Use of an Antisense Strategy to Dissect the Signaling Role of Protein-tyrosine Phosphatase α*

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The protein-tyrosine phosphatase PTPα has been proposed to play an important role in controlling the dephosphorylation of a number of key signaling proteins and in regulating insulin signaling. To examine the potential cellular functions and physiological substrates of PTPα, a potent phosphorothioate oligonucleotide-based antisense strategy was developed that specifically depleted endogenous PTPα from 3T3-L1 adipocytes. The antisense probe, αAS1, achieved PTPα depletion levels normally of ≥85% and which varied up to levels where PTPα was not detected at all. Elimination of PTPα by 85% inhibited c-Src activity by 80%. Abolishing PTPα to levels undetected did not alter the tyrosine dephosphorylation of the insulin receptor or insulin receptor substrate proteins. Moreover, the ability of insulin to activate ERK2 or to stimulate DNA synthesis was not altered by αAS1. It is concluded that endogenous PTPα is a key regulator of c-Src activity in 3T3-L1 adipocytes and that PTPα is not required for the dephosphorylation of the insulin receptor or the insulin receptor substrate proteins or for the regulation of several downstream insulin signaling events in 3T3-L1 adipocytes. Finally, the development of the antisense probe, αAS1, provides an important molecular tool of general applicability for further dissecting the roles and precise targets of endogenous PTPα.

The large family of protein-tyrosine phosphatases (PTPs) identified over recent years represents a potentially important regulatory mechanism for cellular signal transduction through the modulation of protein tyrosine phosphorylation status. However, at present relatively little is known regarding the cellular functions of individual PTPs. Identification of PTP substrates is of importance in defining the physiological role of PTPs. However, in vivo substrates for many of the PTPs have yet to be established. Thus, there is an urgent need to develop methods capable of establishing the specific targets and roles of individual PTPs in physiologically relevant systems.

PTPα is a widely expressed receptor-like PTP characterized by a short (123 amino acids) glycosylated extracellular domain and two cytoplasmic catalytic domains (1, 2). The N-terminal catalytic domain has been shown to possess a majority of PTP activity toward exogenous phosphotyrosine-containing substrates, whereas the C-terminal domain exhibits low PTP activity (3, 4). PTPα has been shown to be constitutively phosphorylated on both serine and tyrosine residues (4–6). Phosphorylation of specific serine residues has been suggested to play a role in the regulation of PTPα activity (5, 6).

PTPα has been implicated as a positive controller of c-Src activity (7–10) and negative regulator of insulin signaling (11–13). Most of these studies have used overexpression systems. Thus overexpression of PTPα enhanced the activation of c-Src and the dephosphorylation of the C-terminal regulatory phosphotyrosine residue Tyr-527 of c-Src (7, 8). Similarly the insulin receptor (IR) was suggested as a putative substrate for PTPα since overexpression of PTPα in baby hamster kidney cells already overexpressing the IR rescued the cells from insulin-stimulated growth inhibition, and IR tyrosine phosphorylation of IR transiently overexpressed in human embryonic kidney 293 cells was diminished by transiently overexpressing PTPα as well (11–13). Overexpression of PTPα in cell lines may, however, lead to a shift in the expression pattern of seemingly unrelated tyrosine kinases or PTPs (see Ref. 12 and refs therein) and may lead to nonspecific effects. Although in the case of c-Src, PTPα−/− mice have very recently been reported to contain less c-Src activity in brain and fibroblasts (9, 10), the possibility, as stated by the authors (9), that additional mechanisms caused the altered c-Src activity was not excluded.

