Threading analysis suggests that the obese gene product may be a helical cytokine

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Abstract **The *ob* gene encodes a protein that, in mutant form, is associated with obesity and type II diabetes in mice. Sequence analysis has revealed no similarities to other proteins, however, and no clues as to possible functions. The possibility nonetheless remains that *ob* is functionally or ancestrally related to other proteins, whose sequences are divergent to the point that only a comparison of three-dimensional structures might detect relationship. To explore this possibility, we conduct a 'threading' search of a 3-dimensional structure database, to determine whether the *ob* protein might adopt a fold similar to any known structure. This search reveals that the *ob* sequence is compatible, at a significance level of $P < 0.05$, with structures from the family of helical cytokines that includes interleukin-2 and growth hormone. A structural model of *ob* based upon these results is physically and biologically plausible and leads to testable predictions, including the prediction that *ob* may activate the JAK-STAT pathway, via binding to a receptor resembling those of the cytokine family.**

Key words: ob Gene; Leptin; Protein threading; Structure prediction

1. Introduction

The end result of a successful positional cloning project is a DNA sequence, most commonly one that encodes a protein [1]. Sequence analysis is then expected to yield clues as to the gene product's biochemical function in normal and perhaps pathological states. Database homology searching [2] is universally used for this purpose, with the goal of finding a sequence or sequences that have known function or phenotype, and which will shed light on the function of the newly-cloned gene. This strategy has resulted in a number of dramatic discoveries in recent years [1], and greatly contributed to analysis of sequences derived from projects which comprehensively survey expressed genes [3]. Sometimes, however, the results of the homology search are negative, and one finds no significant similarities that provide clues as to a protein's biological function. The product of the mouse *ob* gene, and its human homolog, is a case in point. Mice that are homozygous for mutations in *ob* develop morbid obesity and type II diabetes [4]. The *ob* gene was recently isolated by positional cloning methodologies, and its product found to encode a 167-residue protein that is 84% identical between mice and humans. Sequence analysis revealed a putative signal peptide, suggesting that *ob* encodes an extracellular, secretory protein [4]. Database homology searches were entirely negative, however, and continue to be so at the time of writing.

The finding of homology between two sequences implies descent from a common ancestral gene and presumed structural and functional similarity. In some cases, however, evolutionary divergence may be so great that no significant sequence similarity remains, even though the protein products nevertheless continue to share a common three-dimensional structure [5]. Thus, if structural similarity is found, one may also sometimes infer homology and similarity of biological function. Because of this possibility the development of computational methods for automatic structure–structure comparison has received considerable recent attention [6]. For the same reason computational biologists are actively pursuing the development of 'threading' methods which attempt to detect similarity to a known structure given only the sequence of a newly discovered protein, and in the process to predict its tertiary structure [7,8,9,10,11,12,13].

Threading methods are essentially a way to automatically generate and test a large number of molecular models, each similar in some way to an experimentally determined 3-dimensional structure. The sequence under investigation is 'threaded' through alternative structures in a database, meaning that its amino acid residues are aligned in various ways with the coordinates occupied by residues from the known structures. The hypothetical models generated in this way are then evaluated by means of empirical energy functions which are sensitive to the chemical nature of the amino acid side chains, such as their tendency to be buried in the protein interior, or their tendency form contacts with one another. Since they rely on physical complementarity of sequence and structure, threading methods may potentially detect relationships over large evolutionary distances, where few or no residues remain the same, and it is only the residue-environment 'signature' of the 3-dimensional fold that is preserved. Precisely because of this feature, however, threading methods entail a considerable possibility for false positives, particularly when very many alternative, gapped alignments of sequence and structure are considered. The strength of the evidence offered by a threading search must therefore be evaluated carefully, using appropriate statistical tests [14].

In the present study we apply a protein threading technique [15,16] to an analysis of the mouse and human *ob* gene products. We conduct a search of unique structures drawn from the Protein Data Bank [17], looking for any which provide a physically and biologically plausible 3-dimensional model for the *ob* gene product. From the results of these computations we suggest that the *ob* gene product may adopt a 3-dimensional struc-

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ture similar to the helical cytokines, proteins which function as mediators of cell-to-cell communication [18]. We present a threading-derived alignment of the *ob* gene product and the common structural core of the helical cytokines, and discuss the implications of this putative structural model with respect to the biological function of *ob*.

