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A ligand-receptor fusion of growth hormone forms a dimer and is a potent long-acting agonist

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Cytokine hormones have a short plasma half-life and require frequent administration. For example, growth hormone replacement involves daily injections. In common with other cytokines, the extracellular domain of the growth hormone receptor circulates as a binding protein, which naturally prolongs the biological half-life of growth hormone. Here we have studied the biological actions of a ligand-receptor fusion of growth hormone and the extracellular domain of its receptor. The genetically engineered ligand-receptor fusion protein was purified from mammalian cell culture. In rats, the ligand-receptor fusion had a 300-times reduced clearance as compared to native growth hormone, and a single injection promoted growth for 10 d, far exceeding the growth seen after administration of native growth hormone. The ligand-receptor fusion forms a reciprocal, head-to-tail dimer that provides a reservoir of inactive hormone similar to the natural reservoir of growth hormone and its binding protein. In conclusion, a ligand-receptor fusion of cytokine to its extracellular receptor generates a potent, long-acting agonist with exceptionally slow absorption and elimination. This approach could be easily applied to other cytokines.

Growth hormone is an anabolic cytokine hormone important for linear growth in childhood and normal body composition in adults[1]. The current therapeutic regimen for growth hormone replacement requires once-daily subcutaneous injections, which are inconvenient and expensive. Several approaches have been taken to create long-acting preparations, including pegylated hormones[2] and sustained-release formulations[3–5]. Pegylation has the disadvantage that it reduces the affinity of a hormone for its receptor[2], and chemical modification with subsequent purification is expensive. Sustained-release formulations show efficacy[4–7], but such growth hormone preparations are characterized by a dominant early-release profile, which causes supraphysiological growth hormone levels[3], their manufacture is expensive and injections may be painful[4]. There is a need for cytokine formulations that minimize manufacturing costs, have good pharmacokinetic profiles, are easy to administer, and are acceptable to individuals.

Growth hormone acts through a cell-surface type 1 cytokine receptor (GHR; **Fig. 1a**,**b**). In common with other cytokine receptors, the extracellular domain of the GHR is proteolytically cleaved and circulates as a binding protein (GHBP; ref. 8). Under physiological conditions, growth hormone is in part bound in the circulation in a 1:1 molar complex with GHBP, and this complex seems to be biologically inactive and protected from clearance and degradation[9,10]. A cross-linked complex of growth hormone with GHBP has delayed clearance but no biological activity[11]. Co-administration of separately purified GHBP and growth hormone in a 1:1 ratio can augment the anabolic actions of growth hormone[12]. Thus, similar to many hormonal systems, binding in the circulation provides a circulating reservoir of inactive hormone in equilibrium with active free hormone[13].

We initially sought to make a growth hormone antagonist, and hypothesized that a ligand–receptor fusion of cytokine and the extracellular domain of its receptor might interfere with receptor conformation and block signaling. This idea was based on our observation that a truncated extracellular domain variant of GHR acts as a dominant-negative inhibitor of GHR (refs. 14,15). Unexpectedly, the first ligand-receptor fusion of growth hormone that we made was an agonist. Here we show that a ligand-receptor fusion of growth hormone is a potent long-acting agonist.

RESULTS

Design and characterization of the ligand-receptor fusion

We generated a recombinant gene encoding human growth hormone linked to the A and B domains of the GHR extracellular domain (exGHR1–238) by a flexible $(Gly_4Ser)_4$ linker (**Fig. 1**). We expressed this ligand-receptor fusion in Chinese hamster ovary (CHO) cells and purified it to >95% purity by using an affinity column comprising monoclonal antibody to growth hormone (**Fig. 2a**). We screened the ligand-receptor fusion by ELISA using 16 conformationally sensitive monoclonal antibodies. All of these monoclonal antibodies bound the

