Generation of Soluble Leptin Receptor by Ectodomain Shedding of Membrane-spanning Receptors in Vitro and in Vivo*

Received for publication, June 12, 2002, and in revised form, August 16, 2002
Published, JBC Papers in Press, September 20, 2002, DOI 10.1074/jbc.M205825200

Hongfei Ge‡§, Lu Huang‡§§, Tiffany Pourbahrami‡§, and Cai Li‡§§**

From the ‡Touchstone Center for Diabetes Research, Departments of §Physiology and ¶Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, Texas 75390-8854

Leptin is an adipocyte-derived hormone with potent effects on food intake and body weight. Genetically obese rodents with mutations of leptin or leptin receptor develop morbid obesity and diabetes. The receptor for leptin, OB-R, is alternatively spliced to at least five transcripts, encoding receptors designated OB-Ra, -b, -c, -d, and -e. OB-Re does not encode a transmembrane domain and is secreted. However, soluble leptin receptor does circulate in human plasma and represents the major leptin-binding activity. In this report, we attempted to determine whether the soluble leptin receptor may also be derived from membrane-spanning receptor isoforms by ectodomain shedding. Using stable cell lines expressing both OB-Ra, the most abundantly expressed leptin receptor isoform, and OB-Rb, the signaling form of the leptin receptor, we demonstrate that soluble leptin receptor protein can indeed be generated by proteolytic cleavage of these two receptor isoforms in vitro. Experiments using adenoviruses expressing dually tagged OB-Ra or OB-Rb also demonstrate that soluble leptin receptor may be derived from ectodomain shedding of both receptor isoforms in vivo. Because our earlier and other studies have shown that the soluble receptors modulate the levels as well as activity of leptin, our findings suggest that regulated shedding of the ectodomain of membrane-spanning leptin receptors may represent a novel mechanism of modulating leptin’s biological activity.

* This work was supported in part by Grant 0255737Y from the American Heart Association, Texas Affiliate, Welch Foundation Grant I-1470, and National Institutes of Health Grant DK 60137. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Present address: Shanghai Huaqia Technology Development Co. Ltd., Shanghai 200235, China.
‡ Recipient of a career development award from the American Diabetes Association. To whom correspondence should be addressed. Tel.: 214-648-3340; Fax: 214-648-9191; E-mail: Cai.Li@UTSouthwestern.edu.

-442-648-3340; Fax: 214-648-9191; E-mail: Cai.Li@UTSouthwestern.edu.

This paper is available on line at http://www.jbc.org

This paper is available on line at http://www.jbc.org
main shedding of both OB-Ra and OB-Rb is detected by a
polyclonal antibody recognizing the FLAG epitope tag fused
in-frame with the amino terminus of recombinant receptor
protein. Stable 293 cells expressing OB-Ra and OB-Rb release
the soluble leptin receptor under serum-free conditions, sug-
gest- ing that the cleavage of membrane-bound receptor may be
mediated by proteases resident in the cell. Because the soluble
leptin receptor affects leptin’s effect on food intake and body
weight in ob/ob mice (19), regulated ectodomain shedding of
membrane-spanning receptor proteins may represent a novel
mechanism of regulating leptin’s biological activity.

EXPERIMENTAL PROCEDURES

Generation of Stable Cell Lines Overexpressing OB-Ra and OB-Rb—cDNA of OB-Ra or OB-Rb in the expression vector
pcDNA3.1—→MycHisA was digested with Pme I and ligated into
the same site of pcDNA5/FRT (Invitrogen). The resulting vectors contain-
ing OB-Ra or OB-Rb were co-transfected with pOG44 into a Flp-InTm
host cell line (Invitrogen) using FuGene 6 Reagent (Roche Molecular
Biochemicals) following the instructions by the respective manufactur-
ers. Stable integrants were selected in Dulbecco’s modified Eagle’s
medium containing 10% fetal bovine serum and 100 μg/ml hygromycin
B (Clontech) for about 3 weeks. pcDNA5/FRT containing chlorampheni-
ocol (CAT) as an antibiotic was also transfected and selected as
a negative control in subsequent experiments.

