Monitoring leptin activity using the chicken leptin receptor

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Abstract

We report on the construction of a leptin bioassay based on the activation of chicken leptin receptor in cultured cells. A human embryonic kidney (HEK)-293 cell line, stably transfected with the full-length cDNA of chicken leptin receptor together with a STAT3-responsive reporter gene specifically responded to recombinant human and *Xenopus* leptins. The observed higher sensitivity of chicken leptin receptor to the former is in agreement with the degree of sequence similarity among these species (about 60 and 38% identical amino acids between humans and chickens, and between humans and *Xenopus* respectively). The specific activation of signal transduction through the chicken leptin receptor, shown here for the first time, suggests that the transition of Gln269 (implicated in the Gln-to-Pro *Zuckerfatty* mutation in rats) to Glu in chickens does

not impair its activity. Analysis of leptin-like activity in human serum samples of obese and lean subjects coincided well with leptin levels determined by RIA. Serum samples of pre- and post partum cows showed a tight correlation with the degree of adiposity. However, specific activation of the chicken leptin receptor in this assay was not observed with serum samples from broiler or layer chickens (representing fat and lean phenotypes respectively) or with those from turkey. Similar leptin receptor activation profiles were observed with cells transfected with human leptin receptor. Further work is needed to determine whether the lack of leptin-like activity in the chicken serum samples is due to a lack of leptin in this species or simply to a serum level of leptin that is below the detection threshold.

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Introduction

The chicken leptin receptor (CLEPR) gene, which has been cloned in our laboratory (Horev *et al.* 2000) and by others (Ohkubo *et al.* 2000), was the first leptin receptor (LEPR) cloned in a nonmammalian organism. It exhibits about 60% sequence similarity to its mammalian orthologues at both the nucleotide and amino acid levels. The turkey LEPR gene has also been cloned and found to share more than 90% predicted amino acid similarity with that from chicken (Richards & Poch 2003). Mapping the CLEPR to chromosome 8 (Dunn *et al.* 2000) demonstrated its syntenic position relative to that of the human LEPR. In general, the tissue-specific pattern of expression of the chicken and turkey LEPRs parallels that of mammalian LEPRs, with some variations (Horev *et al.* 2000, Ohkubo *et al.* 2000, Richards & Poch 2003).

The recent cloning of leptin and LEPR genes in *Xenopus laevis* (Crespi & Denver 2006) and leptin's demonstrated role in energy balance in frogs show that this signaling pathway is ancient, likely arising very early in the evolution of vertebrates. In chickens, understanding the control of energy balance at the molecular level is of great interest for both agricultural and

academic research, as it relates to a number of commercial interests, such as food intake, fat accumulation, and reproductive efficiency, among others. The study of this control mechanism in avian species lags behind due to the conflict in the identification of chicken leptin (Friedman-Einat *et al.* 1999, Amills *et al.* 2003, Richards & Proszkowiec-Weglarz 2007). Nevertheless, as birds clearly possess genes orthologous to mammalian and frog LEPRs, it is likely that a gene for the ligand also exists in the bird genomes. Here, we describe the construction of a leptin bioassay based on the forced expression in cultured cells of CLEPR. We show that recombinant leptins originating from both humans and frogs activate CLEPR with high potency. This bioassay is expected to be valuable for the identification of CLEPR natural ligands, and for further study of this signaling pathway's role in avian growth and physiology.

Materials and Methods

Cytokines

Human leptin was kindly donated by Prof. Arie Gertler (The Hebrew University, Rehovot, Israel). *Xenopus* leptin was prepared as described previously (Crespi & Denver 2006).

Interferon- α (IFN- α) was a kind gift from Dr Daniela Novic and Dr Menachem Rubinstein at the Weizmann Institute of Science, Rehovot, Israel.

Constructs

The reporter construct pAH32, an expression vector transactivated by leptin, has been previously described (Rosenblum *et al.* 1998). This construct contains a trimer of the STAT3-binding element derived from interferon regulatory factor 1, preceding the herpes simplex virus thymidine kinase minimal promoter and the luciferase cDNA. The expression vector pgkPuro, containing the puromycin resistance gene, was kindly provided by Prof Moshe Oren at the Weizmann Institute of Science. The expression vector pCMV-NSV was contains the cytomegalovirus promoter and the SV 40t-intron and early polyadenylation site.

