nent longer than previously thought. The work leading to the formal recognition of the disease was begun in November 1975 (*5*). Subsequently, earlier cases were identified retrospectively that fulfilled the clinical criteria for Lyme disease in Wisconsin and on Cape Cod, Massachusetts (*40, 41*). Reports from senior medical practitioners in eastern Long Island indicate that "Montauk knee" and "Montauk spider-bite" have been in the vernacular of the local residents for many years (*42*). These descriptive terms could have referred respectively to the arthritic and dermatologic manifestations of Lyme disease, but they seem not to have been linked to each other or to tick-bite. Application of the same technology to preserved, archived human samples, such as paraffin-embedded tissue sections, may someday provide additional clues to the antiquity of Lyme disease.

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44. We thank H. Levi and A. Johnston of the Department of Invertebrates at the Harvard Museum of Comparative Zoology and J. E. Keirans of the Smithsonian Institution for specimens from their repository of acarines. J. Anderson, S. Barthold, W. Weisberg, C. Pretzman, and P. Duffy provided isolates of *Borreliae* that demonstrated the specificity of our probes. We also thank J. Anderson and R. Flavell for a critical reading of this manuscript, G. Van Blaricom for technical assistance, and P. Jatlow for support of this project. Supported by NIH grants AM40452, AM10493, AM07107, and AI 19693. D.H.P. is a Mayo Foundation Scholar.

8 May 1990; accepted 18 July 1990

An Insertion in the Human Thyrotropin Receptor Critical for High Affinity Hormone Binding

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Thyrotropin (TSH), lutenizing hormone (LH), and chorionic gonadotropin (CG) are structurally related glycoprotein hormones, which bind to receptors that share a high degree of sequence similarity. However, comparison of the primary amino acid sequences of the TSH and LH-CG receptors reveals two unique insertions of 8 and 50 amino acids in the extracellular domain of the TSH receptor. The functional significance of these insertions were determined by site-directed mutagenesis. Deletion of the 50–amino acid tract (residues 317 to 366) had no effect on TSH binding or on TSH and thyroid-stimulating immunoglobulin (TSI) biological activities. In contrast, either deletion or substitution of the eight–amino acid region (residues 38 to 45) abolished these activities. This eight–amino acid tract near the amino terminus of the TSH receptor appears to be an important site of interaction for both TSH and TSI.

THYROTROPIN (TSH) REGULATES thyroid cell function by interacting with the TSH receptor on the plasma membrane (*1*). Auto-antibodies against the TSH receptor (thyroid-stimulating immunoglobulins, TSI) are important in the development of the hyperthyroidism associated with Graves' disease (*2*). The sites on the TSH receptor that interact with TSH and TSI are unknown. The TSH and LH-CG receptors each bind specific members of a family of glycoprotein hormones that are structurally related, suggesting that the receptors may be highly similar. The molecular cloning of the complementary DNA that encodes these receptor proteins has allowed comparison of the primary amino acid sequences of the TSH (*3–6*) and LH-CG (*7, 8*) receptors. The two receptors are highly related except for two unique insertions of 8 and 50 amino acids in the extracellular domain of the 764–amino acid TSH receptor (*3*). We have therefore focused on these two regions and used site-directed mutagenesis to determine their functional significance.

The cDNA that encodes the two unique segments in the extracellular domain of the human TSH receptor, amino acids 38 to 45 and 317 to 366 (*3*), were deleted by site-directed mutagenesis, singly or together (Fig. 1). These segments are near the NH_2- and COOH-terminal regions of the 418–amino acid extracellular domain of the TSH receptor (*3*), respectively (Fig. 1). Chinese hamster ovary (CHO) cells were stably transfected with the cDNAs that encoded mutated TSH receptors and were then tested for their ability to bind TSH and to respond to TSH stimulation, measured by an increase in intracellular cyclic adenosine monophosphate (cAMP) content.

