PTP-1B Dephosphorylation of the Insulin Receptor Occurs in a Perinuclear Endosome Compartment in HEK 293 Cells

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Abbreviations:
BRET, bioluminescence resonance energy transfer; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERC, endocytic recycling compartment; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HEK, human embryonic kidney; IR, insulin receptor; MEF, mouse embryonic fibroblast; pNPP, p-nitrophenylphosphate; PDGFR, platelet derived growth factor receptor; PTP-1B, protein tyrosine phosphatase-1B; RTK, receptor tyrosine kinase.

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ABSTRACT

Protein tyrosine phosphatase-1B (PTP-1B) is a negative regulator of insulin signaling. It is thought to carry out this role by interacting with and dephosphorylating the activated insulin receptor (IR). However, little is known regarding the nature of the cellular interaction between these proteins, especially since the IR is localized to the plasma membrane and PTP-1B to the endoplasmic reticulum. Using confocal microscopy and fluorescence resonance energy transfer (FRET) the interaction between PTP-1B and the IR was examined in co-transfected HEK 293 cells. Biological activities were not significantly affected for either PTP-1B or the IR with the fusion of W1B-Green Fluorescent Protein (GFP) to the N-terminus of PTP-1B (W1B-PTP-1B) or the fusion of Topaz-GFP to the C-terminus of the IR (Topaz-IR). FRET between W1B and Topaz was monitored in cells transfected with either wild type PTP-1B (W1B-PTP-1B) or the substrate trapping form PTP-1B\texttextsubscript{D181A} (W1B-PTP-1B\texttextsubscript{D181A}) and Topaz-IR. Co-expression of W1B-PTP-1B with Topaz-IR resulted in distribution of Topaz-IR to the plasma membrane but no FRET was obtained upon insulin treatment. In contrast, co-expression of W1B-PTP-1B\texttextsubscript{D181A} with Topaz-IR caused an increase in cytosolic Topaz-IR fluorescence and in some cells, a significant basal FRET signal, suggesting that PTP-1B is interacting with the IR during its synthesis. Stimulation of these cells with insulin resulted in a rapid induction of FRET that increased over time and was localized to a perinuclear spot. Co-expression of Topaz-IR with a GFP labeled RhoB endosomal marker and treatment of the cells with insulin identified a perinuclear endosome compartment as the site of localization. Furthermore, the insulin-induced FRET could be prevented by the treatment of the cells with a specific PTP-1B inhibitor. These results suggest that PTP-1B appears not only to interact and dephosphorylate the insulin-stimulated IR in a perinuclear endosome compartment but also is involved in maintaining the IR in a dephosphorylated state during its biosynthesis.
INTRODUCTION

The insulin receptor is a heterotetramer plasma membrane protein that consists of two extracellular α subunits and two membrane spanning intracellular β subunits that contain inherent tyrosine kinase domains (1). The receptor is initially synthesized as a single polypeptide α-β proreceptor that undergoes processing in the endoplasmic reticulum (ER). While in the ER the proreceptor dimerizes, undergoes disulfide bond formation, glycosylation and proteolysis before transfer to the plasma membrane (2-4). Binding of insulin to the receptor on the plasma membrane results in activation of the β-subunit tyrosine kinase activity and once activated, autophosphorylation on three critical tyrosine residues in the kinase activation loop occurs, resulting in full activation of the tyrosine kinase activity (1). The activated IR then begins to phosphorylate its various substrates to initiate the insulin signaling cascade but at the same time undergoes internalization into endosomes, where it remains activated (5,6). However, acidification of the endosome lumen causes insulin dissociation from the receptor, which prevents further activation and the receptor then undergoes dephosphorylation at which time it is then sorted back to the plasma membrane (5). Identification of the protein tyrosine phosphatases (PTPs) involved in the dephosphorylation of the activated IR is of critical interest since inhibitors directed towards these PTPs could be used in the treatment of insulin resistance and Type 2 diabetes.

Using a mouse gene knockout, PTP-1B was shown to be a crucial phosphatase involved in the in vivo dephosphorylation of the activated IR (7, 8). The results from the PTP-1B null mouse were key in establishing the role of PTP-1B in the negative regulation of insulin signaling (9). In addition to having enhanced insulin sensitivity, these mice were also resistant to diet induced obesity. Moreover, insulin-induced tyrosine phosphorylation of the receptor was increased and
prolonged in muscle and liver of these animals suggesting that the role of PTP-1B is to dephosphorylate the activated IR (7). Although these studies have highlighted the importance of PTP-1B as a negative regulator in insulin signaling, it is conceivable that these results could possibly be due to an indirect effect of PTP-1B on insulin signaling and not due to a direct interaction between PTP-1B and the IR. PTP-1B has been shown to be located on the cytoplasmic surface of the ER (10), whereas the IR is located at the plasma membrane; where and how these two proteins interact within the cell has not been demonstrated. Recently, Boute et al (11) reported on the interaction between PTP-1B and the IR in cells using bioluminescence resonance energy transfer (BRET), even though these authors could show very nicely that PTP-1B and the insulin-activated IR do interact, where in the cell this interaction takes place was not reported. In order to investigate where PTP-1B and the IR interact within the cell we have fused PTP-1B and the IR to mutant derivatives of the green fluorescent protein (GFP) and employed fluorescence resonance energy transfer (FRET) to monitor the interaction (12). FRET is a non-destructive and non-invasive technique for effectively measuring protein-protein interactions in the range of 10–100 Å (12). By utilizing this technique we show that PTP-1B rapidly interacts with the insulin-activated IR in a specific subcellular compartment. Furthermore, this interaction can be disrupted by the addition of a specific PTP-1B inhibitor.
MATERIALS AND METHODS

Materials.

