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Effect of Tumor Necrosis Factor-α on the Phosphorylation of Tyrosine Kinase Receptors Is Associated With Dynamic Alterations in Specific Protein-Tyrosine Phosphatases

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Abstract Tumor necrosis factor-α (TNF-α) can modulate the signalling capacity of tyrosine kinase receptors; in particular, TNF-α has been shown to mediate the insulin resistance associated with animal models of obesity and noninsulin-dependent diabetes mellitus. In order to determine whether the effects of TNF-α might involve alterations in the expression of specific protein-tyrosine phosphatases (PTPases) that have been implicated in the regulation of growth factor receptor signalling, KRC-7 rat hepatoma cells were treated with TNF-α, and changes in overall tissue PTPase activity and the abundance of three major hepatic PTPases (LAR, PTP1B, and SH-PTP2) were measured in addition to effects of TNF-α on ligand-stimulated autophosphorylation of insulin and epidermal growth factor (EGF) receptors and insulin-stimulated insulin receptor substrate-1 (IRS-1) phosphorylation. TNF-α caused a dose-dependent decrease in insulin-stimulated IRS-1 phosphorylation and EGF-stimulated receptor autophosphorylation to 47–50% of control. Overall PTPase activity in the cytosol fraction did not change with TNF-α treatment, and PTPase activity in the particulate fraction was decreased by 55–66%, demonstrating that increases in total cellular PTPase activity did not account for the observed alterations in receptor signalling. However, immunoblot analysis showed that TNF-α treatment resulted in a 2.5-fold increase in the abundance of SH-PTP2, a 49% decrease in the transmembrane PTPase LAR, and no evident change in the expression of PTP1B. These data suggest that at least part of the TNF-α effect on pathways of reversible tyrosine phosphorylation may be exerted through the dynamic modulation of the expression of specific PTPases. Since SH-PTP2 has been shown to interact directly with both the EGF receptor and IRS-1, increased abundance of this PTPase may mediate the TNF-α effect to inhibit signalling through these proteins. Furthermore, decreased abundance of the LAR PTPase, which has been implicated in the regulation of insulin receptor phosphorylation, may account for the less marked effect of TNF-α on the autophosphorylation state of the insulin receptor while postreceptor actions of insulin are inhibited. J. Cell. Biochem. 64:117–127.

Key words: insulin receptor; epidermal growth factor receptor; insulin resistance; tyrosine phosphorylation; TNF-α

With recent advances in our understanding of reversible tyrosine phosphorylation in growth factor signalling, interest has developed in further characterizing how these pathways are regulated in normal physiology and also in identifying the mechanisms of the cellular and molecular defects that lead to the alterations of these signalling systems in disease states. One of the most intensively studied examples of this problem is the pathological resistance to the biological actions of insulin that is commonly observed in the general population with Type II diabetes and related syndromes [1]. In particular, the relationship between obesity and the development of insulin resistance is widely appreciated, but the underlying mechanism is poorly understood [2,3].

Recently, tumor necrosis factor (TNF)-α has been shown to be associated with the insulin resistance of obesity and noninsulin-dependent diabetes mellitus in animal models and in human subjects [reviewed in 4]. Administration of

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TNF-α to rats has profound physiological effects in the body by altering lipid and protein metabolism in insulin-sensitive tissues, and it has been shown to impair insulin action on peripheral glucose disposal and hepatic glucose output [5–8]. Cellular studies have shown that exposure of 3T3-L1 adipocytes to 5 nM TNF-α blocks insulin-stimulated hexose uptake due to the suppression of glucose transporters GLUT4 and GLUT1 [9]. The mechanism of action of TNF-α is unknown, although it can initiate a cascade of signal transduction that includes the activation of phosphoprotein phosphatases as well as kinases A and C and MAP kinase [10–13] and possibly additional effects that result in alterations of the phosphorylation state of multiple cell proteins. Reversible tyrosine phosphorylation of signalling proteins in the action of insulin and other growth factors has been shown to be modulated by the effects of TNF-α in adipose tissue and hepatoma cells [14–17], which may be part of a paracrine signalling loop as TNF-α is synthesized locally in adipose cells and increases in insulin-resistant states [18–20]. The mechanism for the modulation of insulin receptor kinase activity, thought to be of central importance to the action of TNF-α on insulin signalling, is not known with certainty. The available data supports a hypothesis that TNF-α effects on the insulin receptor are mediated by the activation of a serine kinase that phosphorylates IRS-1, and possibly also the insulin receptor itself, resulting in an attenuation of receptor kinase activity and reduced tyrosine phosphorylation of IRS-1, which effectively blocks postreceptor insulin signalling [17].

One potential mechanism for the action of TNF-α on insulin signal transduction which has not been explored in detail involves modulation of cellular protein-tyrosine phosphatases (PTPases) that have been shown in recent studies to regulate the activation state of the insulin receptor and its endogenous substrate proteins, such as IRS-1 [21,22]. By dephosphorylating the regulatory domain of the insulin receptor, PTPases can attenuate the catalytic activity of the receptor tyrosine kinase [23]. In addition, PTPases can modulate postreceptor signalling by dephosphorylating the phosphotyrosyl form of cellular substrate proteins for the insulin receptor such as IRS-1 and Shc, effectively blocking their ability to complex with src homology-2 (SH2) domain-containing downstream enzymes and preventing their activation [24]. Work in our laboratory and others has implicated the tandem domain transmembrane PTPase called leukocyte common antigen-related, or LAR [25], and the intracellular single domain enzymes PTP1B [26] and SH-PTP2 [27,28] as candidate PTPases for the regulation of insulin action pathway [reviewed in 22]. In particular, recent studies have shown that manipulation of the abundance or activity of LAR or PTP1B in situ has direct effects on insulin signalling in intact cells [29,30], and blocking the activity of SH-PTP2 or preventing its complex formation with endogenous proteins can attenuate mitogenic signalling by insulin [31–34].

In order to determine whether the effect of TNF-α to induce insulin resistance might also be mediated by alterations in cellular PTPase activities, we measured overall PTPase activity as well as the abundance of candidate PTPases that impact on the insulin signalling system in well-differentiated rat hepatoma cells treated with TNF-α. Our data suggest that at least part of the TNF-α effect on pathways of reversible tyrosine phosphorylation may be exerted through the dynamic modulation of the expression of specific PTPases.

