BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 676–682 (1997)
ARTICLE NO. RC977355

LMW-PTP Is a Negative Regulator of Insulin-Mediated Mitotic and Metabolic Signalling

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Received July 27, 1997

To understand the physiological role of low Mr weight phosphotyrosine protein phosphatase (LMW-PTP) in insulin mediated signaling, we established clonal cell lines overexpressing the dominant negative (C12S mutant) LMW-PTP (dnLMW-PTP) from NIH3T3 murine fibroblasts expressing insulin receptor. Upon insulin stimulation we observe an association between the dnLMW-PTP and the β-subunit of the insulin receptor. This association is dependent on the tyrosine phosphorylation of the insulin receptor since it is not observed in unstimulated cells. Furthermore, in vitro binding experiments between dnLMW-PTP and the insulin receptor reveal that the interaction is mediated by the LMW-PTP catalytic site, as indicated by competition with orthovanadate. DnLMW-PTP overexpression influences both the mitogenic and the metabolic bioeffects of insulin. In particular, in cells overexpressing dnLMW-PTP we observe an increase in the glycogenosynthesis rate and in mitosis as indicated by glucose incorporation into glycogen and thymidine incorporation into DNA, respectively. Moreover, we studied the insulin mediated signal transduction pathways starting from insulin receptor, such as the Src kinase, the p21Ras/ERK, and the PI3K routes. Our findings are consistent with a specific regulation of mitogenesis by LMW-PTP through a pathway involving c-Src kinase but independent by both PI3K and ERK. These data strongly suggest that LMW-PTP acts as a negative regulator of both mitogenetic and metabolic insulin signalling. © 1997 Academic Press

Key Words: **phosphotyrosine protein phosphatase; insulin; insulin receptor; Src-kinase.**

Low molecular weight phosphotyrosine protein phosphatase (LMW-PTP) (1) is a cytosolic enzyme without extensive sequence homology with the other two classes of PTPases (2, 3, 4). Its crystal structure revealed a tridimensionally folded phosphate binding loop that is structurally identical to that contained in the human placenta PTP1B and Yersinia PTP (5, 6). LMW-PTP contains a CXXXXXR motif which is the active site signature of all PTPases members. Both arginine and cysteine are essential for catalytic activity of the LMW-PTP, as well in the other PTPases: most likely all PTPases share a common catalytic mechanism in which is involved a covalent cysteinyl phosphate intermediate (7). We have previously demonstrated that mutation of the cysteine residue in the signature motif to serine (C12S mutant), causes the complete loss of catalytic activity. On the other hand the same mutant phosphatase is still able to bind substrates (8, 9). Overexpression of the active phosphatase causes a reduction of cell proliferation and DNA synthesis, whereas the C12S mutant phosphatase (dnLMW-PTP) induces a remarkable increase in both this parameters, showing that this latter molecule behaves as a dominant negative *in vivo* (10). In addition we have demonstrated that this phenotype is associated with a specific and direct interaction between the active site of dnLMW-PTP and the activated PDGF-R (11), probably due to a slower PDGF receptor inactivation, resulting in an enhanced mitogenic signal.

Insulin receptor belongs to the tyrosine kinase receptor superfamily and presents an heterotetrameric structure. Ligand binding results in the autophosphorylation of tyrosine residues within the β-subunit of the receptor itself, activating receptor tyrosine kinase activity toward endogenous substrates (12). Although several substrates for the insulin receptor tyrosine kinase have been characterised, insulin receptor substrate 1 (IRS1) and Shc, which associate with intracellular molecules containing SH2 domains, play a major role in insulin receptor signalling through interactions with molecules containing SH2 domains (13, 14). IRS1,

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Abbreviations: LMW-PTP, low molecular weight phosphotyrosine protein phosphatase; dnLMW-PTP, dominant negative LMW-PTP; ERK, extracellular signal regulated kinase; IR, insulin receptor; IRS1, insulin receptor substrate; PDGF, platelet derived growth factor; PDGF-R, PDGF receptor; PI3K, phosphatidilinositol-3 kinase; STAT, signal transducer and activator of transcription.

