Note

Evolutionary, Structural and Biochemical Evidence for a New Interaction Site of the Leptin Obesity Protein

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ABSTRACT

The Leptin protein is central to the regulation of energy metabolism in mammals. By integrating evolutionary, structural, and biochemical information, a surface segment, outside of its known receptor contacts, is predicted as a second interaction site that may help to further define its roles in energy balance and its functional differences between humans and other mammals.

THE Leptin protein has been a focus of energy metabolism and obesity studies since its discovery in obese mice that are lacking a functional *leptin* gene (Zhang *et al.* 1994; Friedman and Halaas 1998; Mantzoros 1999). In these mice, as in several other mammalian species, injections of exogenous Leptin lead to significant weight loss, a result that has generated much interest in the hormone as a potential antiobesity drug (Friedman 1998). However, most obese human patients show an excess of Leptin, rather than deficiency, thereby implying that its detailed actions in energy metabolism are different in us *vs.* other mammals (Chicurel 2000; Hofbauer and Huppertz 2002). This possibility is bolstered by evolutionary studies that provide evidence of positive (adaptive) selection on the Leptin of hominoids (humans and great apes; Benner *et al.* 2002; Siltberg and Liberles 2002).

To better understand these functional differences, we extended these earlier comparative studies by integrating our evolutionary results with the available structural and biochemical information for Leptin. The same multiple alignment and phylogeny for the coding DNA sequences of mature Leptin (146 residues), as used before (Benner *et al.* 2002; Siltberg and Liberles 2002), were analyzed with PAML (Yang 1997). This evolutionary analysis included the estimation of the nonsynonymous (NS) to synonymous (SYN) rate ratios (amino acid *vs.* silent substitution rates or ω) and the reconstruction of ancestors and their inferred substitutions. The amino acid replacements for the NS substitutions were then mapped onto the known tertiary structure of human Leptin and evaluated against the functional evidence that a specific segment of this protein is primarily responsible for appetite suppression and weight loss in obese mice (Zhang *et al.* 1997; Grasso *et al.* 1999a,b).

Starting with ω free to vary across branches, an evolutionary model with different transition *vs.* transversion rates and the same but unequal base frequencies for all three codon positions was selected by the likelihood ratio tests (LRTs; Table 1; Huelsenbeck and Rannala 1997). Given this model, ω was estimated as <1 for all branches with more than five inferred substitutions, except for the hominoid stem (Figure 1). Values of $\omega < 1$, $= 1$, and > 1 are indicative of negative selection, neutral evolution, and positive selection, respectively (Yang and Bielawski 2000). Thus, the stem hominoid with $\omega = 1.66$ corresponds to the most likely episode of positive selection, underlying the known functional differences between human and mouse (Benner *et al.* 2002; Siltberg and Liberles 2002). This hypothesis is corroborated by the significant increase of NS to SYN substitutions in the stem hominoid relative to all other mammals and to its immediate primate ancestor and descendant hominoid clade (Table 2, A and B). More importantly, it is congruent with available structural and biochemical information for mammalian Leptin (see below).

The administration of synthetic Leptin peptides identifies positions 85–119 as critical for appetite suppression and weight loss in obese mice (Grasso *et al.* 1999a,b). Segment 85–119 includes the end of α-helix C and intervening C/D loop with helix E (Figure 2A) and is outside the region where Leptin contacts its receptor (interface of α-helices A and C; Hiroike *et al.* 2000). Removal of segment 85–119 from the evolution-

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TABLE 1

LRTs between the codon-based evolutionary models for *leptin*

Evolutionary model	Ln likelihood (Ln L) score	Δ d.f.	P	ω[a]
F_{eq}	−1930.03			1.31
F_{eq} + κ	−1887.95	1	<0.05	1.71
F1 × 4 + κ	−1882.65	3	<0.05	1.66
F3 × 4 + κ	−1880.86	6	>0.05	2.18

The evolutionary models are listed in their order of testing from the more simple to complex. These models assume equilibrium codon frequencies that are equal (F_{eq}) or that are calculated from the overall mean base frequencies for all three codon positions (F1 × 4) or from the separate averages for each position (F3 × 4). Kappa (κ) allows for different transition *vs.* tranversion rates. Δ d.f. specifies the difference in the numbers of free parameters between the more complex and simpler models in each comparison. The F1 × 4 + κ model is supported for *leptin* by these LRTs, since its Ln L score is not significantly worse than that for the more parameter-rich F3 × 4 + κ.