Although the 50–amino acid deletion (hTSHR-D1) represented 12% of the 418–amino acid extracellular domain of the TSH receptor, high-affinity TSH binding to the recombinant human TSH receptor was not reduced (Table 1). Consistent with this high-affinity TSH binding, TSH addition stimulated cAMP production in cells that

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expressed hTSHR-D1, with an EC_{50} that was similar to values observed for cells that expressed the wild-type receptor. In contrast, deletion of the eight–amino acid insertion near the NH_2-terminus (hTSHR-D2) completely abolished high-affinity TSH binding. Again, consistent with the absence of high-affinity TSH binding, TSH stimulation did not increase intracellular cAMP concentrations. The CHO cells transfected with a TSH receptor cDNA that lacked 50– and 8–amino acid segments (hTSHR-D3) had neither high-affinity TSH binding nor a cAMP response to TSH stimulation.

Because the eight–amino acid deletion in hTSHR-D2 might have altered the overall protein conformation, the observed loss of biological activity may not be attributable to the loss of a critical hormone binding site, but to formation of an abnormally folded protein. We therefore constructed a mutant receptor (hTSHR-S1) in which the eight critical amino acids were substituted with eight residues (Fig. 1) that would not be expected to alter the generally hydrophilic nature of this region or its likely presence on the surface of the globular protein. There is evidence that normal folding may be unaffected by substantial alterations of hydrophilic regions at the surface of globular proteins (*9*), as suggested by the 50–amino acid deletion mutant (hTSHR-D1). As with hTSHR-D2, hTSHR-S1 expressed neither high affinity TSH binding nor a cAMP response to TSH stimulation.

Sera from patients with Graves' disease that contain thyroid-stimulating immunoglobulin (TSI) stimulated the mutant TSH receptor clones in a manner similar to that of TSH. Thus, only the wild-type TSH receptor and the TSH receptor with the 50–amino acid deletion (hTSHR-D1) exhibited a cAMP response to a pool of sera from four patients with Graves' disease, which contained potent TSI activity as measured in the conventional TSI bioassay (*10*) (Fig. 2). Deletion or substitution of the eight–amino acid tract (residues 38 to 45) in the TSH receptor abolished the cAMP response to TSI stimulation. These data indicate that the 50–amino acid tract did not contain an epitope that was important for TSI action. In contrast, as for TSH action, the eight–amino acid segment appeared to be an important site of TSI function.

Because the 8– and 50–amino acid segments in the TSH receptor are absent from the LH-CG receptor (*3, 8*) it was conceivable that their deletion from the TSH recep-

Table 1. Hormone binding and function in CHO cells that stably expressed the TSH receptor mutants in Fig. 1. Each value represents the mean of data obtained with pools of clones from two separate transfections, each transfection measured in duplicate. The range from the mean did not exceed 25%. TSH binding studies were performed as described (*13*) with the exception that TSH was labeled with ^{125}I to ~80 μCi of protein per milligram with the Bolton-Hunter reagent. Nonspecific ^{125}I-labeled TSH binding was determined in the presence of unlabeled TSH (10^{-6} M), and this value was subtracted from total binding to yield specific TSH binding. Measurements of the intracellular cAMP response to hormone stimulation (1 hour at 37°C) was as described (*14*). K_d, dissociation constant; M, mutant; WT, wild type; EC_{50}, effective concentration of hormone that produces a 50% response; ND, not detectable; pSV2-NEO-ECE, vector without cDNA insert.

Receptor construct	High-affinity TSH binding		TSH stimulation of cAMP	
	K_d (nM)	K_d-M / K_d-WT	EC_{50} (nM)	EC_{50}-M / EC_{50}-WT
hTSHR-WT	0.3	1	4.0	1
hTSHR-D1	0.2	0.7	7.0	1.4
hTSHR-D2	ND	ND	ND	ND
hTSHR-D3	ND	ND	ND	ND
hTSHR-S1	ND	ND	ND	ND
pSV2-NEO-ECE	ND	ND	ND	ND