It is important to obtain direct evidence over whether the putative cellular roles of PTPα are functions of PTPα in physiological systems and at normal expression levels. Achieving this requires the development of methods that work against native PTPα in suitable cell types. Toward this end we now describe an antisense technique that enables the specific depletion of endogenous PTPα from intact cells. Moreover, the antisense strategy works on a cell type that is highly insulin-responsive and a major model for insulin signaling, namely, 3T3-L1 adipocytes. We have applied the antisense method to dissect facets of the signaling role of endogenous PTPα.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**3T3-L1 fibroblasts were a kind gift from Dr. G. Gould, University of Glasgow, Scotland. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 1-gluteraldehyde, Life Technologies, Inc.) and 10% fetal bovine serum (Sigma) at 37 °C in the presence of 5% CO₂. Medium was changed at 48-h intervals. For adipocyte preparation, 3T3-L1 fibroblasts were used at passage 6–9 in 22-mm dishes. At 2–3 days post-confluence cells were differentiated as described previously (14).

**Oligonucleotide Treatment of Cells—**Phosphorothioate-modified oligodeoxynucleotides (ODNs) were kindly synthesized by Dr. M. Pickett, Dept. of Microbiology, Southampton General Hospital. ODN preparation was as described previously (14). The phosphorothioate ODNs used were antisense αAS1 (CCA TGC TGA CCG AGC CGG) and the corresponding sense (CGG GCT CCG TCA GCA TGG), scrambled (CAC CGG
GCT GAC CTC GCA), and mismatch (CAA TGA TGA ACG AGA CCG) sequences. Lipofectin reagent (Life Technologies, Inc.) was used to facilitate the introduction of phosphorothioate ODNs into cells. 3T3-L1 adipocytes were routinely transfected between days 8 and 14 post-differentiation. Lipofectin reagent was allowed to preincubate with DMEM for 30–45 min. Appropriately diluted ODNs (100 μM of DMEM (no additions) and incubated with 100 μM of DMEM containing Lipofectin reagent (120 μg/ml) at room temperature for 15 min. During this time, cells were washed (3 × 1 ml) with prewarmed DMEM (no additions; 37 °C). Cells were then incubated with the mixture (200 μl) together with an equal volume (200 μl) of DMEM (no additions). With the no-ODN conditions, the mixture was still added to the cells, except where stated otherwise, but it contained no ODN. 3T3-L1 adipocytes were incubated in the presence of Lipofectin for 24 h. The transfection medium was then replaced with DMEM (supplemented with 0.25% (w/v) bovine serum albumin), and the ODN concentration was maintained. Additional medium changes were performed every 48 h. The ODN concentrations and incubation times utilized were routinely 15 μM and 7 days for aAS1 and corresponding control ODNs unless stated otherwise in figure legends. Cells were incubated with or without insulin at 37 °C as indicated. Cells were extracted as described previously (14).

Western Blotting—The monoclonal anti-mitogen-activated protein kinase antibody was from Zymed Laboratories Inc. The monoclonal anti-enolase, monoclonal c-Src antibody, and SHP-2 antibody were from Upstate Biotechnology, Inc. The anti-PTPα antibody was raised against the sequence KVQVEYIDAFSDFYANKF (residues 777–794 of the PTPα sequence in Ref. 2). In addition to the PTPα band at 130 kDa, this antibody detected a band at 140 kDa in Western blots of cell homogenates (e.g. see Fig. 1 and 2); both bands were competitively removed by the peptide used for the immunization. Because the peptide sequence used for immunization is significantly conserved in PTPs, we tested whether the 140-kDa band was PTPα. Three different antisense phosphorothioate oligonucleotides (17- or 18-mers) specific to distinct regions of the PTPα sequence depleted the 140-kDa band up to a level that was not detected, identifying the 140-kDa band as PTPα. The identification of the 140-kDa band as PTPα is supported by studies of Elson and Leder (15), who showed that PTPα is glycosylated in a tissue-specific manner, generating different molecular mass species depending on the tissue, including a major immunoreactive species of 140 kDa. The monoclonal anti-LAR antibody was from Transduction Laboratories. Western blotting was undertaken as described previously (anti-mitogen-activated protein kinase and anti-PTPα; Refs. 14, 16, and 17) or by following the manufacturer’s protocol (anti-c-Src, anti-phospho-tyrosine, anti-SHP-2, and anti-LAR). Immunoreactive bands were visualized using ECL reagent and Hyperfilm-MP (Amersham Pharmacia Biotech).