2. Materials and methods

We present a brief summary of our threading technique, which is described in detail elsewhere [14,15,16]. We employ a library of 'core motifs' based on structures from the Brookhaven Protein Data Bank [17] April 1994 release, a subset of 435 proteins with no more than 35% pairwise sequence identity. The core motifs allow us to sample models with tertiary structures closely related to any of these proteins, meaning those with a similarly arranged collection of helices and strands, but with intervening loops which may differ in length and conformation. The precise boundaries of core elements and constraints on the maximum lengths of loops are defined automatically based on geometrical criteria [16]. We identify the most favorable sequence–structure alignments by means of a Gibbs sampling algorithm which aligns chain-continuous subsequence blocks with core elements, and also allows 'recruitment' of additional residue coordinates by chain-continuous addition to either the amino- or carboxy-terminus of core elements [16]. The derived models thus contain a nucleus defined by the core motif, but may optionally include further coordinates derived from extensions of strands or helices, including extensions into adjacent loops. The fitness of alternative sequence–structure alignments is judged according to the sum of pairwise residue contact energies, as judged by a statistically-derived contact potential, with no use of arbitrary gap penalties [15].

Threading contact energies are corrected for sequence-composition bias by random shuffling of the aligned residues. The resulting 'Composition Corrected Threading Scores' shown in Fig. 1 correspond to the quantity $Z(r|m)$ described previously [15]. The probability of obtaining a given threading score by chance, with a random sequence, is primarily a function of the number of alternative alignments possible, which is in turn a function of the number and lengths of core elements in the structure and the length of the sequence [14]. To evaluate statistical significance we therefore generate 100 random permutations of the *ob* sequence, and repeat the alignment optimization procedure as many times for each core motif. From the distribution of threading scores obtained we then compute the 'Chance Occurrence Z Score' as shown in Fig. 1, which expresses the threading score of the *ob* sequence, in standard deviation units, relative to the distribution obtained for random sequences, when optimally threaded onto the same core motif. These scores are comparable across different structural models for the *ob* protein, and allow us to rank the alternatives on the basis of the