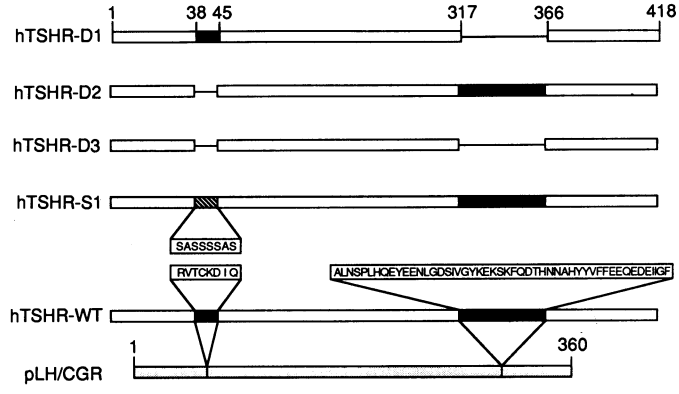

Fig. 1. Schematic representation of the extracellular domains of the mutant human TSH receptors. The solid bars represent segments in the TSH receptor that are not present in the LH receptor. Lines interrupting the open bars represent deletions in the TSH receptor. The hatched bar indicates substitution rather than deletion of eight amino acids in the TSH receptor. The amino acids are shown in the single letter code (*15*). Numbers indicate amino acid positions in the wild-type human TSH receptor (*3*). The full-length human TSH receptor cDNA in Bluescript (Stratagene) was subjected to oligonucleotide-directed mutagenesis (Muta-gene kit, Bio-Rad) (*16*). Nucleotides that coded for amino acids 38 to 45 and 317 to 366 were deleted, separately and in combination, with oligonucleotides with bases complementary to the nucleotides flanking these regions (5′-CGGTAAGCTGGGGATGCGGAAGTCCTCCTCCTGATG-3′ and 5′-GGGTTTTTGAGCTCCTGGCCATTCACAGATTTTCTCTGGC-3′, respectively). Amino acids 38 to 45 (RVTCKDIQ) were substituted with the sequence SASSSSAS with the oligonucleotide, 5′-AAGCTGGGGATGCGTGAAGCAGAACTGCTGGACGCACTGAAGTCCTCCTCCT-3′, that contained 14 bases complementary to the nucleotides flanking either side of the substituted sequence. The nucleotide sequences of the mutagenized and adjacent regions were determined (*17*) and confirmed to be correct. The cDNAs that encoded the mutagenized TSH receptor were excised with Eco RI and subcloned into the expression vector, pSV2-NEO-ECE (*3*). These mutants, the wild-type receptor in the same vector and a vector without a cDNA insert, were transfected by the calcium-phosphate method (*18*) into CHO cells. Surviving colonies were selected by G418 (400 μg/ml) and pooled for study of their ability to bind to TSH and increase intracellular cAMP.

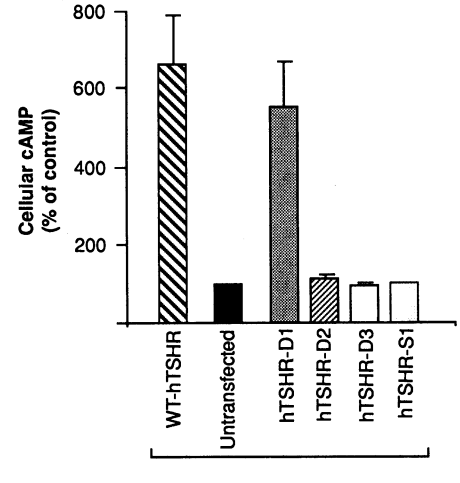

Fig. 2. Measurement of the intracellular cAMP response to TSI stimulation in pooled clones of stably transfected CHO cells. WT-hTSHR, wild-type human TSH receptor; the structures of hTSHR-D1-3 and hTSHR-S1 are shown in Fig. 1. Each bar (with the exception of hTSHR-S1, which was tested once in duplicate) represents the mean ± standard error of values obtained in duplicate dishes, in three separate experiments, with immunoglobulin G prepared (*19*) from pools of four individual TSI-positive sera or TSI-negative sera (the control value). TSI stimulation was for 2 hours at 37°C. Intracellular cAMP concentrations were measured as described (*14*).

tor would alter its hormone binding specificity and allow it to respond to human CG (hCG). This was not the case. As with the wild-type TSH receptor, these mutant TSH receptors were unresponsive to 10^{-7} M hCG, as measured by cAMP production (*11*).