Assays—c-Src activity was assayed by a standard immunocomplex kinase assay. For this, anti-c-Src antibody IgG was coupled to protein A-agarose beads through a bridging layer of anti-mouse IgG (18). Plates of cells were washed rapidly with ice-cold phosphate-buffered saline and extracted into modified radioimmune precipitation buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 100 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell extracts from 4 identical wells were pooled and homogenized by repeated expulsion through a 25-gauge needle. 40 μl of the extract was removed for Western blotting against the anti-PTPα antibody. The remainder was centrifuged (10,000 × g, 10 min), and the supernatant was removed, adjusted to a final protein concentration of 1 mg/ml with phosphate-buffered saline, and incubated with 50 μl of protein A-agarose beads coated with anti-c-Src IgG for 18 h at 4 °C with constant agitation (10 rpm). Samples were subjected to Western blotting and standard kinase assay against enolase (acid-denatured; Ref. 19) after extensive washing (8).

For dephosphorylation assays of the IR and IRS proteins, cells were challenged with 100 ng insulin for 15 min, then washed with DMEM (no additions; 3 × 1 ml) and incubated in DMEM (no additions; 37 °C) for the required time. Short time courses were performed because the M and 7 days for PTPα treatments achieved from 3T3-L1 adipocytes was normally 99.9% (levels quantitated as 99.9% by metric scanning of amido black-stained nitrocellulose membranes) for the Specific Depletion of PTPα from Intact 3T3-L1 Adipocytes—The antisense ODN used in this study was selected using specially designed computer programs (20) according to the criteria described by Sale et al. (14). Modified phosphorothioate ODNs were used since they display an increased resistance to nuclease degradation. These were combined with the use of cationic liposomes (Lipofectin), which have been shown to enhance ODN uptake into cells and improve the subcellular distribution (21). Analysis of the murine PTPα sequence (from Ref. 1) revealed a region close to the initiation codon of low secondary structure with particular antisense potential, namely, nucleotides 12–29 of sequence 5′-CGG CCT CGG TCA GGA TGG-3′, where ATG is the initiation codon. The corresponding antisense probe, called aAS1, was of sequence 5′-CCA TGC TGA CGG AGC CGG-3′. Additionally, corresponding sense (CGG CCT CGG TCA GCA TGG), scrambled (CAC CGG GCT GAC CTC GCA), and mismatch (mismatches, CAA TGA TGA ACG AGA CCG) phosphorothioate ODNs were utilized.

The efficiency of PTPα antisense treatment was assessed by quantitative Western blot analysis using an anti-peptide antibody against PTPα. In addition to the PTPα band at 130 kDa, this antibody detected a second band in Western blots of cell homogenates (e.g. see Fig. 1 and 2); this band was of 140 kDa and was identified as the highly related PTPα (see "Experimental Procedures"). Treatment of cells with Lipofectin reagent alone had no affect on cellular levels of PTPα (data not shown). Pilot experiments showed that aAS1 was highly effective in depleting PTPα from 3T3-L1 adipocytes.

