A human leptin mutant induces weight gain in normal mice

Sandra A.B.W. Verploegen, Geert Plaetinck, René Devos*, José Van der Heyden, Yves Guisez

Roche Research Gent, Jozef Plateaustraat 22, B-9000 Ghent, Belgium

Received 27 January 1997

Abstract Leptin, a fat secreted hormone, regulates ingestive behaviour and energy balance by binding to a specific receptor. Using site-directed mutagenesis, we screened for single amino acid residues in human leptin which are critical for receptor binding and biological activity. Here we report that one of these mutants has *in vivo* antagonistic properties. An Arg to Gln substitution at position 128 of human leptin does not affect receptor binding but knocks out biological activity. Repeated injection of R128Q in normal C57BL/6J mice results in a progressive increase in body weight. This demonstrates that R128Q is able to interfere with the negative feedback control of endogenous leptin. This mutant could be of therapeutic use for wasting disorders, such as anorexia and cachexia, where weight gain would be beneficial.

Key words: Leptin; Antagonist; Mutagenesis; Body weight; Anorexia; Cachexia

1. Introduction

Leptin, the product of the *ob* gene, plays a central role in the control of body adiposity. Genetic defects that lead to leptin deficiency (*ob/ob* mice) or leptin resistance (*db/db* mice, *fa/fa* rats) both cause severe obesity [1–3]. Administration of recombinant leptin decreases food intake and increases energy expenditure in *ob/ob* mice and WT mice [4–6]. The weight reducing effects are likely mediated through a signaling leptin receptor in the hypothalamus [7,8]. Leptin itself displays no apparent sequence similarity to any other existing protein. Yet structure prediction and comparison algorithms unmistakably identify leptin as a member of the haemopoietic cytokine family that adopt a four α-helix bundle fold [9,10]. This observation is further corroborated by the identification of a leptin receptor that resembles other haemopoietic-class receptors such as the IL-6 signal transducing chain, gp 130 [7].

In order to investigate the interaction with its receptor we constructed a series of point mutations in human leptin. Mutant proteins were tested for binding and *in vitro* biological activity. In the present report we show that one mutant, R20Q, affects receptor binding. Three other mutants, D40N, S127D and R128Q, although they bind normally to the mouse leptin receptor, show reduced biological activity. One of these, R128Q, is unable to trigger intracellular signaling. As such, R128Q has the characteristics of a competitive inhibitor. In order to investigate antagonistic properties of R128Q *in vivo*, we treated lean C57BL/6 mice with this mutant. Here we show that R128Q induces an obese phenotype in these animals and increases serum insulin levels, features reminiscent of leptin-deficient mice.

2. Materials and methods

2.1. Mutagenesis

Human leptin cDNA was subcloned into the pSV-SPORT1 vector (BRL-Gibco) and site-directed mutagenesis was carried out according to the method of Deng and Nickoloff [11] with a Transformer kit (Clontech). Mutant proteins were expressed in COS1 cells as described (DEAE dextran method [12]). R20Q, D40N, S127D and R128Q were also subcloned in the pVL1393 vector for expression in Sf9 insect cells (Baculogold, Pharmingen) and purified by immunoaffinity chromatography using a mAb (2A5) directed against human leptin.

2.2. Competition binding assay.

COS1 cells were transfected with murine leptin receptor cDNA [7]. 2×10^5 cells were incubated in 200 μl Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS for 3–5 h at 4°C with 1 nM ^{125}I-labelled human leptin in combination with increasing concentrations of unlabelled leptin or leptin mutants. Bound ligand was separated from free radioactivity by centrifugation through a phthalate oil cushion and γ emission was counted.