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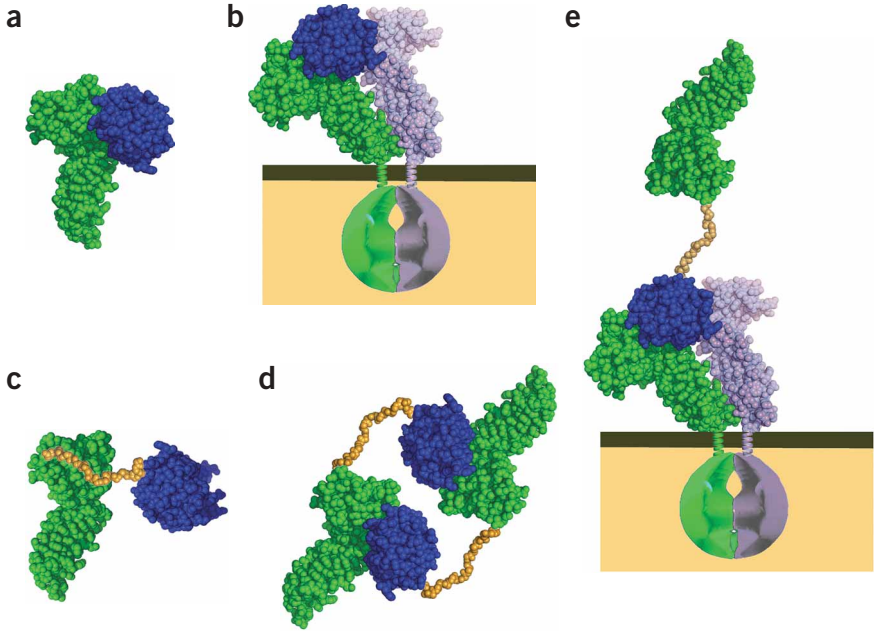

Figure 1 Relationship between growth hormone, GHBP, the ligand-receptor (LR) fusion and the GHR on the basis of published structures[27] (pdb3HHR). (**a**) Natural configuration of growth hormone (blue) binding to the GHBP (green) in a 1:1 complex. (**b**) Growth hormone (blue), released from the GHBP complex, binds to the GHR (green and blue-gray) on the cell surface. Black horizontal line represents the cell membrane; yellow-brown region indicates the cytoplasmic space. (**c**) The LR fusion molecule in monomeric form with growth hormone (blue) linked (gold) to exGHR (green). (**d**) Model of the LR fusion forming a reciprocal head-to-tail dimer in which growth hormone (blue) in one molecule binds to exGHR (green) in the other molecule. (**e**) The LR fusion in monomeric form is capable of binding and activating the GHR.

ligand-receptor fusion with affinity comparable to that for GHBP derived from human serum.

Coomassie staining and immunoblotting of SDS-PAGE gels showed that the ligand-receptor fusion protein separated as a doublet of approximately 75 kDa with a difference of about 5 kDa between the two bands. Native PAGE gel analysis showed no evidence of aggregation (**Fig. 2b**). The ligand-receptor fusion appeared as two distinct forms. We excised these distinct protein forms, fast and slow, from the native PAGE gel and reanalyzed them by SDS-PAGE under reducing conditions. Both the fast and slow forms from the native PAGE gel consisted of the 75-kDa doublet (**Fig. 2c**). We confirmed the existence of two forms of ligand-receptor fusion in solution by analytical gel filtration (**Fig. 2d**). These results are consistent with the idea that the ligand-receptor fusion exists as a dimer in solution. This idea was confirmed by analytical ultracentrifugation, which verified the size of the monomer as 75 kDa.

In vitro bioassay and pharmacokinetics

We tested the *in vitro* bioactivity of the ligand-receptor fusion using a growth hormone–specific luciferase reporter assay[16]. The ligand-receptor fusion had roughly 10% of the bioactivity of growth hormone in this static assay system, but it stimulated a maximal response, albeit at a higher concentration than growth hormone (**Fig. 2e**). We examined the pharmacokinetic profile of the ligand-receptor fusion in normal rats after a single subcutaneous or intravenous injection (**Fig. 3**). The ligand-receptor fusion showed delayed clearance irrespective of the route of administration and delayed absorption after subcutaneous administration. After an intravenous bolus, the terminal half-life of the ligand-receptor fusion was 21 ± 2 h and clearance was 3.3 ± 0.9 ml h^{-1} kg^{-1}. The clearance of the ligand-receptor fusion was 300 times slower than that of growth hormone (refs. 2,12). After a single subcutaneous administration, the ligand-receptor fusion had a delayed peak as compared with growth hormone (30 versus 1 h). The ligand-receptor fusion was still detectable at 8 d, whereas growth hormone was undetectable at 6 h.