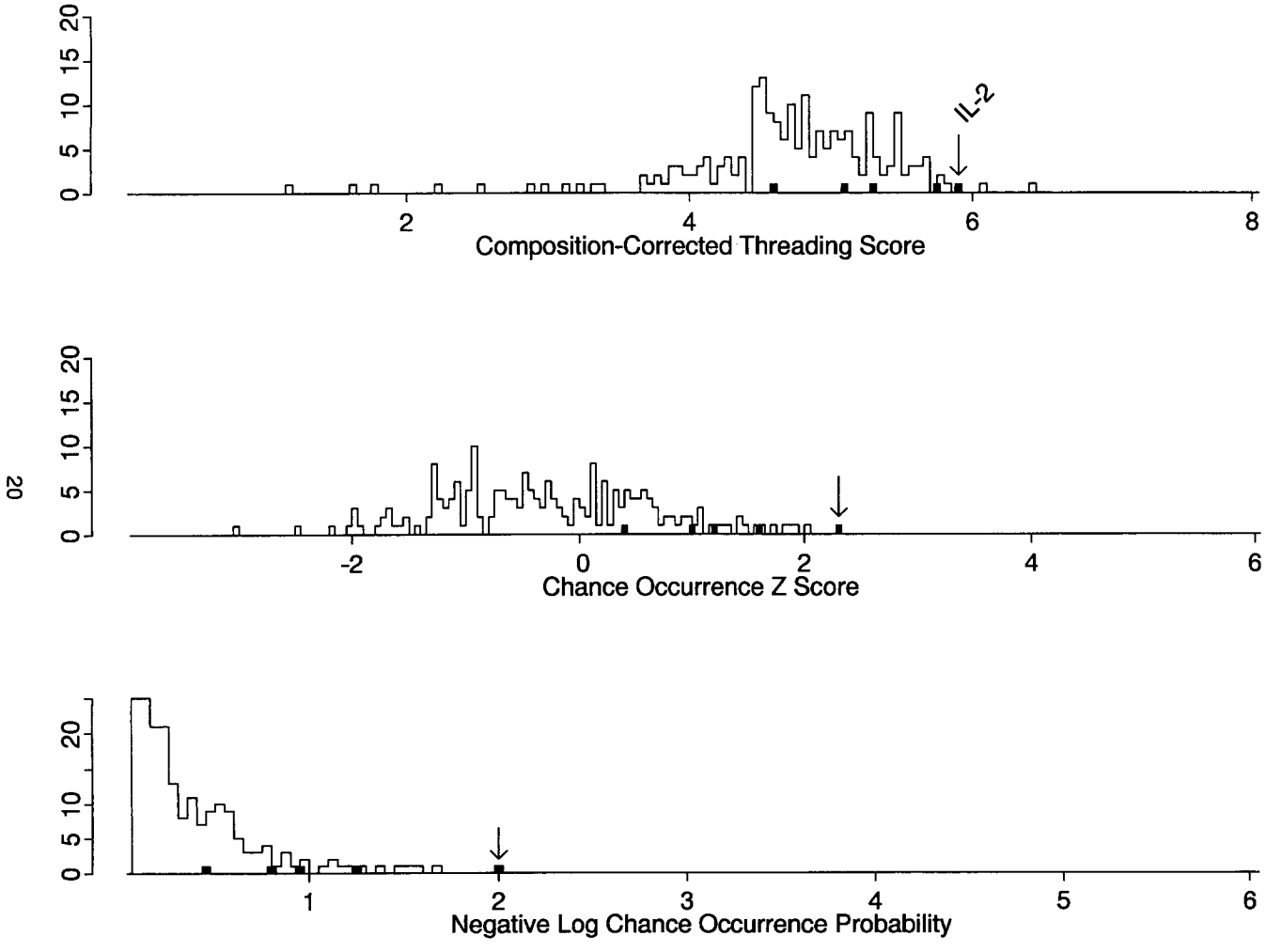

Fig. 1. Threading search of the known structure database using the mouse *ob* protein sequence. Scores for helical cytokine structures are indicated by the shaded bars, and those for interleukin-2 are indicated. The upper panel displays threading scores corrected for composition effects. The central and lower panels show tests for statistical significance, Z scores which rank the threading scores relative to values for randomly shuffled sequences, and chance-occurrence probabilities calculated from these Z scores, respectively. Sequence–structure alignments which assign less than 50% of the *ob* gene product sequence to explicit coordinates rather than loops of unknown structure have been omitted.

inverse likelihood that they represent a chance sequence–structure complementarity. Threading Z scores follow approximately a standard normal distribution, and we may thus calculate from them the probability that the *ob* threading scores would be observed by chance, in comparison of a randomly chosen sequence and core motif [14]. Chance occurrence probabilities calculated in this way are shown in Fig. 1 as their negative logs to the base 10. We note that these values are conservative, in that we apply no constraints on the fraction of aligned residues for randomized sequences, as we have for *ob*.

Mouse and human *ob* gene sequences and translations are taken from Genbank accession numbers U18812 and U18915, respectively. For threading experiments we omit the putative N-terminal signal sequence of 21 residues [4]. The structural alignment of helical cytokines shown in Table 2 is based on that presented by Rozwarski et al. [18], with extension to the long-chain and interferon-like subclasses by manual superposition using the program Insight, from Biosym Technologies Inc. Root mean square superposition residuals for all models with complete backbone coordinates are under 3 Å. Coordinates for known cytokines were taken from the Protein Data Bank [17], with accession codes as indicated in the header to Table 1. Residue numbering for known cytokines as shown in Table 2 corresponds to that in the Protein Data Bank file. For consistency with cytokine numbering residue numbers for *ob* in Table 2 begin with the first residue of the putative mature product.

3. Results

Fig. 1 provides a summary of the threading search which suggests the helical cytokines as candidate structural models for the *ob* gene product. The histograms diisplay threading scores for three-dimensional models for the *ob* protein, each based on the best possible alignment of the *ob* sequence with a known structure in the database. Scores adjusted for statistical significance identify interleukin-2 (IL-2) as the protein most likely to have structural similarity to the *ob* protein. The approximate *P*-value is 0.01, indicating the odds are only 1-in-100 that a random match of sequence and core motif would obtain a score this favorable. A false positive at this *P*-value is by no means impossible, because we have searched a large database, and because threading scores in this range may identify a structural similarity that is not sufficiently extensive to infer common descent or function [14]. Identification of IL-2 would be a striking coincidence, however, when one considers that this is an inter-cellular signaling protein, precisely the presumed biological function of *ob*. Other helical cytokines with structures similar to IL-2 are also identified as favorable models, and the contact energy scaffold for the predicted *ob*-protein model resembles that for IL-2, as shown in Fig. 2. These observations also suggest that a structural model based on the helical cytokines is physically plausible.