Time course and dose-response experiments (Fig. 1) were performed to optimize the antisense protocol. Exposure of 3T3-L1 adipocytes to aAS1 (15 μM) caused a time-dependent reduction in levels of PTPα, with near complete depletion of PTPα after 7 days (Fig. 1, A and B). Depletion of PTPα by aAS1 (7-day incubation) occurred in a dose-dependent fashion with near complete depletion of PTPα achieved with 15 μM aAS1 (Fig. 1, C and D). Thus the optimum protocol for obtaining PTPα depletion from 3T3-L1 adipocytes with aAS1 was a 7-day incubation at a concentration of 15 μM, and this protocol was used hereinafter. With this protocol, the depletion of PTPα achieved from 3T3-L1 adipocytes was normally ≥85% (e.g. Fig. 1) and varied up to levels where PTPα was not detected (e.g. Fig. 6) with a mean ± S.E. depletion of 91% ± 3% for blotts herein. The corresponding control ODNs (sense, scrambled, and mismatched) had no significant affect on the level of PTPα expression when compared with no-ODN controls (Fig. 2, A and B), suggesting that aAS1 was acting specifically in these cells. Further support for the specificity of aAS1 (also see "Discussion") comes from observations that aAS1 treatment of cells did not significantly affect the expression of several internal control proteins. These included a number of PTPs, namely, the highly related PTPs as well as LAR and SHP-2 (Figs. 1–3 and 6, respectively), the expression of the protein kinases ERK1, ERK2, and p90rsk (e.g. Fig. 8 and data not shown) and the levels of all other major cellular proteins as determined by densitometric scanning of amido black-stained nitrocellulose membranes were unaltered by aAS1 (levels quantitated as 99.9% ± 0.3% for aAS1, 100% ± 0.3% for scrambled ODN, 100.8% ± 2.1% for sense ODN, and 101.6% ± 1.7% for mismatch ODN-treated cells, where 100% was taken to be the level of protein in the no-ODN-treated cells). Moreover, aAS1 did not affect cell.
morphology or cell viability and did not cause disruption of cell function, since cells exhibited the characteristic cellular responses to insulin (discussed later). The depletion of PTPα elicited by αAS1 was reversible. Washing out the αAS1 resulted in full recovery of the level of PTPα in no-ODN condition. Dose response: cells were incubated for 7 days with the indicated αAS1 concentration; C, representative anti-PTPα Western blot; D, quantitation of Western blots from 3 experiments (means ± S.E.), where 100% was taken to be the level of PTPα in no-ODN controls. The PTPα and PTPε bands were of 130 and 140 kDa, respectively.

**c-Src**—To examine whether endogenous PTPα plays a role in the regulation of c-Src activity in 3T3-L1 adipocytes, control and αAS1-treated cells were subjected to immunoprecipitation with an anti-c-Src antibody. Immunocomplex assays showed that c-Src activity from cells treated with αAS1 was reduced by 80% compared with that in no-ODN control cells (Fig. 5). The level of c-Src recovery in the immunocomplexes was not affected by αAS1 treatment (Fig. 5). Western blotting of whole cell lysates from the same cells showed that αAS1 had caused significant inhibition of PTPα expression (~85% depletion of PTPα protein) when compared with no-ODN controls (Fig. 5). Experiments with control ODNs confirmed that they did not affect PTPα expression or c-Src phosphorylation (see above and data not shown). These results indicate that PTPα plays an important role in the regulation of c-Src activity in 3T3-L1 adipocytes.
Insulin Signaling—The IR is an insulin-activated tyrosine kinase that catalyzes the autophosphorylation of tyrosine residues in its β-subunit (reviewed in Ref. 22). Autophosphorylation results in activation of the insulin receptor tyrosine kinase and leads to the phosphorylation of endogenous substrates (23). PTPs have been postulated to be important for the regulation of insulin signaling because the IR does not possess autodephosphorylation activity (24–26). Indeed, dephosphorylation of the IR by cellular PTPs has been shown to return insulin receptor tyrosine kinase activity to basal levels, with the tyrosine 1150 domain of the IR β-subunit in tris-phosphorylated form being exquisitely sensitive to PTP action (27, 28). In addition, PTPs can potentially regulate insulin signaling at the level of the IR substrates. These include IRS-1, IRS-2, and Shc, whose dephosphorylation modulates the post-receptor pathways of insulin action by impairing the docking and activation of SH2-containing proteins. IRS-1 and IRS-2, which comigrate on SDS-polyacrylamide electrophoresis gels and are collectively termed "IRS proteins" stand out as a well characterized major band of 185 kDa on anti-phosphotyrosine Western blots of insulin-stimulated cells (29). Previous studies have shown that the tyrosine phosphorylation state of the IRS proteins is closely regulated in vivo (26), but the PTPs responsible for the dephosphorylation of the IRS proteins have not yet been identified.