0006-291X/97 $25.00

upon its tyrosine phosphorylation, can physically associate with several molecules, including Grb2 adapter protein, phosphatidil inositol 3 kinase and Syp phosphatase (14, 15). Insulin receptor undergoes tyrosine dephosphorylation upon removal of the hormone, and also the insulin-stimulated tyrosine phosphorylations of IRS1 and Shc are transient. The adaptability requires an efficiently and properly controlled system of protein tyrosine dephosphorylation. Within this system, the role of specific PTPases is counterbalanced by that of protein-tyrosine kinases, yet very little is known about the identity of the PTPases involved and about their specific functions within these signalling cascades.

Aim of this study is to establish the physiological role, if any, of LMW-PTP in insulin signalling. We analysed the effect of overexpression of the dominant negative form of LMW-PTP on early signalling events regulating mitogenesis and on ligand-stimulated glucose incorporation into glycogen, a distal metabolic response induced by insulin. In this paper we present evidences that LMW-PTP is a critical regulator of the insulin signal transduction pathway in fibroblasts as it negatively regulates both the mitogenic and metabolic response to insulin.

EXPERIMENTAL

Cell culture and transfections. NIH3T3-IR cells (kindly provided from Alan Saltiel) were routinely cultured in DMEM additioned with 10% fetal calf serum and 75 units/ml hygromycin, in 5% CO_2 humidified atmosphere (16). Cells were transfected and clonal lines were screened for expression of the transfected gene as reported previously (10).

Immunoprecipitations and western blot analysis. 1×10^6 cells were seeded onto 10 cm plates in DMEM supplemented with 10% fetal calf serum. Cells were serum starved for 24 h before receiving 100 nM insulin and treated for immunoprecipitation as reported previously (10, 11). In vitro binding assay was performed as in (11).

Growth kinetic experiments. 2×10^4 cells of NIH-IR and of dnLMW-PTP overexpressing cells were seeded in 24-multiwells plates, serum starved for 24 h and then insulin at increasing concentrations was added for 16 h to stimulate mitogenesis. 1 h pulses with 1 μCi/ml of 3[H]-thymidine were performed.

ERK activity assay. ERK activity was measured by an in vitro kinase assay on myelin basic protein as described by Mihasaka *et al.* (17).

Phosphatidylinositol 3-kinase activity. PI3K assay was performed as described elsewhere (18, 19).

Src kinase assay. It was performed using Src kinase assay kit from Upstate Biotechnology Inc. Briefly, 1.5×10^6 cells were seeded in 10 cm dishes, serum starved for 24 h and then stimulated with 100 nM insulin for 5 min. at 37 °C. Cell were lysed in modified RIPA buffer (11). Lysates were clarified by centrifugation and assayed for protein content. 5 μg of anti-Src antibodies were used for immunoprecipitation (10) from 500 μg lysates. The immunocomplex-beads were then incubated for 15 min at 30°C in 40 μl kinase buffer (25 mM Tris/HCl pH 7.2, 30 mM $MgCl_2$, 6 mM $MnCl_2$, 0.5 mM EGTA, 0.1 mM orthovanadate, 0.5 mM dithiothreitol, 125 μM ATP, 1μCi [γ-^{32}P]ATP (3000 Ci/mmol) containing 10 μg of the Src specific peptide substrate. The reaction was stopped by adding 20 μl of 40% TCA. After centrifugation 25 μl of supernatant were spotted onto 2X2 cm phosphocellulose paper, that was subsequently washed in a 0.75% phosphoric acid solution and then in pure acetone. Radioactivity was quantitated in a scintillation counter.

Glucose incorporation into glycogen. Cells were grown until they reached 60-80% confluence and then incubated 16 h with 0.05% fetal calf serum. Cells were rinsed in the same media plus 1% bovine serum albumine and then incubated in the presence of ^{3}[H]-D-glucose (0.4 μCi/6 cm dish) with or without increasing concentration of insulin. Glycogen was collected and quantitated as previously indicated (20).