Constructing the CLEPR expression vector

PCR fragments encompassing the full-length CLEPR and the primers used for their amplification are described in Fig. 1 and Table 1 respectively. In the forward primer of the first fragment, a 'KOZAK' sequence taken from the chicken β -actin gene was inserted upstream of the ATG codon to enhance efficient translation of the mRNA.

Each fragment of the expected-size PCR product was cloned into pGEM-T vector system from Promega. Several independent clones of each fragment were sequenced with Sp6 and the T7 primers, enabling verification of the complete sequence, including primers from both the 3' and 5' ends of the inserted fragment. For each fragment, about four independent clones were sequenced and the fragments with the correct sequences were gel-purified from pGEM-T. Fragment I was subcloned into the *NotI*-*XmaI* sites of pBluescript (Stratagene). Fragment II was subcloned downstream of fragment I, using the *XmaI* and *EcoRI* sites. Fragment III was cloned downstream of fragment II using the *EcoRI*-*HindIII* sites, and fragment IV was then cloned into the *HindIII*-*KpnI* sites. The full-length CLEPR was excised using *NotI* and *KpnI* and cloned into the *NotI* and *KpnI* sites of a pCMV expression vector between the CMV promoter/enhancer and the SV40 t intron and the 3' untranslated region.

Cell culture

The human embryonic kidney HEK-293 cell line was obtained from the American type culture collection. Cells were grown in growth medium containing Dulbecco's modified eagle medium (DMEM), 10% (w/v) fetal calf serum, 50 μ g/ml streptomycin, and 50 μ g/ml penicillin. Cells were incubated at 37 °C, in an atmosphere containing 5% CO₂. Clones transfected with plasmid DNA were maintained in growth medium with the addition of 2 μ g/ml puromycin.

Transfections

To generate the test clones, HEK-293 cells were transfected with three constructs: CLEPR, pAH32, and pgkPuro, at a molar ratio of 4:4:1 respectively. To generate the control cell lines, the cells were transfected with two constructs: pAH32 and pgkPuro, at a ratio of 10:1 respectively. Transfections were performed using lipofectamine (Gibco/BRL) as described in the manufacturer's protocol. About 40 clones were collected from each transfection and each was tested for the activation of luciferase using human leptin (100 ng/ml) and IFN- α (100 U/ml). Whereas IFN- α is believed to operate through an endogenous receptor, thus revealing the presence of the inducible reporter construct, human leptin requires the presence of exogenous LEPR. Out of about 40 clones isolated, 20 were found to express the reporter construct and about 10 were found to respond well to the recombinant human leptin and to give a positive signal with radioactive CLEPR probe by northern analysis (see Results). One of the clones, which displayed stable and significant leptin-induced luciferase activity, was used for subsequent analyses (test clone). A control cell line (with no LEPR) was chosen among the control clones based on its relative high sensitivity with respect to response to IFN- α .

HEK-293 leptin assay

Test and control cultures, in their log growth phase, were briefly dissociated with trypsin and resuspended in the growth medium described above. Resuspended cells were plated in 24-well tissue-culture plates (Nunc, Danyel Biotech, Rehovot, Israel) at a concentration of 5×10^5 cells per well in a 500 μ l final volume. After 16–20 h, the medium in each well was replaced with 300 μ l DMEM (Gibco/BRL), supplemented with 1 mM CaCl₂. Empirically, this addition of calcium was found to

Figure 1 Schematic drawing of the strategy for building the CLEPR full-length cDNA. Black boxes indicate extended primer sequences to include *NotI* site and a 'KOZAK' sequence in the 5'- as well as a stop codon and *KpnI* site at the 3'-ends of the cDNA. These sequences are indicated in Table 1.