The pituitary-placental glycoprotein hormone receptors represent a distinct subgroup in the G protein–coupled receptor family, whose members contain seven transmembrane spanning regions. The majority of the members of this receptor family, such as the α-adrenergic, β-adrenergic, muscarinic acetyl choline, and dopamine receptors (which we term group A), interact with small ligands and have insignificant extracellular regions. In contrast, the receptors for the very large glycoprotein hormones TSH, LH, CG, and follicle-stimulating hormone (FSH) (group B) have large extracellular domains (348 to 418 amino acids) (*3–5, 8, 12*). The largest extracellular domain is in the TSH receptor, primarily because of the presence of the 50–amino acid insertion. It is remarkable that this 50–amino acid tract that is unique to the TSH receptor was not important for TSH binding or signal transduction (cAMP generation). It is possible that this region serves a negative function, such as to prevent the binding of other ligands to the TSH receptor. However, deletion of the 50–amino acid segment did not allow the receptor to respond to hCG.

The data in this report provide strong evidence for the localization of a site for TSH binding to the eight–amino acid insertion of the TSH receptor. The amino acid sequence of the rat FSH receptor (*12*), reveals a unique ten–amino acid segment in the same general region (residues 21 to 30) as the eight–amino acid segment in the human TSH receptor. However, like the LH receptor, the FSH receptor does not contain the unique 50–amino acid segment of the TSH receptor (*3, 12*). The significance of this FSH receptor segment remains to be determined.

Identification of the epitopes on the TSH receptor that are recognized by auto-antibodies to the receptor in the sera of patients with Graves' disease is a subject of interest. These epitopes, which may or may not be identical to the TSH binding sites, may permit new diagnostic and therapeutic approaches to the management of patients with this common autoimmune disorder. The data in this report identify the eight–amino acid insert as an important site of action for both TSH and TSI.

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20. We recognize the contribution of F. Cohen and S. Presnell for their valued advice during the course of these studies and appreciate the expert administrative assistance of G. dela Calzada. Supported by NIH grants DK-36182 and DK-19289, and the Research Service of the Veterans' Administration.

20 June 1990; accepted 1 August 1990

Anatomy of a Conformational Change: Hinged "Lid" Motion of the Triosephosphate Isomerase Loop

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Triosephosphate isomerase (TIM) is used as a model system for the study of how a localized conformational change in a protein structure is produced and related to enzyme reactivity. An 11-residue loop region moves more than 7 angstroms and closes over the active site when substrate binds. The loop acts like a "lid" in that it moves rigidly and is attached by two hinges to the remainder of the protein. The nature of the motion appears to be built into the loop by conserved residues; the hinge regions, in contrast, are not conserved. Results of molecular dynamics calculations confirm the structural analysis and suggest a possible ligand-induced mechanism for loop closure.

CONFORMATIONAL CHANGES IN proteins are often an essential part of enzyme mechanisms (*1, 2*). Although x-ray crystallography provides structural data for highly populated species, such as the initial and the final states in a conformational change, other experiments and computer simulations are necessary to explore the motions involved. In this report, we combine molecular dynamics (MD) simulations with structural and sequence data to obtain a description of the so-called "loop transition" in the enzyme TIM. We show that the localized loop closure involves an essentially rigid-body type displacement, analogous to that implicated in the hinge bending domain motions of certain proteins (*3, 4*).

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Yeast TIM is a dimer with two identical subunits, each composed of 247 residues. Although the monomers have identical noncooperative sites, only the dimer is catalytically competent. Each subunit contains a loop region (residues 166 to 176) (*5*) that projects into solvent in the unliganded enzyme and closes over the active site when substrate binds (Fig. 1) (*6*). Earlier low-resolution studies (*7*) and simulations (*8*) suggested that the loop was disordered in the open structure. The refined high-resolution structure of native TIM shows a single predominant conformation for the loop with high thermal parameters (*9*). In the refined high-resolution crystal structure of TIM complexed with the transition-state-intermediate analog phosphoglycolohydroxamate (PGH), the loop is closed and appears to be in the same position as in the enzyme-substrate complex (*10*). Mutagenesis experiments have shown that the loop is essential for catalysis (*11*).

When the open and closed structures of TIM are superimposed by least-squares optimization (*12*) of all α-carbons of subunit 1 (excluding residues 166 to 176), the root-