RESULTS

Association of dnLMW-PTP with activated insulin receptor. To assess the role of LMW-PTP on insulin receptor mediated signalling the insulin receptor transfected NIH3T3 cell line (NIH-IR) was chosen. This cell line expresses the insulin receptor at a level of about 10^6 molecules/cell. Expression plasmid containing the coding sequence for the dnLMW-PTP was transfected in NIH-IR. Selection of clones resistant to both neomycin and hygromycin resulted in the establishment of cell lines overexpressing the inactive LMW-PTP. Analysis of six independent clones showed a clear increase in LMW-PTP level, due to the overexpression of the dominant negative form. Two clones (dnLMW-PTP cl. 3 and 11), both showing an average 15-fold increase in LMW-PTP expression levels were chosen for further studies. Analysis of these two clones showed that cell morphology, viability, and protein content was not affected by dnLMW-PTP overexpression. Moreover, we did not notice any difference between growth rates of the two clones. All the experiments described were performed with either clone 3 or 11 with overlapping results.

To study the role of LMW-PTP in insulin stimulated receptor autophosphorylation and activation, we analysed the tyrosine phosphorylation of the insulin receptor upon hormone stimulation. NIH-IR cells overexpressing dnLMW-PTP were stimulated with 100 nM bovine insulin after serum deprivation for 24 h. Anti β-chain insulin receptor antibody immunoprecipitates were analysed by antiphosphotyrosine immunoblotting. Results are presented in Fig. 1A. Insulin stimulation in NIH-IR cells resulted in the rapid stimulation of the 90 kDa β-subunit of the insulin receptor (12). The amount of this phosphorylated protein in the immunoprecipitate appears to be dependent on the overexpression of dnLMW-PTP: scanning densitometry of the film indicates that in cells overexpressing the dnLMW-PTP this increase is about two fold with respect to mock trasfected cells. To assess if the 90 kDa β-subunit of the insulin receptor physically associates with dnLMW-PTP an anti LMW-PTP antibody immunoprecipitation was performed and the samples were subjected to anti insulin receptor β-chain antibodies immunoblot. Fig. 1B shows that dnLMW-PTP actually