A role for PTPs in the negative regulation of insulin signaling has been suggested by studies using overexpression systems (11–13). However, this potential role has not yet been investigated in the major insulin-sensitive tissues or at endogenous levels of PTP expression. To this end, the effect of PTPα depletion on several well characterized insulin responses was examined in 3T3-L1 adipocytes. To rigorously test whether the IR and the IRS proteins are substrates for PTPα in vivo, the effect of PTPα depletion on both their rate of tyrosine dephosphorylation after removal of insulin and on their steady state tyrosine phosphorylation in the presence of insulin were determined.

To study the effect of PTPα depletion on the rate of dephosphorylation of the IR β-subunit and IRS proteins after the removal of insulin, cells were transfected as before prior to challenging with 100 nM insulin for 15 min. A time course of dephosphorylation of the IR β-subunit and IRS proteins was performed by withdrawal of insulin from all cells and incubating them in DMEM (no additions) for specified lengths of time. Preliminary experiments indicated that dephosphorylation of

**Fig. 5.** PTPα depletion suppresses c-Src activity in 3T3-L1 adipocytes. Immunocomplex c-Src kinase activity was assayed against enolase. In addition, anti-PTPα and anti-c-Src Western blots of extracts from the same cells were quantitated by densitometry. In each case, 100% was taken to be the value in the no-ODN control condition.

**Fig. 6.** Dephosphorylation of the IR β-subunit and IRS proteins in 3T3-L1 adipocytes. Cells were transfected without or with the indicated ODNs and challenged with 100 nM insulin for 15 min before incubation with DMEM (no additions) for the indicated times. A, dephosphorylation profile of the IR β-subunit from quantitated anti-phosphotyrosine Western blots. B, dephosphorylation profile of the IRS proteins from quantitated anti-phosphotyrosine Western blots. C, representative anti-PTPα Western blot. In A and B, values are for no ODN (○), αAS1 (■), and scrambled ODN (▲) conditions. Results are expressed as means ± S.E. (n = 3).

both the IR and IRS proteins was very rapid in 3T3-L1 adipocytes (within 5 min). Tyrosine phosphorylation of the IR and IRS proteins was determined by anti-phosphotyrosine Western blotting.

In no-ODN control cells, tyrosine dephosphorylation of the IR β-subunit occurred very rapidly, with a majority of tyrosine phosphorylation lost after 1 min of insulin withdrawal and with maximum dephosphorylation being reached after 2 min (Fig. 6A). This profile of IR β-subunit dephosphorylation is in agreement with a previous study using permeablized rat adipocytes (30). In addition, the rate of tyrosine dephosphorylation of the IRS proteins was rapid, with a majority of dephosphorylation occurring within 2 min (Fig. 6B). A comparison of the rate of dephosphorylation of the IR β-subunit and IRS proteins in no-ODN control cells indicated that the IR β-subunit was dephosphorylated more rapidly than the IRS proteins after insulin withdrawal.

Depletion of a PTP, which plays a negative regulatory role at the level of the IR or IRS proteins, would be expected to slow the rate of dephosphorylation of these proteins. However, in cells treated with αAS1, dephosphorylation of the IR β-subunit
was extremely rapid (a majority of phosphotyrosine was lost after 1 min), at a rate similar to that in the no-ODN and scrambled-ODN controls (Fig. 6A). Furthermore, treatment of cells had no significant effect on the dephosphorylation of the IRS proteins when compared with no-ODN and scrambled-ODN controls (Fig. 6B). Western blotting against the anti-PTPα antibody was performed in each experiment to determine the level of tyrosine phosphorylation at 100 nM insulin in the no-ODN panels. Results are representative of three experiments obtained for PTPα depletion levels of 80–90%.