[a] Estimates of ω for the stem hominoid under each model (Figure 1).

ary analysis reduced ω for the stem hominoid from 1.66 to 0.52 (Figure 1). Of the 11 substitutions for this stem, 5 NS and no SYN changes mapped to this segment, which was significant (Table 2C). Furthermore, segment 85–119 packs onto the folded protein core by hydrophobic interactions between helix E and residues 60, 64, and 68 (Zhang *et al.* 1997). As 2 NS substitutions of the stem hominoid mapped to residues 60 and 68, 7 of its 9 NS changes were thereby associated either directly or indirectly with segment 85–119 (Figure 2). In contrast, no NS substitution of the stem hominoid was directly associated with the Leptin-receptor binding domain.

The evolutionary, structural, and biochemical information implicates segment 85–119 as of special functional significance. The physicochemical properties and finer structural details of its conserved residues now point to a more specific function for this segment. Fourteen of its 15 conserved positions are fixed for charged and strongly hydrophobic residues (Figure 3). This mix of charged and hydrophobic residues, with their outwardly projecting side chains, predicts a second binding site for Leptin-protein interactions, which is separate from that for its receptor (Figure 2; Benner and Gerloff 1991). At least some of the six positions with NS substitutions in the stem hominoid, which are directly or indirectly related to segment 85–119, may then contribute new hydrophobic, charged, and smaller residues that may alter the secondary structure and specific binding properties of this second interaction site. For example, the conserved G118 of hominoids permits a more pronounced turn at the N terminus of this segment

