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Cell Density-Dependent Changes in the Insulin Action Pathway: Evidence for Involvement of Protein-Tyrosine Phosphatases

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Abstract In order to examine alterations in the phosphorylation state of proteins involved in insulin action that might accompany the reduced growth state of density-arrested cells, we measured the insulin-stimulated phosphorylation of the receptor and high M_r cellular substrates of the receptor kinase in rat hepatoma cells at different cell densities. As cell density increased from 2×10^5 to 3.2×10^6 per 35-mm well, the rate of DNA synthesis fell to 22% of control, while insulin-stimulated tyrosine phosphorylation of high M_r receptor substrates ("pp185") was enhanced to 198% of control, without a change in the abundance of insulin receptor substrate (IRS)-1 protein. In anti-IRS-1 immunoprecipitates, tyrosine phosphorylation was increased by only 30%, suggesting that increased tyrosine phosphorylation of additional high M_r proteins (e.g., IRS-2) accounted for much of the observed increase in tyrosine phosphorylation of the receptor substrates. In spite of increased tyrosine phosphorylation of IRS-1 and total pp185-related proteins, however, cells studied at high growth density exhibited a 25% decrease in IRS-1-associated phosphatidylinositol 3′-kinase activity and only a 39% increase in phosphatidylinositol 3′-kinase activity in antiphosphotyrosine immunoprecipitates. To explore the potential role of hepatic protein-tyrosine phosphatases (PTPases) in the hyperphosphorylation of pp185 proteins, we found by immunoblotting that at high cell density the intracellular PTPase PTP1B and the transmembrane PTPase LAR were reduced in abundance by 49% and 55%, respectively, while the abundance of the SH2-domain containing PTPase SH-PTP2 was increased by 48%. These data demonstrate that the attenuation of post-receptor signaling by insulin in hepatoma cells at increasing growth density involves changes in endogenous substrate phosphorylation which may result from alterations in specific PTPases implicated in the regulation of the insulin action pathway.

Key words: cell density, DNA synthesis, M_r receptor substrates, IRS-1 protein, tyrosine phosphorylation

The cellular effects of insulin are propagated by pathways of reversible tyrosine phosphorylation [Rosen, 1987]. Upon insulin binding, insulin receptors are autophosphorylated on multiple tyrosine residues, including those in the receptor regulatory domain which activates the intrinsic tyrosine kinase activity of the receptor towards specific cellular proteins. These include the widely expressed high-molecular-weight substrates of M_r 180–190 kDa, known collectively as "pp185," which consists primarily of insulin receptor substrate (IRS)-1 and IRS-2 [White and Kahn, 1994; Araki et al., 1994]. After tyrosine phosphorylation, the "pp185" protein substrates have been shown to participate in a number of intermolecular associations. Prominent among these interactions is the high-affinity binding of src-homology 2 (SH2) domain-containing proteins, such as the p85 subunit of phosphatidylinositol 3′-kinase, the protein-tyrosine phosphatase (PTPase) SH-PTP2 and the SH2/SH3 adaptor protein GRB-2/ASH. The association of these proteins is dependent on the tyrosine phosphorylation of the pp185 receptor substrates, and in each case, a distal pathway is activated. Thus, the p110 catalytic subunit of phosphatidylinositol 3′-kinase is activated by the association of its noncatalytic subunit with IRS-1 [Backer et al., 1992, 1993], the phosphatase activity of SH-PTP2 is enhanced by ligation

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of the SH2 domains [Kuhné et al., 1993; Sun et al., 1993], and the binding of GRB-2/ASH links insulin signaling to the activation of the ras pathway by the association of GRB-2/ASH with mSOS, a guanine nucleotide exchange protein [Skolnik et al., 1993].