Luciferase Reporter Analysis of Leptin Activation of OB-Ra and OB-Rb Cell Lines—Stable Flp-In cell lines expressing OB-Ra, OB-Rb, or
CAT were seeded into 6-well plates until 50–60% confluent. Cells were trans- ferred with 1 μg of p6x47Luciferase plasmid (containing STAT3
response element and luciferase cDNA) kindly provided by Dr. Jim
Darnell, the Rockefeller University). Twenty-two hours post-transfec-
tion, cells were washed and replaced with serum-free medium for 5 h,
and leptin was added directly to a final concentration of 1 μM (50 nM).
After 6–24 h, cells were collected and luciferase activity measured
using a luciferase assay system (Promega). 21

D3 Leptin Binding to Stably Transfected Cells—Leptin binding to
Flip-In 293 cell lines was performed in 6-well plates as described (20).
Briefly, cells stably transfected with CAT, OB-Ra, or OB-Rb were grown
to about 90% confluence and washed with cold PBS. Cells were incu-
bate d with 60,000 cpm of 125I-leptin (PerkinElmer Life Sciences) in
the presence or absence of an excess of cold leptin (2 μg/ml) for 6 h at
4 °C in a final volume of 1 ml of binding buffer (PBS containing 1%
(w/v) bovine serum albumin (fraction V, Sigma)). The end of incubation,
unbound label was removed by two PBS washes; 1 ml of 1 N NaOH was
added, and radioactivity in lysate was measured using a Canberra
Model 2500 gamma counter.

Construction of Adenoviral Vectors Encoding Dually Tagged Leptin Receptors—The cDNA sequence encoding the signal peptide of OB-R
was synthesized with XhoI and BamHI sites engineered at the 5’ and 3’
ends. Annealed oligonucleotides were ligated to pcDNA3.1—→MycHisA
digested with the XbaI and BamHI to generate the vector
pcDNA3.1—→MycHisA-SS. The cDNA encoding the long-form leptin
receptor (OB-Rb) as well as the short-form receptor (OB-Ra) was PCR-
amplified from full-length expression vectors described earlier (21). The
5’-oligonucleotide contained the restriction site of BglII and the coding
sequence of FLAG epitope (DYKDDDDK) as well as OB-R sequences
immediately downstream of signal peptide. The 3’-oligonucleotide con-
tains a HindIII site. PCR products of both OB-Ra and OB-Rb digested
by BglII and HindIII were then ligated to pcDNA3.1—→MycHisA-SS
digested with BamHI and HindIII. In this fashion, the resulting vectors
contain a FLAG epitope that is inserted between the signal peptide and
the remaining coding sequences of OB-R. The carboxyl terminus is
already fused in frame with a c-myc epitope on the vector, as described
earlier (21). Upon cleavage of the signal peptide during receptor trans-
location from the membrane, the FLAG epitope becomes the amino
terminus of recombinant OB-R protein, while the c-myc epitope be-
comes the carboxyl terminus. Final vectors of both OB-Ra and OB-Rb
were sequenced and transiently expressed in 293T cells to ensure
that no cloning errors were introduced. Primer sequences are the
following: Signal peptide forward (CL317), 5’-CTAGAAGAGATGATGTG-
CTGAGAATCTCTCTGTCGCTCCTCACCACATTTATCCACTGTAGATGCTGACCTTTG-3; reverse (CL318), 5’-GATCCCAAGTGCAGCT-

1 The abbreviations used are: CAT, chloramphenicol acetyltrans-
ferase; PBS, phosphate-buffered saline.
a polyclonal antibody against the extracellular domain of the receptor, followed by Western blotting using a c-Myc monoclonal antibody that recognizes the carboxyl terminus that has been fused in-frame with the coding region of OB-Ra and OB-Rb (21). The two shaded regions in the amino half of each receptor construct are sequences homologous to each other as well as to other cytokine receptors. TMR, transmembrane region. B, Western blotting of receptor protein expressed in OB-Ra or OB-Rb stable cell lines. Cells were lysed with radioimmune precipitation assay buffer, and recombinant OB-R was immunoprecipitated with a rabbit anti-receptor antibody, followed by detection with a monoclonal antibody recognizing the c-Myc epitope tag. C, luciferase reporter assay of leptin activation of OB-Rb. Flp-In 293 cells stably expressing OB-Rb were transfected with luciferase expression construct on a minimal promoter with four STAT3 DNA binding sites. Leptin (50 nM) was added for the duration indicated, and luciferase activity was determined. D, 125I-leptin binding to stably transfected 293 cells overexpressing OB-Ra or OB-Rb. Cells were seeded in 6-well plates in quadruplicate, and labeled leptin was added to determine the number of receptor molecules present on the cell surface that are capable of binding leptin. Specificity of binding was confirmed by competition with an excess amount of cold leptin.