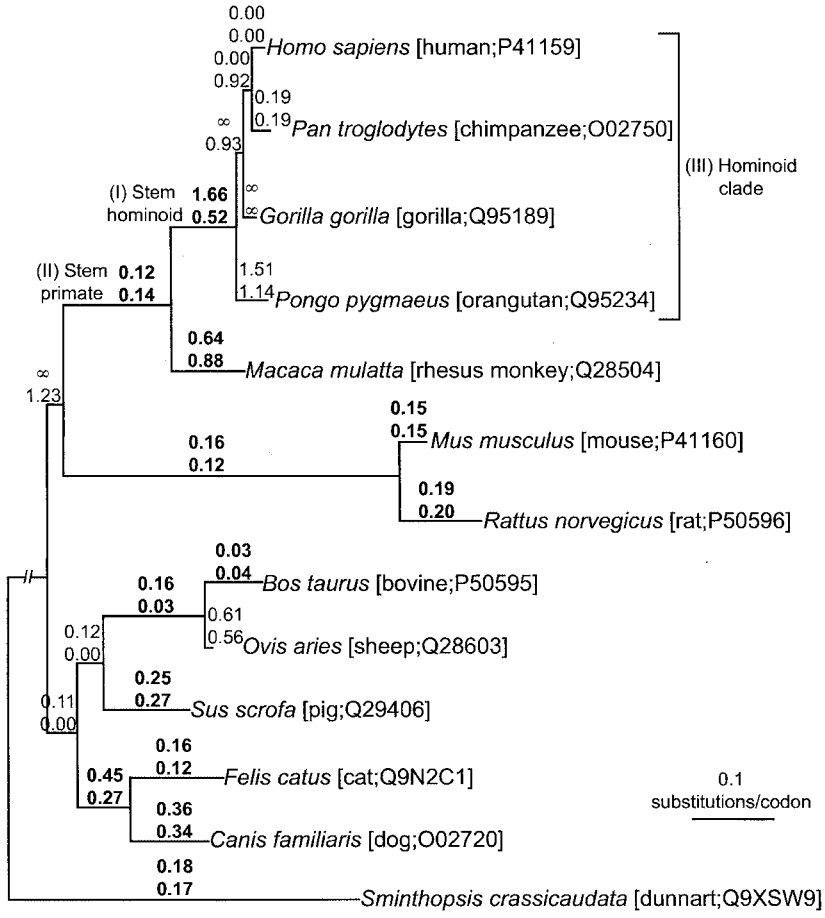


Figure 1.—Accepted phylogeny following earlier evolutionary studies of *leptin* (Benner *et al.* 2002; Siltberg and Liberles 2002). Common names and SWISSPROT accession numbers are given in brackets, and three key periods of primate evolution are labeled I, II, and III (Table 2B). Values of ω, as calculated with and without segment 85–119, are presented in that order for each branch, with boldface type highlighting those estimates based on more than five substitutions. Branch lengths, parameter estimations, and other calculations (Figure 2B) were determined with PAML (Yang 1997). However, as in the earlier evolutionary studies, the available chicken and turkey *leptin*s were not included in this analysis because of persistent concerns about their authenticity (Friedman-Einat *et al.* 1999; Doyon *et al.* 2001). Nevertheless, identical to near-identical results were obtained when these bird sequences were included (results not shown).

TABLE 2

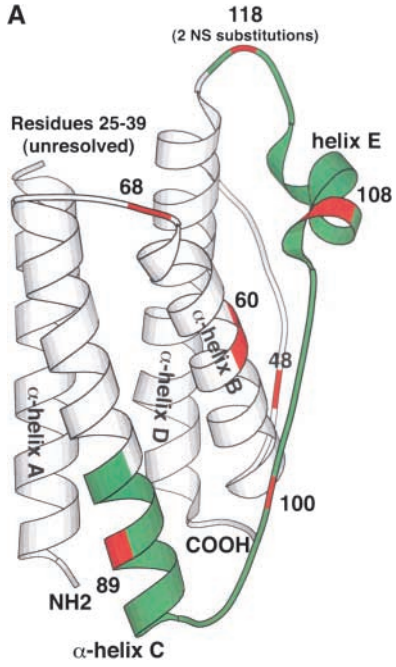
Frequency distributions of stem hominoid substitutions: (A) between this lineage and all other branches; (B) among three key periods of primate evolution; and (C) between segment 85–119 and the rest of Leptin (Figures 1 and 2)

A. Lineage(s)[a]	NS substitutions	SYN substitutions	Totals
Stem hominoid	9	2	11
All other branches	116	262	378
Totals	125	264	389

B. Major primate lineage[b]	NS substitutions	SYN substitutions	Totals
Stem hominoid (I)	9	2	11
Stem primate (II)	5	17	22
Hominoid clade (III)	7	6	13
Totals	21	25	46

C. Segment[c]	Sites w/ 1 NS substitution	Sites w/ 2 NS substitutions	Sites w/ 1 SYN substitution	Sites w/ no substitutions	Totals
85–119	3	1	0	31	35
All others	4	0	2	105	111
Totals	7	1	2	136	146

[a] Fisher's exact test, $P = 0.0008644$ (Nei and Kumar 2000).
[b] $G_{adj[2]} = 10.95$, $P < 0.005$.
[c] Combinatoric test, $P = 0.035$. Here, P corresponds to the proportion of different combinations for a predefined block of 35 residues with 5 to 9 NS and no SYN substitutions, given 7, 1, and 2 positions out of 146 with 1 NS, 2 NS, and 1 SYN changes, respectively (*i.e.*, Leptin).



B

Site	Stem hominoid replacement
35	R (0.99) → K (1.00)
48	V (0.91) → I (1.00)
60	I (0.96) → V (1.00)
68	L (.099) → M (1.00)
89	L (0.99) → V (1.00)
100	L (0.76) → W (1.00)
108	E (0.99) → D (1.00)
118	L (0.99) ⇒ G (1.00) [two NS substitutions]

Figure 2.—(A) Tertiary structure of human Leptin, PDB accession 1AX8 (Zhang *et al.* 1997), as rendered with MOLSCRIPT (Kraulis 1991). (B) Inferred replacements for the eight sites with NS substitutions in the stem hominoid (Figure 1). The posterior probabilities for the reconstructed codons of the primate (I) *vs.* hominoid (II) ancestors are given in parentheses. These same replacements, ancestral reconstructions, and posterior probabilities are also obtained by the other evolutionary models in Table 1 and with the addition of the bird *leptin*s to the analysis (results not shown).

relative to that predicted for L118 of the other mammals. In these ways, segment 85–119 may underlie the functional differences between human and other nonhominoid Leptins (*e.g.*, why this hormone is central to energy expenditure in mice, but apparently not in us; Mantzoros 1999; Hofbauer and Huppertz 2002).