2.3. BAF3 1423 proliferation assay

A chimeric leptin receptor construct was made by fusing the extracellular and transmembrane domain of the murine leptin receptor [7] with the intracellular domain of the human β_c receptor (β_c is a common receptor subunit present in the IL-3, IL-5 and GM-CSF receptor complex [13]). The DNA fragments were linked by PCR generated *Bam*HI sites. IL-3- or GM-CSF-dependent BAF3 cells were transfected with this construct as described [14] and responsive cells were selected in leptin-containing growth medium. One of several independent clones, BAF3 1423, was used to set up a leptin proliferation bioassay. Overnight-starved BAF3 1423 cells were incubated at 1×10^3 cells/200 μl in 96-well microtiter plates with variable concentrations of wild-type leptin or leptin-mutein. After 72 h, 0.5 μCi [^{3}H]thymidine was added for 4 h and the cells were harvested. The incorporated label was counted with a Topcount scintillation counter (Packard, Canberra).

2.4. Injections into ob/ob mice

8–10-week-old *ob/ob* mice were treated for 9 days with human leptin, S127D, R128Q or PBS (control). Injections were given i.p. daily at 5.30 p.m.

15 μg leptin or mutant in combination with 1.8 mg 2A5 was given per animal per day. Body weight was monitored by weighing the animals at 5.00 p.m.

2.5. Injections into C57BL/6J mice

Nine 9–10-week-old C57BL/6J mice were injected twice daily (9.30 a.m. and 5.30 p.m.) with human leptin (100 μg/injection), R128Q (100 μg/injection) or PBS (control) in presence of 1.38 mg 2A5 per injection. Mice were weighed daily at 9.00 a.m.

2.6. Insulin determination

Serum insulin levels were determined using a commercially available RIA (Linco Research).

*Corresponding author. Fax: (32) (9) 2331119.
E-mail: Rene.Devos@Roche.com

PII S0014-5793(97)00192-0

3. Results and discussion

To study the interaction of leptin with its receptor we generated 37 human leptin mutants, among which 29 resulted from changing a charged to an uncharged amino acid residue. Muteins were expressed in COS1 and baculovirus-infected insect cells, and the expression level in the supernatant was quantified by an ELISA using mouse mAbs and polyclonal rabbit IgG directed against human leptin (data not shown). All muteins were tested in a competition binding experiment by measuring displacement of ^{125}I-labelled human leptin from the murine leptin receptor expressed on COS1 cells. The biological activity was evaluated in an in vitro assay based on proliferation of leptin-sensitive BAF3 1423 cells. These cells were obtained after transfection of BAF3 cells with a plasmid encoding a chimeric receptor consisting of the mouse leptin receptor extracellular domain fused to the cytoplasmic do-