EXPERIMENTAL PROCEDURES

Cell Culture

Rat hepatoma KRC-7 cells were kindly provided by Dr. John Koontz (University of Tennessee) and maintained in Dulbecco's modified Eagle's medium (GIBCO/BRL, Gaithersburg, MD) containing 10% fetal calf serum (Sigma, St. Louis, MO) at 37°C in an atmosphere of 5% CO_2 in air. Cells were plated into six-well 35 mm diameter dishes and used at 70% confluence.

Measurement of the Effect of TNF-α on Cell Growth

Cells were plated into 35 mm diameter dishes at a density of 3×10^5 cells/well and cultured overnight. Various concentrations of TNF-α were then added, and the cells were cultured for an additional 72 h before the cells were released by trypsinization, resuspended in culture medium, and the cell number determined by counting on a hemacytometer. Cell viability after incubation with TNF-α for a period of 1–2 h was determined by exclusion of Trypan blue dye (0.4% v/v) (Sigma).

Treatment of Cells With TNF-α and Preparation of Cell Extracts

Hepatoma cells were incubated in culture medium with murine TNF-α (R&D Systems, Minneapolis, MN) for the indicated time intervals and then stimulated with 100 nM insulin or epidermal growth factor (EGF) (Sigma). For Western blot analysis, the medium was removed, and the cells were rapidly frozen in liquid nitrogen and solubilized into 0.25 ml/dish of 50 mM 4-(2-hydroxyethyl-1-piperazine) ethanesulfonic acid (HEPES) buffer, pH 7.6, containing 150 mM sucrose, 2 mM sodium orthovandate, 80 mM α-glycerophosphate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium [ethylene-bis(oxyethylenenitrilo)] tetraacetic acid (EGTA), 2 mM sodium ethylenediamine tetraacetic acid (EDTA), 1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate (SDS), and protease inhibitors including 1 mM phenylmethylsulfonylfluoride (PMSF), 10 µg/ml aprotinin, and 10 µg/ml leupeptin. The solubilized cell material was centrifuged at 12,000*g* for 10 min. Aliquots of the supernatant were assayed for protein content by the method of Bradford [35], and equivalent amounts of protein were loaded onto gels in sample buffer and subjected to electrophoresis in gels containing SDS and 7.5% (w/v) polyacrylamide [36].

For preparation of cytosol and particulate cell fractions for the PTPase enzyme assays, the washed cells were scraped into ice-cold 50 mM HEPES buffer, pH 7.6, containing 150 mM sucrose, 2 mM sodium EDTA, and protease inhibitors including 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, briefly homogenized, and then centrifuged at 1,000*g* for 5 min at 4°C to remove nuclei and cell debris. The supernatant was centrifuged at 100,000*g* for 1 h at 4°C, and the supernatant was taken as the cytosol fraction. The pellet was then solubilized in homogenization buffer containing 1% (v/v) Triton X-100 and recentrifuged at 15,000*g* for 15 min, and the supernatant was taken as the solubilized particulate fraction.

Immunoblotting

Following electrophoresis, proteins were transferred to nitrocellulose filters (0.45 µ pore size) at 100 V for 3 h in a buffer containing 20% (v/v) methanol in 25 mM tris(Hydroxymethyl)-aminomethane (Tris) base and 192 mM glycine at pH 8.3 [37]. Filters were incubated in a blocking buffer containing 150 mM NaCl, 0.05% (v/v) NP-40, 5% (w/v) bovine serum albumin, 1% (w/w) ovalbumin, 0.01% (w/v) sodium azide, and 10 mM Tris-HCl buffer, pH 7.4, with rocking for 1 h at room temperature. Depending on the experiment, an appropriate antibody was added (1.0 µg/ml for PTPase 1B, 0.5 µg/ml for LAR, 1.25 µg/ml for SH-PTP2, 0.8 µg/ml for phosphotyrosine, and 1 µg/ml for the insulin receptor), and rocking was continued for 2 h. Membranes were washed three times for 10 min with blocking buffer alone, followed by incubation with 2 µCi of ^{125}I-protein A (30 mCi/mg) (ICN Biomedicals Inc., Irvine, CA) for 1 h at room temperature, followed by three washes for 10 min each in 150 mM NaCl and 0.1% (v/v) Triton X-100 in 10 mM Tris-HCl buffer, pH 7.4. Immunoreactive proteins were visualized by direct phosphorimager analysis of the immunoblot (Molecular Dynamics, Sunnyvale, CA). Protein migration was calibrated with prestained molecular size standards (Bio-Rad, Melville, NY).

Polyclonal antiserum to the cytoplasmic domain of recombinant rat LAR was obtained by immunization of rabbits with LAR protein purified from a bacterial expression system [38], and the antibodies were affinity-purified using Affi-Gel (Bio-Rad) columns containing the immobilized purified LAR cytoplasmic domain [39]. Polyclonal antiserum to insulin receptors and EGF receptors as well as monoclonal antibodies to SH-PTP2 were obtained from Transduction Laboratories (Lexington, KY). After blotting with the monoclonal antibody, blots were incubated with 150 µl rabbit anti-mouse IgG (Sigma) in 10 ml of blotting buffer and then washed again prior to reaction of the immunoblot with labeled protein A. Polyclonal antibodies to PTP1B were obtained by immunization of rabbits with a peptide corresponding to amino acids 42–56 of the rat PTP1B sequence [40] conjugated to keyhole limpet hemocyanin and injected subcutaneously into rabbits [39]. Antibody reactive towards PTP1B was immunopurified by on an affinity column of recombinant full-length rat PTP1B [38] coupled to Affi-Gel [39].

EGF Receptor Immunoprecipitation

KRC-7 cells treated with TNF-α and controls were treated with 100 nM EGF for 5 mins and solubilized in the extraction buffer indicated

above. EGF receptor monoclonal antibody (4 µg) was added to 250 µg of cell lysate protein and incubated at 4°C for 1 h before 5 µg of rabbit anti-mouse IgG (Sigma) was added for an additional 30 min. The immunocomplexes were precipitated by incubating with 25 µl of 10% (v/v) Trisacryl-Protein A beads (Pierce, Rockford, IL) for 30 min. After pelleting the beads at 12,000*g* for 5 min, the samples were washed three times with cell extraction buffer and resolved on polyacrylamide gels followed by immunoblotting with phosphotyrosine antibody as described above.

