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Protein-tyrosine Phosphatase H1 Controls Growth Hormone Receptor Signaling and Systemic Growth*[S]

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Several protein-tyrosine phosphatases (PTPs) have been implicated in the control of growth hormone receptor (GHR) signaling, but none have been shown to affect growth *in vivo*. We have applied a battery of molecular and cellular approaches to test a family-wide panel of PTPs for interference with GHR signaling. Among the subset of PTPs that showed activity in multiple readouts, we selected PTP-H1/PTPN3 for further *in vivo* studies and found that mice lacking the PTP-H1 catalytic domain show significantly enhanced growth over their wild type littermates. In addition, PTP-H1 mutant animals had enhanced plasma and liver mRNA expression of insulin-like growth factor 1, as well as increased bone density and mineral content. These observations point to a controlling role for PTP-H1 in modulating GHR signaling and systemic growth through insulin-like growth factor 1 secretion.

Growth hormone (GH)[4] is key to mammalian growth and plays additional roles in metabolism, immune surveillance, heart development, and behavior (1), all of which are mediated by the growth hormone receptor (GHR). GHR engagement triggers activation of Janus kinase 2 (JAK2), STAT-3 and -5, and the mitogen-activated phosphatase kinase pathways but is also under the control of negative feedback (1, 2). Such feedback control includes ubiquitin-dependent endocytosis of the GHR (3, 4), transcriptionally induced SOCS (suppressors of cytokine signaling) proteins, PIAS (protein inhibitors of activated STATs) and signaling attenuation by protein-tyrosine phosphatases (PTPs). Of particular interest among these factors are the PTPs, enzymes that, if validated, constitute drugable targets that may be exploited to treat growth-related disorders (2). The human PTP family comprises 37 "classical" PTPs (which exclusively dephosphorylate phosphotyrosine residues) plus 65 "dual specific phosphatases," most of which in addition dephosphorylate serine- and threonine-phosphate residues (5). Some of the classical PTPs have been implicated in GHR signaling, namely Src homology domain 2-containing tyrosine phosphatase-1 and -2 (SHP-1 and -2), PTP-1B, and TC-PTP. The enzymatic activity of SHP-1 limits JAK2 activation upon GH stimulation (6). By contrast, SHP-2 has been shown to act as both a positive and negative regulator of GH signaling, depending on its local concentration and cell context. It is recruited to the GH-induced complex that consists of GHR, JAK2, and SIRP-α (signal-regulatory protein α). GH stimulation induces SHP-2 binding to growth factor receptor-bound protein 2 (7). Mutation of the GHR docking site for SHP-2 prolongs GH signaling (8), but expression of catalytically inactive SHP-2 reduces transcriptional activity downstream of the GH receptor (7).

GHR, JAK2, STAT-5a, and STAT-5b are all physiological substrates for PTP-1B (9, 10). In cells in which the *PTP-1B* gene was deleted, GH induces hyperphosphorylation of JAK2, STAT-3, and STAT-5, whereas PTP-1B overexpression reduces GH-mediated gene expression (11).

TC45 is a nuclear splice form of TC-PTP that dephosphorylates STAT1 (12) and STAT-5 (13). In cells in which the *TC-PTP* gene had been deleted, nuclear dephosphorylation of GH-activated STAT1 and STAT-3, but not STAT-5 or STAT6, was impaired (12).

Cellular and biochemical assays allow screening of large numbers of PTPs for their implication in pathways. We have shown previously that phosphorylated GHR is a good *in vitro* substrate for PTP-1B, TC-PTP, PTP-H1, and Sap-1 (10). One would predict that the absence of a PTP that exerts negative control on GHR signaling leads to enhanced sensitivity to GH and increased systemic growth, such as shown in transgenic animals that overexpress GH (14). Yet mice that lack functional SHP-1 (15–17), SHP-2 (18–20), TC-PTP (21, 22), or PTP-1B (23, 24) fail to show evidence of enhanced growth or other signs of enhanced GH signaling.

In the present study we tested all GH-induced tyrosine-phosphorylated players as potential substrates for a large panel of PTPs in biochemical and cellular studies. Our results implicate PTP-H1 at various points of the GH-induced pathway. We

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[4] The abbreviations used are: GH, growth hormone; GHR, GH receptor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; PTP, protein-tyrosine phosphatase; SHP, Src homology domain 2-containing tyrosine phosphatase; IGF, insulin-like growth factor; GST, glutathione *S*-transferase; siRNA, small interfering RNA; KO, knock-out; HET, heterozygous; WT, wild type; DEXA, dual energy x-ray absorptiometry; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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demonstrate that mice that lack the PTP-H1 catalytic domain are significantly heavier than their wild type littermates and present with increased plasma levels of insulin-like growth factor 1 (IGF-1), consistent with a greater sensitivity to GH and suggesting that PTP-H1 plays a controlling role *in vivo* in GHR signaling and adult body size.

EXPERIMENTAL PROCEDURES

Cells, Media, and Reagents—HEK293 cell line were maintained in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose/L-glutamine (Invitrogen 41965-039) supplemented with 10% fetal bovine serum and Geneticin (10131027 from Invitrogen) when selectin stable transformants. The cells were transfected with full-length rabbit GHR (10) using Lipofectamine 2000 reagent (Invitrogen) or GeneJammer reagent (Stratagene). Growth hormone was from Serono, clinical grade. HepG2 cells were grown in Eagle's minimal essential medium (Invitrogen 21090-022) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium private.

Immunoprecipitation and Western Blot Detection—SDS-polyacrylamide gels (Novex, 4–12%) were run according to the manufacturer's instructions, and proteins were transferred to nitrocellulose membrane (Bio-Rad 162-0112 Trans-Blot) using a dry blot (Bio-Rad) transfer apparatus. The membrane was blocked in phosphate-buffered saline, 0.2% Tween 20, 5% dissolved nonfat milk powder and then incubated overnight at +4 °C with indicated antisera at 1:1,000 dilution. The blots were washed and incubated for 1 h at room temperature with corresponding horseradish peroxidase-conjugated antiserum (goat anti-mouse Ig-horseradish peroxidase (Dako P0447), goat anti-rabbit Ig-horseradish peroxidase (Bio-Rad 170–6515), or donkey anti-goat Ig-horseradish peroxidase (Santa Cruz sc-2020) at 1:2,000 dilution, washed again, and visualized by chemoluminescence (ECL kit, Amersham Biosciences RPN 2106). For the Western blot antibodies, qPCR primers, Luciferase reporter plasmids, and siRNA sequences used, please see supplemental materials.

Luciferase Assays—96-well plate-grown cells were lysed in 50 μl/well of passive lysis buffer 1× (Promega E1941); 10 μl of lysate was mixed with 50 μl of 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM ATP, and 200 μM D-Luciferin (Biotium 10101), and light emission was read for 10 s in a MicroLumat LB96P luminometer (EG&G Berthold). 50 μl of 50 mM ammonium acetate, pH 5.2, 100 mM NaCl, and 2.5 μM coelenterazine (Prolume Ltd. 55 779-48-1) was added, and light production was read for 5 s.

In Vitro Dephosphorylation of GHR, JAK2, and STAT-3 and -5—HEK-GHR cells were starved overnight and stimulated with pervanadate (50 μM) and GH (100 ng/ml) for 30 min. The cells were lysed, and the indicated proteins were immunoprecipitated. The immunoprecipitates were divided and incubated with 1 μg of indicated GST fusions of phosphatase domains for 30 min in 37 °C in phosphatase buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol). Equal GST loading was controlled with Ponceau staining of the nitrocellulose membrane.