As tyrosine phosphorylation of the receptor and its substrates activate the insulin signaling pathway, cellular PTPases have been shown to reverse the activation of components in the insulin signaling pathway and return them to the basal state [Goldstein, 1993]. The PTPases are members of a large superfamily of enzymes with varied structures, of which the transmembrane, receptor-type enzyme LAR, and the intracellular enzyme PTP1B have both been recently shown to be involved in the negative regulation of insulin signaling [Kulas et al., 1995; Ahmad et al., 1995a]. The available data suggest that these enzymes inactivate the receptor kinase *in situ* by the dephosphorylation of activating phosphotyrosine residues in the receptor regulatory domain, and they may impact on the tyrosine phosphorylation state of post-receptor substrates in the signaling pathway [Goldstein, 1995]. SH-PTP2, on the other hand, has been implicated in the *positive* transmission of signals to growth-stimulating pathways triggered by insulin, although the exact mechanism has not yet been elucidated [Noguchi et al., 1994; Xiao et al., 1994; Yamauchi et al., 1995].

Insulin exerts pleiotropic anabolic effects on cells that involve various aspects of cell growth and metabolism. In order to explore how the insulin action pathway might be downregulated in a state of reduced cell growth rate as nontransformed insulin-sensitive cells become confluent, we examined insulin-stimulated receptor autophosphorylation and the tyrosine phosphorylation of high-molecular-weight cellular substrates of the receptor kinase in KRC-7 rat hepatoma cells plated at low and high cell density. The results reveal dramatic alterations in the phosphorylation state of receptor substrates and evidence for novel regulation of post-receptor insulin action. Increasing cell density was associated with tyrosine hyperphosphorylation of the "pp185" substrates of the insulin receptor, but this increased phosphorylation of pp185 did not lead to enhanced activation of phosphatidylinositol 3′-kinase by insulin to a similar degree. Evidence was also obtained that alterations in the abundance of specific PTPases may influence the steady state tyrosine phosphorylation of intermediates in the insulin signalling pathway as they reach saturating cell density.

MATERIALS AND METHODS

Materials

Phosphatidylinositol was purchased from Avanti Polar Lipids (Alabaster, AL); phosphatidylinositol 4′-phosphate was from Sigma (St. Louis, MO); trisacryl-protein A was from Pierce (Rockford, IL). [^{125}I]protein A (>30 μCi/μg) was purchased from ICN (Costa Mesa, CA). [^{3}H]thymidine and γ-[^{32}P]ATP were from NEN/DuPont (Boston, MA). Rabbit antisera against the recombinant LAR cytoplasmic domain was prepared as described [Ahmad and Goldstein, 1995]. Rabbit antiserum against PTP1B and the insulin receptor were purchased from Transduction Laboratories (Lexington, KY) and antiserum against the SH-PTP2 C-terminus was from Santa Cruz (Santa Cruz, CA). Rabbit anti-IRS-1 polyclonal antibody raised to a recombinant rat IRS-1 protein was a generous gift from Dr. Morris White (Joslin Diabetes Center, Boston). Fetal bovine serum (FBS) and bovine serum albumin (BSA) (fraction V) were purchased from Sigma; DME medium was from Gibco/BRL (Gaithersburg, MD). High range prestained protein markers were from BioRad (Melville, NY). All other reagents were of the highest available grade.

Cell Culture and Lysis

The well-differentiated rat hepatoma cell line KRC-7 was provided by Dr. John Koontz (University of Tennessee). The cells were maintained in DMEM containing 10% fetal bovine serum in a humidified atmosphere composed of 95% air and 5% CO_2. For experiments, cells were seeded in 6-well plates at different cell densities with the same medium and cultured overnight. Cells were washed with DMEM twice, stimulated with 10^{-7} M porcine insulin for 1 min at 37°C in DMEM, and lysed with a buffer (150 mM NaCl, 2 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 1% Triton X-100, 50 mM Hepes, pH 7.5) supplemented with 10 mM PMSF, and 10 μg/ml each of antipain, pepstatin A, aprotinin and leupeptin. Crude cell lysates were clarified at 12,000*g* for 20 min. Protein was measured by the method of Bradford [1976].