**Release of Soluble Leptin Receptor into Medium by Stable 293 Cells Overexpressing Both OB-Ra and OB-Rb**—Although the OB-Re transcript encoding soluble leptin receptor in rodents have been identified, such mRNA species in humans has not been found (26). Because soluble leptin receptor represents the main leptin-binding activity in human blood (27), an unresolved issue is how soluble leptin receptor is generated in humans. An earlier study reported that leptin-binding activity could be detected in the medium of cells transiently transfected with the human leptin receptor cDNA (20). This observation suggests that proteolytic cleavage of membrane-spanning leptin receptors could be the source of soluble leptin receptor in human circulation, analogous to that of the generation of the human soluble growth hormone receptor (28).

To test this possibility further, we determined whether the murine-soluble leptin receptor might also be generated by ectodomain shedding of membrane-spanning receptors, in addition to synthesis from the OB-Re transcript. Stable 293 cells overexpressing CAT, OB-Ra, or OB-Rb were incubated in the presence or absence of serum to determine whether soluble receptor immunoreactivity may be detected after leptin-Sepharose pull-down of culture supernatant and immunoblotting. Fig. 2 shows that a soluble leptin-binding protein with the size of OB-Re is readily detectable in the tissue culture supernatant of both serum-containing and serum-free medium of OB-Ra-expressing cells. Signal is also detectable, although much weaker, from the supernatant of OB-Rb-expressing cells, con-
Ectodomain Shedding of Leptin Receptor

FIG. 2. Release of soluble leptin receptor into medium by stable 293 cells overexpressing Ob-Ra or Ob-Rb. Stable 293 cells overexpressing CAT (C), Ob-Ra (Ra), or OB-Rb (Rb) were incubated in the presence (10% fetal bovine serum) or absence (containing 1% BD ITS Cell Culture Supplement) of serum for 48 h. Medium from each cell line was collected and incubated with leptin-Sepharose resin overnight. Leptin beads were washed with PBS three times and boiled directly in SDS sample buffer. Resin suspension was loaded onto an 8% SDS-PAGE gel and blotted with an anti-leptin receptor polyclonal antibody as described (18). In addition to the soluble leptin receptor protein with a size similar to that of OB-Re, a second band of smaller size (~100 kDa) is also detected, which is likely the product of further proteolytic processing, because it is absent in supernatant of control cells, can bind leptin, and can react with the receptor antibody. The intensity of the smaller band also varies between experiments. Shed receptor is only visible from OB-Rb cells after longer exposure (right). Ra, 1 μl of plasma from a mouse infused with adenovirus overexpressing OB-Re, which is used as a positive control.

FIG. 3. Expression of dually tagged OB-R protein in 293 cells. A, diagram of viral constructs generated. Adenoviruses encoding OB-Ra or OB-Rb were engineered to contain a FLAG epitope tag at the amino terminus immediately following the signal sequence. The carboxyl terminus is fused to a c-Myc tag. Mature OB-R protein on the cell surface should have the FLAG epitope at its amino terminus after removal of the signal peptide. B, detection of virally produced receptor protein with monoclonal antibodies against the amino-terminal FLAG tag or the carboxyl-terminal c-Myc tag. Total cellular extracts of infected 293 cells were prepared by lysis in radioimmune precipitation assay buffer, immunoprecipitated, and blotted using ECL reagents as for Fig. 2.

FIG. 4. Release of soluble leptin receptor into plasma via ectodomain shedding of membrane spanning receptor in vivo. Dually tagged adenoviral expression vectors of OB-Ra (not shown) or OB-Rb were infused into C57Bl/6 mice and allowed to express OB-R protein from the liver. Plasma was obtained from control virus (AdCMV-β-Gal)-injected mice or those that received OB-Rb. Leptin-Sepharose beads were incubated with plasma from each mouse as indicated on the figure and blotted with antibody recognizing the FLAG epitope tag, which is present only in soluble leptin receptor released from membrane-spanning full-length receptor (bottom). Viral expression of OB-R was demonstrated by Northern blotting from similarly loaded RNA samples of treated mice, using OB-R cDNA fragment as probe (top).

Expression of Adenoviral Encoded OB-Ra and OB-Rb in Vivo—To determine whether the soluble leptin receptor may also be generated in vivo by ectodomain shedding, we generated dually tagged adenoviral vectors of OB-Ra and OB-Rb and expressed them in mice. This approach was taken because the size of the soluble leptin receptor released from stably transfected cells is indistinguishable from that produced by OB-Re cDNA (Fig. 2). To overcome this limitation, we inserted an amino-terminal FLAG epitope tag between the signal sequence of endogenous OB-R and the remaining coding sequences (Fig. 3A). If the soluble leptin receptor is cleaved from membrane-spanning receptor isoforms, it will be distinguishable from endogenous OB- Re by virtue of the presence of the FLAG tag, which is detectable by a monoclonal antibody.