Table 1 Primers used for the generation of the full-length CLR

Fragment number	Fragment size (bp)	Exon included	Primer sequence
1	981	3–8	For: 5'-CGAGCGGCCGCCACCCGCCACCAGTTCGCCATGTATCATCAAATCATTCTGACC-3' Rev: 5'-ACAGTGTGCTCCATTCACCTCC-3'
2	1304	8–16	For: 5'-GCTCAAGTGAGGTGCAAGAG-3' Rev: 5'-TGCACAGCAATCACTGTGCTC-3'
3	719	16–20	For: 5'-CCATGGACTGAAAGCACACAC-3' Rev: 5'-GTGAACATGGAATCAAGCAC-3'
4	760	20	For: 5'-AGTGCTGGAAGACATCAGTG-3' Rev: 5'-CTCGGTACCTTACTTATCGTCGTCGCTCTGTCTGTGGTCTC-3'

reduce the activation of the control cells by chicken serum samples. Cytokines (recombinant human leptin, *Xenopus* leptin, or human IFN- α) at the indicated concentrations, or 30 μ l aliquots of serum samples, were added to the incubation medium for 3 h. For incubation with purified proteins (leptins and INF), medium was supplemented with 2% (W/V) albumin (Sigma) for enhancing protein stability. Medium was then aspirated and the cells were lysed by adding 100 μ l Promega cell lysis reagent. A 40 μ l aliquot of each cell lysate was mixed with 100 μ l Promega luciferase assay reagent. Luciferase activity was measured using the TD20e luminometer (Turner Design, Mountain View, CA, USA). The measured luminescence was normalized to the amount of protein in each well. Protein concentration was measured by the Bradford assay according to the manufacturer's protocol (Bio-Rad) following 150-fold dilution of the cell lysate.

Preparation of RNA

RNA was extracted from the hypothalamus of 3-month-old Leghorn chickens using RNAzol B (Tel-Test Inc., Talron, Rehovot, Israel) according to the manufacturer's instructions. Briefly, hypothalamuses from four chickens were extracted in 1 ml RNAzol using a homogenizer, centrifuged at 10 000 g, and the upper phase was extracted with a phenol–chloroform solution (1:1 v/v). After separation of the aqueous phase by centrifugation as described above, the upper phase was treated with 1/10 volume of 10 mM Na-acetate and 2.4-fold ethanol for precipitation of the RNA.

Preparation of RNA from cultured cells was performed from near-confluent cultures in 80 mm plates using 1 ml RNAzol B per plate. The rest of the procedure was as described above.

All experiments on animals were approved by the local Ethics Committee.

Reverse transcription (RT) and PCR

To generate long cDNA templates from the hypothalamus-derived RNA, a thermostable RT (Tth RT, Ambion,

Agentech, Tel-Aviv, Israel) was used following the manufacturer's protocol. Briefly, reactions were incubated in a Hybaid thermal cycler (Hybaid, Tamar, Jerusalem, Israel); elongation was for 50 min at 60 °C, followed by 15 min at 70 °C. For other purposes, RT was performed with 200 U of Superscript II RT (Invitrogen) in a 20 μ l reaction containing 0.15 μ g random hexamers, 1 $\times$ RT buffer (Invitrogen), 10 mM dithiothreitol, and 0.5 mM deoxynucleoside triphosphates. Reactions were incubated in the thermal cycler for 50 min at 42 °C, followed by 15 min at 70 °C.

Conventional PCR and gel electrophoresis were performed on 1 μ l cDNA with 0.5 U recombinant Taq DNA polymerase (Ready Mix Taq DNA Polymerase Purple $\times$ 2, Talron) in a 50 μ l reaction mixture containing the ready mixture and 0.5 p/mol from each of the forward and reverse primers. The reaction was incubated in a Hybaid thermal cycler for 3 min at 94 °C, followed by two 5 cycles of 94 °C for 30 s, 65 °C and 60 °C (for the second 5 cycles set) for 30 s, and 72 °C for 90 s; 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s; and a 10-min final extension step at 72 °C. Amplicons were analyzed by 1.2% (w/v) agarose gel electrophoresis.

Subcloning

PCR fragments were treated with Wizard PCR or gel purification kits (Promega, Biological Industries) and subcloned using either the pGEM-T kit (Promega) or the Promega T₄ DNA ligase kit. Ligation reactions were begun in room temperature water, and placed in a cold room for gradual cooling overnight. Transformation of the ligation products into DH5-competent bacteria (Promega, Biological Industries) and plasmid preparation were performed according to standard procedures (Sambrook 2001).