DNA Synthesis

Cells were seeded in 6-well plates at low and high densities, as described in the text and la-

beled with 1 μCi/ml [^{3}H]thymidine in DMEM containing 10% FBS for 18 h. Cells were washed three times with DMEM and total DNA was precipitated with 20% (w/v) ice-cold TCA; radioactivity in the washed precipitates was determined by scintillation counting. The incorporation of thymidine was normalized by the protein content of the cells in the culture wells.

Western Blot

After normalization of protein concentration, 30 μg lysate protein was treated with gel sample buffer containing 100 mM DTT, heated in a boiling water bath for 4 min, and applied to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), along with prestained protein markers [Laemmli, 1970]. Separated proteins were blotted onto nitrocellulose filters (90 V for 90 min) in 154 mM glycine, 20 mM Tris–HCl, pH 8.3, and 20% methanol with 0.02% SDS [Towbin et al., 1979]. Blots were probed for 1 h each with polyclonal antibodies and 2 μCi [^{125}I]protein A in 5% BSA, 0.9% NaCl, 0.01% NaN_3, 10 mM Tris–HCl, pH 7.2, and quantitated by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation and Phosphatidylinositol 3′-Kinase Assay

Lower- and higher-density cells were washed with DMEM and stimulated with 10^{-7} M porcine insulin for 1 min at 37°C in DMEM and then solubilized in 20 mM Tris–HCl, pH 7.5, containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 1% NP-40, 10% glycerol with 10 mM PMSF and 10 μg/ml each of antipain, pepstatin A, aprotinin, and leupeptin. Insoluble material was removed by centrifugation at 13,000*g* for 20 min. IRS-1-associated phosphatidylinositol 3′-kinase activity was precipitated from the supernatant using the polyclonal IRS-1 antibody and trisacryl protein A. The pellets were successively washed with phosphate-buffered saline (PBS) containing 1% (v/v) NP-40 and 100 μM Na_3VO_4, followed by 100 mM Tris–HCl, pH 7.5 containing 500 mM LiCl and 100 μM Na_3VO_4, and finally in 10 mM Tris–HCl, pH 7.5 containing 100 mM NaCl, 1 mM EDTA and 100 μM Na_3VO_4. Then the pellets were resuspended in 50 μl of 10 mM Tris–HCl, pH 7.5 containing 100 mM NaCl, 1 mM EDTA and 100 μM Na_3VO_4, and mixed with 10 μl 100 mM $MgCl_2$, 10 μl phosphatidylinositol (2 mg/ml) in 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA. The kinase reaction was initiated by the addition of 10 μCi γ-[^{32}P]ATP at a final ATP concentration of 440 μM. After 10 min at 23°C, the reaction was terminated by the addition of 20 μl 8 N HCl and 160 μl $CHCl_3$–methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel thin-layer chromatography (TLC) plate pretreated with 1% potassium oxalate. TLC plates were chromatographed in $CHCl_3$–CH_3OH–H_2O–NH_4OH (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity in spots that comigrated with phosphatidylinositol 4′-phosphate standard was quantitated by phosphorimager analysis.

Data Analysis

Data presented represents the mean ± *SEM* for the average of a minimum of three separate determinations.

RESULTS

Insulin Signaling at High Cell Density

Examination of insulin-stimulated tyrosine phosphorylation of the insulin receptor and its high M_r substrates in well-differentiated hepatoma cells revealed changes in their phosphorylation state that accompanied alterations in the cell growth rate at various cell densities. The growth rate of cells at high plating density (3.2×10^6 cells per 35-mm culture well, estimated by measuring DNA synthesis, was shown to be reduced to 21.6 ± 0.4% (n = 5) of the value in the low-density cells (2×10^5 cells/well). With increasing cell density, the slowed growth rate was accompanied by a striking and progressive enhancement of phosphorylation of pp185 in response to insulin stimulation for 1 min that was increased by up to 98% at the highest density, compared to the less dense control cells (Fig. 1). By contrast, the autophosphorylation of the insulin receptor itself was only increased by 19% in the more dense cells. When stimulated for a longer time (5 min), the results were similar, with essentially no change in insulin receptor autophosphorylation at high density, but a 105% increase in pp185 phosphorylation at high density, compared to control, low-density cells (not shown).