The cDNAs for W1B and Topaz in pRSETA vectors were obtained from Aurora Biosciences. pDNR-1, subcellular localization vectors, (pECFP-Endo, pEYFP-Endo and pEYFP-ER) and anti-GFP antibodies were from Clontech. The pET11a vector was from Novagen, and all other cloning vectors were supplied by Invitrogen. Mouse and human PTP-1B antibodies were from Upstate and the IR antibodies for the α and β subunits were obtained from BD Biosciences and Santa Cruz, respectively. The anti-phospho Tyr-1158 antibody was supplied by Biosource. Anti-rabbit IgG horseradish peroxidase-linked whole antibody was obtained from Amersham. Cell culture media was obtained from Gibco. Poly-d-lysine coated culture slides were obtained from Becton Dickinson, and antifade mounting medium was from Amersham. The W1B-Topaz fusion construct containing the six amino acid linker was kindly provided by Paul Tawa (13). The PTP-1B inhibitor, disodium\{4-[[3’-(aminosulfonyl)-4’-bromobiphenyl-4-yl]methyl\]thio)methyl]-2-bromophenyl\}(difluoro)methyl]phosphonate (see Fig. 5B inset) was synthesized at Merck Frosst. Details of the synthesis can be found in reference 14. All other reagents were supplied by Sigma.

Construction of W1B and Topaz fusion proteins in expression vectors.

W1B-PTP-1B fusion protein. The W1B GFP mutant was amplified from pRSETA-hW1B by polymerase chain reaction (PCR) such that an Nde I site was added at the 5’ end and an Eco RI site at its 3’ end with removal of the stop codon. The fragment was first subcloned into pCR2.1-Topo and then cloned into pDRN-1r using Nde I and Eco RI. Full-length hPTP-1B was amplified to add an Eco RI site at its 5’ end and a Bam HI site at its 3’ end and subcloned into
Following excision with Eco RI and Bam HI, the PTP-1B cDNA was cloned in-frame to the 3’ end of W1B in pDNR-1r to generate a fusion protein containing a two amino acid linker (D,F) between the GFP and PTP-1B. To generate the W1B-PTP-1B substrate-trapping mutant (15), Asp at amino acid position 181 was changed to Ala using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) using pDNR-1r-W1B-PTP1B as a template. For expression of W1B-PTP-1B in bacterial cells the fusion construct was excised from pDNR-1r and cloned into the Nde I/Bam HI sites in pET11a. For mammalian expression, the constructs (either wildtype or mutant) were excised from pDNR-1r with Asp718 and Bam HI and cloned into the appropriate sites in pcDNA3.1.

**Topaz-IR fusion protein.** The Topaz GFP mutant was excised from pRSETA-hTopaz using EcoR1 and Xho1 and cloned into the EcoR1/Xho1 site of pcDNA3.1. The 5’ half of the human IR cDNA was excised from pECE-hIR (kindly supplied by Dr. Bei Zhang) (16) using Hind III and Bam HI digestion, while the 3’ half was amplified by PCR to include the Bam HI site and to add an Eco RI site and remove the stop codon at the 3’ end. The resulting Topaz-IR fusion protein contained a 5 amino-acid linker peptide (AEFAT) between the coding sequence of hIR and the ATG codon of Topaz. Fidelity of the constructs was verified by sequencing.

**Bacterial expression and partial purification of W1B-PTP-1B for kinetic characterization.**

Escherichia coli (E. coli) BL21 cells containing the W1B-PTP-1B-pET11a plasmid were grown in Luria Bertani broth at 37 °C to an optical density (O.D) of 0.7, induced by the addition of 1 mM IPTG and grown for an additional 2 hours. A 50 µl cell pellet of harvested cells was washed twice with PBS, resuspended in 1.5 ml lysis buffer (PBS + 5 mM EDTA) and briefly sonicated. Following addition of 150 µl of 10 % Triton X-100, the cells were left at 4 °C overnight with gentle rocking. Cell lysate was then collected by centrifugation at 12,000×g for
30 minutes and 30 µl of protein G sepharose beads (50% slurry) was added followed by incubation for a further 1 hour at 4 °C to remove non-specific binding. The supernatant was collected and combined with 10 µl of anti-GFP antibody (full-length polyclonal) and 40 µl of protein G sepharose. Following overnight incubation at 4 °C with rocking, the supernatant was removed by centrifugation and the sepharose beads were then washed 5 times with PBS and resuspended in enzyme dilution buffer (100 mM Tris/HCl, 5 mM N,N’-dimethyl-bis(mercatoacetyl)hydrazine (DMH), 2 mM EDTA, 2% glycerol, 0.01% Triton X-100, pH=7.4). Enzyme activity was quantitated as described previously (17).