Serum IGF-1 Quantification—Blood from wild type and PTP-H1 knock-out female and male littermates (four animals per group) were sampled individually and allow to clot for 2 h at room temperature. The serum was removed after a 20-min centrifugation at 1,000 × *g*, and IGF-1 concentration was measured with a mouse IGF-1 Quantikine enzyme-linked immunosorbent assay kit (R & D Systems, MG-100).

PTP Enzyme Selectivity Assay—Assays were performed in a 96-well plate format, using the catalytic core of a human recombinant PTP as the enzyme (25) and 6,8-difluoro-4-methylumbelliferyl phosphate (Molecular Probes, D-6567) as a substrate. The compounds to be tested were dissolved in 100% Me_2SO at a concentration of 2 mM. Subsequent dilutions of the test compounds (to yield concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19, 0.097, and 0.049 μM) were performed in 60% Me_2SO manually. 8 μl of diluted compound or vehicle (60% Me_2SO = control) was distributed to a black Costar 96-well plate. 42 μl of human recombinant PTP enzyme diluted in assay buffer (20 mM Tris HCl, pH 7.5, 0.01% Igepal CA-630, 0.1 mM ethylenediaminetetracetic acid, 1 mM DL-dithiothreitol) was added to the dilutions of compound or vehicle (distributed in a black Costar 96-well plate) preincubated for 10 min at room T, followed by 50 μl of 6,8-difluoro-4-methylumbelliferyl phosphate diluted in the assay buffer. After 60 min at room temperature, the fluorescence intensity (integral or intensity) was measured in a Perkin-Elmer Life Sciences Victor 2 spectrofluorimeter (excitation of 6,8-difluoro-7-hydroxy coumarin at 355 nm, emission at 460 nm, for 0.1 s). The percentage of inhibition is determined by measuring the relative fluorescence in absence of a test compound (PTP inhibitor), *i.e.* with solvent alone (5% Me_2SO).

mRNA Quantification by qPCR—Total RNA was prepared using TRIzol reagent (Invitrogen 15596-026) using the manufacturer's protocol. The procedures for reverse transcription and real time PCRs were described previously (10), using SYBR GREEN PCR master mix (4309155 from Applied Biosystems).

RNA Interference Experiments—Annealed siRNA duplexes (5 nM) were HEK293-transfected using HiPerFect transfection reagent (Qiagen). In a typical siRNA experiment, adherent HEK cells were transiently transfected with GHR-expressing plasmid on day 1 (using Lipofectamine 2000; Invitrogen), trypsinized, and replated in 24-well plates (1×10^5 cells/well) on day 2. Transfection complexes were prepared by 10 min of incubation of 37.5 ng of siRNA and 3 μl of HiPerFect in 100 μl of OptiMEM medium (Invitrogen). The complexes were added to cell suspensions, and the cells were grown for 48 h prior to a 12-h serum starvation on day 4 and stimulation on day 5 as indicated. Efficiency of gene silencing was monitored by reverse transcription-PCR.

In Vitro Dephosphorylation Assay—The assay was performed essentially as described previously (9). Briefly, immunoprecipitated proteins (protein A-Sepharose CL-4B 17-0780-01; Amersham Biosciences) prepared from pervanadate/GHR-treated HEK293 cells overexpressing GHR were washed twice with dephosphorylation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol) and incubated with 1 μg of GST-PTP fusion proteins in dephosphorylation assay buffer for 30 min at 37 °C. The reactions were terminated by adding SDS sample buffer. In the case of JAK2, the JAK2 immune complex agarose (Upstate Biotechnology Inc., 14-134) was autophos-

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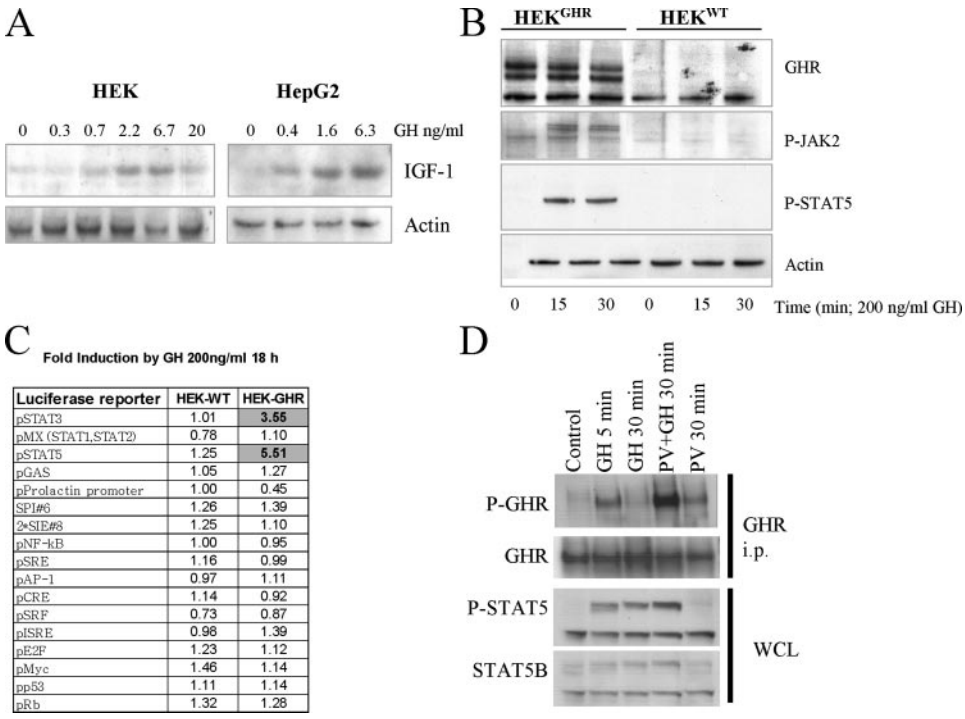
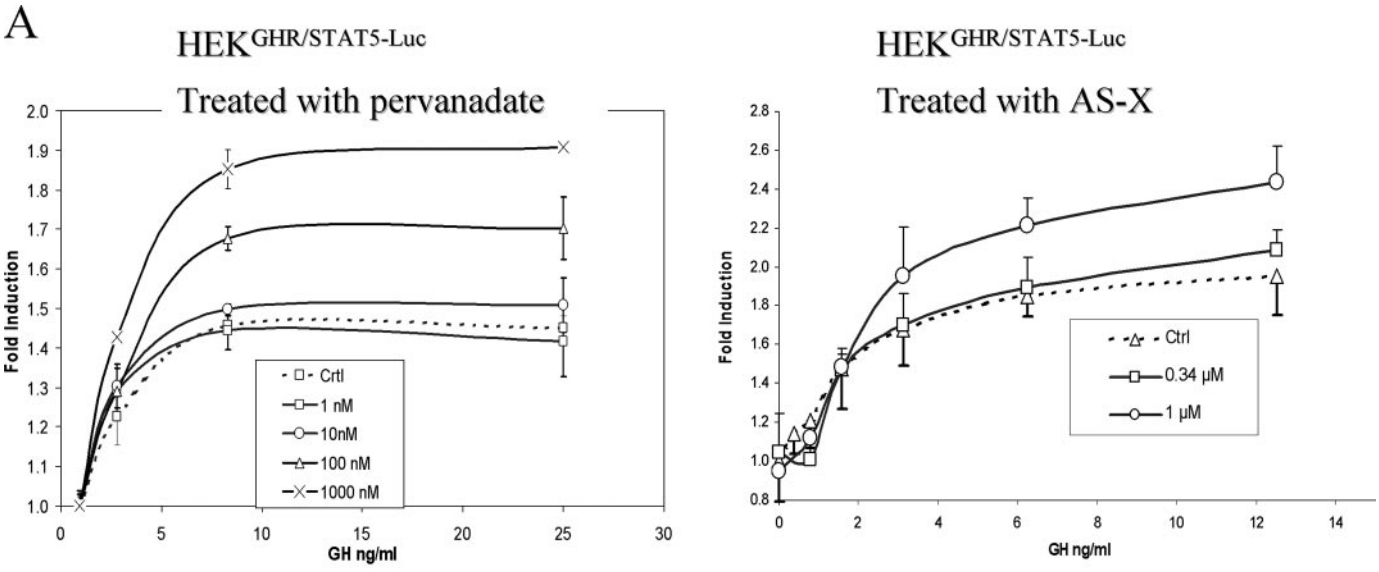


