Supporting Information

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SI Materials and Methods

Peptide Mapping. Tryptic digests were performed as described in the European Pharmacopoeia (2002, 4th Edition, p 1938) with the following modifications: All samples (WHO hGH [hGH reference standard from the World Health Organization], and PEGylated Y35pAcF-hGH) were dialyzed overnight in 50 mM Tris-HCl, pH 7.5. Samples were then incubated with trypsin (Worthington Biochemical Corp.) for 4 hours at 37 °C. To stop the digestion reaction, samples were incubated on ice for several minutes and subsequently maintained at 4 °C during HPLC analysis. Digested samples (approximately 200 μg) were loaded onto a 25 × 0.46 cm Vydac C-8 column (Grace) in 0.1% trifluoroacetic acid and eluted with a gradient from 0 to 80% acetonitrile over 70 min at a flow rate of 1 mL/min at 30 °C. The elution of tryptic peptides was monitored by absorbance at 214 nm.

Cloning and Purification of the Rat GH Receptor. The gene encoding the extracellular domain of rat GH receptor (GHR ECD, amino acids S29-T238) was cloned into pET20b vector (EMD4Biosciences). An L43R mutation was introduced to further approximate the human GH receptor binding site (1). Recombinant protein was produced in BL21(DE3) *Escherichia coli* cells (EMD4Biosciences) by induction of cell culture (OD_{600} = 0.8–1.0) with 0.4 mM IPTG at 30 °C for 4–5 hours. Cells were lysed by sonication, centrifuged, and the pellet washed four times in 50 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1% triton X-100, and twice in the same buffer without triton X-100. Inclusion bodies consisted of more than 95% GHR (L43R) ECD and were solubilized in 7M guanidine-HCl. GHR (L43R) ECD was refolded at 0.2 mg/mL in 50 mM Tris, pH 8, 0.5 M L-arginine, 10% glycerol. Monomeric GHR (L43R) ECD was purified on HP Q Sepharose columns (GE Healthcare BioSciences), concentrated to 1 to 2 mg/mL, adjusted to 20% glycerol, aliquoted and stored at −80 °C.

Biacore Analysis. Using a standard amine coupling procedure (Biacore, GE Healthcare BioSciences), approximately 500–800 RUs of soluble GHR (L43R) ECD was immobilized on a Biacore CM5 chip. This level of receptor immobilization was sufficient to produce maximal specific GH binding response of about 100–150 RUs. Various concentrations of GH variants (0.1–300 nM) in HBS-EP buffer (Biacore) were injected over the GHR (L43R) ECD surface at a flow rate of 30 mL/min for 4 min, and dissociation was monitored for 12 min postinjection. The surface was regenerated by a 15 sec pulse of 4.5 M $MgCl_2$. A reference cell with serum albumin immobilized in place of the receptor was used to subtract any buffer bulk effects and nonspecific binding. To determine individual rate constants (Table 1), the sensograms were fit to a "Bivalent Analyte" binding model using BiaEvaluation 4.1 software (2).

Measurement of in Vitro Activity. Human IM-9 lymphocytes were purchased from American Type Culture Collection and the IL-3 dependent mouse cell line, BAF3, was a kind gift from Markus Warmuth (Genomics Institute of the Novartis Research Foundation). Cells were routinely passaged in RPMI 1640, sodium pyruvate, penicillin, streptomycin, and 10% heat-inactivated fetal calf serum. BAF3 cells were supplemented with 50 uM 2-mercaptoethanol and 10% IL-3 (BD Biosciences). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The BAF3 cell line was used to establish a mutant rat GHR(L43R) stable cell clone as described (3). Human IM-9 cells were starved overnight in assay media containing 1% heat-inactivated, charcoal/dextran treated FBS before stimulation with increasing concentrations of hGH for 10 min at 37 °C. Stimulated cells were fixed, permeabilized and stained for pSTAT5 as recommended by the manufacturer (Cell Signaling Technology). Sample acquisition was performed on a FACSArray (BD Biosciences), with acquired data analyzed using Flowjo software (Tree Star Inc.). Cells derived from a mutant rat GHR(L43R)/BAF3 cell clone were plated at 5×10^4 cells/well in a 96-well plate. Cells were activated with increasing concentration of hGH proteins and labeled with 50 uM BrdU (Sigma-Aldrich). After 48 h in culture, cells were fixed/permeabilized and treated with DNAses to expose the BrdU epitope, followed by staining with an APC-conjugated anti-BrdU antibody for sample analysis on the FACSArray. EC_{50} values were calculated with SigmaPlot (Systat Software, Inc.) from dose response curves plotted with % pSTAT5 and % BrdU positive cells against protein concentration.