Northern hybridization

Northern hybridization was performed on total RNA. The samples were denatured for 5 min at 65 °C, separated in a 1.2% agarose/2.2 M formaldehyde gel, and blotted onto a

Qiabran nylon membrane (Qiagen, Bio-Lab Laboratories Ltd) according to the standard procedures (Sambrook 2001). The membrane was prehybridized for 2 h at 42 °C in a solution containing 5× SSC, 50% (v/v) formamide, 5× Denhardt's solution, 10% (w/v) dextran sulfate, 1% (w/v) SDS, and 100 mg/ml denatured salmon sperm DNA. Hybridization was carried out overnight at 42 °C in the same solution but with the addition of ³²P-labeled full-length CLEPR cDNA (the *NotI*-*KpnI* fragment). Labeling was performed using the random primer labeling kit (Amersham Pharmacia Biotech). Exposure times were 24 h for the transiently transfected clone and 4 days for the stable clones. The size of the detected band was estimated according to the position of the 18S and 28S rRNA following ethidium bromide staining of the gel.

Serum samples

Human serum samples were collected from obese and lean subjects and analyzed for leptin by RIA in Prof Donny Strosberg's laboratory as described previously (Friedman-Einat et al. 2003). Informed consent was obtained from all subjects. Samples from pre- and post partum cows were a gift from Prof Ami Arieli (the Hebrew University, Rehovot, Israel). The chicken serum samples were taken from layer (Lohman) and broiler (Cobb) hens at about 30 weeks of age. Both hen types were purchased from local husbandries when they were a day old and raised with free access to feed and water (according to the NRC Manual) in our experimental farm. Samples from feed-restricted Lohman layers were obtained following 20 h of fasting. Samples from turkeys and feed-restricted broilers were taken from commercial farms.

Figure 2 Characterization of the transfected clones. (A) Northern hybridization using the CLEPR-specific probe showing three LEPR positive clones (lanes 1–3), two clones transfected with the reporter construct only (lanes 4 and 5), the parental line (lane 6) and the parental line 72 h after transient transfection with the CLEPR (lane T). (B) Ethidium bromide staining of the gel before blotting indicating the integrity of the RNA. (C) Reporter gene activation following incubation with IFN- α (IFN). No luciferase activation was detected in the parental line that does not contain the reporter gene (fold induction = 1). Cells were grown in 24-well plates and incubated in triplicates with 100 IU of IFN- α for 3 h. Luciferase activity was measured using luciferin (Promega) and luminometer (Turner Biosystems). Fold induction was calculated by dividing the signal obtained in the presence and absence of IFN- α . Means of triplicate are shown. Standard error was about 10% in all cases (not shown).

Results

Bioassay construction

Generation of full-length CLEPR with the available proof-reading PCR enzymes always resulted in sequences with some mutations and deletions (not shown). Therefore, the full-length

Figure 3 Stimulation of luciferase activity in the stably transfected clones by recombinant human and *Xenopus* leptins and by IFN- α . (A) The CLEPR expressing cells (squares) and the control cells (circles) were incubated with the indicated amounts of either human leptin (filled squares, filled circles) or *Xenopus* leptin (open squares, open circles), as described in Fig. 2. (B) The human-LEPR expressing cells (HLEPR, squares) and control cells (circles) were incubated with the recombinant leptins described in A. (C) The CLEPR expressing cells (squares) and the control cells (circles) were incubated with IFN- α . Luciferase activity was measured as described in Fig. 2. Results represent the average of three measurements $\pm$ s.d. and are representative of three independent experiments.

cDNA was tailored from smaller fragments, each selected by sequencing as described in Materials and Methods. The resultant expression vector was stably introduced into HEK-293 cells together with a reporter construct (pAH32), which included the firefly luciferase gene under the control of the STAT3-responsive promoter (Rosenblum *et al.* 1998). Control cells were transfected with the reporter construct alone for the detection of possible nonspecific activation mediated independent of the LEPR. Several transfected clones selected by neomycin resistance were analyzed for expression of CLEPR mRNA by northern hybridization (Fig. 2) using a CLEPR-specific probe. A specific band of the expected size (about 3.5) was detected in the clones stably transfected with the CLEPR expression vector (lanes 1–3), while no signal could be observed in the clones transfected with the reporter construct only (lanes 4 and 5) or the parental cell line (lane 6). As expected, signal intensity with the CLEPR probe was much stronger in transiently transfected HEK-293 cells (lane T) than in the stable clones. The response of these clones to IFN- α , reflecting the activation of the reporter gene through an endogenous receptor, is shown in Fig. 2C. The five transfected cell lines analyzed responded to IFN- α by induction of luciferase activity, indicating the expected cytokine-induced activity of the reporter luciferase gene. Since the reporter gene promoter is a cytokine-responsive element, control cell lines that do not express LEPR were needed to detect possible nonspecific activation mediated independently of the LEPR.