Luciferase reporter	HEK-WT	HEK-GHR
pSTAT3	1.01	**3.55**
pMX (STAT1,STAT2)	0.78	1.10
pSTAT5	1.25	**5.51**
pGAS	1.05	1.27
pProlactin promoter	1.00	0.45
SPI#6	1.26	1.39
2×SIE#8	1.25	1.10
pNF-kB	1.00	0.95
pSRE	1.16	0.99
pAP-1	0.97	1.11
pCRE	1.14	0.92
pSRF	0.73	0.87
pISRE	0.98	1.39
pE2F	1.23	1.12
pMyc	1.46	1.14
pp53	1.11	1.14
pRb	1.32	1.28

FIGURE 1. **GHR induced IGF1 production and signal transduction in cultured cells.** *A*, GH-induced IGF-1 production in native HEK293 and HepG2 cells (Western blots). *B*, GH-induced JAK2 (*upper band*) and STAT5 phosphorylation in wild type and transiently GHR-transfected HEK cells. *C*, induction of luciferase expression using a set of reporters transfected in WT or stably GHR-transfected HEK293 cells. For each, induction is compared with non-GH-treated cells (as ratios). *D*, PTP inhibitor pervanadate (*PV*, Na_3VO_4, 50 μM) treatment increases GH-induced transient phosphorylation of GHR and prolongs phosphorylation of STAT5. Western blot was performed using immunoprecipitated (intraperitoneal) GHR or whole cell lysate (*WCL*). GH induction was at 100 ng/ml.

phorylated *in vitro* according to the manufacturer's guidelines prior to the dephosphorylation assay. The cDNA cloning of phosphatase domains in pGEX4T3 as well as expression and elution of GST fusions are described elsewhere (26).

Malachite Green Dephosphorylation Assay—250 pmol of NFLMDNA(pY)FCEADA peptide (Jerini, Berlin, Germany) were added to an empty well of the 384-well plate of the Jerini's phosphatase substrate set containing 360 other phosphopeptides at 250 pmol/well (one peptide/well, see list in Appendix 2). Thirty μl of enzyme diluted at 0.33 μg/ml in reaction buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.01% Igepal CA-630, 0.1 mM EDTA) were added to each well, and the plate was incubated for 60 min at 37 °C. Twenty-five μl of Malachite Green reagent (Biomol International LP, Plymouth Meeting, PA) were added per well. After 15 min of incubation at room temperature absorbance was measured at



B

Selectivity profiles of PTP inhibitors

	PTP-1B	TC-PTP	PTP-β	DEP-1	LAR	SHP-1	SHP-2	GLEPP-1	PTP-κ	PTP-μ	VHR	PTP-H1	
Vanadate	0.01	0.04	0.04	0.12	0.03	0.04	0.04	0.04	0.06	0.02	0.04	0.09	IC50 (μM)
AS-X	0.80	5.27	7.71	NT	NT	4.19	3.36	0.86	NT	4.49	1.96	5.30	IC50 (μM)

FIGURE 2. **Both inorganic and organic PTP inhibitors potentiate GHR signaling.** *A*, effect of pervanadate (*left panel*) or an organic PTP inhibitor (AS-X, *right panel*) on GH-induced luciferase induction in HEK293 stably transfected for overexpression of GHR and STAT5-Firefly luciferase and control *Renilla* luciferase reporters. Incubation was for 18 h, and F luciferase values were normalized for R luciferase values. Similar results were obtained in three independent experiments with three data points each. *B*, *in vitro* potency (IC_{50}) of pervanadate and AS-X for a set of human PTPs (catalytic domains).

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640 nm with a fusion (Perkin-Elmer Life Sciences, Boston, MA) instrument.

Construction and Analysis of PTP-H1 Knock-out Mice—The PTP-H1 null mice were generated by Velocigene Technology (27). A mouse/BAC (bacterial artificial chromosome) containing the PTP-H1 gene was modified to replace exons 15–22 of PTP-H1 with an in-frame LacZ reporter sequence and a neomycin-selectable marker and electroporated into embryonic stem cells. F1 heterozygous mice were bred to generate F2 PTP-H1$^{-/-}$ mice. The 5′- and 3′-flanking mouse sequences of the lacZ insert were . . . CTC TCA CGT GTC TTC TAG AGT GAC and AGA CAT CAA ACC CAC CCT TCT CC . . .

PTPH1 knock-out (KO), heterozygous (HET), and wild type (WT) littermates (F2 generation, 87.5% C57Bl/6, 12.5% 129S6SvEv) aged 3–4 months were used for mating. Prior to mating, the mice were housed in separate cages. For mating, one male and one female for each genotype were housed together and maintained in a 12 h light/12 h dark cycle (lights on at 7 am) at 21 ± 1 °C with food and water available *ad libitum*. Weaning of pups occurred at 21st day of age, and they were housed two or three/cage. At 21 days of age a tail snip was taken for genotyping. The pups were weighed every 2 days and at 80 days of age were sacrificed by CO_2 and analyzed by DEXA.

PCR-based Genotyping—Tail snips were digested overnight with proteinase K (Sigma) and passed through a vacuum column (Promega, Wizard® SV 96 genomic DNA purification system A2370) for DNA trapping. Genomic DNA was washed in Wizard SV Wash solution (Promega; containing 95% ethanol) and eluted in 200 μl of water at 65 °C. After protease inactivation at 95 °C, 2 μl of DNA were used for the PCR. Two PCRs were conducted in parallel (TAQ GOLD kit, 0.2 μ/μl final; Applied Biosystems N8080256). A multiple PCR with three primers was used that distinguished WT, KO, and HET mice. The forward primer was designed in the flanking region of the cassette insertion site, the "reverse1" primer was designed in the deleted region, and the "reverse2" primer was inside the cassette (primer sequences: forward, 5′-CTG CTC TCC AGA TGG AGT TG-3; reverse1, 5′GCC ATC TCC ATC GTC ACT CT-3′ (for WT/HET); and reverse2, 5′-CCT AGC TTC CTC ACT GTT TCT-3′ (for KO/HET)). The pair of primers "forward/reverse1" gave an amplification product 254 bp (indicating WT or HET genotypes). The pair of primers "forward/reverse2" gave an amplification product 320 bp (indicating KO or HET genotype). In parallel to the multiple PCR, another PCR for LacZ insert was performed for confirmation to distinguish HET and KO mice from WT mice (primers sequences: forward, 5′-TCA TTC TCA GTA TTG TTT TGC C-3′; and reverse, 5′-CCA CTA TCA GTT GGT CAC TG-3′).

DEXA Analysis—Bone and tissue composition measurements were by DEXA densitometry. The Lunar PIXImus II Densitometer (GE Medical Systems) provides bone mineral and body composition results from total body imaging in less than 5 min.

Statistical Analyses—The growth curves were analyzed by one-way analysis of variance followed by the Fisher post-test or by *t* test. The data regarding DEXA were analyzed by *t* test.

TABLE 1

List of PTPs tested for modulating GH-induced luciferase reporter induction

Nomenclature is as in Ref. 5. HD-PTP (ED) and (EA) are mutants that have the WPE motif modified into WPD and WPA, respectively. Control is the empty parent vector pcDNA 3.1 HisB.