The alterations in tyrosine phosphorylation observed with increasing cell density could result from changes in the specific tyrosine phosphorylation of these proteins or changes in their

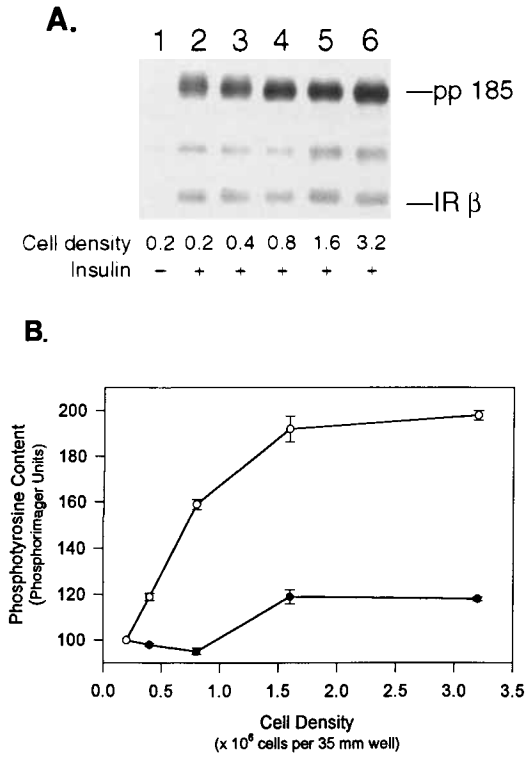

Fig. 1. Changes in insulin-stimulated receptor autophosphorylation and pp185 tyrosine phosphorylation with increasing growth density of hepatoma cells. KRC-7 hepatoma cells were seeded at different densities in DMEM supplemented with 10% FBS. Cells were cultured overnight at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2, then stimulated with 10^{-7} M insulin for 1 min at 37°C. Cell lysate samples, normalized for protein concentration were applied to 7.5% SDS–PAGE, transferred onto a nitrocellulose filter and immunoblotted with a polyclonal antibody against phosphotyrosine. After treatment with ^{125}I-protein A, the bands were visualized by phosphorimager analysis. **A:** Representative phosphorimage of phosphotyrosine blot of hepatoma cells plated at the indicated cell densities ($\times 10^6$ per 35-mm culture well). Migration positions of the 95-kDa insulin receptor (IR) β-subunit and pp185 are shown. **B:** Mean ± SE of data from three experiments similar to that shown in **A** for the IR (●) and pp185 (○).

overall protein abundance. Immunoblot analysis was performed to quantitate the abundance of insulin receptors in the cells at low and high cell density as well as the abundance of IRS-1, a major component of the high M_r substrates of the insulin receptor for which antibodies are available. At high cell density, the abundance of insulin receptors and of IRS-1 protein was actually decreased by 14% and 21%, respectively (Fig. 2A). The protein phosphotyrosine content was also assessed in these same samples by immunoblotting with anti-phosphotyrosine antibodies, demonstrating similar results to those shown in Figure 1 for the increase in phosphotyrosine in the insulin receptor β-subunit band (19%) and in the pp185 proteins (98%) with increasing cell density (Fig. 2B). The ratios of specific phosphotyrosine content per protein mass were then calculated. These data showed a further enhancement in the more densely plated cells of the phosphotyrosine content of the insulin receptor (138%) and the pp185 band normalized to the IRS-1 protein abundance (251%) compared to the less dense control cells (Fig. 2C).