We examined whether these exceptional pharmacokinetics of the ligand-receptor fusion were related to size. We tested two variant ligand-receptor fusion molecules with identical linkers: a ligand-receptor fusion of growth hormone and only the B domain exGHR (55 kDa), and tandem growth hormone molecules (growth hormone linked to growth hormone) linked to exGHR (100 kDa). Both the 55-kDa and 100-kDa proteins showed increased agonist

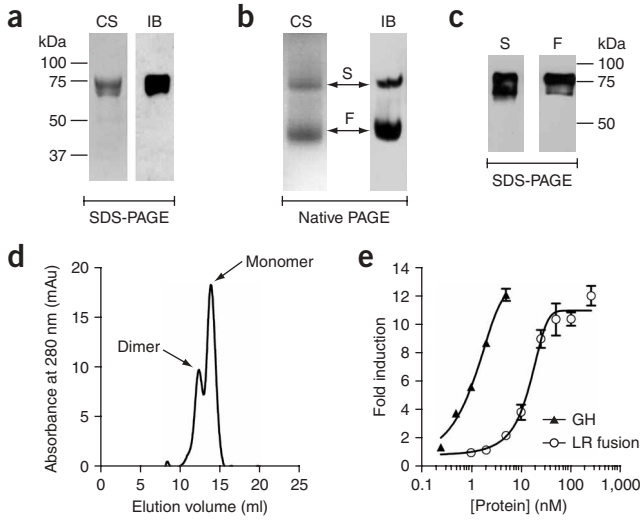

Figure 2 Characterization and bioactivity of the ligand-receptor (LR) fusion. (**a**) SDS-PAGE of the LR fusion, followed by Coomassie staining (CS) and immunoblotting (IB) using antibody to growth hormone (anti-GH). The LR fusion is ~75 kDa and resolves into two bands separated by ~5 kDa. (**b**) Native PAGE of the LR fusion showing that there are two protein forms: fast (F) and slow (S). (**c**) Individual bands from the native PAGE gel were excised and subjected to SDS-PAGE under reducing conditions, followed by immunoblotting using anti-GH. The two bands (F and S) run at ~75 kDa and separate as a doublet, suggesting that they both comprise the 75 kDa LR fusion, which may exist under native conditions as an equilibrium of monomers and dimers. (**d**) Gel filtration elution profile of the LR fusion. The two distinct peaks are again indicative of the existence of the LR fusion as a monomer and dimer in solution. (**e**) Cell-based GHR signaling bioassay of GH and the LR fusion. The *y* axis represents the fold induction of corrected luciferase activity from a Stat5 luciferase reporter assay. The standard curve for GH ranges from 0 to 5 nM; the LR fusion standard curve ranges from 0 to 250 nM. The maximal response to GH is achieved with 5 nM, whereas the maximal response to the LR fusion requires 50–250 nM.

activity in the bioassay as compared with the original 75-kDa ligand-receptor fusion, but the circulating half-life of both was less than 4 h after intravenous administration (precise determination of the half-life was not possible because the sampling protocol that we used expected a longer half-life). The results confirmed that the exceptional pharmacokinetics of the original 75-kDa ligand-receptor fusion was not due solely to molecular weight.

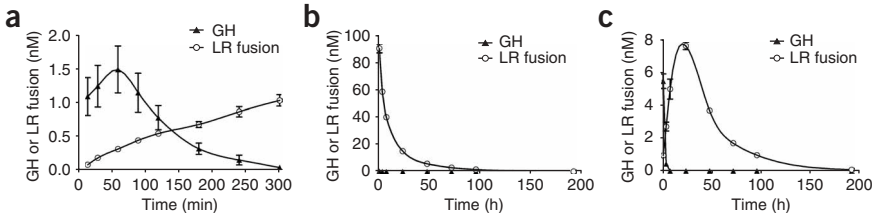

Figure 3 Profiles of growth hormone (GH) and ligand-receptor (LR) fusion measured after subcutaneous and intravenous administration. (**a**) Early phase (5 h) after subcutaneous administration. (**b**) Late phase (8 d) after intravenous administration. (**c**) Late phase after subcutaneous administration.

Superiority of ligand-receptor fusion growth promotion

To test its biological activity, we administered the ligand-receptor fusion and growth hormone to hypophysectomized (growth hormone–deficient) rats. Daily administration of growth hormone induced continuous growth over 10 d. We then compared the ligand-receptor fusion to growth hormone by using alternate-day subcutaneous injections, two injections over 10 d, or a single injection. For all experiments, we used equimolar doses of growth hormone and the ligand-receptor fusion, and gave the same total dose over the 10-d period: namely, 220 μg per kg (body weight) per day (~10 nmol over 10 d), which is similar to the dose previously used to obtain a maximal growth response[12]. The ligand-receptor fusion promoted an increase in weight gain that was greater than that promoted by growth hormone under the same injection protocol and similar to that seen after daily growth hormone injections (**Fig. 4** and **Table 1**). Growth hormone seemed to promote weight gain only in the 24 h following injection. By contrast, the ligand-receptor fusion produced continuous weight gain over 10 d even when given as a single injection. A similar pattern of growth was seen in femur, tibia, thymus, liver and kidney (**Table 1**).