The effect of PTPα depletion on the steady state tyrosine phosphorylation of the IR β-subunit and IRS proteins over a range of insulin concentrations was also determined. Treatment of no-ODN control cells with a range of insulin concentrations (0, 1, 10, and 100 nM) caused an increase in the phosphotyrosine content of the IR β-subunit in parallel with increasing dose (Fig. 7). If PTPα was controlling the phosphotyrosine status of the IR or IRS proteins, αAS1 treatment of cells would be expected to increase their sensitivity to insulin (i.e. shift the dose-response curve to the left). However, treatment of cells with αAS1 did not significantly affect the level of the IR or IRS protein tyrosine phosphorylation at any of the insulin concentrations tested, when compared with the no-ODN controls (Fig. 7). In addition, cells treated with the scrambled ODN exhibited a similar insulin dose-response curve (Fig. 7). Basal tyrosine phosphorylation of the IR and the IRS proteins was also unaffected by αAS1 treatment (Fig. 7). Western blotting against the anti-PTPα antibody confirmed extensive depletion of PTPα in these experiments (see the legend to Fig. 7).

The effect of depletion of PTPα on insulin signaling to more distal sites was next investigated. Activation of the ERK1/ERK2 cascade is a well established post-receptor response to insulin treatment. The effect of αAS1 on insulin-stimulated ERK2 activation was measured by band shift on anti-ERK2 Western blots (17). An insulin concentration was chosen that activated 50% of the cellular ERK2 pool, such that any augmentation in ERK2 activation caused by PTPα depletion would be detected. As illustrated in Fig. 8A, treatment of control cells with insulin for 15 min caused a marked stimulation of ERK2 activation (Fig. 8A, lane 1 compared with lane 4; mean 45%, see Fig. 8B). Insulin stimulation of scrambled-ODN-treated cells resulted in a similar level of ERK2 activation compared to no-ODN controls (mean ± S.E.).
activation to that seen in no-ODN cells (Fig. 8A, lane 6 compared with lane 4; mean 47%, see Fig. 8B). αAS1 treatment of cells caused marked depletion of PTPα when measured by an anti-PTPα Western blot (Fig. 8C), yet this caused no change in the ERK2 band-shift pattern when compared with no-ODN and scrambled-ODN-treated cells (Fig. 8A, lane 5 compared with lanes 4 and 6; average ERK2 activation was quantitated as 47%, see Fig. 8B). These results are consistent with those above showing that PTPα does not affect insulin signaling at the receptor level. Furthermore, these results indicate that PTPα does not regulate the insulin-stimulated activation of ERK2 at a post-receptor site.

A further aspect of post-IR signaling investigated in this work was insulin-stimulated DNA synthesis, as assayed by \(^{3}H\)thymidine incorporation. In comparison with the no-ODN, scrambled-ODN, and mismatch-ODN controls, basal levels of DNA synthesis were not significantly affected by αAS1 treatment of cells (see legend to Fig. 9). This result was of importance since an insulin-independent alteration in DNA synthesis would make it difficult to determine whether PTPα depletion had any affect on the activation of DNA synthesis by insulin. Additionally, a submaximal concentration of insulin was used. Incubation of the no-ODN control cells with insulin caused a ~2-fold increase in the level of \(^{3}H\)thymidine incorporation (Fig. 9A). Moreover, a similar increase in the level of \(^{3}H\)thymidine incorporation was observed after insulin stimulation of cells treated with the scrambled and mismatch control ODNs (Fig. 9A). If PTPα was playing a negative role in regulating this insulin-responsive pathway, then depletion of PTPα should augment the level of insulin-stimulated DNA synthesis. However, marked depletion of cellular PTPα protein (Fig. 9B) did not significantly affect the ability of insulin to increase DNA synthesis when compared with the control conditions (Fig. 9A). Hence, these results complement those indicating that PTPα does not negatively regulate insulin receptor tyrosine kinase activity. In addition, these results suggest that PTPα does not play a role in the pathway ultimately leading to an insulin-stimulated increase in the rate of DNA synthesis.

Collectively, all results obtained in this study are consistent with the hypothesis that PTPα is not necessary for the regulation of insulin signaling, either at the level of the IR or by acting on various downstream components of the insulin-signaling cascade, in 3T3-L1 adipocytes.