In an attempt to separate out the contribution of IRS-1 to the phosphotyrosine content of other proteins migrating at the position of pp185, we examined the specific phosphotyrosine content of IRS-1 using immunoprecipitates of IRS-1 from control and cells at high density. The insulin-stimulated tyrosine phosphorylation of IRS-1 itself was increased by only 30%, suggesting that increased tyrosine phosphorylation of additional high M_r receptor substrates (most likely IRS-2) accounted for the observed doubling in the tyrosine phosphorylation of the pp185 insulin receptor substrates (data not shown).

Insulin-Stimulated Phosphatidylinositol 3′-Kinase Activity

The increased IRS-1 phosphorylation and presumed increase in IRS-2 phosphorylation was unexpected, since the cells plated at higher density were terminating their exponential growth phase. As the cells approached a contact-inhibited growth state, they might have been expected to demonstrate a reduced activation of certain pathways of insulin signaling. In order to assess whether the increased phosphorylation state of the insulin receptor substrates resulted in activation of signaling mediated by IRS-1 and IRS-2, the insulin-stimulated activation of phosphatidylinositol 3′-kinase was measured. The phosphatidylinositol 3′-kinase activity in anti-phosphotyrosine immunoprecipitates of hepatoma cell lysates was very low in the absence of insulin stimulation at either low or high cell density (Fig. 3). With insulin treatment, the phosphatidylinositol 3′-kinase activity in antiphosphotyrosine immunoprecipitates increased only by 39% in the cells plated at high cell density compared to the low density plated cells. This is in contrast to the observed increase of 2.0-fold in the phosphotyrosine content of insulin receptor substrates in the high-density-plated cells (Fig. 1).

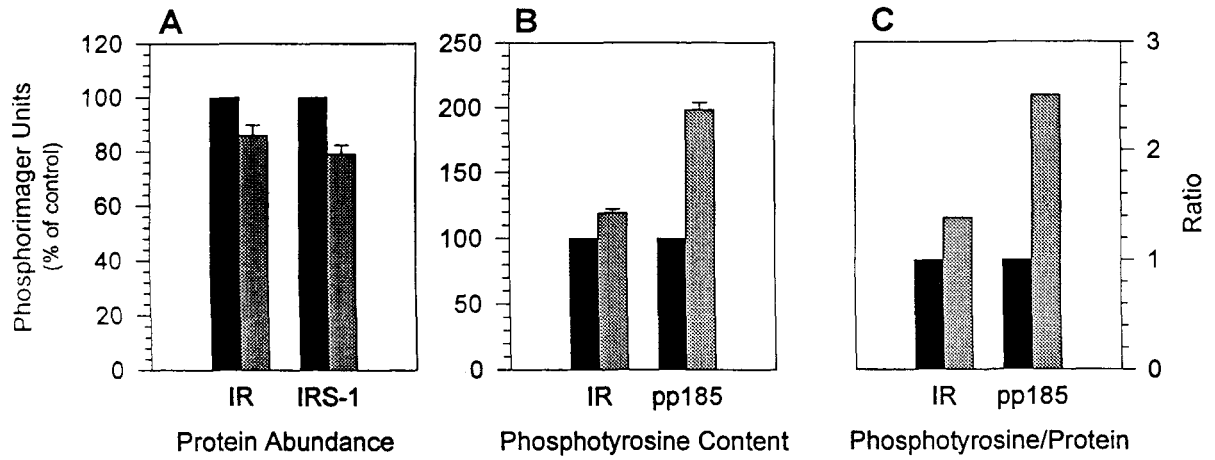

Fig. 2. **A:** Protein abundance for the insulin receptor (IR) β-subunit and IRS-1 determined by immunoblotting and phosphorimager analysis for hepatoma cells cultures at low (0.3×10^6 cells per well; *black bars*) and high cell density (2.7×10^6 cells per well; *gray bars*). **B:** Phosphotyrosine content of hepatoma cell IR β-subunits and pp185 proteins determined by phosphorimager analysis for low- and high-density plated cells as described for **A. C:** Ratio of phosphorimager units for phosphotyrosine content per protein abundance for the IR β-subunits and the IRS-1 component of the pp185 proteins from the data presented in A and B.