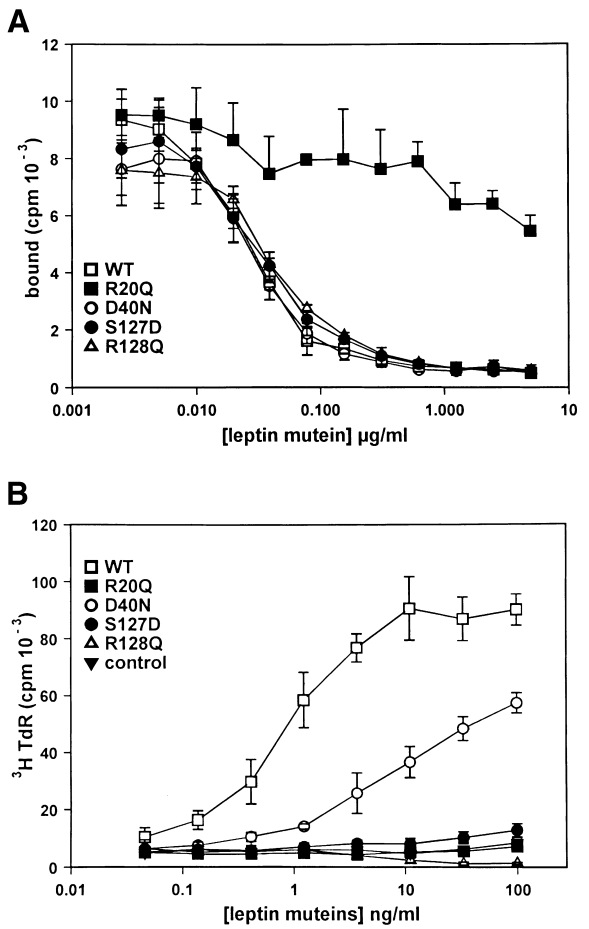

Fig. 1. Analysis of receptor binding and in vitro biological activity of different human leptin mutants. A: ^{125}I-labelled human leptin displacement by cold wild-type leptin (WT) or leptin muteins, on COS1 cells transiently transfected with the murine leptin receptor. Each point represents mean ± S.E.M. cpm bound ^{125}I-labelled leptin ($n = 3$). The binding affinity of the mutants D40N, S127D and R128Q for the receptor was indistinguishable from that of wild-type leptin (K_d: ±1 nM). B: Activities of wild-type leptin and leptin muteins, in the BAF3 1423 proliferation assay. Data are expressed as mean ± S.E.M. cpm incorporated [^{3}H]thymidine ($n = 3$). 1 ng/ml leptin induces 50% of maximal proliferation. D40N has 30-fold less activity than wild type. S127D has only marginal and R128Q no proliferative activity at all.