1	BDP-1
2	CD45/LCA
3	DEP-1
4	GLEPP-1
5	HD-PTP (EA)
6	HD-PTP (ED)
7	He-PTP
8	hVH-5
9	IA-2β
10	KAP-1
11	LAR
12	Meg-2
13	MKP-1
14	MKP-2
15	MKP-3 (rat)
16	MKP-3-1
17	MKP-5
18	PC-PTP1
19	Prl-2
20	PTEN/MMac-1
21	PTP-1B
22	PTP-alpha
23	PTPBAS
24	PTP-β
25	PTP-D1
26	PTP-δ
27	PTP-ϵ
28	PTP-γ
29	PTP-H1
30	PTP-IA2
31	PTP-κ
32	PTP-λ
33	PTP-μ
34	PTP-PEST
35	PTP-ρ
36	PTPS36
37	PTP-σ
38	PTP-ζ
39	Pyst-2
40	SAP-1
41	SHP-1
42	SHP-2
43	TC-PTP
44	VHR
	pcDNA 3.1 HisB (Control)

RESULTS

Phosphatase Inhibitors Potentiate GHR Signaling—HEK293 cells represent a suitable model for GHR signal transduction (28). As shown in Fig. 1*A*, native HEK cells produce IGF-1 when stimulated with GH, approximately as efficiently as liver HepG2 cells, indicating that physiologically relevant GHR signaling pathways are intact in both cell types (29). However, GHR expression limits the HEK response; only upon overexpression of full-length GHR could GH-induced JAK2 autophosphorylation and tyrosine-phosphorylated STAT-5 be detected in total cell lysate (Fig. 1*B*).

To further evaluate signaling events in GHR-overexpressing HEK cells, we individually transfected a range of luciferase reporters and stimulated the cells with GH. Only promoters carrying STAT-3 or STAT-5 elements were significantly activated in this cell line (Fig. 1*C*). Further experiments were performed in a HEK line stably overexpressing GHR, a STAT5 (firefly) luciferase reporter, and a *Renilla* luciferase internal control, which responded to GH in a dose-dependent manner (supplemental material).

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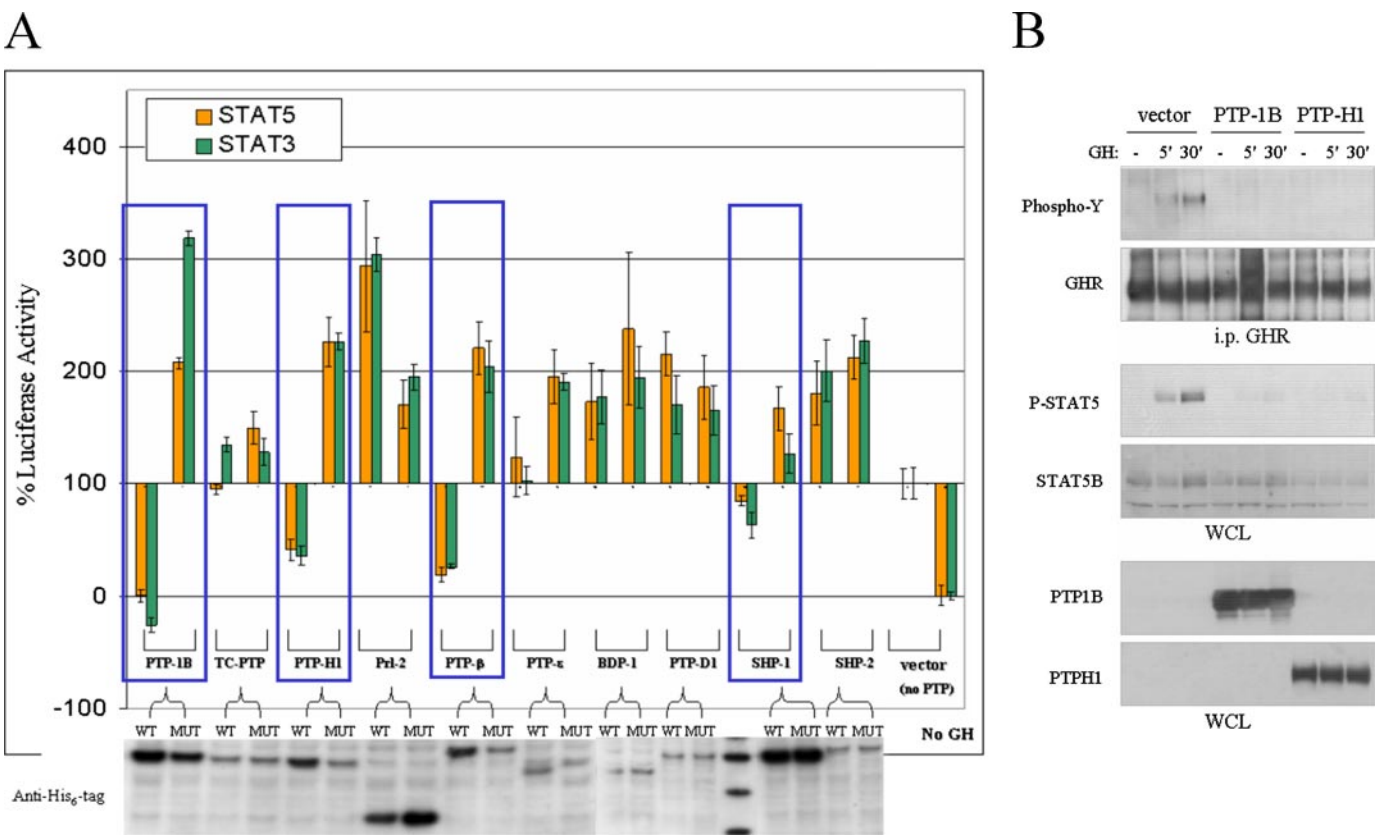

FIGURE 3. **Overexpression of PTPs modulates GHR signal transduction.** *A*, HEK293 were transiently transfected with GHR expression plasmid plus either STAT5- (*orange*) or STAT3 firefly luciferase (*green*) reporters, a control *Renilla* luciferase reporter and one of a set of His_6-tagged WT and trapping mutant (*MUT*) PTPs (see text). L-luciferase signals were normalized for the *Renilla* luciferase control and expressed as percentage of GH-treated cells co-transfected with empty (no PTP cDNA) vector. The l*ast lane* shows empty vector without GH induction. *Lower panel*, Western blot for the His_6-tagged recombinant PTPs. Similar results were obtained in three independent experiments done in quadruplicate. *B*, overexpression of full-length PTP-1B or PTP-H1 abolishes GH-induced phosphorylation of GHR and STAT5. The cells were starved overnight 48 h following PTP plasmid transfection and stimulated with 100 ng/ml GHR.

Treatment of the HEK line with pervanadate, a potent, non-selective inhibitor of phosphatases (30), resulted in phosphorylation of GHR (Fig. 1*D*). GH combined with vanadate acted synergistically, leaving the receptor phosphorylated 30 min after treatment, at which point phosphorylation by GH alone had disappeared (Fig. 1*D*, *third lane*). Phosphorylation of STAT5 was also increased upon vanadate treatment, compared with stimulation by GH alone (Fig. 1*D*, *bottom panel*). These results suggest that GH-induced GHR signaling is normally subdued by native PTPs in this cell line. To see how vanadate treatment affects the transcriptional response, we tested the HEK line for STAT-5-driven luciferase activity using different concentrations of GH (Fig. 2*A*, *left panel*). As expected, the response was enhanced by vanadate in a dose-dependent manner, even when saturating doses of GH were present (above 10 ng/ml). Because vanadate not only inhibits PTPs but also other enzymes (31), we additionally tested a cell-permeable organic PTP inhibitor from a previously described class (Ref. 32 and Fig. 2*B*) that does not inhibit a large panel of other enzymes (data not shown). Because this pharmacological tool presents an activity (Fig. 2*A*, *right panel*) that is very similar to the structurally unrelated vanadate, we can say with confidence that GH signaling potentiation in these experiments is due to PTP inhibition. Both PTP inhibitors increased luciferase responses only in the presence of GH.