The 10-d terminal bleed from all rats was analyzed for the growth hormone–dependent biomarker, insulin-like growth factor-I (IGF-I) and for growth hormone and the ligand-receptor fusion (**Table 1**). IGF-I concentrations were significantly increased after administration of the ligand-receptor fusion, even as a single injection, and were significantly greater than those seen after daily injection of growth hormone. Concentrations of growth hormone were undetectable in the terminal bleed after all injection regimens, whereas the ligand-receptor fusion was detectable 10 d after a single injection.

DISCUSSION

We have shown that a ligand-receptor fusion of growth hormone generates a potent agonist. We propose that the ability of the molecule to form head-to-tail reciprocal dimers (**Fig. 1d**) is responsible for its enhanced *in vivo* bioactivity. The design of the ligand-receptor fusion was based on the known crystal structure of the GHR (ref. 17). We used a flexible Gly_4Ser linker with four repeats (predicted length 80 Å). This long linker was chosen as a relatively flexible tether between growth hormone and the GHR so that the growth hormone moiety could interact with the cell surface GHR (**Fig. 1e**). Similar Gly_4Ser linkers have been used in recombinant single-chain Fv antibody production because of stability and lack of immunogenicity[18].

The ligand-receptor fusion was appropriately folded, appearing on both native PAGE gels and in gel filtration as two distinct species, potentially a monomer and dimer. We confirmed the presence of dimers by analytical ultracentrifugation. We propose that the ligand-receptor fusion forms a reciprocal head-to-tail dimer through intermolecular binding of the growth hormone moiety in each ligand-receptor fusion molecule to the receptor moiety in the other (**Fig. 1d**). The ligand-receptor fusion appeared as two bands on SDS-PAGE, with a molecular weight difference of 5 kDa, presumably as a result of glycosylation[19,20].

The ligand-receptor fusion was more potent than growth hormone *in vivo* but its *in vitro* bioactivity was only 10% of that for growth hormone. This discrepancy can be attributed to dimerization of the ligand-receptor fusion. In a static *in vitro* bioassay, the dimer would be biologically inactive, as seen for the native growth hormone–GHBP complex[21,22]. *In vivo*, however, the dimer provides a reservoir of inactive hormone in equilibrium with biologically active monomer.

After intravenous administration to rats, our ligand-receptor fusion had a 300-times reduced clearance as compared with growth hormone, and a 10–30-times reduced

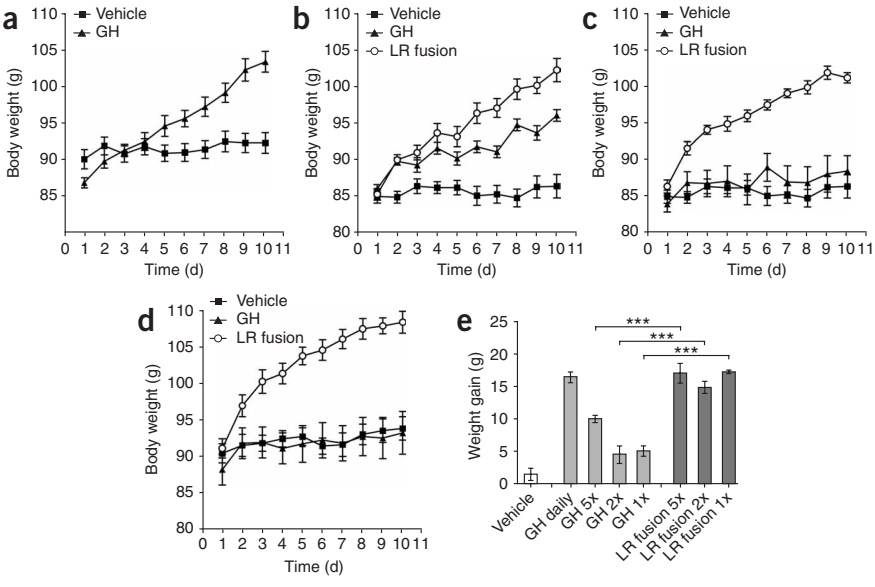

Figure 4 Body weight change in rats after subcutaneous administration of growth hormone (GH) and the ligand-receptor (LR) fusion. (**a**) Change after daily GH and placebo (vehicle only) administration. (**b**) Change after alternate day injections. (**c**) Change after two injections on days 1 and 5. (**d**) Change after a single injection on day 1. (**e**) Summary of changes in body weight after different treatment regimens. ***$P < 0.0001$ GH versus the LR fusion.

