylated receptor incubated in the absence of phosphatases). Values are means $\pm$ S.E.M. of four experiments.

without receptor (lane d). This indicates that potential proteolysis by contaminating proteinases contained in our preparation was unlikely to occur. After autoradiography, the portions of the gels corresponding to the IGF-I receptor β subunit were excised and submitted to amino acid analysis. As observed in Fig. 4, in the basal state (lane A) the WGA-purified receptor was already labelled predominantly on tyrosine residues. Stimulation by 0.1 μ M-IGF-I (lane B), increased by 2–3-fold the signal corresponding to phosphotyrosine residues and induced phosphoserine as well as phosphothreonine labelling. A 30 min incubation in the presence of rat liver phosphatases (lane C) decreased almost totally the phosphotyrosine and phosphoserine signals, with a smaller diminution in phosphothreonine content. A sample containing phosphatases phosphorylated with $[\text{P}^{32}]\text{ATP}$ in the absence of receptor was run as a control (lane D), and showed only a faint phosphothreonine phosphorylation, with no evidence of phosphotyrosine. This eliminates the possibility of the presence of proteins phosphorylated on tyrosine being able to contaminate our phosphatase preparation.

Re-phosphorylation of the dephosphorylated receptor

Having demonstrated that the IGF-I receptor can be dephosphorylated on tyrosine residues by membrane-associated PTPases, we investigated whether this dephosphorylated form could be activated again, i.e. re-phosphorylated. The results are presented in Fig. 5. After incubation of the IGF-I receptor with phosphatases leading to a 70% decrease of autophosphorylation, the receptor could be phosphorylated anew by addition of vanadate to the dephosphorylated receptor solution. The extent of re-phosphorylation was 30% after 10 min and 110% after 30 min incubation in the presence of vanadate. These data

demonstrate that the phosphorylation/dephosphorylation status of the IGF-I receptor can be readily modified *in vitro* and that vanadate is able to re-augment the IGF-I receptor phosphorylation.

Comparative studies of the dephosphorylation of several receptor tyrosine kinases

We have looked at the ability of a membrane-associated fraction of rat liver PTPases to dephosphorylate three different receptor tyrosine kinases on tyrosine residues. As shown on Fig. 6, the IGF-I, insulin and epidermal growth factor receptors were dephosphorylated to 50 % of control values by using 30 m-units of phosphatase activity. With this type of tissue phosphatase preparation, the maximal extent of receptor dephosphorylation, i.e. $79 \pm 4\%$ for the insulin receptor and $90 \pm 6\%$ for the epidermal growth factor receptor, was obtained with a 10-fold higher concentration of phosphatases. These data confirm that the non-radioisotopic immuno-enzymic method that we have developed to characterize the dephosphorylation of the IGF-I receptor can be used successfully to assess the dephosphorylation of other autophosphorylated receptor tyrosine kinases.

DISCUSSION

Protein kinases and phosphatases, by modulating the phosphorylation level of several proteins on serine, threonine and tyrosine residues, regulate a great number of cellular events, such as metabolic pathways, cell division and fertilization processes [38]. Biochemical and molecular characterization of several enzymes of the PTPase family [21,22,32,36] has led to new insights in the study of the regulation of receptor tyrosine kinases. For instance, it has been shown that the intrinsic activity of the insulin receptor is enhanced by autophosphorylation and attenuated by PTPases that dephosphorylate tyrosine residues [25]. A recent study has provided evidence that the fully activated insulin receptor, i.e. phosphorylated on the three closely spaced tyrosine residues 1146, 1150 and 1151, becomes highly sensitive to dephosphorylation by membrane fractions of rat liver PTPases [39].

In view of the high degree of structural and functional similarity of the insulin and IGF-I receptors, we were interested in characterizing cellular PTPase activities with effects on the IGF-I receptor. Over the past 10 years the evaluation of receptor tyrosine kinase dephosphorylation was mostly performed with synthetic peptides or artificial proteins, because of the lack of adequately purified biological substrates [19,40,41]. In the present study, problems inherent in the use of artificial substrates were avoided by taking advantage of intact receptors obtained from NIH 3T3 cells transfected with a plasmid encoding the human IGF-I receptor. In addition, most of the assays developed so far involved the use of radioisotopes to measure the final release of [^{32}P]P_i, which could correspond to the hydrolysis of either phosphotyrosine or phosphoserine residues. These shortcomings are greatly improved in our immuno-enzymic assay based on the e.l.i.s.a. technique and the specific recognition by anti-phosphotyrosine antibodies of the remaining phosphotyrosine residues of intact IGF-I receptors (Fig. 1).

The first evidence of the existence of a phosphotyrosine-dephosphorylation activity was provided by the study of Ushiro & Cohen [42] in A431 cells. PTPase activities have now been identified in a large number of eukaryotic tissues and cell lines, in both soluble and particulate fractions [17]. Using IGF-I receptors as substrates and phosphatase preparations obtained from different tissues and cell lines as sources of PTPases, we observed that the ratio of particulate to cytosolic activity ranged from 2 to 5 depending on the origin of phosphatases (Table 1).