Specific activation of CLEPR by recombinant human and Xenopus leptins

As shown in Fig. 3, recombinant leptins from both humans and *Xenopus* specifically induced luciferase activity in the CLEPR-expressing cell line. The dose–response curves shown in Fig. 3A demonstrate a significant (twofold or greater) induction of luciferase activity at minimum effective doses of 0.7 ng/ml human leptin, when compared with 4 ng/ml *Xenopus* leptin. Induction levels reached a plateau at about 11- and 8-fold for the human and *Xenopus* leptins respectively. The higher sensitivity of the assay to induction by human versus *Xenopus* leptin was

compatible with the higher degree of amino acid similarity between human and chicken LEPRs than between those of chicken and *Xenopus* (Table 2). The control cell line, containing only the reporter construct, did not respond to any of the recombinant leptins (Fig. 3A), indicating that this cell line does not respond to human and *Xenopus* leptins without the introduction of an exogenous LEPR gene. In Fig. 3B, the response of both the control and CLEPR-expressing cell lines to recombinant IFN- α indicated that the control cell line is not less sensitive to luciferase induction than the CLEPR cells when activated through an endogenous receptor. In fact, the control cells responded with even higher sensitivity than the LEPR-expressing cells. An additional control (shown in Fig. 3C) consisted of obtaining a dose–response curve for human and *Xenopus* leptins using a cell line harboring the human LEPR gene instead of the CLEPR. This cell line, described previously (Marikovsky *et al.* 2002, Friedman-Einat *et al.* 2003), exhibited a response similar to that of the CLEPR cell line (Fig. 3A), albeit with somewhat higher sensitivity.

Comparison of human, chicken, and Xenopus LEPR amino acid sequences

Amino acid-sequence comparison of the LEPR genes from humans, chickens, and *Xenopus* is shown in Fig. 4 and Table 2. Of the various known functional motifs, boxes 1 and 2 in the intracellular domain of the LEPRs have been implicated in binding and activation of Jak kinases (Ihle 1995, Ghilardi & Skoda 1997), while box 3 and the three tyrosine residues Y963, Y1077, and Y1138 have been implicated in the activation of STAT3 (White *et al.* 1997a). The predicted single transmembranal domain, characteristic of the class I cytokine receptor family, was found to be highly hydrophobic in all three molecules. The two WS $\times$ WS residues suggested to contribute to ligand-recognition in cytokine receptors, and the cytokine receptor homology (CRH) domains, implicated in leptin binding, were well conserved. The C residues characteristic of the fibronectin type II modules (Zabeau *et al.* 2005) are indicated. Thus, despite the low sequence similarity among these sequences, all of the known functional motifs

Table 2 Amino acid similarity of the LEPRs among chicken, *Xenopus* human, and bovine. Comparisons were done using BLAST

	Identities	Positives	Gaps
Comparison			
A. The full-length LEPR			
Chicken – <i>Xenopus</i>	38% (447/1166)	58% (680/1166)	3% (39/1166)
Chicken – Human	47% (550/1152)	64% (746/1152)	2% (28/1152)
Chicken – Bovine	48% (557/1150)	66% (761/1150)	2% (24/1150)
B. The leptin-binding domain			
Chicken – <i>Xenopus</i>	46% (151/324)	64% (208/324)	1% (5/324)
Chicken – Human	61% (198/322)	77% (248/322)	0% (3/322)
Chicken – Bovine	60% (196/322)	77% (250/322)	0% (3/322)

Accession numbers are: human LR, gi40254464; bovine LR, gi90991698; CLR, gi49170082; *Xenopus* LR, gi13205944.

and domains of LEPR were conserved in terms of both sequence and position.