**DISCUSSION**

At present, relatively little is known regarding the roles of specific PTPs in eukaryotic cell signaling. Antisense ODNs are proving useful in the study of complex signaling pathways, since depletion of one component of a functional system can give information regarding a specific role for that protein in a pathway- and cell-specific context.

In the present study, an effective antisense strategy was developed for the specific depletion of PTPα from 3T3-L1 adipocytes. A phosphorothioate-modified antisense ODN was utilized (called αAS1) that targeted the region of mRNA close to the initiation codon. This sequence was chosen since many antisense studies have used antisense probes against the initiation codon region of the target mRNA with great success (e.g. Refs. 14, 31, and 32).

αAS1 potently depleted PTPα in a time- and concentration-dependent manner (Fig. 1). A micromolar concentration of αAS1 achieved PTPα depletion levels normally of ≥85% (e.g. Fig. 1), which varied up to levels where PTPα was not detected at all (e.g. Fig. 6) with a mean ± S.E. depletion of 91% ± 3%.

Several points of evidence were obtained to indicate that the action of αAS1 in depleting PTPα was specific. First, the expression of PTPα was not significantly affected by the scrambled, sense, or mismatched phosphorothioate ODNs. Second, αAS1 did not alter the level of several internal control proteins including other PTPs (namely the highly related PTPs, LAR, and SHP-2) and several protein kinases (namely ERK1, ERK2, and p90RR). Third, αAS1 did not affect the expression of any major cellular proteins as visualized by staining of membrane filters. Fourth, αAS1 did not impair general cell function. Thus, cells treated with αAS1 retained normal morphology and exhibited characteristic responses to insulin (including normal insulin-stimulated tyrosine phosphorylation of the IR and IRS proteins, ERK2 activation and stimulation of DNA synthesis) when compared with no-ODN control cells. Collectively, these results provide rigorous evidence that the action of αAS1 in depleting PTPα from 3T3-L1 adipocytes was specific.

It is important to obtain direct evidence over whether putative substrates for PTPα are in vivo targets of PTPα in physiological systems and at normal expression levels. Fulfilling this objective requires the development of methods that work with endogenous proteins in suitable cell types. Our antisense technique achieves this objective because it enables the specific depletion of endogenous PTPα from intact cells. Moreover, the antisense method works on a cell type that is highly insulin-responsive and a major model for insulin signaling, namely, 3T3-L1 adipocytes. Subsequent work utilized the antisense
strategy to dissect the involvement of PTPα in a variety of cellular functions.

Depletion of cellular PTPα protein by ~85% decreased the level of c-Src activity by ~80% in 3T3-L1 adipocytes (Fig. 5), demonstrating that c-Src is regulated by endogenous PTPα in 3T3-L1 adipocytes. This result is consistent with a very recently reported decrease in the level of c-Src kinase activity in brain and fibroblasts from PTPα−/− mice (9, 10). The level of c-Src activity remaining after αASI treatment of cells was relatively low (20% of the control), indicating that PTPα is the dominant PTP governing c-Src activity in 3T3-L1 adipocytes.

PTPs have been postulated to play a particularly important role in the regulation of insulin signaling since the IR and its substrates do not have autodephosphorylation activity (reviewed in Refs. 24–26). Since multiple PTPs are expressed in the major insulin-sensitive tissues (i.e. adipose, liver, and muscle), there is a need to ascertain the significance of individual PTPs in each of these tissues. A number of PTPs have been proposed to affect insulin action at receptor or post-receptor sites. These include PTPα, PTPε, LAR, and PTP1B, which have been proposed to negatively regulate IR activity (11–13, 33–40), and SHP-2, which has been shown to have a positive regulatory effect on post-receptor signaling (41–44). PTPα is expressed in the major insulin-sensitive tissues. However, a role for PTPα in insulin signaling in these tissues has yet to be established. With this in mind, experiments were designed to determine whether PTPα regulates insulin signaling in 3T3-L1 adipocytes.