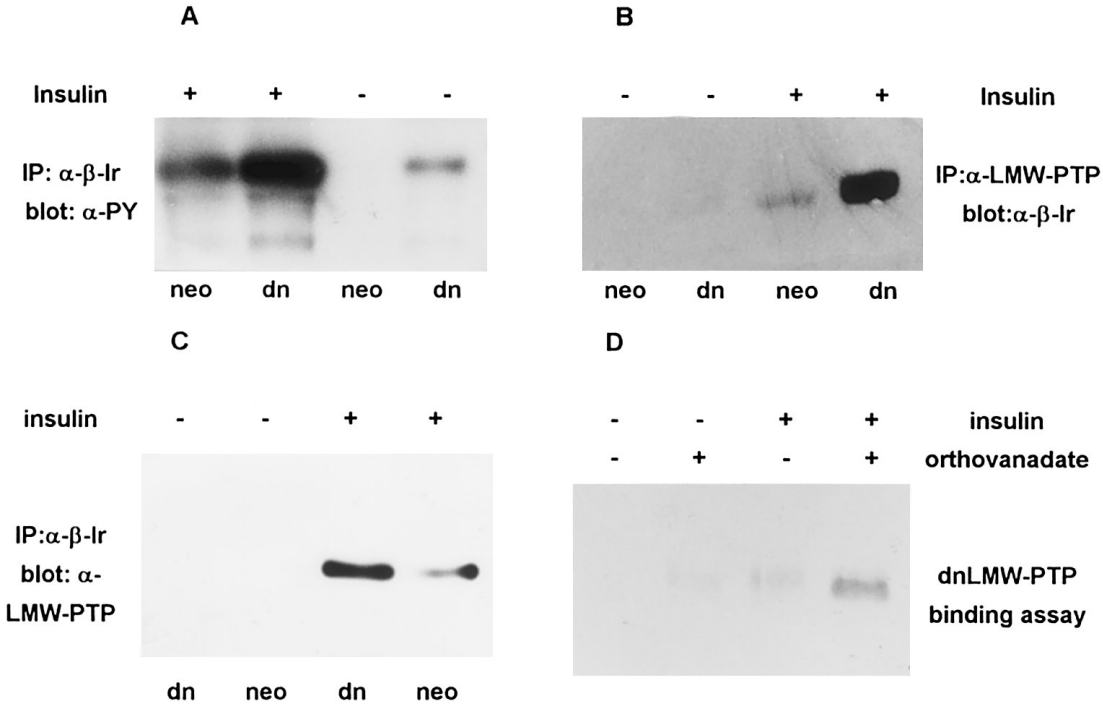

FIG. 1. dnLMW-PTP interacts with the β-chain of insulin receptor. NIH-IR cells, transfected with dnLMW-PTP or neomycin resistance alone (neo), were serum starved for 24 hours and then incubated with 100 nM insulin for 5 min. Cell lysates were immunoprecipitated with the indicated antibodies. IP, immunoprecipitation; neo, NIH-IR-neo cells; dn, dnLMW-PTP overexpressing NIH-IR cells; PY, phosphotyrosine. A, B, and C are immunoblots with the indicated antibodies. D is a dnLMW-PTP binding assay with the immunoprecipitated insulin receptor performed as described in Methods. Results are representative of three independent experiments.

associates with insulin receptor β-chain and that this association is dependent on receptor activation. The association is evident already in stimulated mock transfected cells (lane 3) but the amount of the coimmunoprecipitated insulin receptor is dramatically increased in cells overexpressing dnLMW-PTP (lane 4). In addition, we performed an anti β-subunits antibody immunoprecipitation from cells overexpressing dnLMW-PTP in comparison to mock trasfected cells, after insulin stimulation. Results of anti LMW-PTP immunoblotting (Fig. 1C) again confirmed an association between dnLMW-PTP and the β-subunit of the insulin receptor, and that this association is dependent on receptor stimulation.

Moreover, we were interested in characterising the interaction between LMW-PTP and the β-subunit of the insulin receptor by in vitro binding assay. The β-subunit of the activated receptor was immunoprecipitated after 100 nM insulin stimulation and then an *in vitro* binding assay was performed in presence of orthovanadate, a competitive inhibitor of LMW-PTP. The results, shown in Fig. 1D, reveal that the dnLMW-PTP binds the tyrosine phosphorylated insulin receptor *in vitro* and that this binding is inhibited by orthovanadate: these data suggest that the binding is mediated by the LMW-PTP catalytic site.

Insulin mitogenic cell response analysis. LMW-PTP appears to be involved in the regulation of growth factor dependent mitogenesis: in fact overexpression of the dnLMW-PTP in NIH3T3 cells causes an increased mitogenic response to both PDGF and fetal calf serum (10). Analysis of growth parameters was performed in NIH-IR overexpressing dnLMW-PTP in comparison with mock transfected cells in response to insulin treatment (Fig. 2A). Results showed a clear and significant increase in DNA synthesis in both dnLMW-PTP expressing clone 3 and 11 with respect to mock trasfected cells, as indicated from ^{3}[H] thymidine incorporation in insulin stimulated cells. These results are consistent with an involvement of LMW-PTP in insulin mediated mitogenic signal transduction pathways.

Recent results from our laboratory (data submitted) indicate that LMW-PTP interaction with PDGF receptor results in a selective interaction with only two pathways of PDGF signal transduction: namely the Src and the STATs pathways. In particular, dnLMW-PTP upregulates the Src kinase activity and the DNA binding activity of STATs in PDGF stimulated cells. In contrast, other independent routes starting at phosphorylated PDGF receptor such as PI 3 kinase, PLC-γ1 or Ras activated MAP kinases, remain almost inalterated in response to LMW-PTP overexpression. The insulin