Detection of leptin activity in chicken serum samples

Given that physiological serum leptin concentrations in mammals range from about 3 to 80 ng/ml (Tomimatsu *et al.* 1997, Friedman-Einat *et al.* 2003, Nkrumah *et al.* 2007), we expected to be able to detect leptin activity in the serum samples with this bioassay. As shown in Fig. 5, human serum samples from obese and lean subjects, diluted 10-fold, induced luciferase activity by factors of 6.7 (± 0.5) and 2.5 (± 0.4) respectively. This level of activity is compatible with the amount of leptin immunoreactivity determined in these samples by RIA (Friedman-Einat *et al.* 2003), i.e. 75 and 10 ng/ml, in the original samples (tenfold more concentrated than the incubated doses). Serum samples from pre- and post partum cows also showed the expected high and low leptin-like activities (Andrew *et al.* 1994, Block *et al.* 2001). However, serum samples from broiler and layer chickens and turkeys under free-access feeding, (Fig. 5) or from the same strains but under feed-restricted regimens (not shown) did not exhibit significant induction of reporter activity in this assay. It may be that leptin levels in these poultry samples are below the threshold of detection by the bioassay.

Discussion

In this work, we demonstrated the possibility of employing CLEPR-transfected HEK-293 cells for the detection of leptin activity. CLEPR-expressing cells responded specifically, via the activation of a reporter gene, to recombinant human and *Xenopus* leptins despite high divergence in the predicted amino acid sequence of the LEPR among these species. This *in vitro* cross-CLEPR activation appears to be stronger for human than for frog leptin, compatible with the significantly higher degree of sequence similarity between human and chicken LEPRs.

Besides providing a rapid and simple experimental tool for measuring leptin activity, this assay indicates that CLEPR can bind mammalian and *Xenopus* leptins and activate STAT3-dependent signaling. It is also evident that the transition of Gln269 to Glu in the chicken homologues does not impair CLEPR activity. This is in contrast to the Gln269-to-Pro transition, implicated in the fatty mutation in rats (White *et al.* 1997b).

The response of the CLEPR-harboring cells to the recombinant leptins was similar to that of our previously reported cell line expressing human LEPR (Marikovsky *et al.* 2002, Friedman-Einat *et al.* 2003). It should be noted, however, that the two bioassays cannot be used for accurate comparisons of leptin affinities since each of the LEPR constructs is driven by a different promoter and the copy number of LEPRs in each cell line is probably different as it has not been determined.

Previous analysis of predicted tertiary structures of *Xenopus*, rat, and pufferfish leptins (Crespi & Denver 2006) has shown a four-helix structure that is nearly identical for the three analyzed species, despite the low sequence similarities of their predicted protein sequences (e.g. *Xenopus* leptin shares 35% sequence similarity with mammalian leptins). This suggests that the conserved tertiary structure is important for receptor binding, as has been shown for other type I cytokines (Huising *et al.* 2006). No such structural modeling studies have been performed with the chicken leptin protein, as it does not yet appear to have been unequivocally identified (Friedman-Einat *et al.* 1999, Amills *et al.* 2003, Richards & Proszkowiec-Weglarz 2007). Nevertheless, the above-mentioned structural similarity found in the highly divergent leptins strongly suggests that the putative chicken leptin also shares a similar structure, presumably evolutionarily constrained by the structure of the receptor-binding pocket.

The leptin-binding domain in the LEPR has been localized to residues 323–640 (Fig. 5) of the human sequence (Peelman *et al.* 2006), which contains the second segment of the cytokine receptor domain and the fibronectin type 3 domain (residues 428–635, Fig. 5). This domain is significantly more conserved than the overall LEPR sequences between chickens and either *Xenopus*, human, or bovine (Table 2). This can be taken as another support for the hypothesis regarding the existence of chicken leptin, based on the same consideration of evolutionary constraint mentioned above.