Overexpressed PTPs Interfere with GHR Signaling—As a first step to identify the PTP(s) that exert this control on GHR signaling, we overexpressed wild type or mutant PTPs and observed their effect on the GH response in HEK. PTPs were selected from a classical PTP panel that covers the majority of the human PTP genome (5). The mutated PTPs were "trapping mutants" (33) in which the general acid residue Asp in the conserved WPD motif is replaced by an Ala, functionally resulting in a stable association of the PTP with its substrate (as used, for example, in Ref. 25). In a first run, 44 PTPs were tested (see Table 1 for a complete list). Among the PTPs thus evaluated, 10 showed activity and were retested (Fig. 3*A*). The effect of PTP overexpression on GH-stimulated STAT-3 and STAT-5 luciferase reporters was compared with vector-transfected (no PTP overexpression) cells, stimulated or not with GH (Fig. 3*A*, *last four data points*). All of the overexpressed PTPs could be detected using Western blotting (Fig. 3*A*, *bottom panel*). For nearly all PTPs there was a strong and reproducible correlation between the induction of STAT-3 and STAT-5 reporters. To rule out transcriptional effects on the plasmid backbone, we also tested the noninducible p53 reporter (Fig. 1*C*, pp53). The basal activity of this p53 reporter was not affected by PTP overexpression (data not shown). Remarkably, many PTPs significantly stimulated GH-induced STAT3 and STAT5 reporter expression, either as wild type or as trapping mutant. Overex-

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pression may compromise PTP substrate specificity, and we suspect that a number of these activating PTPs modulate GHR signaling through a similar, as yet unresolved, mechanism. The results from these PTPs run counter to our observation that chemical PTP inhibitors stimulate GHR signaling (Fig. 2). In contrast, four PTPs, namely PTP-1B, PTP-H1, PTP-β, and SHP-1 behaved as predicted, with the wild type PTPs suppressing signaling and the mutants enhancing signaling (*boxed* in Fig. 3*A*). These PTPs had also been identified earlier as recognizing GHR as a substrate (10).

Several of the PTPs tested are known to contain domains that direct defined subcellular localization. To assess the role of full-length PTP-1B and PTP-H1 in this system, the effect of their overexpression in GHR and STAT-5 phosphorylation was evaluated. As shown in Fig. 3*B*, overexpression of full-length PTP-1B or PTP-H1 results in dephosphorylation of the tested substrates, consistent with our earlier results.

In Vitro Selectivity of PTPs for Tyrosine-phosphorylated Substrates That Are Induced by GH—As another "filter" to discover which PTPs might be responsible for limiting the GHR response in intensity and duration in HEK cells, we evaluated key tyrosine-phosphorylated signaling intermediates as potential PTP substrates. For this purpose a set of GST-tagged PTPs was prepared (human catalytic domains). The purity of the enzyme preparations was checked (supplemental materials). GHR, STAT-3, and STAT-5B were immunoprecipitated from HEK cells that had been treated for 30 min with both GH and vanadate to obtain maximal phosphorylation. The immunoprecipitated substrates were then incubated with 1 μg of each of the PTPs, followed by evaluation of the substrate phosphorylation status using Western blotting. As shown in Fig. 4, activated GHR was a very good substrate for PTP-1B, PTP-H1, Meg1/2, PTP-β, and SHP-1/2, in agreement with earlier results (10). STAT-3 was incompletely dephosphorylated by all PTPs, whereas most activity was seen for PTP-1B, Meg1/2, and the SHPs; STAT-5 was partially dephosphorylated by PTP-1B, PTP-H1, Meg1/2, and PTP-β.

Because of the difficulty in obtaining phosphorylated JAK2 from HEK immunoprecipitates, we used *in vitro* autophosphorylated, purified JAK2 kinase. Incubation of phosphorylated JAK2 with the phosphatase array demonstrated similar specificity as for phospho-STAT5, with highest efficiency of PTP-1B, PTP-H1, Meg2, and PTP-β (Fig. 4, *bottom panel*).

The GHR dephosphorylation experiment (Fig. 4) provided no information as to which phosphotyrosine residues were being dephosphorylated by the PTPs, because a generic anti-phosphotyrosine antibody was used. The human GHR has seven intracellular tyrosines that play different roles in signal transduction (34–39) and that are nonequivalent PTP substrates (10). To address this question we tested a combination of PTP-H1 with PTP-1B or TC-PTP, under conditions where dephosphorylation was incomplete. The combination PTP-H1 plus PTP-1B was more effective than either PTP alone in dephosphorylating GHR or STAT-5B (see supplemental materials). This result is consistent with our earlier finding that PTP-H1 and PTP-1B recognize different GHR phosphotyrosines (10).

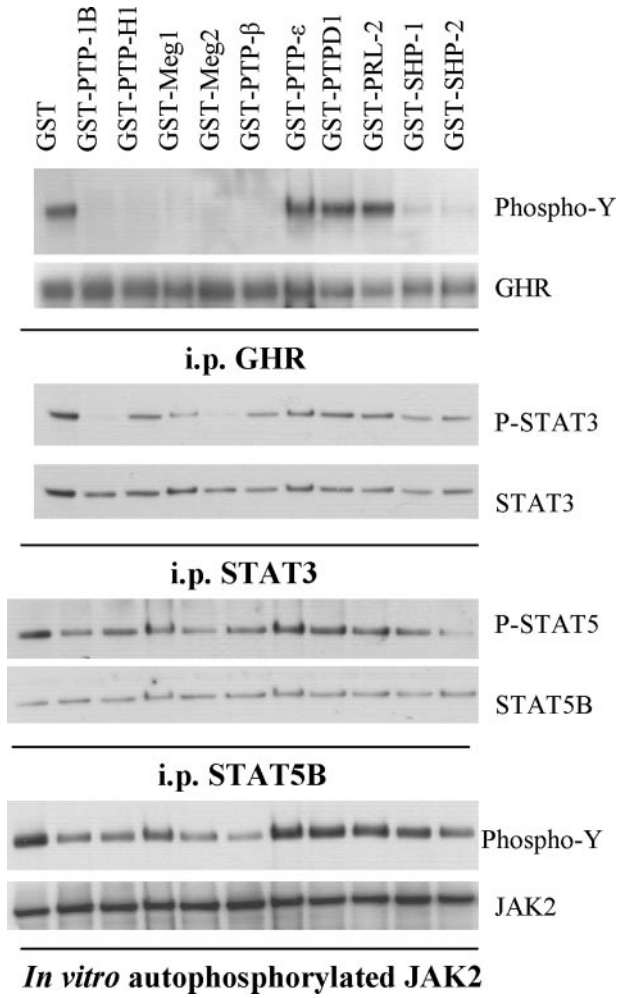

FIGURE 4. **Phosphorylated GHR, STAT3/5, and JAK2 are PTP substrates *in vitro*.** Phosphorylated proteins immunoprecipitated (*i.p.*) from GH- and vanadate-stimulated HEK^{GHR} cells were incubated with indicated PTPs. The substrate in the *last panel* was *in vitro* autophosphorylated JAK2.