**Cell cultures and transfections.**

HEK 293 EBNA cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 1 mM sodium pyruvate, 100 Tg/ml penicillin, 100 Tg/ml streptomycin, 0.25 mg/ml G-418 and 10% fetal bovine serum (Invitrogen) in a humidified 37 °C incubator with 6 % CO₂. For immunoprecipitation and Western blot analysis, cells were seeded onto poly-d-lysine coated plates (Biocoat, Becton Dickinson) at a density of 1 × 10⁶ cells per 100-mm plate. Cells were transfected 24-hours later with 13 µg of the appropriate plasmids using Lipofectamine Plus reagent (Invitrogen) following the manufacturer’s protocol. Twenty-four hours post-transfection, the transfection mixture was removed and was replaced with regular growth medium. Cells were maintained under normal growth conditions until ready to use. For confocal microscopy and FRET experiments, transfections were performed as outlined above, except that cells were transfected in poly-d-lysine coated chamber slides. Cells were seeded at a density of 1 × 10⁵ per well of a 4-well chamber slide and grown for 24 hours prior to transfection. The cells were transfected with 120 ng of the appropriate plasmids using
Lipofectamine Plus reagent and maintained under normal growth conditions as described above. For cotransfections, equal amounts of the constructs were added to the transfection mixtures. For “mock” transfections, cells were transfected by following the same protocol, but without DNA.

**Immunoprecipitation and Western blot analysis of HEK 293 cell lysates.**

Transiently transfected HEK 293 EBNA cells were serum starved in DMEM supplemented with 0.15% BSA (no serum) for 4 hours to overnight at 37 °C. Following stimulation with 100 nM insulin, the cells were immediately placed on ice, the media was removed, and the cells were washed twice with ice-cold PBS. Lysates were prepared by scraping the cells into 1 ml of lysis buffer containing 10 mM sodium phosphate pH 7.0, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM sodium pyrophosphate, 100 µM pervanadate, and a Complete™ protease inhibitor cocktail (Roche). Cell debris was removed by centrifugation of the samples for 15 minutes at 13,000 × g. The supernatants were used directly for Western blot analysis or were processed further for immunoprecipitation experiments. In the latter case, the supernatants were diluted to yield a protein concentration of 1 mg/ml and 1 mg of each sample was incubated with 3 µg of anti-PTP-1B antibody (Upstate) overnight at 4 °C with gently rocking. 100 µl of protein G sepharose (50% slurry) was then added to the antibody complexes and the mixtures were incubated for a further 4 hours at 4 °C with gentle rocking. The immunoprecipitates were then harvested by centrifugation and washed twice with lysis buffer followed by two washings with PBS. Immune complexes were resolved by SDS-PAGE on 8% Tris-glycine gels. The procedures for immunoblotting of cell lysates and immune complexes were essentially the same. Briefly, following SDS-PAGE, the resolved proteins were transferred onto nitrocellulose overnight at 16V (75 mA). Membranes were blocked in 5% BSA and incubated with the
appropriate primary antibodies in the presence of 3% BSA followed by anti-rabbit horseradish peroxidase linked whole antibody. Protein bands were detected by chemiluminescence using the Western Lightning™ kit (Perkin Elmer).

**Microscopy, Image Analysis and FRET quantification.**

Cells were transfected and grown on glass slides as described above. Following insulin treatment, cells were washed twice with PBS and fixed with 3.2% paraformaldehyde for 20 minutes at room temperature. Following three more washes (15 minutes each) with PBS, the cells were mounted under glass coverslips with Prolong™ Antifade reagent (Molecular Probes) according to the manufacturer’s instructions and visualized under a Zeiss LSM510 laser-scanning confocal microsome. For detection of W1B, cells were viewed with an excitation filter of 458 nm, a beam splitter of 545 nm, and a 475–525 nm bandpass emission filter. Topaz was detected using an excitation filter of 514 nm, a beam splitter of 545 nm, and a 530–600 nm emission filter. The filters for FRET were an excitation filter of 458 nm, a beam splitter of 545 nm, and a 530–600 nm emission filter. Images were captured using a cooled CCD camera. All images were background subtracted and corrected FRET ($FRET^C$) was calculated as follows (18):

$$FRET^C = I_{FRET} - (I_{W1B} \times a + I_{Topaz} \times b)$$

where $I_{FRET}$, $I_{W1B}$, and $I_{Topaz}$ were the intensities of the images under the FRET, W1B, and Topaz filter sets, respectively. $a$ is the percentage of W1B bleed-through into the FRET filter set, and $b$ was the percentage of Topaz bleed-through into the FRET filter set. There were no bleed-
through signals from W1B under Topaz filter sets and *vice versa*. \( a \) and \( b \) were determined by taking images of cells containing W1B or Topaz alone and determining the relative intensity of emission in the FRET channel and that in the W1B or Topaz channel. A broad range of intensities was examined, and a line of best fit was drawn through the data to obtain correction factors. Exposure settings were identical for all images.
RESULTS

Construction of GFP fusion proteins and analysis of protein expression.