Insulin Receptor Dephosphorylation

Partially purified insulin holoreceptors were obtained by wheat germ agglutinin-agarose chromatography [41] of solubilized plasma membranes from transfected Chinese Hamster Ovary cells overexpressing the recombinant human insulin receptor [23]. Aliquots of 4 mg protein were autophosphorylated in a 0.45 ml reaction containing 1 mM insulin, 5 mM $MnCl_2$, 0.1 mM ATP, 180 µCi of γ-[^{32}P]ATP (3,000 Ci/mmol) (Amersham, Arlington Heights, IL), and 0.1% (v/v) Triton X-100 in 50 mM HEPES buffer at pH 7.6 at 4°C for 120 min. Unincorporated [^{32}P]-ATP was removed by a Bio-Gel P6 spin column, and 25 µl aliquots of the labeled receptors were incubated with 40 µl of the cell lysates in a 125 µl reaction containing 1 mM dithiothreitol (DTT) and 2 mM EDTA in 50 mM HEPES, pH 7.6, at 30°C. The reactions were terminated by the addition of 0.5 ml of a chilled stop solution containing 10 mM ATP, 10 mM sodium pyrophosphate, 4 mM EDTA, 100 mM NaF, 2 mM sodium vanadate, 0.1 mg/ml aprotinin, and 2 mM PMSF in 50 mM HEPES buffer, pH 7.6. After boiling in gel sample buffer containing 100 mM DTT, samples were subjected to electrophoresis in gels containing sodium dodecyl sulfate and 7.5% polyacrylamide [36]. Dephosphorylation of the 95 kDa β-subunit of the insulin receptor was analyzed by direct phosphorimager analysis of the dried gel (Molecular Dynamics).

PTPase Assay With *p*NPP and Phosphotyrosyl Lysozyme

For the hydrolysis of *para*-nitrophenylphosphate (*p*NPP), aliquots of cell lysates were incubated in a final volume of 100 µl at 37°C for 10–30 min in reaction buffer containing 10 mM *p*NPP (Sigma) in 50 mM HEPES buffer, pH 7.0, containing 1 mM DTT and 2 mM EDTA. The reaction was stopped by the addition of 50 µl of 1 M NaOH, and the absorption was determined at 410 nm in a Dynatech microplate reader. A molar extinction coefficient of 1.78×10^4 M^{-1} cm^{-1} was used to calculate the concentration of *p*-nitrophenolate ion produced in the reaction [42].

For use as a PTPase substrate, reduced, carboxamidomethylated, maleyated lysozyme (RCM-lysozyme) (Sigma) was phosphorylated using partially purified rat liver insulin and EGF receptor kinases [43] and γ-[^{32}P-ATP] as described by Tonks et al. [44]. PTPase activity was assayed using 20 µl of PTPase preparation, diluted to less than 1 unit/ml in buffer, and preincubated for 5 min at 30°C. The reaction was initiated by the addition of 20 µl of 10 mM phosphotyrosyl RCM-lysozyme, and the reaction was terminated by the addition of 0.9 ml of acidic charcoal mixture (0.9 M NaCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4 and 4% (v/v) Norit A). After microcentrifugation, the amount of radioactivity in 0.4 ml of supernatant was measured by Cerenkov counting in a liquid scintillation counter. One unit of PTPase activity was defined as the amount releasing 1 nmole of phosphate per minute.

Data Analysis

The results from at least three separate experiments were used for quantitative data analysis, which is presented in graphical form as the mean ± standard error of the mean.

RESULTS

Effect of TNF-α on Cell Growth

Incubation of KRC-7 rat hepatoma cells with TNF-α over a period of 72 h slowed down the rate of cell division, resulting in a dose-dependent decrease in the number of cells per culture dish (Fig. 1). This cell line is sensitive to the antiproliferative effect of TNF-α, and the concentration of the cytokine required to inhibit cell division by 50% (IC_{50}) is estimated to be 0.4 nM. During the shorter incubation times of 1–2 h with TNF-α employed in the more acute treatment studies described below, there was no apparent loss of cell viability as estimated by exclusion of Trypan blue dye (data not shown).

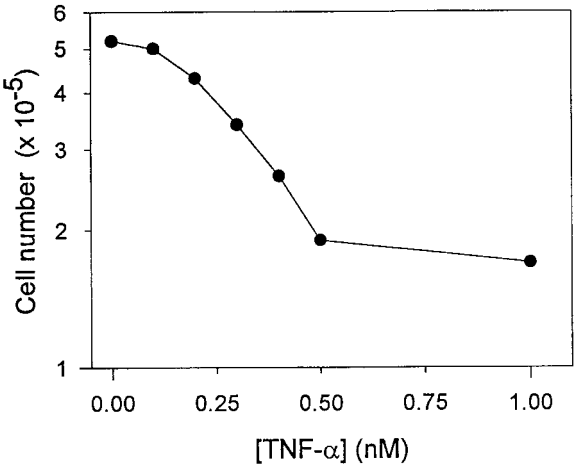

Fig. 1. Effect of TNF-α on the growth of KRC-7 rat hepatoma cells. After we incubated KRC-7 cells with the indicated concentration of TNF-α for 72 h, the cell number was determined by counting the resuspended cells from each well of a 35 mm six-well culture plate in a hemacytometer.

Effect of TNF-α on Tyrosine Phosphorylation of the Insulin Receptor and IRS-1

In order to determine the effect of TNF-α treatment on the insulin signalling system in KRC-7 hepatoma cells, the cells were treated with increasing doses of TNF-α for 1 h prior to assessment of insulin-stimulated receptor autophosphorylation and IRS-1 phosphorylation by immunoblotting with antibodies to phosphotyrosine. No change in the autophosphorylation of insulin receptors was found after incubation of cells with TNF-α at concentrations up to 2 nM (Fig. 2). No significant effect on insulin receptor abundance was observed during this time period as measured by immunoblotting with insulin receptor antibodies (data not shown). However, under these conditions, TNF-α treatment lead to a 49% decrease in the tyrosine phosphorylation of IRS-1 (Fig. 2). These results suggested that the effect of TNF-α on insulin signalling in the hepatoma cells was mediated primarily by effects on insulin receptor kinase activity or by altering the ability of IRS-1 to be tyrosine-phosphorylated in response to insulin.