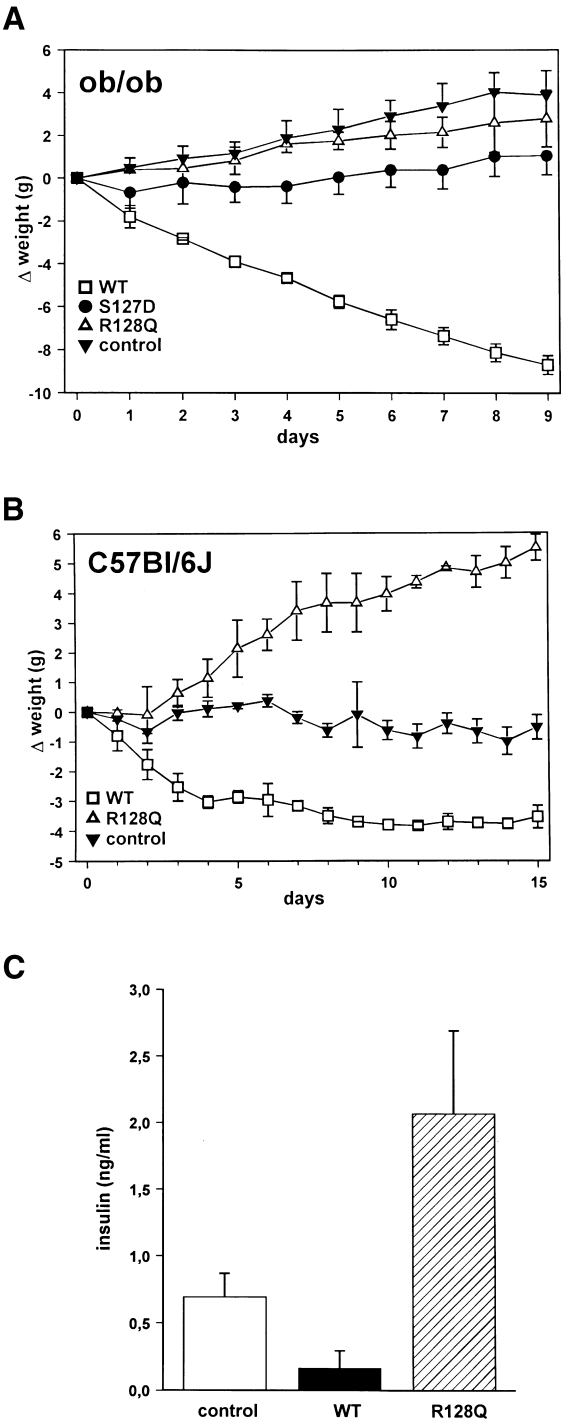

Fig. 2. In vivo biological effects of mutants and wild-type leptin. A: Effect on body weight in *ob/ob* mice. Control group was injected with PBS. Data expressed in the figure are mean weight change ± S.E.M. ($n = 3$). Initial body weight of each treated group was for wild type: 39.4 ± 2.2 g, S127D: 40.6 ± 1.2 g, R128Q: 41.0 ± 2.9 g and control: 41.5 ± 4.2 g. B: Effect on body weight in lean C57BL/6J mice. Mice were injected twice daily with relatively high amounts (100 µg/injection) of R128Q, wild-type leptin or control. Data in the figure display mean change of body weight per group ± S.E.M. ($n = 3$). Initial body weight for each group was: wild type: 21.6 ± 0.6 g, R128Q: 21.5 ± 0.9 g and control: 21.0 ± 1.0 g. C: Serum insulin levels of C57BL/6J mice after 15 days of treatment with R128Q, wild-type leptin or PBS (control). Two serum samples per mouse were tested and the data represent mean insulin concentration ($n = 6$) of each group.

main of the human common β receptor. Most of the mutants showed similar binding and biological activity as wild type (data not shown). R20Q was unable to bind to the receptor (Fig. 1A). As expected, this mutant was also inactive in the proliferation assay (Fig. 1B). Interestingly three single point mutations, D40N, S127D and R128Q, did not affect receptor binding (Fig. 1A) but had profound effects on biological activity. Although normal receptor binding was observed, D40N showed reduced and S127D a very weak proliferative response, while R128Q had no biological activity at all (Fig. 1B). BAF3 1423, in contrast to untransfected cells, exhibited some growth factor-independent proliferation probably through constitutive activation of the chimeric receptor. Interestingly, R128Q completely inhibited this background proliferation at concentrations above 10 ng/ml (Fig. 1B). We also found that S127D and especially R128Q at high doses can antagonise the proliferative stimulus of wild-type human leptin (data not shown).

Loss of biological activity despite normal receptor binding suggests that these muteins could prevent receptor oligomerisation. Homodimerisation of the extracellular domain of the leptin receptor is most likely responsible for leptin-mediated signaling [15,19] and leptin receptor homodimers can be observed after chemical cross-linking (Devos *et al.*, in preparation). However, no different cross-linking patterns relative to wild-type leptin were seen with D40N, S127D and R128Q (data not shown). Alternatively, the mutated residues might be crucial for receptor activation by inducing allosteric changes in the receptor upon binding. Finally it cannot be excluded that S127, R128 and to a lesser extend D40 are involved in binding of an as yet unidentified leptin receptor accessory chain.

Treatment of obese C57BL/6J (*ob/ob*) mice with exogenous recombinant leptin decreases food intake, increases energy expenditure and decreases plasma insulin levels [4–6]. This results in a net reduction of body weight. We have previously observed that co-administration of human leptin with a monoclonal antibody (2A5) directed against human leptin enhances the leptin effect (Van der Heyden *et al.*, in preparation). The mechanism of this potentiation is unclear at present. The antibody could increase the half-life of leptin in serum, alternatively it might affect tissue distribution or transport to the brain. The biological activity of the muteins S127D and R128Q was tested in *ob/ob* mice by i.p. administration together with 2A5 (Fig. 2A). As expected, treatment with wild-type human leptin led to significant weight loss (up to 1 g day^{-1}), while no effect was observed in animals injected with S127D and R128Q. Taken together, the injections into *ob/ob* mice unambiguously demonstrate the lack of biological activity of S127D and R128Q, which is in accordance with the inability of these muteins to trigger a proliferative response in BAF3 1423 cells.

Since R128Q can compete with leptin for receptor binding it is conceivable that, at a high dose, it should be able to block the activity of resident leptin *in vivo*. To test this hypothesis lean C57BL/6J mice were i.p. injected twice a day with 100 μg R128Q. Control groups were treated with an equal dose of wild-type leptin or carrier without leptin. To potentiate the activity of leptin or the mutant, 2A5 mAb was co-injected. As can be seen from Fig. 2B, animals treated for 15 days with R128Q gradually gained about 25% of their initial body weight. We observed a significant increase in white fat deposits in these animals (not shown). In contrast, animals that were injected with wild-type leptin rapidly lost circa 15% of their initial body weight and maintained this plateau as long as the injections lasted. When the treatment was stopped both the R128Q- and the wild-type leptin-treated animals returned to control body weight levels (not shown). We also measured serum insulin levels after 15-day treatment (Fig. 2C). Insulin concentrations of R128Q-injected mice were significantly increased relative to controls. Treatment with wild-type leptin showed an opposite effect. From these observations it can be concluded that R128Q behaves as a potent antagonist of the endogenous mouse leptin.