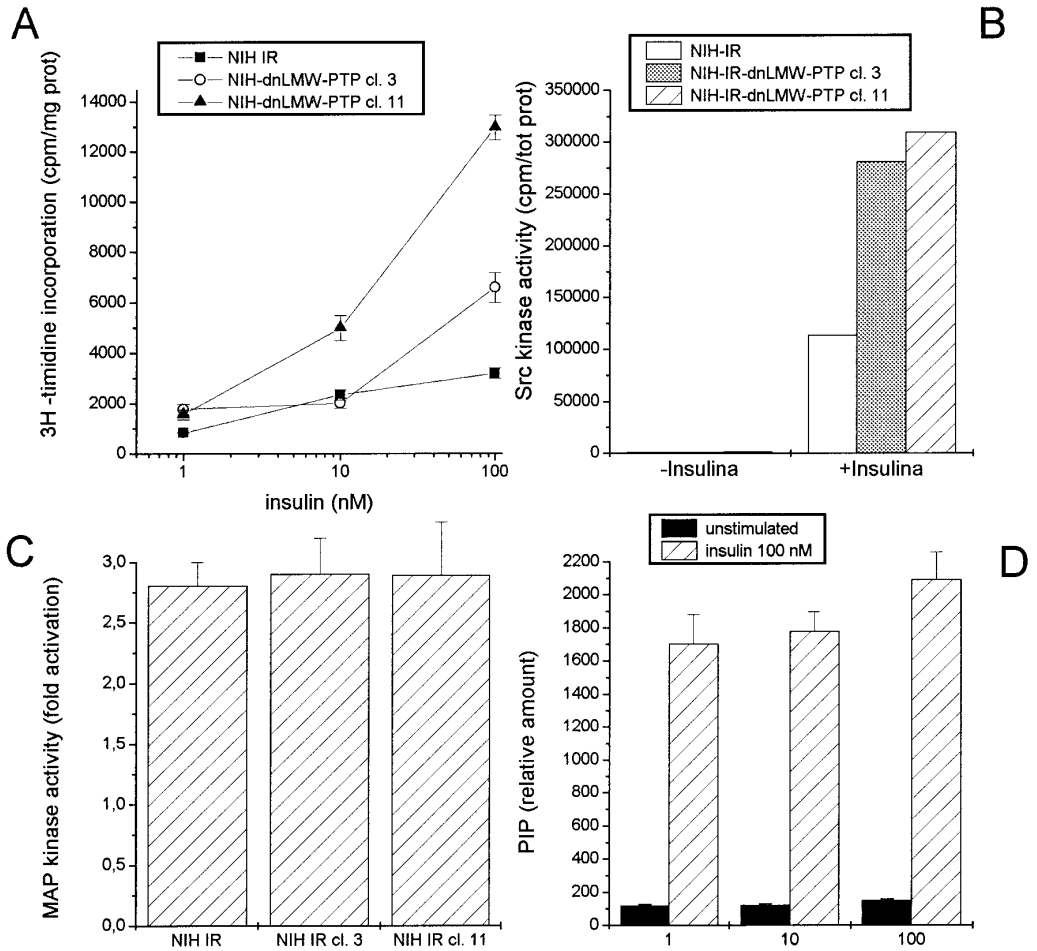

FIG. 2. dnLMW-PTP affects insulin mediated mitogenesis through Src kinase activity. (A) *Thymidine incorporation:* Cells were serum starved for 24 hours and then incubated with increasing concentration of insulin for 16 h. 3[H]-thymidine incorporation was taken as an index of DNA synthesis and data are normalised on the basis of total protein content. (B) *Src kinase activity assay:* Serum starved cells were incubated for the indicated time with 100 nM insulin. Cell lysates were immunoprecipitated with anti-Src antibodies and Src activity was assayed as described in Methods. Data are the mean ± SD of three independent experiments. (C) *ERK kinase activity assay:* NIH-IR cells, transfected with dnLMW-PTP or neomycin resistance alone (neo), were serum starved for 24 hours and then incubated with 100 nM insulin for 5 min. Cells were lysed and 50 μg of total proteins was immunoprecipitated with polyclonal anti-ERK1 antibodies. The immunobeads were washed and used for an immune complex assay for ERK1 kinase activity. (D) *PI3K assay:* 24 h serum starved cells were stimulated with 100 nM insulin for 10 min. 150 μg of total proteins from cell lysate was immunoprecipitated with antiphosphotyrosine antibodies and used for PI3K assay as described in Methods. The spots corresponding to PIP in the autoradiogram were quantitated by laser scanning the film. Data shown in all panels are representative of three independent experiments (mean ± SD).

receptor signalling system appears to resemble, at least in part, the signal transduction pathway of PDGF receptor (12). The importance of Src for starting DNA duplication and promoting G1/S phase transition is largely accepted (20), while the significance of the STATs activation remains extremely controversial and the role of these transcription factors in insulin mediated mitogenesis is not jet established (21, 22). We have explored if the changes described in PDGF-R signalling are unique for this system or rather if they apply to other receptors such as insulin receptor. NIH-IR cell overexpressing dnLMW-PTP were analysed for Src kinase activity in comparison to mock transfected cells. Serum starved cells were stimulated with 100 nM insulin and anti-Src immunoprecipitates were tested for their ability to phosphorylate a Src specific peptide as described in Material and Methods. Overexpression of the dnLMW-PTP (Fig. 2B) greatly increased Src activity upon stimulation with insulin in comparison to mock transfected cells. No significant changes were observed in the Src activity of serum-starved, unstimulated cells.