The phosphatidylinositol 3′-kinase specifically associated with IRS-1 was also studied in the insulin-stimulated hepatoma cells at low and high cell density (Fig. 3). At high growth density, a 25% decrease in the phosphatidylinositol 3′-kinase activity specifically associated with IRS-1 was observed compared to the cells plated at low density. This result was also unexpected, since the tyrosine phosphorylation state of IRS-1 was increased by 30% in the cells at high cell density. Taken together, these data demonstrate that the increased tyrosine phosphorylation either of IRS-1 or of other high-molecular-weight insulin receptor substrates in the cell did not lead to enhanced signalling by activation of the phosphatidylinositol 3′ kinase enzyme. In fact, the activation of the enzyme was relatively attenuated, compared to the activation level in cells plated at lower cell density.

PTPase Abundance at High Cell Density

A potential mechanism for increases in the phosphorylation state of the insulin receptor and the pp185 receptor substrates might involve alterations in the abundance of specific cellular PTPases implicated in the regulation of the insulin action pathway. To evaluate this possibility, the abundance of three candidate PTPases in the insulin action pathway (PTP1B, LAR, and SH-PTP2) was determined by immunoblotting in hepatoma cells plated at low and high cell density. Compared to cells plated at low cell density, the abundance of PTP1B and LAR was decreased by 49% and 55%, respectively, in the cells at high growth density (Fig. 4). These results are of interest since we have recently shown that both PTP1B and LAR have a negative regulatory impact on insulin action in hepatoma cells. The reduction in expression of these PTPases might effectively lead to the observed enhancement of the phosphorylation state of the insulin receptor and its tyrosine phosphorylated substrates in the cells. By contrast, the abundance of SH-PTP2 was actually increased by 48% in the cells grown at high cell density. SH-PTP2 is thought to act as a positive regulator of insulin signaling; however, since its exact site of action in the insulin action pathway is unclear, it is difficult to predict the potential effects of this increase in the mass of SH-PTP2 at high cell density on the insulin action pathway.

DISCUSSION

As the major anabolic hormone in the body, insulin affects not only the metabolic processes of is target tissues, such as glucose transport, macromolecule synthesis, and the storage of fuels, but also growth effects mediated by stimulation of pathways of DNA synthesis and cell division [Rosen, 1987; Lee and Pilch, 1994]. One of the central questions now being explored in studies on the action of various hormones is how the stimulation of a single receptor can trigger a