The helical cytokines are a diverse family in which there is no detectable sequence similarity between its members, but where all possess a common 'core' substructure containing a 4-helix bundle with a distinctive 'up-up-down-down' topology [18]. Proteins in this family for which 3-dimensional structures are available include interleukin-2 [19] (IL2), interleukin-4 [20] (IL-4), granulocyte-macrophage colony-stimulating factor [21] (GMCSF), macrophage colony-stimulating factor [22] (M-CSF), granulocyte colony-stimulating factor [23] (G-CSF), growth hormone [24] (GH), and interferon-β [25] (IFN-β). Division into subclasses based on structural similarities beyond the 4-helix bundle places IL-2, IL4, GM-CSF and M-CSF into the 'short-chain' subclass, G-CSF and GH in the 'long-chain' subclass, and IFN-β in the 'interferon-like' subclass [18]. The finding that the *ob* protein sequence is compatible with the IL-2 structure suggests that it should to some extent be compatible with the structures of the other helical cytokines. The existence of multiple structure data allows us to test this hypothesis, and also to test whether threading scores for *ob* are indeed characteristic of a member of the helical cytokine family.

To conduct this analysis have constructed a structural alignment of the helical cytokines, and from this alignment defined the 'core' substructure common to the known family members. This definition of the cytokine core better reflects the substructure one might expect to be conserved in a new family member, and corrects for inaccuracies in the core definitions employed in the initial search, which were of necessity constructed automatically and without reference to multiple structures [16]. We have also included additional cytokines omitted from the initial search due to incompleteness of coordinate data. Using the corrected core definitions we thread each known cytokine sequence through each structure, to verify that the threading method is indeed sufficiently sensitive to detect structural similarity within this family. We also thread the mouse and human *ob* sequences through the corrected cytokine cores, to determine whether the threading scores obtained with *ob* are similar to those of known cytokines.

The results of this analysis are shown in Table 1, where we present approximate *P*-values, calculated as in Fig. 1, for pairwise sequence–structure comparisons of the cytokines and *ob*. Columns indicate the cytokine structure considered and rows the sequences threaded through each core structure. One may see that cross-comparison of known cytokine sequences and structures yields *P*-values frequently less than 0.05, in particular for comparisons within the cytokine subclasses. These positive controls indicate that threading can indeed detect structural similarity within the helical cytokine family. The method is near the limit of its sensitivity, however, which is not

Table 1
Cytokine–cytokine and *ob*-cytokine threading *P*-values

Structure:	GM-CSF	IL-2	IL-4	M-CSF	G-CSF	GH	IFN-β
PDB code:	1GMF-A	3INK-C	1ITL	1HMC-A	1BGC	3HHR-A	1IFA
GM-CSF	<0.001	0.005	0.358	0.040	0.035	0.285	0.017
IL-2	0.036	<0.001	0.026	0.012	0.002	0.010	0.004
IL-4	0.036	<0.001	<0.001	0.035	0.003	0.006	0.013
M-CSF	0.009	0.069	0.183	<0.001	0.073	0.248	0.062
G-CSF	<0.001	0.053	0.154	0.043	<0.001	0.002	0.021
GH	0.060	0.022	0.032	0.065	<0.001	<0.001	<0.001
IFN-β	0.463	0.093	0.252	0.091	0.096	0.048	<0.001
MOb	0.031	0.016	0.016	0.157	0.012	0.052	0.116
HOb	0.094	0.034	0.027	0.591	0.025	0.107	0.144

Fig. 2. 'Energy scaffolds' for interleukin-2 [19] and a structural model for the *ob* gene product based on threading alignment with interleukin- 2. Coordinates present in the core motif or recruited by the threading algorithm are indicated by blue coloring of the backbone 'worm'. Pairwise contact energies are indicated by the thickness and color of the cylinders connecting C_α coordinates, with favorable interactions shown by thick, magenta-colored cylinders. The figure is prepared using the program GRASP, by Anthony Nicholls [38].

surprising given that as few as 30% of the residues in between-subclass comparisons fall at structurally analogous, superimposable coordinates. It is also clear from Table 1 that the *ob* sequences behave in this experiment much as do other cytokines. Threading scores for *ob*, with *P*-values generally less than 0.05, are as favorable as one would expect for a new member of the cytokine family. They are not consistently lower for any one subclass, suggesting that the similarity of *ob* to the helical cytokines may not extend beyond their common, 4-helix-bundle core structure.