clearance compared with that previously reported for a growth hormone–GHBP complex or conjugate[11,12]. We tested two other ligand-receptor fusion variants of 55 kDa and 100 kDa. Neither protein showed the same delayed clearance. We therefore conclude that monomeric size alone is not responsible for delayed clearance of the ligand-receptor fusion. The renal contribution to growth hormone clearance has been estimated to be 25–53% in humans[23] and 67% in rats[24]. Therefore, reducing renal clearance alone can be predicted only to approximately halve growth hormone clearance[2]. Because clearance of growth hormone is relatively independent of a GHR mechanism[25], it is presumed that proteolysis is a main contributor to this clearance. We propose that the greatly reduced clearance of our ligand-receptor fusion is attributable to both reduced renal clearance and a conformation that prevents proteolysis.

In hypophysectomized rats, a single injection of our ligand-receptor fusion produced an increase in weight over 10 d similar to that seen with daily injections of growth hormone. It has previously been shown that GHBP coadministered as 1:1 molar complex with growth hormone augments growth[12]. Using the same protocol, we found that our ligand-receptor fusion protein promoted growth over 10 d after a single injection, whereas the growth hormone–GHBP complex required daily injections; in addition, our ligand-receptor fusion

Table 1 Results after 10 d of treatment with growth hormone or the ligand-receptor fusion in hypophysectomized rats[a]

Variable at 10 d	Placebo	Treatment	Growth hormone (GH)	Ligand-receptor fusion	*t*-test *P* (GH versus ligand-receptor)
Weight (g)	86.3 ± 1.6	Daily injections	103.3 ± 1.4	n.a.	n.a.
		Injections every 2 d	95.9 ± 0.8	102.2 ± 1.6	<0.0001
		Injections every 5 d	88.4 ± 2.1	101.1 ± 0.7	<0.0001
		Single injection	93.2 ± 2.9	108.3 ± 1.5	<0.0001
Change in weight from baseline (g)	1.43 ± 0.96	Daily injections	16.4 ± 0.8	n.a.	n.a.
		Injections every 2 d	9.9 ± 0.5	17 ± 1.5	0.0003
		Injections every 5 d	4.5 ± 1.3	14.8 ± 0.9	<0.0001
		Single injection	5.0 ± 0.1	17.2 ± 1.1	<0.0001
Change in femur length (mm)	0.00 ± 0.25	Daily injections	0.83 ± 0.26	n.a.	n.a.
		Injections every 2 d	0.99 ± 0.18	1.08 ± 0.07	0.667
		Injections every 5 d	0.44 ± 0.21	1.29 ± 0.22	0.0194
		Single injection	n.a.	n.a.	n.a.
Change in tibia weight (g)	0.00 ± 0.02	Daily injections	0.03 ± 0.01	n.a.	n.a.
		Injections every 2 d	0.06 ± 0.02	0.05 ± 0.01	0.52
		Injections every 5 d	0.01 ± 0.01	0.07 ± 0.02	0.027
		Single injection	n.a.	n.a.	n.a.
Change in thymus weight (mg)	0.00 ± 21	Daily injections	79 ± 20	n.a.	n.a.
		Injections every 2 d	43 ± 6	142 ± 22	0.0054
		Injections every 5 d	35 ± 12	120 ± 15	0.0132
		Single injection	−13 ± 22	117 ± 21	0.0017
Change in liver weight (mg)	0 ± 167	Daily injections	123 ± 170	n.a.	n.a.
		Injections every 2 d	362 ± 74	587 ± 206	0.056
		Injections every 5 d	402 ± 236	407 ± 116	0.073
		Single injection	n.a.	n.a.	n.a.
Change in kidney weight (mg)	0 ± 11	Daily injections	51 ± 22	n.a.	n.a.
		Injections every 2 d	45 ± 26	75 ± 21	0.0053
		Injections every 5 d	5 ± 26	67 ± 12	0.0273
		Single injection	7 ± 22	78 ± 15	0.0062
IGF-I (ng ml^{-1})	51 ± 12	Daily injections	92 ± 30	n.a.	n.a.
		Injections every 2 d	92 ± 30	329 ± 35	0.0005
		Injections every 5 d	55 ± 15	205 ± 5	<0.0001
		Single injection	18 ± 2.5	198 ± 66	0.0146
GH or ligand-receptor by ELISA (nM)	n.d.	Daily injections	n.d.	n.a.	n.a.
		Injections every 2 d	n.d.	44 ± 15	0.015
		Injections every 5 d	n.d.	23 ± 5	0.0015
		Single injection	n.d.	3.2 ± 1.2	0.0193

[a]Results are the mean ± s.e.m. n.d., not detectable; n.a., not analyzed.

generated IGF-I concentrations higher than those seen after growth hormone–GHBP administration. Growth hormone is biologically inactive when conjugated to GHBP, and the noncovalently linked complex lacks the stability of the ligand-receptor fusion[11,12]. The greater biological action of the ligand-receptor fusion may relate to its increased stability and its ability to activate the GHR in monomeric form.