FRET analysis using mutant derivatives of GFP fused to PTP-1B and the IR was used to investigate the cellular interactions between PTP-1B and IR. In order to confirm that PTP-1B retained normal catalytic activity when fused to GFP at its N-terminus, W1B-PTP-1B was expressed and purified from bacterial cells and its ability to hydrolyze p-nitrophenylphosphate (pNPP) was characterized. W1B-PTP-1B displayed normal phosphatase activity with respect to pNPP hydrolysis ($K_m = 1.4 \text{ mM}$ and $k_{cat} = 24 \text{ sec}^{-1}$), which could be abolished by treatment with BzN-EJJ-amide, a potent PTP-1B inhibitor (19) (data not shown). To increase the steady-state population of PTP-1B and IR complexes for optical analysis of cellular fluorescence, we examined PTP-1B-IR interactions using the D181A substrate-trapping form of PTP-1B (W1B-PTP-1B$_{D181A}$) (15).

The expression of W1B-PTP-1B$_{D181A}$ and Topaz-IR co-transfected into HEK 293 EBNA cells was examined using Western blot analysis. Figures 1A and D show the presence of an 80 kDa band following blotting with anti-GFP and anti-PTP-1B antibodies respectively, which corresponds to the expected molecular mass of the W1B-PTP-1B fusion protein. Similarly, blotting with anti-GFP and anti-IR-β subunit antibodies (Fig. 1A and B, respectively) revealed the presence of a 120 kDa band corresponding to the molecular mass of the β-subunit of the IR fused to Topaz. The blots also revealed the presence of a ~ 250 kDa band that in addition to cross reacting with the GFP and β-subunit antibodies (Fig. 1 A,B) also cross reacted with an antibody raised against the α subunit of the IR (IR-α) (data not shown), indicating that the band corresponds to the unprocessed α-β-Topaz form of the receptor. The presence of the unprocessed IR in transfected HEK 293 cells has been previously observed (20). These authors
also found that both the β-subunit and the α-β precursor became tyrosine phosphorylated even in the absence of ligand (20). We also observed similar results in this study where a significant level of basal phosphorylation was observed on Tyr-1158 for both the β-Topaz-subunit and the α-β-Topaz precursor (Fig. 1C, lane 3). Following treatment with 100 nM insulin the level of phosphorylation on the β-subunit-Topaz fusion protein increased at least 2-fold whereas, there was no change in the phosphorylation level of the unprocessed IR (Fig. 1C, lane 4). This result is again consistent with the previous report where insulin treatment increased the tyrosine phosphorylation level of the β-subunit ~2 fold but had no affect on the level of precursor phosphorylation (20).

More importantly, the addition of the GFPs to PTP-1B and IR did not hinder their interaction as the protein complexes could be immunoprecipitated from cell lysates with an anti-PTP-1B antibody (Fig. 1E). In the lysates W1B-PTP-1B interacted with both the β-subunit Topaz fusion protein as well as the unprocessed α-β-Topaz form of the receptor. However, treatment of the cells with 100 nM insulin only resulted in an increase in the amount of the β-subunit Topaz fusion protein that was pulled down in complex with W1B-PTP-1B_{D181A} (Fig. 1E lane 4).

**Localization of W1B-PTP-1B and Topaz-IR in transfected HEK 293 cells.**

GFPs are soluble proteins that when expressed in mammalian cells are normally distributed throughout the cell (12). This typical distribution of GFP is shown in Figure 2A where a W1B-Topaz fusion protein with a 6 amino acid linker was used (13). Because the GFPs are linked they show extensive co-localization and FRET throughout the cell (Fig. 2A). However, fusion of W1B to either wild type (Fig. 2B, 2C) or the substrate trapping mutant of PTP-1B (Fig. 2D, 2E) results in distribution of the W1B fluorescence to a region of the cell surrounding the nucleus. A
similar cellular distribution for YFP-PTP-1B in HEK cells was recently reported and this distribution is consistent with PTP-1B’s association with the ER (11). Support for the localization of W1B-PTP-1B to the ER was obtained when W1B-PTP-1B_{D181A} was co-transfected with a GFP-calreticulin ER targeting vector (Fig. 2D). Both W1B-PTP-1B and GFP-calreticulin show similar distribution and extensive co-localization.