We first verified the correct expression of dually tagged OB-R adenoviral vectors using 293 cells. 293 cells were infected with

AdCMV-β-Gal, AdCMV-OB-Ra, or AdCMV-OB-Rb. Cells were lysed with detergent-containing buffer. Virally expressed OB-R protein was immunoprecipitated with a polyclonal antibody against the extracellular region of the receptor, followed by sequential detection with monoclonal antibodies against the amino-terminal FLAG tag or the carboxyl-terminal c-Myc tag. Fig. 3B shows that bands of the same size were recognized by both antibodies, demonstrating that correct processing of both OB-Ra and OB-Rb has taken place.

Release into Plasma of Soluble Leptin Receptor by Ectodomain Shedding from Membrane-spanning Receptors—We pro-
Ectodomain Shedding of Leptin Receptor

 Ceceded to infuse adenoviruses encoding β-galactosidase, dually tagged AdCMV-OB-Ra or -OB-Rb into the tail vein of C57Bl/6 mice. Four days after virus administration, plasma was obtained from each mouse, and leptin beads were used to pull down soluble receptor protein present in plasma. Consistent with observations from Flip-In 293 cells stably transsected by OB-Ra or OB-Rb, soluble leptin receptor protein recognized by the amino-terminal FLAG antibody is detected in plasma of mice that received AdCMV-OB-Ra and AdCMV-OB-Rb but not in AdCMV-β-Gal mice, demonstrating that the soluble leptin receptor may indeed be generated by ectodomain shedding of membrane-spanning receptor isoforms in vivo (Fig. 4). The cleavage is not leptin signaling-dependent because both OB-Ra and OB-Rb viruses are capable of releasing cleaved soluble leptin receptor (data not shown).

Taken together, our data demonstrate that the soluble leptin receptor may be generated by ectodomain shedding from membrane-spanning receptor isoforms both in vitro and in vivo. Because soluble leptin receptor stabilizes circulating leptin and affects its biological activity in vitro (19), controlled shedding of soluble leptin receptor under physiological conditions or dysregulation of this process under pathophysiological conditions may have important implications regulating leptin's weight-reducing and other biological effects.

Discussion

In our earlier studies, we demonstrated that the soluble leptin receptor circulates in plasma and is capable of binding to leptin (18). Our recently published results also demonstrated that the soluble receptor might play key roles in determining the amount of total leptin in circulation (19). Other studies also suggest that expression and plasma concentration of the soluble leptin receptor may be regulated (29, 30). However, the origin of circulating soluble leptin receptor in plasma has not been clearly defined, especially as the mRNA splice variant encoding this receptor isoform in humans has not been found (26).

Because no post-translational modification of leptin occurs in vivo (31), soluble leptin receptor may be an important factor regulating leptin's availability and bioactivity. This hypothesis is supported by findings that in human plasma, soluble leptin receptor represents the major leptin binding activity (27).

The main site of expression of the mRNA splice variant encoding the soluble leptin receptor in vivo remains unknown. Previously, we failed to detect a signal for OB-Re when Northern blot analysis was performed (17). Available data have demonstrated that in mice, OB-Re is expressed by the placenta. Its expression starts at day 14 of pregnancy, peaking just before parturition to about 40-fold the level found in non-pregnant mice (29, 32). In rats and humans, the pregnancy-associated rise of circulating leptin and its soluble receptor is relatively modest, achieving only a 2-fold increase versus a more than 40-fold increase in mice (33).

Our current study provided evidence of an additional mechanism for the generation of soluble leptin receptor by ectodomain shedding of membrane-spanning receptor, both in vitro and in vivo. This result is also in agreement with an earlier report on the generation of human soluble leptin receptor from transiently expressed membrane-spanning receptors in tissue culture (20).