In humans, IGF-I is a good biomarker of growth hormone activity. In hypophysectomized rats, however, IGF-I concentrations do not always reflect the growth response to growth hormone (refs. 2,12). Administration of the ligand-receptor fusion resulted in a clear increase in IGF-I as compared with growth hormone injection. We suggest that the growth hormone dose–related responses of growth and IGF-I differ in hypophysectomized rats. Thus, the dose of ligand-receptor fusion used in our study was in excess of that required to promote a maximal growth response, but still stimulated generation of IGF-I. Rats show more rapid renal clearance than humans, making it difficult to predict the dosing regimen that will be required in humans. We expect that the ligand-receptor fusion might be used at lower doses and much less frequently than growth hormone.

Fusions of cytokine hormones with serum albumin and pegylation have been used to prolong circulating half-life[2,26]. Our ligand-receptor fusion molecule has clear advantages over these two approaches. Pegylation is highly effective at delaying the clearance of proteins, but requires chemical modification and reduces the affinity of ligand for its receptor[2]. Thus, with pegylated hormone a greater dose is required, whereas with our ligand-receptor fusion a similar dose has a greater effect than native growth hormone. Relatively little is known about the growth hormone fusion with albumen, Albutropin, because as we understand this formulation has been withdrawn from clinical studies. In one pharmacokinetic study[26], Albutropin was found to have a six times longer terminal half-life than GH when given subcutaneously, whereas our ligand-receptor fusion protein has a 100-times longer terminal half-life when given intravenously as compared with the value published for GH (ref. 12). (For native human GH: clearance value of 18.6 ml min kg = 1,116 ml h kg and volume of distribution (V_d) = 336 ml kg; thus, $t_{1/2} = 0.693 \times 336/1116 = 0.21$ h.) Growth hormone naturally binds to circulating exGHR and therefore our ligand-receptor fusion is unlikely to be immunogenic as compared with fusions with other proteins; in addition, extensive *in silico* T-cell epitope screening showed no sites in the ligand-receptor fusion molecule (data not shown).

The attraction of the ligand-receptor fusion concept is its relative simplicity for manufacture and its native configuration, which might be anticipated to be less immunogenic. A clear implication of this work is that this technology can potentially be applied to other cytokine hormones used in the therapy of a wide range of human disease.

METHODS

Use of rats and human samples. Use of human samples was approved by the local ethics committee and all individuals gave informed consent. All of the experiments were conducted in compliance with the French laws (Council Directive No. 86/609/EEC of 24 November 1986) relating to the protection of animals used for experimental or other scientific purpose.

Materials. All materials were purchased from Sigma unless otherwise stated. Recombinant growth hormone was purchased from Pfizer, recombinant *Escherichia coli*–derived human GHBP used in binding assays was a gift from DSL Research Reagents, and iodinated growth hormone was a gift from NovoNordisk Park. Monoclonal antibodies to growth hormone and GHR used for purification and characterization were made in-house (C.J.S.), except for monoclonal antibodies B07b and B24a, which were a gift from L. Skriver (NovoNordisk Park) and monoclonal antibody 263, which was from AbD Serotec.

Purification of growth hormone–exGHR ligand-receptor fusions. We amplified cDNAs encoding human growth hormone and GHR by RT-PCR from human pituitary gland and liver, respectively, and cloned them into the vector pSecTag-V5–FRT–Hist-TOPO (Invitrogen) with a human growth hormone secretion signal sequence. We used four repeats of a Gly_4Ser linker to link the native C terminus of human growth hormone to the native amino terminus of the human GHR. We made stable clones in CHO Flp-In cells (Invitrogen), which were adapted to protein-free media and grown in suspension culture. We confirmed expression of the ligand-receptor fusion by an in-house ELISA. We performed affinity purification with a growth hormone monoclonal antibody column.

Transcription bioassays. We performed transcription bioassays in human 293 cells stably expressing the human GHR as described[16].