Effect of PTP Knockdown on GH Signaling—Given the sensitizing effect of PTP inhibitors (Fig. 2), we investigated the effect of reducing individual cellular PTP mRNAs on GHR signaling components. Fig. 5*A* shows the effect of PTP siRNA transfection on GH-induced IGF-1 mRNA expression. The efficacy of the siRNAs in reducing PTP mRNA was evaluated for PTP-1B and SHP-2 using Western blotting and for all PTPs by quantitative reverse transcription-PCR (Fig. 5*A*, *lower panels*). As compared with the controls (no siRNA, or using an siRNA for green fluorescent protein; Fig. 5*A*, *last lanes*), siRNAs that reduced mRNA expression of SHP1/2, PTP-1B, or PTP-H1 increased IGF-1 mRNA expression. Surprisingly, PTP-D1 and Meg2 siRNAs decreased IGF1 mRNA induction. We also evaluated the effect of RNA interference knockdown on GH-induced STAT5 phosphorylation (Fig. 5*B*). In this experiment, reduction of PTP-1B, Prl2, PTP-β, and Meg2 resulted in stimulation of STAT5 phosphorylation. Results for PTPH1 and SHP2 were ambiguous, with not all siRNAs showing activity.

PTP-H1 Is a Selective PTP with GHR as a Preferred Substrate—A summary of activities for PTPs found in our assays (taken from Figs. 3–5, and data not shown) extends earlier findings and excludes some PTPs while potentially implicating others

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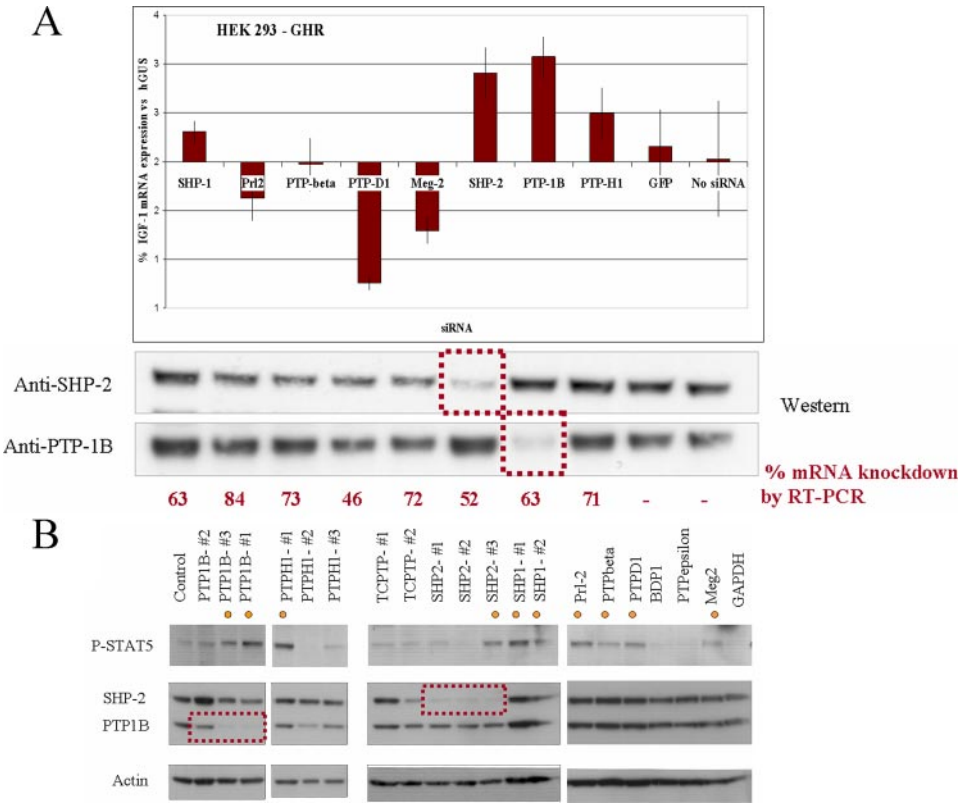

FIGURE 5. **Effect of PTP siRNA transfection on GH-induced IGF-1 mRNA induction in HEK.** *A*, GH-induced IGF1 mRNA levels were normalized to human glucuronidase β mRNA. The cell extracts were examined by Western blotting for SHP2 and PTP1B expression (*bottom panel*; cognate lanes are indicated with *boxes*). PTP mRNA for the cognate siRNAs (same PTP for RNA interference and mRNA measurement) was quantified by reverse transcription-PCR (*bottom*, expressed as percentage reduction). The results shown are the averages of three independent experiments. *B*, effect of PTP siRNA transfection on GH-induced STAT5 phosphorylation. The *numbers* refer to individual siRNAs. The same cellular extracts were also evaluated for SHP2, PTP1B, and actin protein expression (*bottom panel*). Cognate lanes are indicated with boxes. Yellow dots indicate siRNAs that increase STAT-5 phosphorylation as compared with controls.

(supplemental materials). PTP-H1 displayed intriguing activity in a number of cellular GHR signal transduction assays, suggesting that this PTP may play a role in GHR signaling *in vivo*. Although earlier work showed that the GHR is a uniquely good substrate for PTP-H1, not much is known about PTP-H1 substrate selectivity. We tested, therefore, as PTP-H1 substrates a set of 360 phosphopeptides corresponding to known or suspected protein phosphorylated sites, alongside a GHR peptide that contained phosphorylated Tyr^{534} (GHR numbering as in 10), namely NFLMDNA(pY)FCEADA. Among all peptides tested, this GHR peptide was the preferred substrate for PTP-H1 (supplemental materials). Based on our combined results, we decided to evaluate the phenotype of mice that lack the PTP-H1 catalytic domain.

Construction and Analysis of PTP-H1 Knock-out Mice—The human and predicted mouse PTP-H1 orthologs are well conserved (840 of 913 amino acid identities, or 92% at the protein level). The mouse PTP-H1 coding sequences are spread out over 24 exons (Fig. 6*A*). Using the Velocigene® strategy (27), exons numbered 14–23 that encode the PTP-H1 catalytic domain were replaced by a LacZ (β-galactosidase)-encoding cassette. Genetic disruption was confirmed using a standardized "loss of native allele" procedure (Ref. 27 and supplemental materials). Transcript mapping on RNA from various organs from wild type and knock-out animals confirmed loss of expression of the PTP-H1 catalytic domain but showed that the modified transcript was expressed (supplemental materials). Animals carrying one or two copies of the mutant gene were healthy, reproduced normally and showed no obvious phenotype. Because of the in-frame lacZ insertion, PTP-H1 promoter activity could be followed by X-gal staining in adult heterozygous animals. Strong staining was observed in striated muscle, the diaphragm, and colon lining. Weaker staining was observed in brain areas, kidney, liver, and heart (Fig. 6, *B–H*). Histology indicated that muscular staining was nonuniform (Fig. 6*I*), with a pattern that reflected the distribution of slow and fast oxidative fibers.

We noted that among adult (13 weeks old) animals, homozygous knock-out animals were larger in size than their wild type littermates, with the difference being particularly significant for males (Fig. 7*A*). We subsequently followed growth from birth onwards in new litters, which confirmed our observation (Fig. 7*B*). The gap between wild type and homozygous knock-out animals (as measured by weight) started to develop at the onset of sexual maturation around 28 days and was again more marked for males than for females. The difference between wild type and mutant animals became especially prominent after weaning (on day 21) and was therefore not a result of parental behavior and/or litter competition.

The key endocrine mediator of GH is IGF-1 (40). To further understand the PTP-H1 knock-out growth phenotype, we evaluated expression of IGF-1 mRNA in liver and blood plasma IGF-1 from wild type and mutated animals. As shown in Fig. 7*C*, IGF1 mRNA and plasma protein levels were significantly increased in mutant *versus* wild type animals, again with the gap being the widest for male animals. Finally, we evaluated body composition using DEXA. Mutant male animals showed significantly increased bone mineral content and a trend toward increased lean body mass and bone mineral density (Fig. 7*D*).