These data are consistent with several observations [18,20,40], showing that PTPase activity towards the insulin receptor is 3–4-fold higher in membrane-associated than in cytosolic fractions. On the basis of these findings, the present study was performed with membrane-associated phosphatase fractions. Besides their cellular localization, the IGF-I-related PTPases exhibit differences in activity as a function of their origin. In general, cellular PTPases appeared 20–25 % more active than the enzymes prepared from tissue extracts, suggesting that distinct PTPases might exist in the individual preparations and that each one could have its own specificity towards different phosphorylation sites within the IGF-I receptor. In addition, the elevated phosphatase activity observed in glioma cells raises the possibility that PTPase activities towards the IGF-I receptor might be modified by malignant transformation. This would agree with data obtained in transformed cells showing that a constitutive kinase activity of the EGF receptor might be associated with an increase in tyrosine phosphatase activities [14,17]. The present findings also show that large variations in IGF-I binding activity between the different cells and tissues examined are not likely to interfere with the dephosphorylation process of the receptor (Table 1).

The inhibition profile of rat liver and glioma-cell phosphatase preparations by zinc and vanadate indicates that they are both inhibited to the same extent by these compounds (Table 2). Similar to data obtained with most PTPases purified so far [21,43,44,45] the present results contribute to establishing that the crude phosphatase preparations contain genuine PTPase activity. The stimulatory effect of EDTA at low concentration, i.e. 5 mM, also accords with previously published data [21,43]. At higher concentrations (50–100 mM), the different degree of inhibition observed for glioma cells and rat liver could result from cell specificity. In accordance with previous data, the strong dependency on thiol-containing compounds of our phosphatase extracts also strengthens the finding that tyrosine phosphatase activity is present in our preparations [19].

The characterization of IGF-I-receptor dephosphorylation by two methods based on the use of anti-phosphotyrosine antibodies demonstrates that the receptor is dephosphorylated on tyrosine residues. The dephosphorylation on tyrosine residues was further strengthened by amino acid analysis of the IGF-I-receptor β subunit (Fig. 4). Altogether, these results point out that the different phosphatase preparations used in these studies contain one or more tyrosine phosphatases, which have effect on the IGF-I receptor. In addition, these preparations might also contain some serine and threonine phosphatase activities which have been shown to represent distinct classes of enzymes [32,46]. A unique advantage of our immuno-enzymic assay is that such activities do not interfere with the quantification of receptor dephosphorylation on tyrosine residues.

The effects of phosphatases on the kinase activity of the IGF-I receptor were investigated to understand better the process of receptor dephosphorylation. The kinase activity towards the exogenous substrate poly(Glu-Tyr) (4:1) and the autophosphorylation were decreased after incubation of the receptors in the presence of phosphatases prepared from glioma cells (Table 3). These data provide the first evidence that the dephosphorylation of the IGF-I receptor is associated with a decrease in its intrinsic kinase activity. To the best of our knowledge, the dephosphorylation of the IGF-I receptor has not been investigated in detail, except for one study which mentions that autophosphorylation of the IGF-I receptor could be attenuated in the presence of placental phosphatases [31]. Our observation that the inhibition of the kinase activity follows the same pattern of inhibition as that of autophosphorylation suggests that dephosphorylation of the IGF-I receptor results in

a decrease in the receptor activity. These findings are in agreement with data concerning the mechanisms of dephosphorylation/deactivation of the insulin receptor [39]. It is therefore tempting to speculate that comparable mechanisms might be involved in the dephosphorylation process of both insulin and IGF-I receptors. The finding that, after partial inhibition, the intrinsic kinase activity of the IGF-I receptor can be activated again through the addition of vanadate (Table 3, Fig. 5) indicates that *in vitro* the action of PTPases is reversible. This is compatible with the possibility that activation/deactivation of the IGF-I receptor modulated by its state of phosphorylation plays a role in the overall regulation of IGF-I receptor activity. Finally, we would like to emphasize that the present study has been carried out with a non-radioactive immuno-enzymic assay which appears to be suitable to assess tyrosine dephosphorylation of different substrates. As shown in Fig. 6, this quantitative and highly sensitive assay can be used successfully to determine the level of dephosphorylation of several receptor tyrosine kinases by PTPases. Our assay might serve as a powerful tool in future studies of dephosphorylation of IGF-I or other receptor tyrosine kinases.

In conclusion, this study provides the first evidence that the human IGF-I receptor can be dephosphorylated *in vitro* by PTPase contained in membrane-associated fractions from various tissues and cell types. This dephosphorylation process is associated with a decrease in the kinase activity of the receptor, which can be re-augmented through the action of vanadate. Taken as a whole, the present results support the view that different PTPases regulate the phosphorylation state of the IGF-I receptor. To understand better the phosphorylation/dephosphorylation reactions implicated in the mechanism of IGF-I action, potential modifications of PTPase activity directed against the IGF-I receptor under various physiological conditions could be investigated.

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