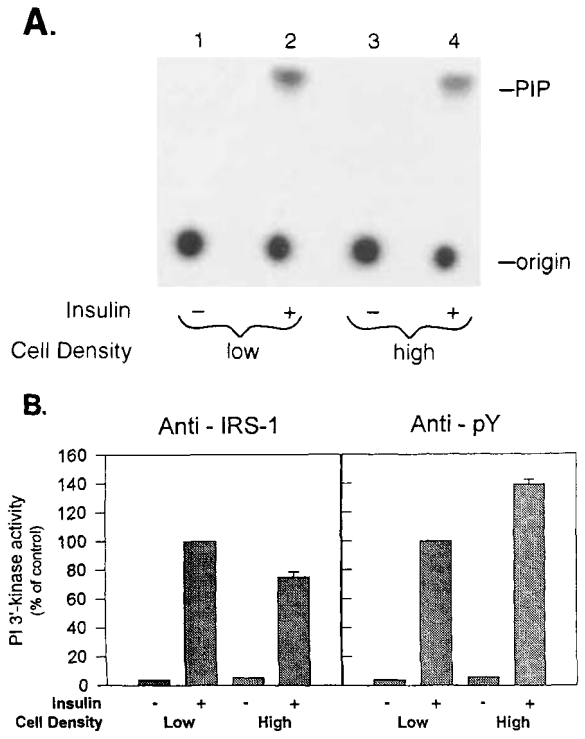

Fig. 3. **A:** Insulin-stimulated phosphatidylinositol 3-kinase activity in IRS-1 immunoprecipitates of KRC-7 hepatoma cells cultured at low and high density, as described in Figure 2. Assay of phosphatidylinositol 3′-kinase in immunoprecipitates of cell lysates was performed as described in Materials and Methods. The reaction product comigrating with the phosphatidylinositol 4′-P standard was quantitated by phosphorimager analysis. **B:** Replicate experiments of phosphatidylinositol 3-kinase activity in IRS-1 immunoprecipitates (*lefthand panel*) and antiphosphotyrosine immunoprecipitates (*righthand panel*) of hepatoma cell lysates at low- and high-density with and without insulin stimulation.

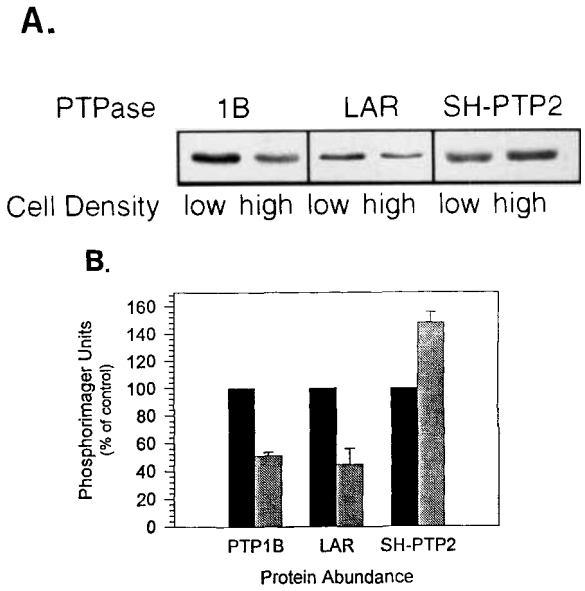

Fig. 4. **A:** immunoblots of three PTPases—PTP1B, LAR, and SH-PTP2—in normalized samples of lysates of hepatoma cells grown at low and high cell density. **B:** Data from replicate experiments of protein abundance for the three indicated PTPases in cells at low density (*black bars*) and high density (*gray bars*).

pleiotropic cellular response that leads to the activation of pathways of both cell growth and metabolism. A deeper issue also involves not only the activation of these various pathways, but how they might be differentially regulated so that individual target cells can respond in a manner appropriate to the physiological situation.

At a later stage of their growth cycle, well-differentiated hepatoma cells that are contact-inhibited in monolayer growth must have an alteration of their insulin-stimulated growth response, with the ability to still respond to the metabolic effects of the hormone, as do mature liver cells in the context of the architecture of the hepatic organ. In order to begin to answer some of these questions, we assessed changes in protein-tyrosine phosphorylation in the early steps of insulin action in cells plated at low and high cell density. These studies were designed to identify alterations in the insulin signaling pathway that might provide some insight into how various aspects of insulin's pleiotropic actions might be regulated within the same cell type.

The data obtained in the present work indicate that increasing density of hepatoma cells is associated with tyrosine hyperphosphorylation of "pp185" substrates of the insulin receptor (including IRS-1 and IRS-2), an interesting effect that paradoxically does not lead to a commensurate enhancement of the activation of phosphatidylinositol 3′-kinase in cells as they reach saturating growth density. This attenuation of post-pp185 signaling may be a way in which the hepatoma cells can deactivate specific cellular pathways that might have been important in the less dense cells and are required to a lesser degree in the more confluent cells. One mechanism by which the docking of certain pp185-associated enzymes could be attenuated in the cells grown at high density might involve the tyrosine phosphorylation of additional residues of the pp185 proteins not usually phosphorylated at low cell density. This effect might lead to the recruitment of new substrate proteins to dock to IRS-1 or IRS-2, which can sterically block some SH2 domain-containing dock-

ing proteins, or initiate signaling to new pathways that are required for cell function in the high density, growth-arrested state.