Effect of TNF-α on Epidermal Growth Factor Receptor Autophosphorylation

To evaluate whether the effect of TNF-α was specific for insulin signalling, we also measured the effect of 1 h exposure to TNF-α on tyrosine autophosphorylation of the EGF receptor in the

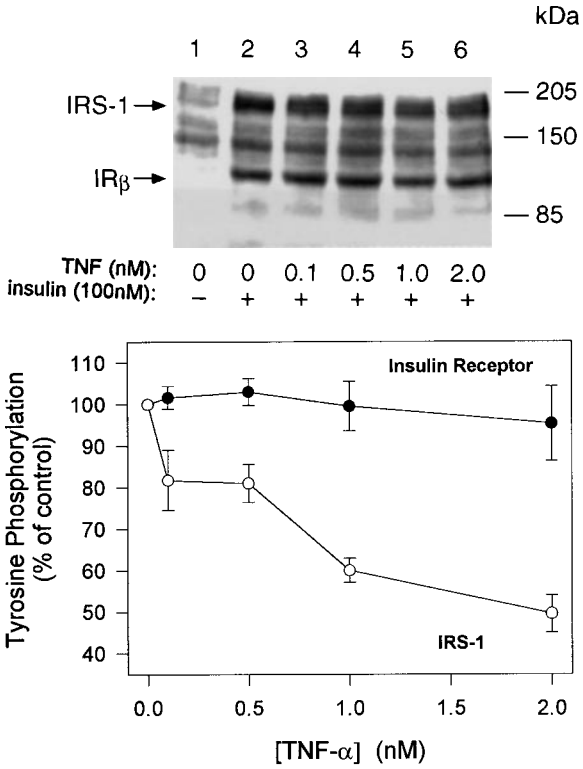

Fig. 2. Effect of TNF-α on insulin-stimulated autophosphorylation of the insulin receptor and IRS-1 in KRC-7 hepatoma cells. **above:** Antiphosphotyrosine immunoblot of hepatoma cell extracts following treatment with the indicated concentration of TNF-α for 1 h followed by stimulation with 100 nM insulin for 1 min (*lanes 2–6*). Migration of the insulin receptor β-subunit (IR-β) and IRS-1 was visualized by phosphorimager analysis after incubation of the immunoblot with ^{125}I-protein A. Protein migration was calibrated with prestained molecular size standards. **below:** Quantitation of insulin-stimulated autophosphorylation of the insulin receptor and IRS-1 by phosphorimager analysis of hepatoma cells treated with TNF-α and stimulated with insulin as described above.

KRC-7 cells (Fig. 3). Treatment with TNF-α resulted in a dose-dependent decrease in EGF-stimulated receptor autophosphorylation to 47% of control at 1 nM, indicating that the cellular effects of this cytokine are not limited to the insulin signalling system but also involve the inhibition of tyrosine kinase activity by other growth factor receptors.

PTPase Enzyme Activity in Subcellular Tissue Fractions

To evaluate the hypothesis that TNF-α might disrupt tyrosine kinase signalling through the modulation of cellular PTPase enzymes, we measured PTPase activity in cytosolic and particulate subcellular fractions of TNF-α–treated

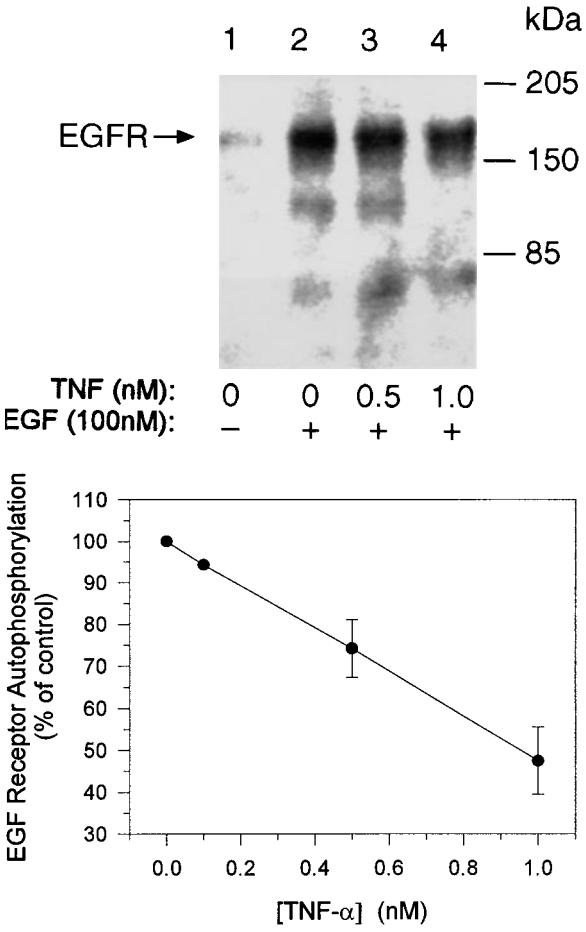

Fig. 3. Effect of TNF-α on EGF-stimulated autophosphorylation of the EGF receptor in KRC-7 hepatoma cells. **above:** Antiphosphotyrosine immunoblot of hepatoma cell extracts following treatment with the indicated concentration of TNF-α for 1 h followed by stimulation with 100 nM EGF for 1 min and immunoprecipitation with anti-EGF receptor antiserum (*lanes 2–4*). Migration of the ~170 kDa EGF receptor was visualized by phosphorimager analysis after incubation of the immunoblot with ^{125}I-protein A. Protein migration was calibrated with prestained molecular size standards. **below:** Quantitation of EGF-stimulated receptor autophosphorylation by phosphorimager analysis of immunoblots represented by the image shown in A.

hepatoma cells using various PTPase substrates (Fig. 4). In addition to the using the autophosphorylated insulin receptor kinase domain as a physiologically relevant substrate, the dephosphorylation of derivatized lysozyme as well as the hydrolysis of *p*NPP was evaluated. Following treatment with 2 nM TNF-α for 1 h, there was no significant change in the overall PTPase activity in the cytosol fraction with any of the substrates, while the solubilized particulate fraction demonstrated a significant 55–66% decrease in PTPase activity, depending on the substrate.

Abundance of Specific PTPases in TNF-α–Treated KRC-7 Cells

Although liver cells contain a variety of PTPases, the modulation of individual PTPase enzymes may have important effects on specific signal transduction pathways. Since the PTPases LAR, PTP1B, and SH-PTP2 are major PTPases in liver cells and have been implicated in insulin signalling, we measured the abundance of these enzymes in hepatoma cells treated with TNF-α to investigate whether the insulin resistance induced by TNF-α might be associated with changes in the level of the protein mass of these individual enzymes.