DISCUSSION

PTPs play important roles in modulating the timing and intensity of phosphorylation-mediated signaling (5). Disease-relevant examples are control of insulin receptor signaling by PTP1B (23), PTEN antagonism of phosphoinositidephosphate-mediated signaling in cancer (41), and (cyclosporin target) the role of protein phosphatase 2B in immune responses. Yet, so far

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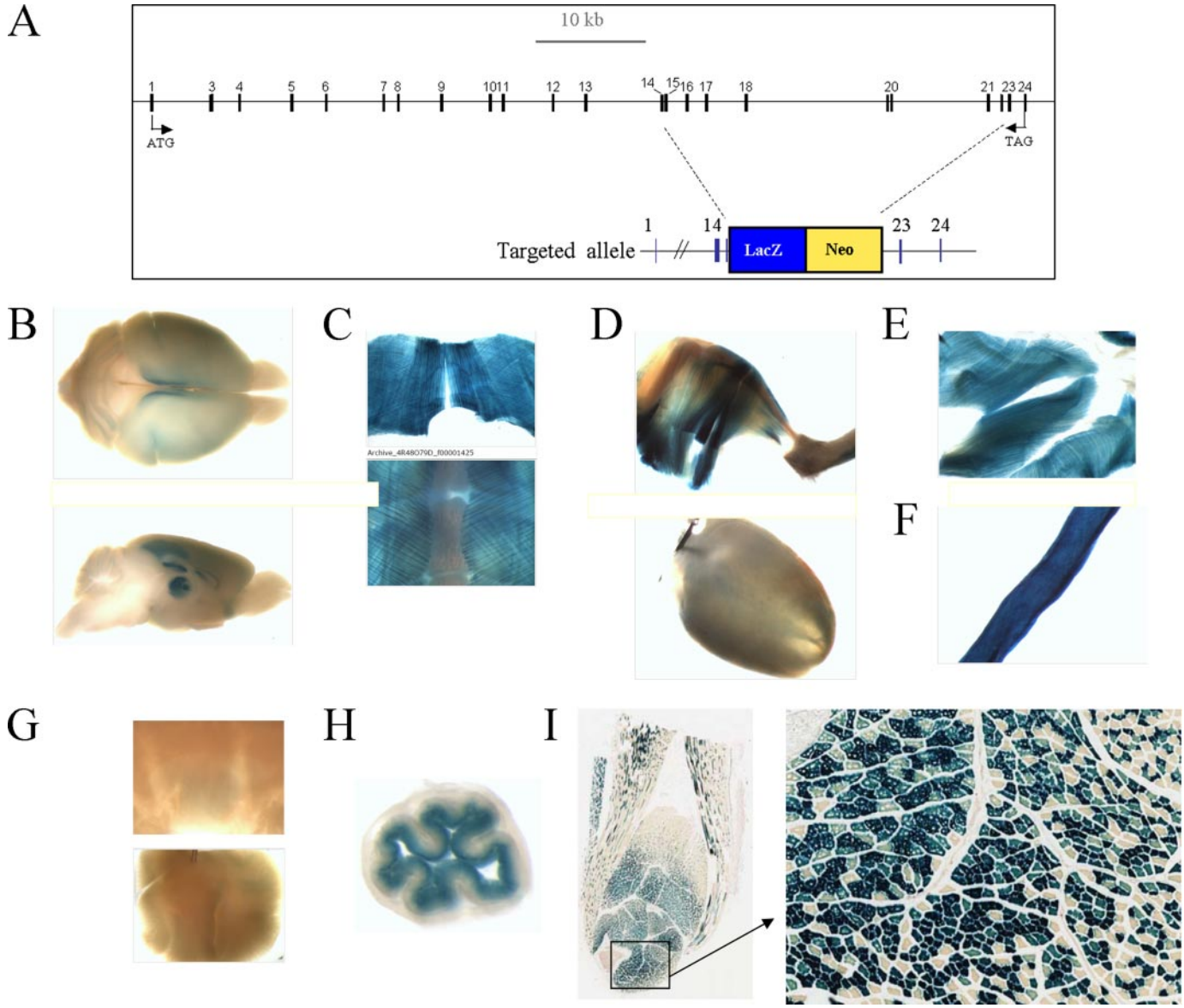

FIGURE 6. **Construction of mice that lack the PTP-H1 catalytic domain and transgenic LacZ reporter expression.** *A*, schematic representation of the predicted mouse PTP-H1 gene intron-exon organization and deleted exons 15–22, corresponding to the catalytic domain. The LacZ coding sequence was inserted in-frame with upstream PTP-H1 cDNA. Shown are stained cryostat sections of PTP-H1 mutant (heterozygous) adult mice. Staining for LacZ enzyme was with X-gal, counterstain Neutral Red. *B*, staining in the hippocampus and around corpus callosum (septum and cortex) and in the thalamus. *C*, abdominal wall (muscle, top); intracostal muscle (*bottom*). *D*, hind limb (muscle); heart. *E*, diaphragm. *F*, esophagus. *G*, kidney (*top*); liver (*bottom*). *H*, colon. *I*, hind limb muscle; detail.

little work has been done on identifying PTPs that functionally control GHR function and growth.

Cell-free and cellular assays may implicate PTPs that play a minor role or no role in GHR control. A parallel can be drawn with the discovery of PTP-1B control of insulin receptor signaling. Before this was demonstrated in knock-out animals (24, 42), seven other PTPs, namely PTP-SHP2 (43–46), PTP-LAR (47–51), PTP-ϵ (52), LMW-PTP (53), PTEN (54), TC-PTP (25), and PTP-α (52, 55), had all been associated with insulin signaling. It is possible that these other PTPs play accessory roles, *i.e.* they may contribute to insulin signaling to lesser extent than PTP1B or in a different cellular or hormonal context. Similarly, cells that lack either DEP1 (56), TC-PTP (57), or PTP1B (58) all show enhanced signaling of platelet-derived growth factor receptor, whereas LMW-PTP (59) and PTP-α (60) have been implicated in platelet-derived growth factor receptor signaling as well.

To date, ~39 PTP knock-out and mutant mouse phenotypes have been described (see supplemental materials), none of which show enhanced growth. Although PTP-1B has been strongly linked to some aspects of GHR signaling, PTP-1B mutant mice are in fact smaller than wild type animals because of their lower fat content, which is linked to increased energy expenditure (24). PTP-H1 is the first PTP whose mutation results in increased body weight and that affects GHR signaling and IGF-1 secretion *in vivo*.

Several observations indicate that the increased body size observed in PTP-H1 knock-out animals is related to enhanced GHR sensitivity. IGF-1, a key effector of GH, was overexpressed in livers (as mRNA), and increased IGF-1 protein was observed