This integrative study of Leptin calls for new experiments for the greater understanding of its roles in the energy metabolism of humans and other mammals (Friedman and Halaas 1998; Mantzoros 1999). The prediction of a new binding site, separate from that for its receptor, argues for experimental assays of Leptin-protein interactions (*e.g.*, with yeast two-hybrid systems; Von Mering *et al.* 2002). Such experiments can test whether the regulation of metabolic rate *vs.* feeding depends on the same or different protein-protein interactions and domains of Leptin, while documenting further its specific roles in energy metabolism (Grasso *et al.* 1999a,b). Furthermore, the inferred amino acid replacements of the stem hominoid supplement the known mutations in human and mouse Leptin and thereby offer additional targets for site-directed muta-

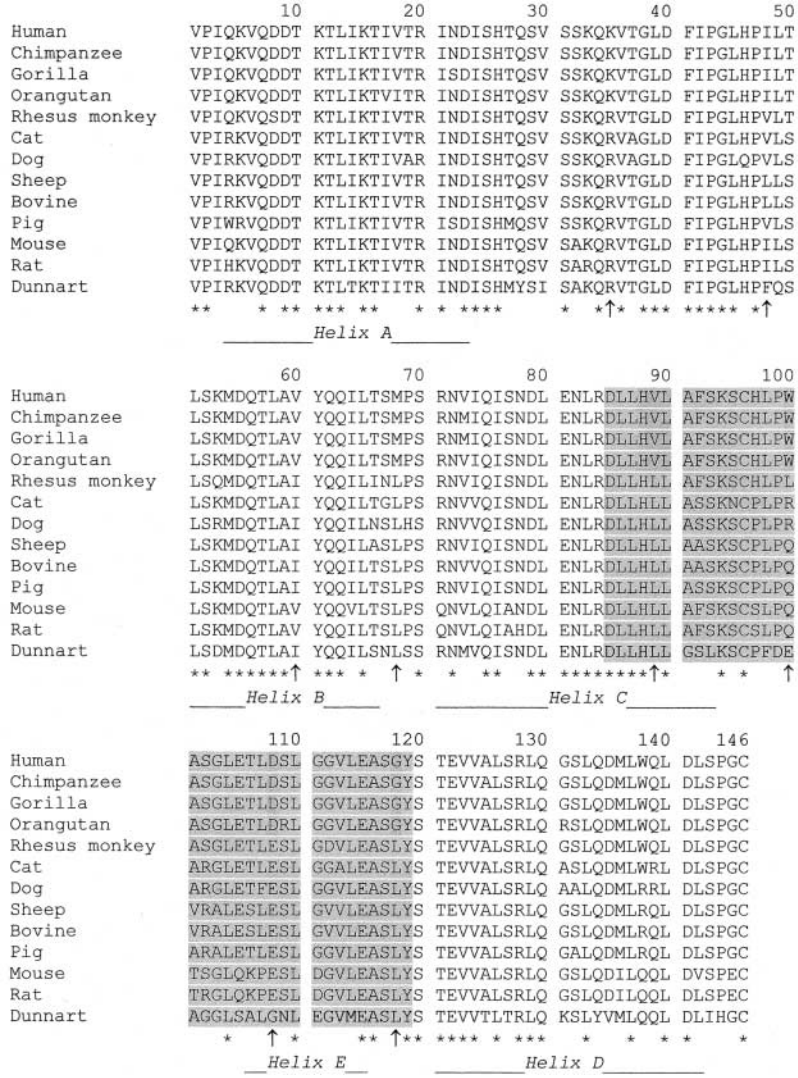


FIGURE 3.—Multiple Leptin alignment. Asterisks highlight conserved residues and arrows mark those sites with the NS substitutions of the stem hominoid (Figure 1). The two SYN substitutions of the stem hominoid occur at residues 37 and 121. Segment 85–119 is shaded, whereas helices A–E are labeled (Figure 2).

genesis of its function (VERPLOEGEN *et al.* 1997). Finally, as mammalian Leptin remains largely under purifying selection outside of hominoids, it may still prove beneficial to develop it as a weight control drug for domesticated animals (HOSSNER 1998).

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