Immunoblotting of cell extracts with an antibody to the LAR PTPase cytoplasmic domain revealed a single protein band of 85 kDa, representing the processed enzyme "P" subunit corresponding to the transmembrane and intracellular catalytic domains of LAR [45,46]. The abundance of the transmembrane PTPase LAR was significantly reduced in a dose-dependent fashion after a 1 h treatment of cells with TNF-α (Fig. 5). At 1 nM TNF-α, the abundance of LAR was reduced by 49% compared to untreated cells.

Using a monoclonal antibody, the 67 kDa SH-PTP2 protein was easily visualized in the cell extracts (Fig. 6). In contrast to the effect of TNF-α to decrease the abundance of the transmembrane PTPase LAR, the mass of SH-PTP2 was significantly increased to a maximum of 2.5-fold over the basal level in response to 2 nM TNF-α treatment for 1 h.

The intracellular PTPase, PTP1B, was quantitated by measuring the abundance of the full-length 50 kDa form found in the cell extracts (Fig. 7). Interestingly, the abundance of PTP1B protein was not significantly changed by treatment with TNF-α for 1 h up to a dose of 2 nM.

DISCUSSION

In the present work, we tested the hypothesis that TNF-α might modulate the signal transduction pathway of tyrosine kinase receptors by affecting cellular PTPase activity and/or the abundance of specific candidate PTPases that have recently been shown to be involved in the regulation of signalling through certain growth factors. The possibility of tyrosine phosphatase

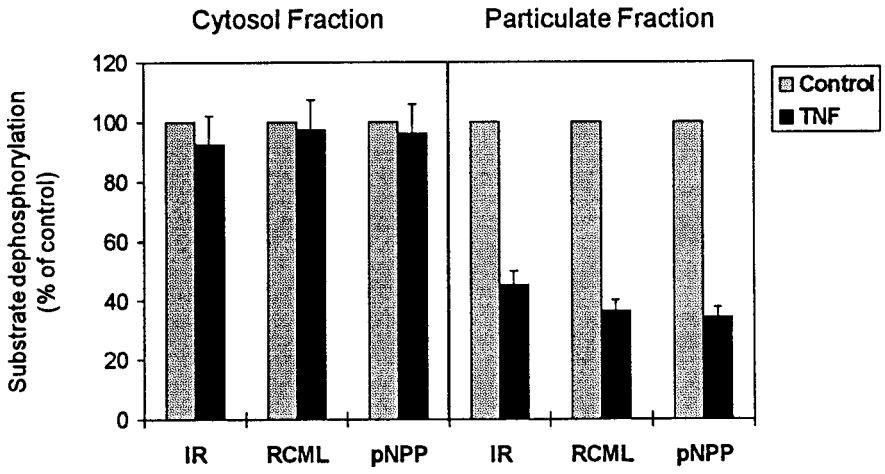

Fig. 4. Effect of TNF-α on PTPase enzyme activity in subcellular fractions of KRC-7 hepatoma cells. Following treatment of hepatoma cells with 2 nM TNF-α for 1 h, subcellular particulate and soluble (cytosol) cell extracts were prepared in the absence of PTPase inhibitors as described in Experimental Procedures. The results of PTPase assays for each subcellular fraction are shown for the substrates tested, including the intact recombinant human insulin receptor (IR), reduced, carboxamidomethylated, maleyated lysozyme (RCML), and *para*-nitrophenyl phosphate (pNPP).

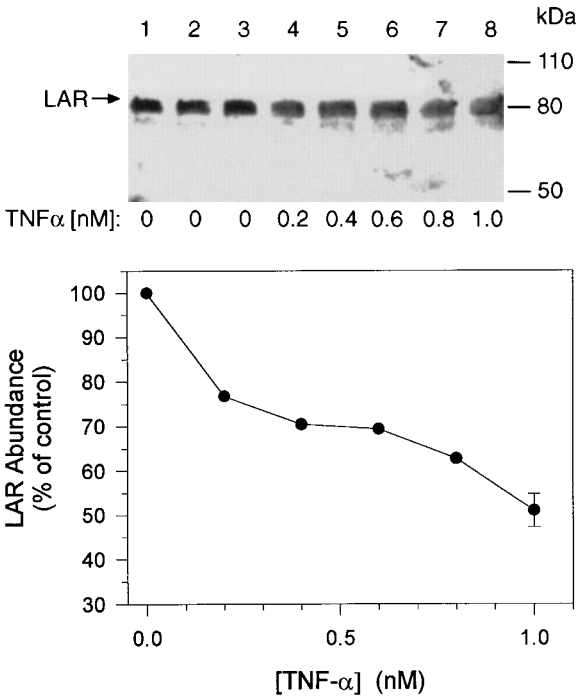

Fig. 5. Effect of TNF-α on the abundance of LAR in KRC-7 hepatoma cells. **above:** Anti-LAR immunoblot of hepatoma cell extracts following treatment with the indicated concentration of TNF-α for 1 h. Migration of the ~85 kDa LAR "P" subunit was visualized by phosphorimager analysis after incubation of the immunoblot with ^{125}I-protein A. Protein migration was calibrated with prestained molecular size standards. **below:** Quantitation of LAR abundance by phosphorimager analysis of immunoblots represented by the image shown in A.

involvement in the mechanism of action of TNF-α was first suggested by Totpal et al. [47], who demonstrated that the antiproliferative effects of TNF-α on a murine connective tissue cell line were blocked by orthovanadate, an inhibitor of PTPases.