This analysis also produces sequence–structure alignments of the *ob* proteins with each cytokine core structure. We find that these alignments are mutually consistent, in the sense that they place the 4 helices of the of the cytokine fold in the same regions of the *ob* sequences, no matter which structure serves as the template for threading (not shown). The 'signal' indicating location of the 4 helices within the *ob* sequences would thus appear to be robust, in that alignments with the structurally diverse, intervening regions are uniformly rejected. Individual sequence–structure alignments are nonetheless uncertain, since alternatives which displace helices by a turn or two preserve their amphipathic orientation and are of nearly equal energy. This uncertainty is expected in a protein family where structural similarity is limited to a small common core, and superposition residuals are in the 2.5 Å range [16]. In Table 2 we present the consensus of the alignments of the mouse and human *ob* gene product sequences with the cytokine core structures, that is, the most probable location of the core helices within the *ob* sequences, as predicted by Boltzmann probabilities of the alternative threading alignments. Consensus threading alignments of the *ob* sequences with the conserved β strand regions (s-1, s-2) of the short-chain cytokines are also shown, though it is unclear, given the *P*-values above, that these represent a more accurate model than do alignments with members of the long-chain or interferon-like families. Complete sequences are shown in Table 2, but we emphasize that consensus alignments of *ob* with the helical cytokines are not possible outside the structurally conserved regions, and the alignments shown for non-conserved regions (nc) are arbitrary.

By computational methods we cannot further verify that the *ob* model structure predicted by this sequence–structure alignment in Table 2 is correct, but we note that it is consistent with two features of the *ob* sequences that are not considered in the threading analysis, disulfide bond formation and location of mouse/human *ob* sequence differences. The two cysteines in the *ob* sequence align close to one of two disulfide bonded pairs in the GM-CSF structure (residues 88 and 121), and we find that a disulfide bond may be readily modeled in a structurally analogous location. Disulfide bonding patterns within the helical cytokines are not highly conserved, but the location of this putative disulfide bond is certainly consistent with *ob* adopting a helical cytokine fold. Of 19 non-conservative residue substitutions between the mouse and human *ob* proteins, 6 occur in the putative signal sequence we omit from the threading experi-

Table 2
Cytokine structural alignment and *ob* sequence–structure alignment

	n	nc	helix-1	nc	s-1	nc
GM-CSF	5	spspstqpwe	HVNAIQEARRLLNLS	rdtaaemne----------	TVEV	----------isemfdlqep
IL-2	6	stkk	TQLQLEHLLLDLQMI	lnginnyknpkltrmltf-----	KFYM	----------pkk
IL-4	0	mhk	CDITLQEIIKTLNSL	teqktlctel----------	TVTD	----------ifaaskn
M-CSF	4	seyc	SHMIGSGHLQSLQRL	idsqmetscqi----------	TFEF	----------vdqeqlkdpv
Ob (H)	1	vpiqkvqdd	TKTLIKTIVTRINDI	shtqsvsskqkvtgld-------	FIPG	----------lhpiltl
Ob (M)	1	vpiqkvqdd	TKTLIKTIVTRINDI	shtqsvsakqrvtgld-------	FIPG	----------lhpilsl
G-CSF	9	slpqsfllk	CLEQVRKIQADGAEL	qerlcaahklchpeelmllrhsl	----	-----gipqaplsscssqslqlrgcl
GH	1	fptiplsrlfdn	AMLRAHRLHQLAFDT	yqefeeayipkeqkysflqnpqt	----	slcfsesiptpsnreetqqksnlell
IFN-B	3	ykqlql	QERTNIRKCQELLEQ	lngkinltyr----------	----	----------adfkipmemtekmqks

	n	helix-2	nc	helix-3	nc
GM-CSF	53	TCLQTRLELYKQGL	rg----------s	LTKLKGPLTMMAS	hykqhcpp----------
IL-2	50	ATELKHLQCLEEEL	kpleevlnlaq--------sknfhlrprdl	ISNINVIVLELKG	settfm----------
IL-4	39	TTEKETFCRAATVL	rqfyshhekdtrclgataqqfhrhkqlirf	LKRLDRNLWGLAG	lnsc----------
M-CSF	48	CYLKKAFLLVQDIM	edtmrfr----------dntpnaia	IVQLQELSLRLKS	cftkdyee----------
Ob (H)	52	SKMDQTLAVYQQIL	tsmpsrn----------viqisnd	LENLRDLLHVLAF	skschlpwasgletl---------
Ob (M)	52	SKMDQTLAVYQQVL	tslpsqn----------vlqiand	LENLRDLLHLLAF	skscslpqtsglqkp---------
G-CSF	77	NQLHGGLFLYQGLL	qalagis----------pelapt	LDTLQLDVTDFAT	niwlmedlgaapavqptq------
GH	77	RISLLLIQSWLEPV	qflrsvfa----------nslvygas	DSNVYDLLKDLEE	giqtlmgrledgsprtgqifkq--
IFN-B	50	YTAFAIQEMLQNVF	lvfrnnfsst----------gwnetivvr	LLDELHQQTVFLK	tvleekgeerltwemsstalhlks