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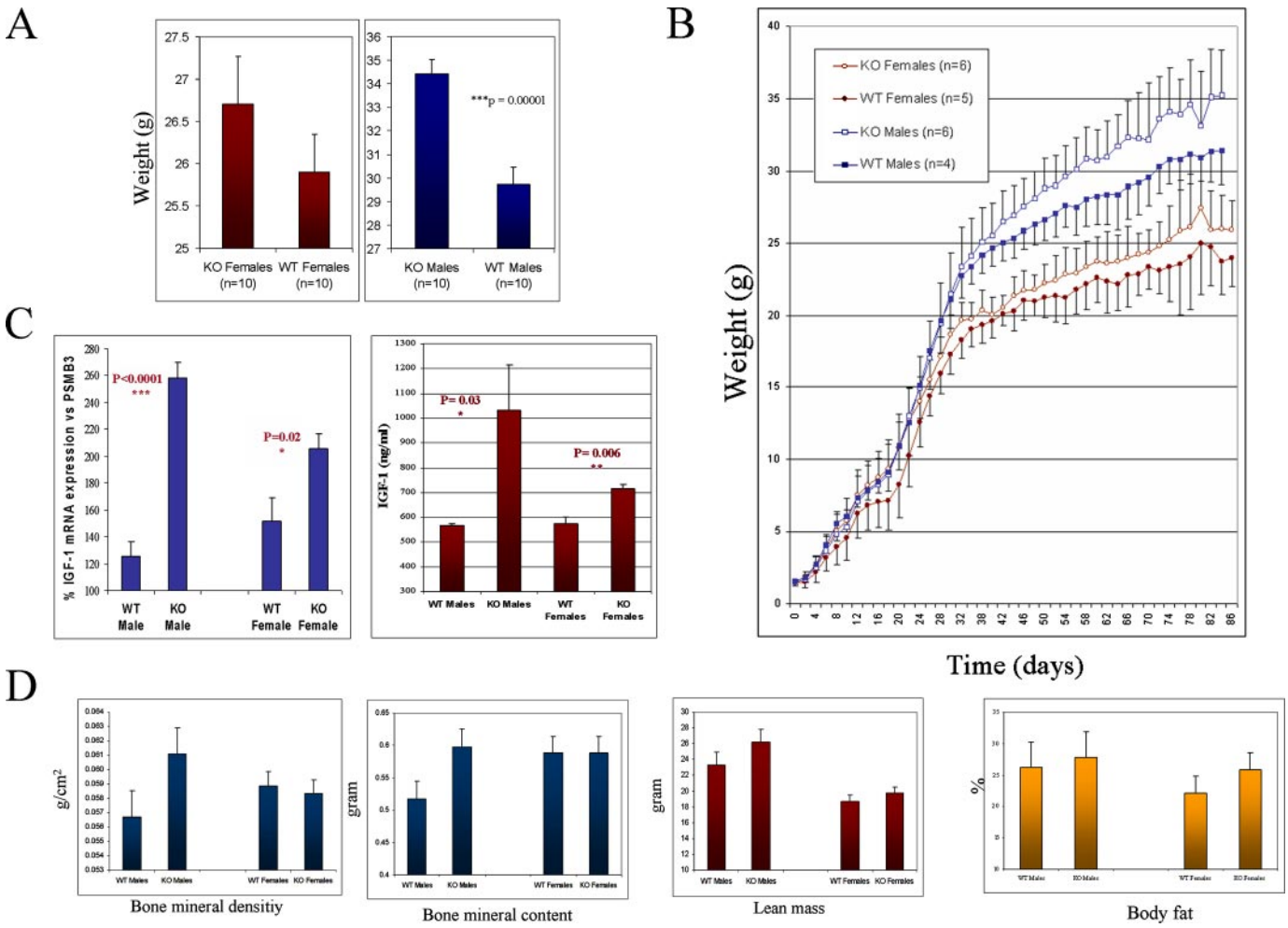

FIGURE 7. **Phenotype of PTP-H1 mutant mice.** *A*, weight variation of 13 weeks old homozygous mutant and wild type littermate animals, females (*left panel*) and males (*right panel*; 10 animals/data point, all born from heterozygous parents). *B*, growth curve of mutant and wild type animals (separate litters from *A*, born from homozygous parents; six female mutant and five female wild type animals, six wild type and four mutant males). *C*, expression of IGF-1 mRNA in liver (*left panel*) and IGF-1 protein in serum (*right panel*) from wild type and knock-out male and female littermates (four animals/group, sampled individually). *D*, bone mineral density, mineral content, lean mass, and body fat as determined by DEXA (four animals/data point).

in serum of the knock-out animals. IGF-1 plays a pivotal role in determining adult body size, as attested by the finding that IGF-1 genetic variation is strongly associated with the large variation in dog breed sizes (61). In PTP-H1 KO mutant mice, the gender difference, with a more pronounced phenotype in males, was seen for all readouts. In humans, women secrete higher amounts of GH than men (62) but as compared with men respond with decreased IGF-1 secretion when given equal doses of GH, to the extent that half the GH dose to produce a given IGF-1 response is sufficient in male as compared with female subjects (63, 64). The observation that males are more sensitive to GH may be linked to our finding that PTPH1 KO male mice show a more prominent growth-related phenotype than female mice.

In addition, GH treatment is known to enhance bone mineral density and lean body mass in humans as measured by dual energy x-ray absorptiometry (65), as we found in PTP-H1 knock-out mice.

GH is known to indirectly affect growth through IGF-1 and to directly bind to adipocyte GHRs resulting in reduced fat deposits (reviewed in Refs. 66 and 67). The PTP-H1 mutant mice showed no clear signs of reduced body fat (Fig. 7*D*). One may speculate that PTP-1B is instead involved in GHR signaling in adipocytes, for PTP-1B mutant mice almost completely lack fat (24). However, PTP-1B has also been reported to exert negative feedback on signaling by leptin, which also reduces fat stores (68–71).

The transgenic LacZ reporter analysis shows that the PTP-H1 promoter is specifically expressed in the slow oxidative (Type I) fibers, which have smaller diameters than Type II fibers. Type I fibers are mitochondria-rich and adapted to long term muscle efforts. This finding is intriguing in the context of GHR signaling, because GH treatment is known to increase the proportion of type-1 fibers in skeletal muscle, whereas hypophysectomy (and GH depletion) reduces the number of type 1 fibers by half (72). Accordingly, GHR mRNA abundance is higher in type 1 slow twitch oxidative muscles (73). The co-expression of PTP-H1 and GHR suggests that PTP-H1 plays a role in GH-induced control of type 1 muscle fiber differentiation.

The relatively modest effect of PTP-H1 mutation on overall growth, *e.g.* as compared with GH overexpression (14), may be

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related to the unequal distribution pattern of PTP-H1, resulting in GHR signaling not being affected by PTP-H1 in some tissues, where other PTPs may take over. PTP-H1 expression is relatively low in liver, a major source of IGF-1, yet the fact that hepatic IGF-1 expression directly depends on the GHR (74) and our finding that plasma IGF-1 is increased in mutant animals suggest a direct role for PTP-H1 in GH-induced IGF-1 secretion. From a drug discovery perspective, although PTP-H1 would seem a safe target, finding a phosphatase inhibitor that affects growth may be a challenge. We tested bioavailable PTP inhibitors with combined selectivity for PTP-1B and PTP-H1 (and *in vitro* potency of 3–5 μM IC_{50}) in hypophysectomized young (26–28 days old) female rats over 4 days, with or without a half-maximal efficacy dose of GH (0.025 IU rhGH) but saw no PTP-inhibitor-related effect on weight gain or tibial growth plate thickness (data not shown). Perhaps more potent, or paradoxically, less selective PTP-H1 inhibitors will show efficacy (75).

Previous molecular studies had implicated PTP-H1 with T-cell receptor signaling (76–78), cancer (79, 80), cardiac sodium channel regulation (81), control of TNF-α shedding by TACE (82), and endoplasmic reticulum assembly (83). While this work was in process, it was reported that PTP-H1 knock-out animals display normal T-cell signal transduction and also appear normal in other processes in which PTP-H1 was identified in the above studies (84). We concur with the conclusion of this study that enhanced weight of knock-out animals is not obvious unless specifically looked at by gender. Also, we cannot exclude that the effect of weight that we observed depends on the genetic background of the animals or the precise nature of disrupted sequences.

Our results show that PTP-H1 plays a nonredundant role in growth and is at least partly responsible for the difference in size between male and female animals (because the impact of loss of PTP-H1 is larger in males). The knock-out phenotype might therefore have been more striking in a species that displays more prominent gender size dimorphism, a characteristic that correlates with polygyny and territorial behavior. This hypothesis is supported by the finding that IGF-1, downstream of GH and PTP-H1 in our model, is genetically linked with sexual size dimorphism in mammals (85).

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