Initially, we demonstrated that TNF-α inhibited the viability of cultured hepatoma cells, an effect that was associated with alterations in the tyrosine phosphorylation state of the insulin receptor and its major cellular substrate. There was a more significant decrease in the insulin-stimulated tyrosine phosphorylation of IRS-1 than that of the insulin receptor itself, in agreement with published work [15,16]. Also, in previous work, the inhibitory effects of TNF-α on insulin receptor and IRS-1 phosphorylation were associated with reduced insulin stimulation of biological effects [15]. We also found, interestingly, that ligand-stimulated EGF receptor autophosphorylation was significantly inhibited by TNF-α treatment in the hepatoma cells, suggesting that the inhibitory mechanism of the TNF-α effect was generalized to multiple signalling pathways involving reversible tyrosine phosphorylation. Previous reports in human tumor cells have shown a variety of TNF-α effects on the EGF receptor, including increased abundance of EGF receptor protein in choriocarcinoma cells [48] and an actual stimulation of EGF receptor kinase activity by TNF-α in cervi-

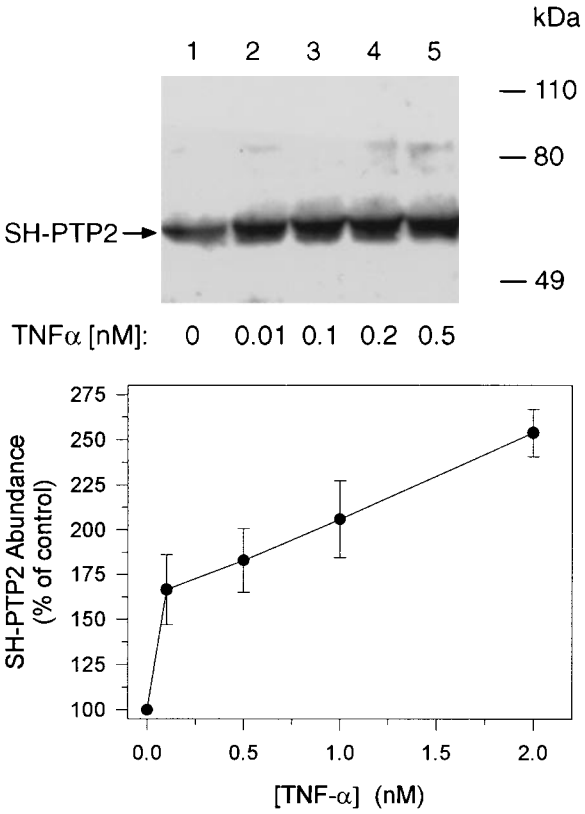

Fig. 6. Effect of TNF-α on the abundance of SH-PTP2 in KRC-7 hepatoma cells. **above:** Anti-SH-PTP2 immunoblot of hepatoma cell extracts following treatment with the indicated concentration of TNF-α for 1 h. Migration of the ~67 kDa SH-PTP2 protein was visualized by phosphorimager analysis after incubation of the immunoblot with ^{125}I-protein A. Protein migration was calibrated with prestained molecular size standards. **below:** Quantitation of SH-PTP2 abundance by phosphorimager analysis of immunoblots represented by the image shown in A.

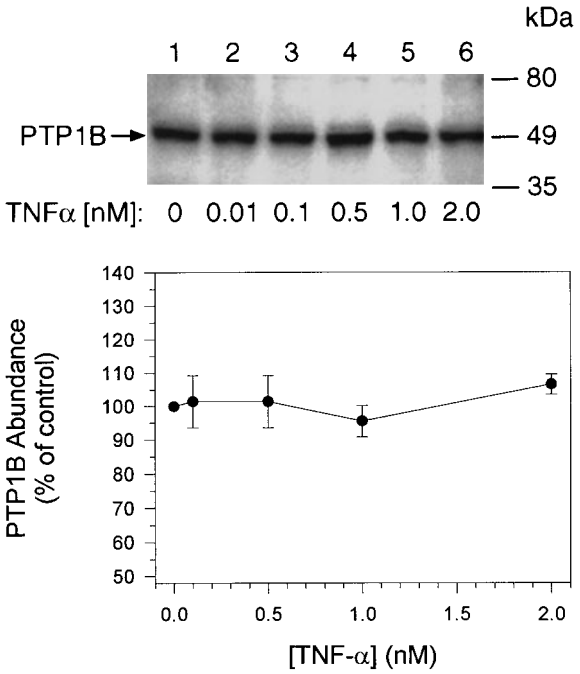

Fig. 7. Effect of TNF-α on the abundance of PTP1B in KRC-7 hepatoma cells. **above:** Anti-PTP1B immunoblot of hepatoma cell extracts following treatment with the indicated concentration of TNF-α for 1 h. Migration of the ~50 kDa PTP1B full-length protein was visualized by phosphorimager analysis after incubation of the immunoblot with ^{125}I-protein A. Protein migration was calibrated with prestained molecular size standards. **below:** Quantitation of PTP1B abundance by phosphorimager analysis of immunoblots represented by the image shown in A.

cal carcinoma cells [49,50], suggesting that cell-type or tissue-specific differences might exist for TNF-α action. Our results in the hepatoma cells that tyrosine phosphorylation of the EGF receptor was affected in addition to the insulin signalling pathway, however, suggested that a common mechanism for the influence of TNF-α might involve cellular PTPases, a possibility that has not been carefully evaluated in previous work in this area.

Following TNF-α treatment, there was no significant change in the cytosol fraction PTPase activity, and the solubilized particulate fraction was decreased by more than 50%. These results showed that the mechanism of the TNF-α effect on tyrosine kinase signalling does not involve a straightforward increase in the overall PTPase activity in the treated cells. The abundance of three candidate PTPases for the insulin action pathway was then examined. SH-PTP2 was found to be significantly increased. This PTPase has been shown to complex with autophosphorylated EGF receptors and tyrosine-phosphorylated IRS-1 and is capable of dephosphorylating both of these proteins in vitro [28,51,52]. Thus, it is possible that although total measured PTPase activity is unchanged or decreased, a specific increase in SH-PTP2 may contribute to the observed inhibition of EGF receptor and IRS-1 phosphorylation in the treated cells. With regard to its potential involvement in insulin signalling, the effects of SH-PTP2 have been perplexing. Blocking SH2 domain/phosphotyrosine complex formation of SH-PTP2 with its cellular substrates or transfection of a catalytically inactive mutant can block insulin signalling, although overexpression of the native SH-PTP2 is without effect on insulin action [31,32,53,54]. Further studies are necessary to decipher the cellular role of SH-PTP2 in the regulation of insulin action and the way the increase in this enzyme by TNF-α

treatment might mediate the inhibition of insulin signalling by this cytokine.

The abundance of the transmembrane PTPase LAR was significantly reduced after the treatment of KRC-7 cells with increasing concentration of TNF-α. As a candidate PTPase for the regulation of insulin signalling, LAR has been shown by Serra-Pagès et al. [55] to be largely found in the cell plasma membrane where the efficient dephosphorylation of insulin receptor occurs [56,57], and our previous work has demonstrated that the LAR cytoplasmic PTPase domain has a catalytic preference for the regulatory phosphotyrosines of the insulin receptor kinase domain in vitro [23]. Importantly, we have also recently demonstrated that reduction of LAR mass by overexpression of antisense mRNA in rat hepatoma cells leads to an amplification of insulin receptor and IRS-1 phosphorylation as well as enhanced postreceptor signal transduction, providing strong evidence that LAR is a physiological regulator of insulin action in intact cells. The decreased abundance of LAR protein associated with diminished PTPase enzyme activity in the cell particulate fraction may protect the autophosphorylation state of the insulin receptor, while IRS-1 and postreceptor actions of insulin are blocked.