Distribution of the Topaz-IR co-transfected with the wild type W1B-PTP-1B resulted in the localization of the Topaz-IR fluorescence primarily to the plasma membrane (Fig 2B, 2C). There was minimum co-localization and no FRET was detected with wild type W1B-PTP-1B either in the absence or presence of insulin (Fig. 2B, and 2C respectively). In contrast, co-transfection of Topaz-IR with the substrate trapping form of PTP-1B, W1B-PTP-1B_{D181A}, resulted in a significant increase in Topaz-IR intracellular fluorescence (Fig. 2E). In a number of the Topaz-IR and W1B-PTP-1B_{D181A} co-expressing cells extensive co-localization was observed as well as a significant basal level of FRET (Fig. 2E). The insulin-independent FRET in these cells appeared to have an ER type of distribution. Based on the results from four independent experiments, where a total of 202 (~ 50 cells examined per experiment) co-expressing cells were examined, 56 or 28% (SD ± 9%) of co-expressing cells display FRET in the absence of insulin.

**Insulin-induced FRET between W1B-PTP-1B and Topaz-IR.**

The basal FRET observed in HEK cells co-expressing W1B-PTP-1B_{D181A} and Topaz-IR is consistent with the trapping of the Topaz-IR by W1B-PTP-1B_{D181A} as it is being processed in the ER. This enhanced basal interaction between the substrate trapping form of PTP-1B and the IR was also recently reported by Boute et. al. who utilized BRET to investigate the interaction between the IR and PTP-1B (11). At the basal level, as stated above, about 28% of the co-
expressing HEK cells displayed FRET whereas the remainder, although co-expressing both W1B-PTP-1BD181A and Topaz-IR did not (Fig. 3A). However, treatment of these cells with 100 nM insulin resulted in about a doubling of the number of the cells displaying a FRET signal. Similarly, as described above, in four independent experiments where 409 (~100 cells per experiment) co-expressing cells were examined after insulin treatment, 208 or 51% (SD±14%) displayed a FRET signal. The insulin-induced FRET signal appeared as early as 5 minutes after insulin treatment as a small perinuclear spot (Fig. 3B) and increased in size and intensity over time (Fig. 3C, 3D). Thirty minutes after insulin treatment the FRET signal was quite intense and was still concentrated in a perinuclear region (Fig. 3D). The cellular location of this insulin-induced FRET signal is very analogous to that of the endosome-recycling compartment (ERC) (21,22). Receptors that are endocytosed and recycle back to the plasma membrane traffic through this compartment (21). Ligand binding to fluorescently labeled receptors results in their trafficking to the ERC which, in many cell types appears as a fluorescent spot in a peri-nuclear location (22-24). To determine if the IR and PTP-1B can localize to an endosomal perinuclear compartment both the Topaz-IR and W1B-PTP-1BD181A were co-transfected with RhoB fused to various GFP mutants. In the absence of insulin, Topaz-IR fluorescence primarily localizes to the plasma membrane (Fig. 4A) and there is minimum co-localization with ECFP-RhoB which localizes to multiple endosome vesicles (25). Upon insulin treatment, there is significant internalization of the Topaz-IR fluorescence as well as a clustering of ECFP-RhoB fluorescence to a perinuclear endosomal compartment, where there is an intense co-localization with Topaz-IR (Fig. 4A). W1B-PTP-1BD181A fluorescence, in either the presence or absence of insulin, is ER localized and although EYFP-RhoB after insulin treatment again localizes to a perinuclear
endosome compartment its co-localization with W1B-PTP-1B<sub>D181A</sub> is much more diffused (Fig. 4B).

*Insulin-induced FRET between W1B-PTP-1B<sub>D181A</sub> and Topaz-IR can be blocked with a specific PTP-1B inhibitor.*

To determine if the FRET signal between W1B-PTP-1B<sub>D181A</sub> and Topaz-IR was sensitive to PTP-1B inhibition, co-transfected HEK 293 cells were treated with a potent and selective PTP-1B inhibitor (Fig. 5B inset). This is a novel (14) reversible active site-directed inhibitor that has an IC<sub>50</sub> of 8 nM on PTP-1B and is selective compared to other PTPs (ie. IC<sub>50</sub> CD45 = 20 µM) except for TC-PTP (IC<sub>50</sub> = 8 nM). HEK 293 cells expressing W1B-PTP-1B<sub>D181A</sub> and Topaz-IR were treated with 100 µM of the PTP-1B inhibitor for 1 hour prior to insulin stimulation. Irrespective of insulin treatment, there was a similar change in the pattern of Topaz-IR localization when cells were treated with the PTP-1B inhibitor (Fig. 5A). Topaz-IR appeared to be more localized to the plasma membrane and displayed a reduced co-localization with PTP-1B. This distribution is reminiscent of the Topaz-IR co-expressed with wild type W1B-PTP-1B (Fig. 2B, 2C). Nevertheless, not all W1B-PTP-1B<sub>D181A</sub> and Topaz-IR interactions were disrupted by treatment with the PTP-1B inhibitor since the percentage of cells displaying background FRET signals (ie. FRET in the absence of insulin) were not reduced with inhibitor treatment. However, the insulin-induced FRET signal was completely blocked when cells were treated with 100 µM of the inhibitor. In fact, the insulin-induced FRET signal was inhibited in a dose-dependent manner by treatment of the cells with increasing concentrations of the PTP-1B inhibitor (IC<sub>50</sub> ~15 µM) (Fig. 5B). These results suggest that the PTP-1B inhibitor binds to the active site of W1B-PTP-1B<sub>D181A</sub> and blocks its interaction with Topaz-IR, however, if W1B-PTP-1B<sub>D181A</sub> and
Topaz-IR are already in a complex (ie. basal FRET) this interaction is then resistant to the inhibitor.
DISCUSSION