1. Souza SC, et al. (1995) A single arginine residue determines species specificity of the human growth hormone receptor. *Proc Natl Acad Sci USA* 92:959–963.
2. Wells JA (1996) Binding in the growth hormone receptor complex. *Proc Natl Acad Sci USA* 93:1–6.
3. Dinerstein H, et al. (1995) The proline-rich region of the GH receptor is essential for JAK2 phosphorylation, activation of cell proliferation, and gene transcription. *Mol Endocrinol* 9:1701–1707.

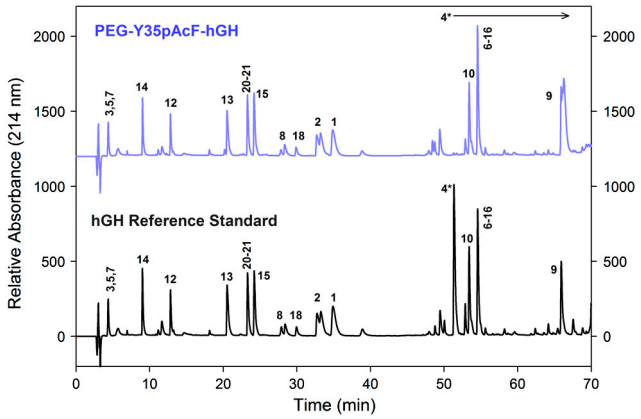

Fig. S1. Tryptic peptide mapping of hGH variants. To demonstrate both site-specific incorporation of pAcF into hGH and site-selective PEGylation, proteins were analyzed by tryptic peptide mapping. The tryptic peptide map of WHO hGH is shown in black, and PEGylated Y35pAcF-hGH in blue. Complete overlap between the two maps is apparent in the figure with exception of peptide 4. The elution time for tryptic peptide 4 in PEGylated Y35pAcF-hGH is altered by the attachment of PEG at position 35, causing this peak to elute at approximately 65 min compared to 51 min. Similar results were obtained when analyzing other hGH variants containing pAcF substitutions.

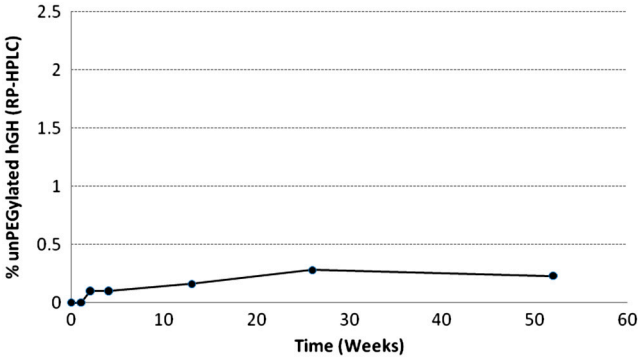

Fig. S2. Stability of PEGylated Y35pAcF-hGH at physiological pH. PEGylated protein was formulated at approximately 12 mg/mL (30 mM Histidine, pH 7.3, 4% mannitol, 2% trehalose, 0.1% pluronic F68) and stored at 5 °C. Samples were taken at the times indicated and a RP-HPLC method was used to measure the degree of dePEGylation.

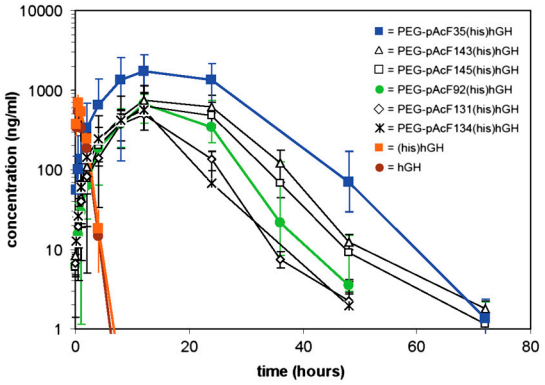

Fig. S3. PEGylation site-dependent serum concentration. Six different PEGylated pAcF-His_6hGH analogs were injected subcutaneously at 1 mg/kg into normal rats (male, Sprague–Dawley, $N = 3$–6 per group). All six displayed longer terminal $t_{1/2}$ values (4–6 h) relative to native WHO hGH standard and His_6-hGH. Error bars represent standard deviation.