ELISA. An in-house growth hormone and ligand-receptor fusion ELISA has been established on the basis of the sandwich ELISA format. In brief, we incubated standards (growth hormone or ligand-receptor fusion), controls and unknowns with biotin-labeled mouse antibody to human growth hormone (monoclonal antibody 10A7) in wells precoated with a mouse antibody to human growth hormone antibody (monoclonal antibody 7F8). The detection limit for the assay was 2.5 pg, and the intra- and interassay coefficients of variance were $<10\%$. We purchased the IGF-I ELISA from DSL Research Reagents (DSL-10-2900 ACTIVE mouse/rat IGF-I kit).

Pharmacokinetic studies. We used 7-week old normal Sprague Dawley rats from Janvier for pharmacokinetic studies. We conducted subcutaneous or intravenous administration (penile vein) and blood withdrawal (orbital sinus) under isoflurane anesthesia. We injected the rats ($n = 4$–6 per group) intravenously or subcutaneously with of 0.1 mg per kg (body weight) of recombinant human growth hormone or ligand-receptor fusion. We collected blood samples from the retro-orbital plexus. We collected serum and stored it at -70 °C until assay. We estimated pharmacokinetic parameters by fitting values of hormone concentration versus time to compartmental models using nonlinear least-squares regression analysis. We normalized clearance values to animal weight. We calculated clearance rate per rat weight and terminal half-lives by using the coefficient and exponents obtained from the intravenous bolus model fits.

Growth studies. The growth studies were performed on Sprague Dawley rats from Charles River Laboratories. Rats were hypophysectomized under isoflurane anesthesia at 4 weeks of age by the breeder and delivered 1 week after selection by body weight criteria for successful surgery. We caged rats individually and gave them another week of rest before the experimental phase. The injection solutions of excipient, recombinant human growth hormone and ligand-receptor fusion never exceeded 2 ml per kg (body weight). We weighed the rats daily and, depending on the administration protocol, the rats received injections of the test substances for 10 d.

Characterization of ligand-receptor fusions. We examined conformation of the ligand-receptor fusion by using 16 conformationally sensitive human growth hormone receptor monoclonal antibodies. We used denaturing, native gels and immunoblotting to analyze the ligand-receptor fusion and performed immunoblotting with nonconformationally sensitive antibodies. We defined the form of the ligand-receptor fusion protein in solution by gel filtration using a Superose G200 analytical column and by analytical ultracentrifugation. The latter was performed by sedimentation velocity (courtesy of the analytical service provided by A. Barron at Leeds University).

Statistics. Two groups were compared with a Student's *t*-test if their variance was normally distributed or by a Student-Satterthwaite's *t*-test if their variance was not normally distributed. We tested distribution with an *F*-test. We used one-way analysis of variance to compare the means of three or more groups and, if the level of significance was $P < 0.05$, we performed individual comparisons with Dunnett's tests. All statistical tests were two-sided at the 5% level of significance and no imputation was made for missing values.

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AUTHOR CONTRIBUTIONS

I.R.W., S.L.P. and S.J. cloned, purified, tested and analyzed the fusion proteins. Z.W., K.C.L. and C.J.S. contributed to the characterization of the fusion proteins. E.F., M.T., C.S. and C.T. performed and analyzed the *in vivo* studies. E.F., P.J.A., J.R.S. and R.J.R. were responsible for the concepts, data analysis and writing of the manuscript. R.J.R. supervised the project.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine.