Ectodomain shedding by proteolysis to yield soluble intercellular regulators has been observed for many proteins, such as tumor necrosis factor α and transforming growth factor α (34). The responsible protease for these proteins, tumor necrosis factor α-converting enzyme, is a member of the ADAM (a disintegrin and metalloproteinase) family of metalloproteinases. Molecules that are capable of inhibiting metalloproteinases are being tested as promising reagents for cancer therapy (35). At the present time, the modulation of the soluble leptin receptor on leptin biology is not well understood. Initial evidence of the presence of free and bound leptin in human circulation was obtained by gel-filtration chromatography of plasma containing added 125I-leptin tracers (36). This and later studies showed that obesity is associated with decreasing levels of the circulating soluble leptin receptor in humans, whereas weight loss increases it (37–40). In others studies, obese and normal weight individuals were found to have similar amount of total circulating soluble leptin receptor; yet in the obese most soluble leptin receptor is bound to leptin, whereas in lean individuals a much smaller percentage of soluble leptin receptor is bound to leptin (75% in the obese versus 33% in the lean); thus, the inability to up-regulate circulating soluble leptin receptor could be a factor in the pathogenesis of obesity (36, 38, 41). However, a larger percentage of leptin circulates in the free form in the obese (41). Opposite modifications of circulating levels of leptin and its soluble receptor also occur across the eating-disorder spectrum, with plasma levels of soluble leptin receptor significantly increased in patients with anorexia nervosa or bulimia nervosa, but decreased in patients with binge-eating disorder or those who are obese but are non-binge-eating (42). Soluble leptin receptor levels are also regulated by gender, adiposity, hormones, and rHLepTest administration (43). In aggregate, these studies suggest that the soluble leptin receptor may have important implications for the biological activity of leptin. Preliminary results of ongoing work in our laboratory using transgenic mice overexpressing the soluble leptin receptor support the hypothesis that increased soluble leptin receptor in wild-type mice is associated with decreased adiposity as well as decreased body weight. It is perceivable that future studies will shed more light on the usefulness of developing methods to regulate the generation of the soluble leptin receptor for therapeutic benefit.

Acknowledgments—We thank Dr. J. Darnell (Rockefeller University) for kindly providing us the STAT3 luciferase reporter construct, Drs. T.-C. He and B. Vogelestein (Johns Hopkins University) for pAdEasy vectors to generate adenoviruses, and Drs. R. H. Unger, C. B. Newgard, and G. Yang for insightful discussions and/or a critical reading of the manuscript.

REFERENCES

1. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) Nature 372, 455–462
2. Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R., and Burn, P. (1995) Science 269, 546–549
3. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Ballone, R. L., Burley, S. K., and Friedman, J. M. (1995) Science 269, 543–546
4. Pellemounter, M. A., Cullen, M. J., Baker, B. M., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) Science 269, 540–543
5. 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
6. Lee, G. H., Proenca, R., Montez, J. M., Carroll, K. M., Darvishzadeh, J. G., Lakey, N. D., Culpepper, J., Moore, K. J., Breithart, R. E., Duyk, G. M., Tepper, R. I., and Morgenstern, J. P. (1996) Cell 84, 491–495
7. Giuliani, N., Zeigler, S., Wietschel, A., Stoffel, R., Hein, M. H., and Skoda, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6231–6235
8. Friedman, J. M. (2000) Nature 404, 632–634
9. Cowley, M. A., Smart, J. L., Husty, M., Cerdan, M. G., Diano, S., Horvath, T. L., Cone, R. D., and Low, M. J. (2001) Nature 411, 480–484
10. Kim, Y. B., Uotani, S., Pierroz, D. D., Flier, J. S., and Kahn, B. B. (2000) Endocrinology 141, 2328–2339
11. Lord, G. M., Matarrese, G., Howard, J. K., Baker, R. J., Bloom, S. R., and Friedman, J. M. (1996) Nature 380, 632–635
12. Hofstetter, A., Winter, G., Stoffel, R., Hein, M. H., and Skoda, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6231–6235
13. Friedman, J. M. (2000) Nature 404, 632–634
14. Cowley, M. A., Smart, J. L., Husty, M., Cerdan, M. G., Diano, S., Horvath, T. L., Cone, R. D., and Low, M. J. (2001) Nature 411, 480–484
15. Kim, Y. B., Uotani, S., Pierroz, D. D., Flier, J. S., and Kahn, B. B. (2000) Endocrinology 141, 2328–2339
16. Lord, G. M., Matarrese, G., Howard, J. K., Baker, R. J., Bloom, S. R., and Lechler, R. I. (1998) Nature 394, 897–901
17. Sierras-Homigana, M. R., Nach, A. K., Murakami, C., Garcia-Cardena, G., Papapetropoulos, A., Sessa, W. C., Madge, L. A., Schechter, J. S., Schwab, M. B., Polverini, P. J., and Flores-Riveros, J. R. (1998) Science 281, 1683–1686
18. Unger, R. H., Zhou, Y. T., and Orci, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2327–2332
19. Huang, L., and Li, C. (2000) Cell Res. 10, 81–92