The kinase cascade originating by activation of Ras results in ERK activation, which migrate to the nucleus and phosphorylate different transcription factors. The Ras/ERK pathway has been shown to be necessary for growth-factor dependent-DNA synthesis. Therefore, we evaluated whether the observed effects of LMW-

TABLE 1

LMW-PTP Modulates Glucose Incorporation into Glycogen

Insulin	NIH-Ir neo	NIH-Ir dnLMW-PTP/3	NIH-Ir dnLMW-PTP/11
0	1,002 ± 40	1,207 ± 105	890 ± 47
0.1	3,030 ± 55	5,600 ± 204	5,980 ± 99
1	23,023 ± 5,021	78,902 ± 19,978	68,000 ± 10,120
10	40,009 ± 10,124	194,967 ± 4,996	220,890 ± 20,543

Note. NIH-IR cells, transfected with dnLMW-PTP or neomycin resistance alone (neo), were serum starved for 24 hours and then incubated with increasing concentration of insulin for 2 h in the presence of 0.4 mCi/dish 3[H]-glucose. Glycogen was collected from 500 μg of total proteins from cell lysates as indicated in Methods. The samples are counted by liquid scintillation. Data reported are representative of three independent experiments (mean ± SD).

PTP on cell proliferation could be mediated by actions on this signalling pathway. We measured ERK activity in immunoprecipitates obtained with the use of anti-ERK antibodies. An immune complex kinase assay using myelin basic protein as a substrate was performed (Fig. 2C). Results obtained revealed that no significant difference is present in the different samples.

Furthermore, PI3K has been shown to contribute in transducing the mitogenic signal originating from insulin receptor activation (15, 23). In order to investigate if LMW-PTP overexpression caused perturbations in these pathways, we measured PI3K activity. Insulin induced a dramatic upregulation of PI3K activity (Fig. 2D), but this variation was not significantly influenced by the overexpression of dnLMW-PTP in comparison to mock transfected cells. Taken together, these data indicate that the phenotypic effect of the LMW-PTP on insulin mitogenic response is not mediated by its interference on the Ras/ERK or in the PI3K pathway.

Glucose incorporation into glycogen. We were interested in determining whether biological functions at the distal end of the insulin pathway, such as glycogen synthesis, could be affected by LMW-PTP. The comparative dose-response curves for the hormone-stimulated incorporation of ^{3}[H]-glucose into glycogen of clones overexpressing dnLMW-PTP and control cells are shown in Tab. 1. Cells were serum starved for 24 h to reduce basal glucose incorporation and then stimulated with increasing concentrations of insulin in the presence of ^{3}[H]-glucose. Already at a concentration of 1 nM, insulin is able to induce a rapid and sustained glycogenosynthesis as indicated in Table 1. Both NIH-IR overexpressing dnLMW-PTP cl. 3 and 11 have a four-fold increased glucose incorporation rate with respect to mock transfected cells either with 1 nM or 10 nM insulin. These results suggest that LMW-PTP may act as a physiological regulator of insulin metabolic signalling.

DISCUSSION

In this paper we describe the involvement of LMW-PTP in negative regulation of insulin signalling system. Previously, this phosphatase has been shown to interact by means of its catalytic site with PDGF receptor and to downregulate the mitogenic signals starting with PDGF receptor activation. The insulin signal transduction system in part resemble that of PDGF receptor (12). However, the intracellular signalling pathway between the insulin receptor and the cellular targets is not well defined. Insulin is known to have essentially two distinct biological effects: membrane ruffling and mitogenesis on one hand and the stimulation of glucose transport and the regulation of both gluconeogenesis and glycolysis on the other (24). One signalling molecule predicted to play an essential role in insulin mediated effects is the major insulin receptor substrate IRS1. Interaction of insulin receptor with the agonist results in the rapid phosphorylation in multiple phosphotyrosines of IRS1 (13). Recent data demonstrated that the IRS1, while mediates many of the effects leading to mitosis, is not an essential component of the metabolic insulin signalling pathway that leads to glucose transporter translocation and distal functions regulation in glucose metabolism (24).