PTP-1B acts as a negative regulator of insulin signaling by dephosphorylating the activated IR. Although there is much support for PTP-1B in this role, how it carries this out in the cell is unclear, particularly since PTP-1B resides in the ER and the IR is located in the plasma membrane. In order to understand how this interaction occurs, confocal microscopy and FRET analysis were used to visualize this interaction within the cell. FRET has become a valuable tool to visualize protein-protein interactions in cells since it is only elicited when the fluorophores are less than 100 Å apart (12,26-28). The donor (W1B) and acceptor (Topaz) GFPs have been used previously, and are highly suited for FRET measurements due to significant overlap in their emission spectra, low Ro factors, high quantum yields, and resistance to photobleaching (13). Using this approach, we have demonstrated that PTP-1B not only directly interacts with the IR within an endosomal compartment in an insulin dependent manner but they also appear to interact in an insulin independent manner in the ER whereby PTP-1B is required to assist in the trafficking of the IR to the plasma membrane.

The involvement of PTP-1B in controlling the tyrosine phosphorylation state of the IR during biosynthesis has been previously postulated (20). Lammers et. al. showed that over expression of the IR as well as various receptor tyrosine kinases (RTK) in HEK 293 cells resulted in a significant level of ligand independent receptor tyrosine phosphorylation (20). This phosphorylation occurred not only on the β-subunit but also on the α-β receptor precursor. Treatment with insulin caused ~ 2 fold increase in β-subunit tyrosine phosphorylation, without any effect on the precursor. Most interestingly was the observation that when PTP-1B was co-transfected with the IR the precursor phosphorylation in the presence and absence of insulin disappeared. We observed similar results when Topaz-IR was co-expressed with W1B-PTP-
PTP-1B D181A cannot dephosphorylate the IR, thus both the precursor and β-subunit exhibited a basal level of phosphorylation in the absence of insulin. The loss of proper processing of the IR probably accounts for the increased intracellular Topaz-IR distribution. This may also account for the background FRET observed in a percentage of the co-expressing cells due to the more stable W1B-PTP-1B D181A unprocessed Topaz-IR complex. It is also possible that the trapping of Topaz-IR as it trafficks through the ER to the plasma membrane by W1B-PTP-1B D181A results in the accumulation of the trapped product in the ER, accounting for the intense background FRET in some cells. Because the background FRET signal results from a stable pre-existing complex between W1B-PTP-1B D181A and Topaz-IR, it would be resistant to disruption by a PTP-1B inhibitor would explain why the addition of the PTP-1B inhibitor had no effect on the background FRET level. In contrast, addition of the PTP-1B inhibitor would prevent free uncomplexed W1B-PTP-1B D181A from interacting with Topaz-IR, resulting in the improved distribution of Topaz-IR to the plasma membrane in these cells. Similarly, the blockade of the insulin-induced FRET would be due to inhibitor bound PTP-1B preventing an interaction with the activated IR.

It is not clear why only 28% of the co-expressing cells display the background FRET, since it may be expected that if PTP-1B interacts with the IR during biosynthesis then a higher percentage of cells would have a FRET background. HEK 293 cells do express an endogenous PTP-1B that would be able to contribute to the processing of Topaz-IR. Perhaps in the cells with the background FRET, Topaz-IR is expressed at a higher level, beyond the capacity of the endogenous PTP-1B, thus allowing for increased interactions with W1B-PTP-1B D181A. In contrast, co-expression of Topaz-IR with W1B fused to wildtype PTP-1B results in the correct processing of the IR and localization of Topaz-IR to the plasma membrane. Boute et. al. using
BRET to monitor the interaction between the IR and the substrate trapping mutant of PTP-1B also found an insulin independent basal BRET signal that they attributed to PTP-1B interacting with the IR during biosynthesis (11). In their study either the removal of the ER localization signal from PTP-1B or treatment of cells with tunicamycin resulted in a significant decrease in basal BRET which, would support a role for the ER localized PTP-1B in the biosynthesis of the IR.