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1. Woodhouse, L.J., Mukherjee, A., Shalet, S.M. & Ezzat, S. The influence of growth hormone status on physical impairments, functional limitations, and health-related quality of life in adults. *Endocr. Rev.* **27**, 287–317 (2006).
2. Clark, R. *et al*. Long-acting growth hormones produced by conjugation with polyethylene glycol. *J. Biol. Chem.* **271**, 21969–21977 (1996).
3. Cook, D.M. *et al*. The pharmacokinetic and pharmacodynamic characteristics of a long-acting growth hormone (GH) preparation (nutropin depot) in GH-deficient adults. *J. Clin. Endocrinol. Metab.* **87**, 4508–4514 (2002).
4. Reiter, E.O. *et al.* A multicenter study of the efficacy and safety of sustained release GH in the treatment of naive pediatric patients with GH deficiency. *J. Clin. Endocrinol. Metab.* **86**, 4700–4706 (2001).
5. Jostel, A., Mukherjee, A., Alenfall, J., Smethurst, L. & Shalet, S.M. A new sustained-release preparation of human growth hormone and its pharmacokinetic, pharmacodynamic and safety profile. *Clin. Endocrinol.* **62**, 623–627 (2005).
6. Laursen, T. *et al.* Long-term effects of continuous subcutaneous infusion versus daily subcutaneous injections of growth hormone (GH) on the insulin-like growth factor system, insulin sensitivity, body composition, and bone and lipoprotein metabolism in GH-deficient adults. *J. Clin. Endocrinol. Metab.* **86**, 1222–1228 (2001).
7. Laursen, T., Jorgensen, J.O., Jakobsen, G., Hansen, B.L. & Christiansen, J.S. Continuous infusion versus daily injections of growth hormone (GH) for 4 weeks in GH-deficient patients. *J. Clin. Endocrinol. Metab.* **80**, 2410–2418 (1995).
8. Muller-Newen, G., Kohne, C. & Heinrich, P.C. Soluble receptors for cytokines and growth factors. *Int. Arch. Allergy Immunol.* **111**, 99–106 (1996).
9. Baumann, G., Amburn, K.D. & Buchanan, T.A. The effect of circulating growth hormone-binding protein on metabolic clearance, distribution, and degradation of human growth hormone. *J. Clin. Endocrinol. Metab.* **64**, 657–660 (1987).
10. Baumann, G. Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. *Endocr. Rev.* **12**, 424–449 (1991).
11. Baumann, G., Shaw, M.A. & Buchanan, T.A. *In vivo* kinetics of a covalent growth hormone-binding protein complex. *Metabolism* **38**, 330–333 (1989).
12. Clark, R.G. *et al.* Recombinant human growth hormone (GH)-binding protein enhances the growth-promoting activity of human GH in the rat. *Endocrinology* **137**, 4308–4315 (1996).
13. Baumann, G. Growth hormone binding protein—errant receptor or active player? *Endocrinology* **136**, 377–378 (1995).
14. Ayling, R.M. *et al.* A dominant-negative mutation of the growth hormone receptor causes familial short stature. *Nat. Genet.* **16**, 13–14 (1997).
15. Ross, R.J. *et al.* A short isoform of the human growth hormone receptor functions as a dominant negative inhibitor of the full-length receptor and generates large amounts of binding protein. *Mol. Endocrinol.* **11**, 265–273 (1997).
16. Ross, R.J.M. *et al.* Binding and functional studies with the growth hormone receptor antagonist, B2036-PEG (pegvisomant), reveal effects of pegylation and evidence that it binds to a receptor dimer. *J. Clin. Endocrinol. Metab.* **86**, 1716–1723 (2001).
17. Cunningham, B.C. *et al.* Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science* **254**, 821–825 (1991).
18. Huston, J.S., Tai, M.S., McCartney, J., Keck, P. & Oppermann, H. Antigen recognition and targeted delivery by the single-chain Fv. *Cell Biophys.* **22**, 189–224 (1993).
19. Herington, A.C., Smith, A.I., Wallace, C. & Stevenson, J.L. Partial purification from human serum of a specific binding protein for human growth hormone. *Mol. Cell. Endocrinol.* **53**, 203–209 (1987).
20. Frick, G.P., Tai, L.R., Baumbach, W.R. & Goodman, H.M. Tissue distribution, turnover, and glycosylation of the long and short growth hormone receptor isoforms in rat tissues. *Endocrinology* **139**, 2824–2830 (1998).
21. Mannor, D.A., Winer, L.M., Shaw, M.A. & Baumann, G. Plasma growth hormone (GH)-binding proteins: effect on GH binding to receptors and GH action. *J. Clin. Endocrinol. Metab.* **73**, 30–34 (1991).
22. Lim, L., Spencer, S.A., McKay, P. & Waters, M.J. Regulation of growth hormone (GH) bioactivity by a recombinant human GH-binding protein. *Endocrinology* **127**, 1287–1291 (1990).
23. Haffner, D., Schaefer, F., Girard, J., Ritz, E. & Mehls, O. Metabolic clearance of recombinant human growth hormone in health and chronic renal failure. *J. Clin. Invest.* **93**, 1163–1171 (1994).
24. Johnson, V. & Maack, T. Renal extraction, filtration, absorption, and catabolism of growth hormone. *Am. J. Physiol.* **233**, F185–F196 (1977).
25. Veldhuis, J.D. *et al.* Impact of experimental blockade of peripheral growth hormone (GH) receptors on the kinetics of endogenous and exogenous GH removal in healthy women and men. *J. Clin. Endocrinol. Metab.* **87**, 5737–5745 (2002).
26. Osborn, B.L. *et al.* Albutropin: a growth hormone-albumin fusion with improved pharmacokinetics and pharmacodynamics in rats and monkeys. *Eur. J. Pharmacol.* **456**, 149–158 (2002).
27. de Vos, A.M., Ultsch, M. & Kossiakoff, A.A. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**, 306–312 (1992).