In summary, our results suggest that complex changes in the abundance of specific PTPases may contribute to the cellular effects of TNF-α on signalling through growth factor receptors. The observed increase in abundance of SH-PTP2 may lead to accelerated dephosphorylation of the EGF receptor and IRS-1, while the decrease in LAR abundance may account for the relative lack of TNF-α effect on the phosphorylation state of the insulin receptor itself. Changes in the activity of additional specific tyrosine phosphatases, not measured in these studies, could also occur and play a role in the cellular effects of this cytokine to block insulin and EGF receptor signalling in the hepatoma cells. Unfortunately, the lack of suitable antibodies has prevented us from characterizing whether changes in the PTPase specific activity occur for the individual enzymes considered in this study.

The present work contributes to an increasingly complex picture of TNF-α effects on cells which can impact on metabolic regulatory pathways. As the mechanisms of TNF-α action in cells are elucidated, the primary and secondary effects of this cytokine on pathways of reversible tyrosine phosphorylation in cells will ultimately be determined. From the studies presented here, it is apparent that modulation of specific cellular tyrosine phosphatases accompanies the action of TNF-α. Because of the close connection between PTPases and reversible tyrosine phosphorylation in the insulin action pathway, alterations in PTPases may influence the degree of insulin resistance developed in target tissues in response to TNF-α and may also play a primary role in its mechanism of action.

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REFERENCES

1. Kahn CR (1994): Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes 43:1066–1084.
2. Caro JF (1991): Clinical review 26: Insulin resistance in obese and nonobese man. J Clin Endocrinol Metab 73:691–695.
3. Olefsky JM, Nolan JJ (1995): Insulin resistance and non-insulin-dependent diabetes mellitus: Cellular and molecular mechanisms. Am J Clin Nutr 61:S980–S986.
4. Hotamisligil GS, Spiegelman BM (1994): Tumor necrosis factor alpha: A key component of the obesity-diabetes link. Diabetes 43:1271–1278.
5. Fong Y, Lowry SF (1990): Tumor necrosis factor in the pathophysiology of infection and sepsis. Clin Immunol Immunopathol 55:157–170.
6. Feingold KR, Grunfeld C (1987): Tumor necrosis factor-a stimulates hepatic lipogenesis in the rat in vivo. J Clin Invest 80:184–190.
7. Van der Poll T, Romijn JA, Endert E, Borm JJ, Buller HR, Sauerwein HP (1991): Tumor necrosis factor mimics the metabolic response to acute infection in healthy humans. Am J Physiol 261:E457–E465.
8. Lang CH, Dobrescu C, Bagby GJ (1992): Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. Endocrinology 130:43–52.
9. Stephens JM, Carter BZ, Pekala PH, Malter JS (1992): Tumor necrosis factor a–induced glucose transporter (GLUT-1) mRNA stabilization in 3T3-L1 preadipocytes. Regulation by the adenosine-uridine binding factor. J Biol Chem 267:8336–8341.
10. Zhang YH, Lin JX, Yip YK, Vilcek J (1988): Enhancement of cAMP levels and of protein kinase activity by tumor necrosis factor and interleukin 1 in human fibroblasts: Role in the induction of interleukin 6. Proc Natl Acad Sci U S A 85:6802–6805.
11. Vilcek J, Lee TH (1991): Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. J Biol Chem 266:7313–7316.