To rigorously test whether PTPα is required for the tyrosine dephosphorylation of the IR β-subunit, the effect of PTPα depletion on both the rate of IR tyrosine dephosphorylation and on the steady state tyrosine phosphorylation of the IR at a variety of insulin concentrations was measured. The kinetics of IR β-subunit dephosphorylation after removal of insulin were not significantly altered by elimination of PTPα to levels not detected (Fig. 6). The steady state phosphorylation of the IR was also measured because even low levels of depletion of a PTP that dephosphorylated the IR would be expected to shift the insulin dose-response curve by increasing the sensitivity of IR tyrosine phosphorylation to insulin. Depletion of PTPα protein levels by up to 90% did not significantly affect either the level of basal IR β-subunit phosphorylation or the insulin dose-response curve (Fig. 7).

In addition, substantial evidence was obtained to suggest that PTPα does not modulate insulin action at the level of the IRS proteins. The rate of dephosphorylation of the IRS proteins after withdrawal of insulin was not affected by depletion PTPα to levels undetected (Fig. 6). Similarly, reduced PTPα expression had no effect on either basal or steady state insulin-stimulated tyrosine phosphorylation of the IRS proteins (Fig. 7). These results show that endogenous PTPα is not required for the dephosphorylation of the IR β-subunit or the IRS proteins in vivo in 3T3-L1 adipocytes.

Insulin-stimulated ERK2 activation and DNA synthesis are both known downstream effects of IRS-1 and Shc phosphorylation (23). Depletion of PTPα had no effect on the level of acute ERK2 activation achieved in response to stimulation of cells with insulin (Fig. 8), on the basal level of DNA synthesis, or the insulin-stimulated increase in DNA synthesis (Fig. 9). This supports the above results with the IR and IRS proteins. Moreover, these results suggest that PTPα is not required for regulating other components of the signaling pathway.

Any residual PTPα activity remaining after αASI treatment of cells is unlikely to account for the absence of effects on insulin signaling for the following reasons. First, depletion of PTPα by ~85% caused a parallel inhibition of c-Src activity and, thus, the residual PTPα was insufficient to maintain c-Src activity.

Second, depletion levels in the insulin-signaling experiments were high, e.g. dephosphorylation of the IR and IRS proteins was unperturbed by ablating PTPα to levels that were undetected. Third, the phosphorylation status of the IR and IRS proteins has been shown to be relatively sensitive to a reduction in levels of expression PTPs, which have been implicated as candidates for the negative regulation of insulin signaling. For example, antisense-mediated depletion of LAR protein (63% reduction) has been shown to cause a marked increase in IR (150%) and IRS-1 (350%) phosphorylation in rat hepatoma cells (34).

Results from the present study are in contrast with overexpression studies in baby hamster kidney cells (11) and human embryonic kidney 293 cells (12–13), where insulin signaling was investigated, and illustrate the need to experiment with endogenous proteins in physiologically relevant systems. Our conclusions are supported by analysis of the specificity of PTPα for the various IR phosphotyrosine residues. It is believed that the PTPs responsible for the deactivation of the insulin receptor tyrosine kinase exhibit selectivity for the tris-phosphorylated tyrosine 1150 domain species of the IR β-subunit, but PTPα displays no such catalytic preference (27, 28, 45). Therefore, the parallel overexpression of PTPα and the IR in the system of Ulrich and co-workers (11–13) may result from interactions that do not normally occur under physiological conditions. Our conclusions are also supported by the results of Jacob et al., (46) who overexpressed PTPα in GH4 pituitary cells and reported that although PTPα inhibits insulin-increased prolactin gene expression, this was not due to reduced tyrosine phosphorylation of the IR, IRS1 or Shc.

In summary, results from this study demonstrate that endogenous PTPα is an important regulator of c-Src activity in 3T3-L1 adipocytes and that PTPα is not required for the dephosphorylation of the IR or IRS proteins or for the regulation of certain downstream insulin-signaling events in 3T3-L1 adipocytes. Additionally, the development of the antisense probe αASI against PTPα provides an important molecular tool for general applicability for further dissecting the roles and precise targets of endogenous PTPα in physiologically relevant cell types.

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