	n	nc	s-2	helix-4	nc
GM-CSF	91	----------tpetscat	QIIT	FESFKENLKDFLLVI	pfdcwe
IL-2	105	----------ceyad	ETAT	IVEFLNRWITFAQSI	istlt
IL-4	100	----------pvkea	NQST	LENFLERLKTIMREK	yskcss
M-CSF	98	----------hdkacvrt	FYET	PLQLLEKVKNVFNET	knlldkdwnifskncnnsfaecssqgh
Ob (H)	108	--------dslggvleasgyst	EVVA	LSRLQGSLQDMLWQL	dlspgc
Ob (M)	108	--------esldgvleaslyst	EVVA	LSRLQGSLQDILQQL	dvspec
G-CSF	136	-------gamptftsafqrrag	----	GVLVASQLHRFLELA	yrglryla
GH	142	--tyskfdtnshnddallknyg	----	LLYCFRKDMDKVETF	lrivqcrsvegscg
IFN-B	120	yywrvqrylklmkynsyawmvv	----	RAEIFRNFLIIRRLT	rnfqn

ments, and 7 of the remaining 13 in a loop region between helices 3 and 4, in the threading alignments. This region is subject to considerable variation in sequence and structure among the known cytokines, and concentration of mouse/human sequence differences there is again consistent with the hypothesis that *ob* is a member of the helical cytokine family.

4. Discussion

Recent evidence indicates that the *ob* gene product (also known as leptin) acts as a polypeptide hormone [26,27,28]. Therefore it must bind to a cell surface receptor in some target tissue, perhaps in the central nervous system [26], and trigger an intracellular signalling pathway. In the absence of other information one may only guess as to which receptor and which pathway, however. Our threading experiments suggest that the *ob* gene product may be related in structure to the family of helical cytokines which includes IL-2 and growth hormone (GH). This result implies that leptin may exert its effect(s) in a manner similar to Class I cytokines, that bind to receptors activated by dimerization or oligomerization [29,30] and ultimately regulate the transcription of specific genes in the nucleus [31]. A number of residues from the helical core region have been implicated in growth hormone/growth hormone receptor (GHR) binding, as judged from the known crystal structure of the complex [24]. To the extent that *ob*/*ob*-receptor system is analogous, the alignment in Table 2 allows one to predict *ob* residues that may be involved in receptor binding, a prediction which may be testable by binding assay of site-specific *ob* mutants.

Signal transduction by GH/GHR involves tyrosine phosphorylation by protein kinases, followed by subsequent intracellular events which culminate in transcription of specific genes. Because GHR has no intrinsic kinase activity, GHR signal transduction is mediated through a cytoplasmic kinase known as JAK2 [32]. In response to GHR aggregation and binding of JAK2 to the cytoplasmic domain of GHR, a variety of tyrosine phosphorylation reactions occur, including autophosphorylation of JAK2, phosphorylation of the GHR cytoplasmic domain, and phosphorylation of other cellular substrates that come to be associated with the GHR–JAK2 complex. These other kinase substrates have recently been shown to be protein transcription factors known as STATs (signal transducers and activators of transcription), specifically STAT1 and STAT3 [33,34,35,36]. These STATs activate transcription of several genes including c-*fos* and insulin-like growth factor I [37]. IL-2 signalling appears to be more complex, being mediated by a heterotrimeric receptor, and multiple, downstream protein tyrosine kinases including JAK1 AND JAK3 [29]. These JAKs activate the transcription factors STAT3 and STAT5 [29].

Structural similarity among IL-2, GH and the *ob* gene product would thus suggest that the latter may also utilize the JAK-STAT pathway. Experiments designed to detect the phosphorylation of specific STATs, in the presence of wild type and mutant *ob*, are thus likely to be the most easily testable predictions of the *ob* structural model we present.

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