12. Van Lint J, Agostinis P, Vandevoorde V, Haegeman G, Fiers W, Merlevede W, Vandenheede JR (1992): Tumor necrosis factor stimulates multiple serine/threonine protein kinases in Swiss 3T3 and L929 cells. Implication of casein kinase-2 and extracellular signal-regulated kinases in the tumor necrosis factor signal transduction pathway. J Biol Chem 267:25916–25921.
13. Saklatvala J, Rawlinson LM, Marshall CJ, Kracht M (1993): Interleukin 1 and tumour necrosis factor activate the mitogen-activated protein (MAP) kinase in cultured cells. FEBS Lett 334:189–192.
14. Hotamisligil GS, Budavari A, Murray D, Spiegelman BM (1994): Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes—central role of tumor necrosis factor-a. J Clin Invest 94:1543–1549.
15. Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM (1994): Tumor necrosis factor alpha inhibits signaling from the insulin receptor. Proc Natl Acad Sci U S A 91:4854–4858.
16. Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A (1993): Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. J Biol Chem 268:26055–26058.
17. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM (1996): IRS-1–mediated inhibition of insulin receptor tyrosine kinase activity in TNF-a and obesity-induced insulin resistance. Science 271: 665–670.
18. Hofmann C, Lorenz K, Braithwaite SS, Colca JR, Palazuk BJ, Hotamisligil GS, Spiegelman BM (1994): Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. Endocrinology 134:264–270.
19. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995): Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 95:2409–2415.
20. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB (1995): The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest 95:2111–2119.
21. Goldstein BJ (1993): Regulation of insulin receptor signalling by protein-tyrosine dephosphorylation. Receptor 3:1–15.
22. Goldstein BJ (1996): Protein-tyrosine phosphatases and the regulation of insulin action. In LeRoith D, Olefsky JM, Taylor SI (eds): "Diabetes Mellitus: A Fundamental and Clinical Text." Philadelphia: Lippincott, pp 174–186.
23. Hashimoto N, Feener EP, Zhang WR, Goldstein BJ (1992): Insulin receptor protein-tyrosine phosphatases—leukocyte common antigen-related phosphatase rapidly deactivates the insulin receptor kinase by preferential dephosphorylation of the receptor regulatory domain. J Biol Chem 267:13811–13814.
24. White MF, Kahn CR (1994): The insulin signalling system. J Biol Chem 269:1–4.
25. Streuli M, Krueger NX, Hall LR, Schlossman SF, Saito H (1988): A new member of the immunoglobulin superfamily that has a cytoplasmic region homologous to the leukocyte common antigen. J Exp Med 168:1523–1530.
26. Charbonneau H, Tonks NK, Kumar S, Diltz CD, Harrylock M, Cool DE, Krebs EG, Fischer EH, Walsh KA (1989): Human placenta protein-tyrosine-phosphatase: Amino acid sequence and relationship to a family of receptor-like proteins. Proc Natl Acad Sci U S A 86: 5252–5256.
27. Freeman RM, Plutzky J, Neel BG (1992): Identification of a human src homology 2–containing protein-tyrosine-phosphatase—a putative homolog of drosophila corkscrew. Proc Natl Acad Sci U S A 89:11239–11243.
28. Ahmad S, Banville D, Zhao ZZ, Fischer EH, Shen SH (1993): A widely expressed human protein-tyrosine phosphatase containing src homology-2 domains. Proc Natl Acad Sci U S A 90:2197–2201.
29. Kulas DT, Zhang WR, Goldstein BJ, Furlanetto RW, Mooney RA (1995): Insulin receptor signalling is augmented by antisense inhibition of the protein-tyrosine phosphatase LAR. J Biol Chem 270:2435–2438.
30. Ahmad F, Li PM, Meyerovitch J, Goldstein BJ (1995): Osmotic loading of neutralizing antibodies defines a role for protein-tyrosine phosphatase 1B in negative regulation of the insulin action pathway. J Biol Chem 270:20503–20508.
31. Yamauchi K, Milarski KL, Saltiel AR, Pessin JE (1995): Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling. Proc Natl Acad Sci U S A 92:664–668.
32. Milarski KL, Saltiel AR (1994): Expression of catalytically inactive Syp phosphatase in 3T3 cells blocks stimulation of mitogen-activated protein kinase by insulin. J Biol Chem 269:21239–21243.
33. Noguchi T, Matozaki T, Horita K, Fujioka Y, Kasuga M (1994): Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated ras activation. Mol Cell Biol 14:6674–6682.
34. Xiao S, Rose DW, Sasaoka T, Maegawa H, Burke TR, Roller PP, Shoelson SE, Olefsky JM (1994): Syp (SH-PTP2) is a positive mediator of growth factor–stimulated mitogenic signal transduction. J Biol Chem 269: 21244–21248.
35. Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.
36. Laemmli EK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
37. Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354.
38. Hashimoto N, Zhang WR, Goldstein BJ (1992): Insulin receptor and epidermal growth factor receptor dephosphorylation by three major rat liver protein-tyrosine phosphatases expressed in a recombinant bacterial system. Biochem J 284:569–576.
39. Harlow E, Lane D (1988): "Antibodies: A Laboratory Manual." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
40. Guan KL, Haun RS, Watson SJ, Geahlen RL, Dixon JE (1990): Cloning and expression of a protein-tyrosine-phosphatase. Proc Natl Acad Sci U S A 87:1501–1505.
41. Kasuga M, White MF, Kahn CR (1985): Phosphorylation of the insulin receptor in cultured hepatoma cells and a solubilized system. Methods Enzymol 109:609–621.

42. Pot DA, Woodford TA, Remboutsika E, Haun RS, Dixon JE (1991): Cloning, bacterial expression, purification, and characterization of the cytoplasmic domain of rat LAR, a receptor-like protein tyrosine phosphatase. J Biol Chem 266:19688–19696.
43. Pike LJ, Kuenzel EA, Casnellie JE, Krebs EG (1984): A comparison of the insulin- and epidermal growth factor–stimulated protein kinases from human placenta. J Biol Chem 259:9913–9921.
44. Tonks NK, Diltz CD, Fischer EH (1991): Purification of protein-tyrosine phosphatases from human placenta. Methods Enzymol 201:427–442.
45. Serra-Pages C, Saito H, Streuli M (1994): Mutational analysis of proprotein processing, subunit association, and shedding of the LAR transmembrane protein tyrosine phosphatase. J Biol Chem 269:23632–23641.
46. Yu Q, Lenardo T, Weinberg RA (1992): The N-terminal and C-terminal domains of a receptor tyrosine phosphatase are associated by non-covalent linkage. Oncogene 7:1051–1058.
47. Totpal K, Agarwal S, Aggarwal BB (1992): Phosphatase inhibitors modulate the growth-regulatory effects of human tumor necrosis factor on tumor and normal cells. Cancer Res 52:2557–2562.
48. Steller MA, Mok SC, Yeh J, Fulop V, Anderson DJ, Berkowitz RS (1994): Effects of cytokines on epidermal growth factor receptor expression by malignant trophoblast cells in vitro. J Reprod Med 39:209–216.
49. Donato NJ, Gallick GE, Steck PA, Rosenblum MG (1989): Tumor necrosis factor modulates epidermal growth factor receptor phosphorylation and kinase activity in human tumor cells. Correlation with cytotoxicity. J Biol Chem 264:20474–20481.
50. Donato NJ, Rosenblum MG, Steck PA (1992): Tumor necrosis factor regulates tyrosine phosphorylation on epidermal growth factor receptors in A431 carcinoma cells: Evidence for a distinct mechanism. Cell Growth Differ 3:259–268.
51. Kuhné MR, Pawson T, Lienhard GE, Feng GS (1993): The insulin receptor substrate-1 associates with the SH2-containing phosphotyrosine phosphatase Syp. J Biol Chem 268:11479–11481.
52. Lechleider RJ, Freeman RM, Neel BG (1993): Tyrosyl phosphorylation and growth factor receptor association of the human corkscrew homologue, SH-PTP2. J Biol Chem 268:13434–13438.
53. Vogel W, Lammers R, Huang JT, Ullrich A (1993): Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science 259:1611–1614.
54. Kuhné MR, Zhao ZZ, Rowles J, Lavan BE, Shen SH, Fischer EH, Lienhard GE (1994): Dephosphorylation of insulin receptor substrate 1 by the tyrosine phosphatase PTP2C. J Biol Chem 269:15833–15837.
55. Serra-Pages C, Kedersha NL, Fazikas L, Medley Q, Debant A, Streuli M (1995): The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein colocalize at focal adhesions. EMBO J 14:2827–2838.
56. Bernier M, Liotta AS, Kole HK, Shock DD, Roth J (1994): Dynamic regulation of intact and C-terminal truncated insulin receptor phosphorylation in permeabilized cells. Biochemistry 33:4343–4351.
57. Mooney RA, Anderson DL (1989): Phosphorylation of the insulin receptor in permeabilized adipocytes is coupled to a rapid dephosphorylation reaction. J Biol Chem 264:6850–6857.