Upon binding of insulin, the tyrosine kinase activity of the IR becomes activated, resulting in autophosphorylation to fully activate the receptor which, then becomes quickly internalized into endosomes (5,6). These events occur within the first few minutes of insulin binding and the internalization allows not only for the IR to phosphorylate substrates that are spatially distinct from those at the plasma membrane but also for attenuating the insulin signal (5,6). Once in the endosome dissociation of insulin from the receptor takes place, preventing further receptor activation. At some point dephosphorylation occurs and the receptor is sorted to recycle back to the plasma membrane or sent to the lysosome for degradation. In this study insulin induced FRET between W1B-PTP-1B_{D181A} and Topaz-IR was observed as early as 5 minutes after insulin treatment suggesting that the interaction between IR and PTP-1B occurs fairly rapidly after IR activation and internalization. These results are in agreement with the time course for the activation and dephosphorylation of the IR as well those reported for the BRET interaction between the IR and PTP-1B that can be detected within 30 seconds of insulin addition (5,11,29). The observation that the FRET signal increases over time after insulin addition probably indicates that W1B-PTP-1B_{D181A} is trapping Topaz-IR in the endosome compartment preventing its dephosphorylation and recycling back to the plasma membrane. Blockage of IR dephosphorylation by peroxovanadium compounds has been shown to cause an accumulation of
the IR in endosomes and inhibition of recycling to the plasma membrane (6). Over expression of Topaz-IR and treatment of cells with insulin results in intense co-localization of Topaz-IR with RhoB, an endosomal protein that is found in early endosomes and prelysosomal compartments but not in mature lysosomes (25). The localization of Topaz-IR with Rho B in a perinuclear endosome compartment and the fact that the insulin-induced FRET observed between W1B-PTP-1B$_{D181A}$ and Topaz-IR also occurs in the same cellular location suggests that PTP-1B interacts with the IR in this perinuclear endosome compartment. It has been previously shown that receptor recycling in HEK 293 cells utilizes a perinuclear endocytic recycling compartment whose distribution is very similar to that seen for the insulin-induced FRET (22-24). Although we cannot say unequivocally that PTP-1B interacts with the IR in this endocytic recycling compartment, it is very likely the case.

The BRET analysis of the PTP-1B-IR interaction showed that insulin can stimulate a BRET signal between YFP-PTP-1B$_{D181A}$ and IR fused to Renilla luciferase but where this interaction occurs was not demonstrated (11). The authors showed that the ER localized form of the PTP-1B substrate trapping mutant produced a higher insulin-induced BRET signal than the cytosolic form and that the ER form of PTP-1B required internalization of the IR for BRET to occur. Posner and colleagues have carried out detailed analyses on IR internalization and signaling (5,6,29). The results presented here are consistent with their previous studies on the activation of the IR and its internalization into endosomes. They also found that PTP-1B was not present in rat liver endosomes containing the activated IR (29). Although we find some co-localization of PTP-1B with Rho B, it is unlikely that PTP-1B is localized to the endosome but rather this may be due to the fact that both the ER and endosome come in close proximity. Thus the interaction between PTP-1B and the endosome localized activated IR takes place between the endosome and
ER. Recently FRET was also used to analyze the interaction of PTP-1B with the epidermal- (EGFR) and platelet- (βPDGFR) derived growth factor receptors and suggested that the interaction occurred in punctate structures on the surface of the ER (30). The PTP-1B, IR interaction described here suggests that the ER localized PTP-1B binds to and dephosphorylates the IR in a single perinuclear endosome compartment. This difference in the interaction between PTP-1B and the IR compared with EGFR and PDGFR could be related to the cell types used in the studies, HEK 293 versus a mouse PTP-1B−/− fibroblast cell line, or possible differences in growth factor receptor recycling. For example both EGF and PDGF do not dissociate from their respective receptors in endosomes thus favoring sorting of the receptor to lysosomes (21). Whereas, insulin readily dissociates from the IR in endosomes and the receptor is rapidly recycled (5,6). Another difference may lie in the timing of the PTP-1B, RTK interaction. In the studies presented here FRET between PTP-1B and the IR was detected as early as 5 min after receptor activation, (earlier time points were not performed) whereas FRET between EGFR and PDGFR could be detected at 10 min but was more evident at 30 min. The fairly rapid detection of the PTP-1B, IR interaction described here is consistent with the recent BRET publication (11). This probably reflects the more direct and less invasive method of FRET production (between two GFPS) utilized in this paper, compared to cell permeabilization and incubation with a conjugated PTP-1B antibody used in the other report.

In summary, we have shown that insulin-induced activation of the IR results in a rapid and direct interaction between PTP-1B and the IR in a perinuclear endosome compartment in HEK 293 cells. However, with the development and characterization of these unique tools, it will now be possible to determine if similar interactions occur in additional insulin sensitive cell lines, especially those derived from liver, muscle and fat.
FOOTNOTES:

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FIGURE LEGENDS

Figure 1: Expression of W1B-PTP-1B and Topaz-IR in HEK 293 EBNA cells. Cells were transfected with W1B-PTP-1B and Topaz-IR and stimulated with or without 100 nM insulin for 5 minutes at 37 °C. Cell lysates were prepared and immunoblotted (IB) with A) anti-GFP B) anti IR-β (C19) C) anti-pTyr-1158 and D) anti-PTP-1B antibodies (bands at ~60 and ~110 kD are non-specific). Lanes 1 and 2 correspond to mock transfected cells, lanes 3 and 4 are lysates from cells transfected with W1B-PTP-1B_{D181A} and Topaz-IR, and lane 5 corresponds to a CHO-IR cell lysate. The β-subunit of the Topaz-IR (~120 kDa) and W1B-PTP-1B (~ 80 kDa) are indicated by arrows. E) Immunoprecipitation (IP) of Topaz-IR with anti-PTP-1B antibody from transiently transfected HEK-293 EBNA cell lysates. Immunoprecipitates were probed with an anti-IR-β subunit antibody. Lanes 1 and 2 correspond to mock transfected cells, lanes 3 and 4 are lysates from co-transfected cells, and lane 5 is lysate prepared from cells transfected with Topaz-IR.

Figure 2: Cellular localization of W1B-PTP-1B and Topaz-IR in transfected HEK 293 cells. A) Cellular localization and FRET of the W1B-Topaz fusion protein, W1B fluorescence is shown in green, Topaz fluorescence is shown in red, regions of co-localization (Merge) appear yellow and the corrected FRET showing intense FRET (red/orange, see scale on right) distributed uniformly throughout the cell. B,C) Representative images of cells co-transfected with wild type W1B-PTP-1B and Topaz-IR constructs before (B) and 15 minutes after insulin treatment (C). D) Co-localization of W1B-PTP-1B_{D181A} with the EYFP-calreticulin ER-localization vector. E) Representative images of insulin-independent FRET in W1B-PTP-1B_{D181A} and Topaz-IR co-transfected cells.
Figure 3: Insulin-induced FRET between W1B-PTP-1B<sub>D181A</sub> and Topaz-IR. Representative images of co-transfected HEK 293 cells before and at various times after insulin treatment. W1B-PTP-1B<sub>D181A</sub> fluorescence appears green, Topaz-IR fluorescence appears red, regions of co-localization appear orange/yellow and corrected FRET is shown in the far right panels for each series. Images were collected prior to insulin treatment (A), and following a 5 (B), 15 (C) and 30-minute (D) stimulation with 100 nM insulin. The highest FRET signals (red) are indicated by an arrow and the weakest signals (background) appear blue.

Figure 4: Topaz-IR co-localizes with the endosome localization vector ECFP-RhoB after insulin treatment. A) HEK 293 cells co-transfected with Topaz-IR and the endosome localization vector ECFP-RhoB and stimulated with or without 100 nM insulin for 15 minutes. ECFP-RhoB fluorescence shown is in green, Topaz-IR fluorescence is shown in red and co-localization appears yellow. B) HEK 293 cells co-transfected with W1B-PTP-1B<sub>D181A</sub> and EYFP-RhoB vector and stimulated with or without 100 nM insulin for 15 minutes. W1B-PTP-1B<sub>D181A</sub> fluorescence is shown in green, EYFP-RhoB fluorescence is shown in red and co-localization appears yellow.

Figure 5: Inhibition of insulin-induced FRET by a specific PTP-1B inhibitor. A) Representative images of HEK 293 cells co-transfected with W1B-PTP-1B<sub>D181A</sub> and Topaz-IR, and then treated with 100 µM of the PTP-1B inhibitor for 1 hour followed by stimulation with 100 nM insulin for 15 minutes. B) PTP-1B inhibitor (Inset) dose titration of insulin-induced FRET. Percent inhibition of the insulin-induced FRET was determined for various concentrations of the PTP-1B inhibitor. The values are the average of 2-3 experiments ± SEM.
Figure 1

A
IB: anti-GFP
Insulin: - + - +

B
IB: anti-IR-β
- + - +

C
IB: anti-pTyr1158
- + - +

D
IB: anti-PTP-1B
- + - +

E
IP: anti-PTP-1B
IB: anti-IR-β
- + - +

+ Topaz

Figure 1

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Figure 2

A

W1B

Topaz

Merge

FRET

B

W1B-PTP1B

Topaz-IR

Merge

FRET

C

W1B-PTP1B

Topaz-IR

Merge

FRET

D

W1B-PTP1B_D181A

EYFP-Calreticulin

Merge

E

W1B-PTP1B_D181A

Topaz-IR

Merge

FRET
Figure 3

A

W1B-PTP1BD18A  Topaz-IR  Merge  FRET

0 min

B

5 min

C

15 min

D

30 min
Figure 4

A

- ECFP-RhoB
- Topaz-IR
- Merge
- – Insulin
- + Insulin

B

- W1B-PTP1B
- EYFP-RhoB
- Merge
- – Insulin
- + Insulin
Figure 5

A

W1B-PTP1B<sub>D181A</sub>  Topaz-IR  Merge  FRET

B

Inhibition of Insulin-Induced FRET

% Insulin-Induced FRET

0  1  10  100

µM Compound
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Yolanda Romsicki, Mark Reece, Jacques-Yves Gauthier, Ernest Asante-Appiah and Brian P. Kennedy

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