The overexpression of dnLMW-PTP in cells expressing the insulin receptor results in enhanced ligand stimulated tyrosine phosphorylation of the insulin receptor. The precise mechanism for the increase tyrosine phosphorylation is not clear. dnLMW-PTP could bind to tyrosine phosphorylated insulin receptor, protecting it from dephosphorylation by endogenous LMW-PTP (10, 25, 26, 27). The interaction between the β-subunit of the insulin receptor and dnLMW-PTP, as shown by means of immunoprecipitation assay, appears to be dependent on receptor autophosphorylation since it is dramatically reduced in unstimulated cells. In addition, this association is likely to be mediated by LMW-PTP catalytic site as indicated by the in vitro binding assays in the presence of the competitive inhibitor orthovanadate.

The increased phosphorylation of the β-subunits of the insulin receptor in dnLMW-PTP overexpressing cells is likely to be accompanied by downstream events. In fact we observe increased cell growth rates in dnLMW-PTP overexpressing cells with respect to mock transfected cells, as indicated by DNA synthesis quantitation. The analysis of mitotic cell response in cells overexpressing the dnLMW-PTP revealed a selective action of this phosphatase on the Src kinase activity accompanied by an enhancement of mitogenic signalling. On the contrary, other relevant mitogenic signal transduction pathways such as PI3K and p21Ras/ERK routes remain almost unaltered by the overexpression of dnLMW-PTP. Taken together, these data suggest that the interference of LMW-PTP with insulin signal-

ling is at least in part similar to that observed for PDGF signalling. In fact, also in the case of the signalling pathways originating with PDGF receptor autophosphorylation, the Src pathway is influenced by the overexpression of dnLMW-PTP (data submitted). Furthermore, the exact role for the Src tyrosine kinase in insulin signalling is still debatable. Recent data confirm the involvement of a Src-regulated/focal adhesion kinase pathway during insulin-mediated mitosis (30), although the direct association of Src with insulin receptor or IRS1 has not been demonstrated. In NIH3T3 cells the Src pathway is essential for Myc transcription factor activation and appears to be required for S-phase DNA synthesis (20). Our data demonstrate that Src kinase activation is a direct consequence of insulin receptor stimulation. To our knowledge this is the first time that such an evidence has been produced. Our findings on the regulation of mitogenesis by LMW-PTP suggest that the increased cell growth rate observed in dnLMW-PTP overexpressing cells could be due to an upregulation of the Src kinase activity.

In addition, according to our data LMW-PTP is likely to affects another fundamental function of insulin such as the regulation of glucose-incorporation into glycogen. Glycogenosynthesis in dnLMW-PTP overexpressing cells is greatly incremented upon insulin stimulation with respect to control cells. The glucose influx and metabolism is directly under the control of the activated insulin receptor, and it is independent from IRS1 receptor recruitment and phosphorylation (24). Recent data revealed that there is a major PI3K pathway controlling glucose uptake and metabolism that involves IRS1, and a redundant route sufficient to activate PI3K-mediated glucose metabolism when IRS1 is inhibited (31, 32). The insulin dependent effect of LMW-PTP on glucose metabolism seems not to be mediated by PI3K activation. It is likely that LMW-PTP acts through a PI3K independent pathway starting with receptor phosphorylation and directly leading to the control of glucose metabolism. For what the involvement of LMW-PTP in the insulin mitogenic effect is concerned, it is likely that it is mediated by the regulation of Src tyrosine kinase. It cannot be excluded that Src activity is also implicated in the metabolic effect of insulin.

In conclusion, our data indicate that LMW-PTP affects both the metabolic and the mitogenic effects of insulin and provide direct support for the growing interest in LMW-PTP as a critical regulator of tyrosine kinase receptor family. In particular, defects in LMW-PTP expression and/or activity could have profound effects on cellular responsivity to insulin.

ACKNOWLEDGMENTS

This work was supported by the Italian Association for Cancer Research (AIRC) and in part by the Ministero della Università e Ricerca Scientifica e Tecnologica (MURST) and by Consiglio Nazionale delle Ricerche (CNR). F.M. was supported by grants from MURST (liver cirrhosis and viral hepatitis project) and from the Italian Liver Foundation.

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