Reversible tyrosine phosphorylation of certain cell proteins is appreciated to be a key feature of regulation of the cell cycle and unregulated cell growth in transformed cells can be elicited by oncogenic tyrosine kinase activity. Since PTPases can potentially act as negative regulators of cellular protein-tyrosine phosphorylation, they have been implicated in the attenuation of the cell growth response in a few studies, including growth conditions impeded by cell-cell contact. Swiss 3T3 fibroblasts harvested at high density exhibited an eightfold increase in membrane PTPase activity that was attributed to a 37-kDa enzyme that was not further identified [Pallen and Tong, 1991]. The level of the low M_r cytosolic PTPase in fibroblasts also increases as the cells reach a contact-inhibited state [Ramponi et al., 1992]. Recently, Östman et al. [1994] have also identified a receptor-type, single domain PTPase called DEP-1, whose expression in cultured fibroblasts increases dramatically in dense cultures compared to sparsely cultured cells. These studies provide evidence for the modulation of a number of specific PTPase enzymes under conditions of varying cell growth density.

As potential mediators of the observed hyperphosphorylation of the pp185 proteins, however, we have found that two key PTPases for the insulin action pathway, PTP1B and LAR, are actually down-regulated in hepatoma cells grown at high cell density. Recent work from our laboratory has also provided evidence that both of these PTPases are involved in negative regulation of the insulin action pathway [Kulas et al., 1995; Ahmad et al., 1995b]. Thus, a potential mechanism involved in the increase in tyrosine phosphorylation of pp185 proteins at high cell density is a decrease in the abundance of the PTPases that serve as physiological regulators of reversible tyrosine phosphorylation of proteins in the insulin action pathway. The available data also suggests that some of these changes may be specific for certain cell types. In Swiss 3T3 fibroblasts, for example, LAR mRNA expression has been reported to increase by approximately twofold as cells grow from 50% to 100% confluence, although effects on the actual protein mass of the LAR enzyme were not reported [Longo et al., 1993]. Also, in cultured fibroblast cells, little change was observed in the abundance of PTP1B protein expression with increasing cell density [Ostman et al., 1994].

In order to provide further evidence that reduction of LAR expression is a potential mechanism for the observed changes in phosphorylation of the insulin receptor and pp185 proteins in the KRC-7 cells at high density, we performed studies using antisense oligonucleotides to complement our previous publication in McArdle hepatoma cells using stable expression of LAR antisense mRNA to reduce LAR protein levels [Kulas et al., 1995]. Exposure of KRC-7 cells plated at low density to an antisense oligonucleotide corresponding to positions 42–56 of the LAR mRNA protein coding region resulted in a reduction of LAR protein abundance to 23% of control levels. This reduction of LAR expression in the KRC-7 cells led to an increase in the insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit by 47% and that of pp185 proteins by 38%. These results substantiate the potential physiological relevance of the observed reduction in LAR expression on protein tyrosine phosphorylation in the hepatoma cells at high density.

In summary, the results presented here demonstrate that increasing cell density is associated with tyrosine hyperphosphorylation of pp185 insulin receptor substrates (IRS-1 and IRS-2), without a concomitant enhancement of activation of phosphatidylinositol 3′-kinase. These effects modulate post-receptor signaling by insulin in a manner that may be essential for the metabolic functions of growth-arrested cells. Evidence is also provided further supporting the hypothesis that PTP1B and LAR may have a physiological role in controlling the steady-state level of reversible tyrosine phosphorylation of intermediates in the insulin signalling pathway, in normally dividing cells, as we have shown previously, as well in growth at high cell density. Further work will help define additional alterations in post-receptor insulin signaling in cells at high density that may provide insight into regulatory mechanisms for the pleiotropic actions of insulin.

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