The inability to detect significant leptin-like activity in serum samples of chickens and turkeys was surprising considering the expected higher affinity of the chicken leptin to its receptor. However, taking into account the percentages of body fat, these results are in agreement with the tight correlation, found in mammals, between the amount of body fat and the level of serum leptin (Mahabir *et al.* 2007). The 'obese' broiler breeder hen (Cobb) raised with free access to food has only about 5% body fat, while a lean man and woman have about 15 and 20% body fat respectively (Funkhouser *et al.* 2000).

The demonstrated specific activation of CLEPR by human leptin is in agreement with both *in vitro* protein-binding assays

Figure 4 Multiple comparisons of the predicted amino acid sequences of the human, chicken, and *Xenopus* leptins. Amino acid sequence of the human, chicken, and *Xenopus* LEPRs (accession numbers: gi40254464; gi49170082; and gi113205944 respectively) are denoted by the single-letter code. Alignment was performed using the ClustalX program. '*' Indicates residues or nucleotides identical in all sequences in the alignment. ':' Indicates conserved substitutions and '.' indicates semi-conserved substitutions. Functional domains, motifs, and critical amino acids found necessary for LEPR activity in mammals are indicated as follows: Box 1, 2, and 3 are indicated by white letters and black background. The Y residues (Y963, Y1077, and Y1138) are indicated by white letters on a gray background. Cysteine residues at positions 672 and 751 of the fibronectin type III domain are indicated by white letters on gray background. The glutamine (Q) at position 269 of the human sequence, implicated in the fatty mutation in rats (transition from glutamine to proline; White *et al.* 1997b) is denoted by gray background.

Figure 5 Detection of leptin-like activity in serum samples of human, cow, and chickens. Serum samples of the indicated sources were incubated with the CLEPR transfected cells (A) or the human LEPR transfected cells (B) and the control cell line, as described in Fig. 3. The indicated serum samples were diluted tenfold in DMEM enriched with 1 mM CaCl_2 . The sensitivity of the assay to physiological leptin levels of humans and cows but not to that of chickens is demonstrated. The experiment was done in triplicates; the mean values are of three representative experiments. Vertical lines indicate S.E.M.

that used a recombinant fragment of CLEPR containing the leptin-binding domain (Niv-Spector *et al.* 2005), and studies involving the administration of human leptin to chickens (Denbow *et al.* 2000, Kuo *et al.* 2005). However, some discrepancies exist within the later reports with respect to the possibility that chicken strains selected for rapid growth are leptin resistant, and with an additional publication (Bungo *et al.* 1999) demonstrating nonresponsiveness of young chicks to administration of mouse leptin. Therefore, the more direct demonstration of CLEPR activation of signal transduction shown here provides an important support for its functionality also *in vivo*.

Given that the controversial chicken leptin is highly similar to mouse leptin (95% identical amino acids), it is not surprising that this recombinant preparation facilitated leptin-like activity in our bioassay (data not shown) and bound a recombinant fragment containing the leptin-binding domain of the CLEPR *in vitro* (Niv-Spector *et al.* 2005). Administration of this leptin in chickens resulted in inhibition of food intake (Dridi *et al.* 2000, Cassy *et al.* 2004). Additional studies have demonstrated the effect of this leptin on the immune response (Lohmus *et al.* 2004), the onset of sexual maturity (Paczoska-Elasiewicz 2006), egg production during and after fasting (Paczoska-Elasiewicz *et al.* 2003), and downregulation of heat shock protein-70 (Figueiredo *et al.* 2007).

In summary, the confusion regarding the identity of chicken leptin (Friedman-Einat *et al.* 1999, Amills *et al.* 2003, Richards & Proszkowiec-Weglarz 2007) strengthens the need for a specific bioassay for leptin activity that employs the native CLEPR. Our results add an important strength to previous reports demonstrating chickens' response to exogenous administration of various leptins (Raver *et al.* 1998, Denbow *et al.* 2000, Paczoska-Elasiewicz *et al.* 2003, 2006, Lohmus *et al.* 2004, Kuo *et al.* 2005), by demonstrating for the first time a specific induction of signal transduction via

CLEPR in response to human and frog leptins *in vitro*. It is possible that our inability to detect leptin activity in bird serum samples was due to below-detection threshold leptin levels, which could still be of physiological significance, rather than to a lack of natural ligand for CLEPR. Therefore, fractionation and purification of poultry serum proteins could enable the detection of leptin activity using this bioassay.

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