Several compounds with appetite- and weight-enhancing effects have been developed and are being investigated for potential use in patients with anorexia and cachexia [16]. Recently a role for leptin in anorexia caused by infection was suggested. Endotoxin and inflammatory cytokines such as TNF and IL-1, which mediate anorexia, have been shown to induce leptin expression [17,18]. Further study is warranted to investigate if R128Q could restore body weight in animal models of anorexia and cachexia. In this context, a leptin mutein with *in vivo* antagonistic effects could be therapeutically useful to prevent weight loss during chronic diseases, such as anorexia nervosa, cancer and AIDS.

Acknowledgements: The authors thank I. Faché, T. Tuypens, F. Vanhoutte, A. Verhee and J. Bostoen for excellent technical assistance. Thanks are also due to Prof. W. Fiers, Drs. M. Steinmetz and J. Tavernier for continued support and interest.

References

[1] Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J.M. (1994) Nature 372, 425–431.
[2] Lee, G.-H., Proenca, R., Montez, J.M., Caroll, K.M., Darvishzadeh, J.G., Lee, J.I. and Friedman, J.M. (1996) Nature 379, 623–635.
[3] Chen, H., Charlat, O., Tartaglia, L.A., Woolf, E.A., Weng, X., Ellis, P., Lakey, N.D., Culpepper, J., Moore, K.J., Breitbart, R.E., Duyk, G.M., Tepper, R.I. and Morgenstern, J.P. (1996) Cell 84, 546–549.
[4] Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R. and Burn, P. (1995) Science 269, 546–549.
[5] Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K. and Friedman, J.M. (1995) Science 269, 543–546.
[6] Pelleymounter, M.A., Cullen, M.J. Baker, M.B., Hecht, R., Winters, D., Boone, T. and Collins, F. (1995) Science 269, 540–543.
[7] Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Woolf, E.A., Monroe, C.A. and Tepper, R.I. (1995) Cell 83, 1263–1271.
[8] Mercer, J.G., Hoggard, N., Williams, L.M., Lawrence C.B., Hannah, L.T. and Trayhurn, P. (1996) FEBS Lett. 387, 113–116.
[9] Madej, T., Boguski, M.S. and Bryant, S.H. (1995) FEBS Lett. 373, 13–18.
[10] Rock, F.L., Altmann, S.W., van Heek, M., Kastelein, R.A. and Bazan, J.F. (1996) Horm. Metab. Res. 28, 649–652.
[11] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81–88.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[13] Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van der Heyden, J., Fiers, W. and Plaetinck, G. (1991) Cell 66, 1175–1184.
[14] Cornelis, S., Plaetinck, G., Devos, R., Van der Heyden, J., Tavernier, J., Sanderson, C.J. and Fiers, W. (1995) EMBO J. 14, 3395–3402.

[15] Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.-F. and Tartaglia, L.A. (1996) Proc. Natl. Acad. Sci. USA 93, 8374–8378.
[16] Plata-Salaman, C.R. (1996) Nutrition 12, 69–78.
[17] Grunfeld, C., Zhao, C., Fuller, J., Pollock, A., Moser, A., Friedmann, J. and Feingold, K.R. (1996) J. Clin. Invest. 97, 2152–2157.
[18] Sarraf, P., Frederich, R.C., Turner, E.M., Ma, G., Jaskowiak, N.T., Rivet III, D.J., Flier, J.S., Lowell, B.B., Fraker, D.L. and Alexander, H.R. (1997) J. Exp. Med. 185, 171–175.
[19] White, D.W., Kuropatwinski, K.K., Devos, R., Baumann, H. and Tartaglia, L.A. (1997) J. Biol. Chem. 272, 4065–4071.