hybrids retaining: human Xq24–qter, Xq26–qter, Xpter–q26.3, Xpter–q27.1, and Xpter–q27, respectively; RJK734 [A. F. Scott, J. A. Phillips, B. R. Migeon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4563 (1979)] and Lowe3 [D. S. Reilly, R. A. Lewis, D. H. Ledbetter, R. L. Nussbaum, *Am. J. Hum. Genet.* **42**, 748 (1988)] are human-mouse hybrid cell lines containing human Xq26–qter and Xq25–qter, respectively.
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21. All labeling was carried out with [α-^{32}P]dCTP by random oligodeoxynucleotide priming [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)]. DNA probes containing repetitive sequences were preannealed with total human DNA before hybridization (0.5 mg/ml, 65°C, and 30 min). Hybridization was carried out in 1% SDS, 1 M NaCl, 10% dextran sulfate, at 65°C overnight, with 0.5 mg of herring sperm or human placental carrier DNA per milliliter. After hybridization, filters were washed at room temperature in 2× saline sodium citrate (SSC) and 0.1% SDS, and at 65°C in 2× SSC and 0.1% SDS or in 0.1× SSC and 0.1% SDS. cDNA insert preparation: Plasmid minipreparation DNA (1 μl, that is, ~100 ng) was used as template in a PCR amplification with the use of M13 universal primer and T7 promoter-derived primer. PCR was performed as described (7). Templates were mixed with 100 pmol of each primer in a total volume of 100 μl containing 50 mM KCl; 10 mM tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin; 200 μM each dNTP; and 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus). Amplification consisted of 35 cycles of denaturation (94°C; 1 min); DNA polymerization (68°C; 2 min); and annealing (60°C; 1 min) in an automated thermal cycler (Perkin-Elmer Cetus) and included an initial denaturation of 7 min at 94°C and a final polymerization at 68°C for 7 min.
22. We thank A. Edwards for the unpublished HPRT sequence; C. Schwartz, K. Davies, and S. Warren for unpublished cell lines; D. Ledbetter for use of somatic cell mapping panel; M. F. Victoria for technical assistance; D. Muzny for DNA sequencing; A. Ballabio for encouragement; and P. Patel for critical reading of the manuscript. Supported by the Howard Hughes Medical Institute and grant #DE-FG05-88ER60692 from the U.S. Department of Energy. J. A. Maley was supported by an appointment to the U.S. Department of Energy Alexander Hollaender Distinguished Fellowship program administered by Oak Ridge Associated Universities.

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Two G Protein Oncogenes in Human Endocrine Tumors

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Somatic mutations in a subset of growth hormone (GH)–secreting pituitary tumors convert the gene for the α polypeptide chain (α_s) of G_s into a putative oncogene, termed *gsp*. These mutations, which activate α_s by inhibiting its guanosine triphosphatase (GTPase) activity, are found in codons for either of two amino acids, each of which is completely conserved in all known G protein α chains. The likelihood that similar mutations would activate other G proteins prompted a survey of human tumors for mutations that replace either of these two amino acids in other G protein α chain genes. The first gene so far tested, which encodes the α chain of G_{i2}, showed mutations that replaced arginine-179 with either cysteine or histidine in 3 of 11 tumors of the adrenal cortex and 3 of 10 endocrine tumors of the ovary. The mutant α_{i2} gene is a putative oncogene, referred to as *gip*2. In addition, *gsp* mutations were found in 18 of 42 GH-secreting pituitary tumors and in an autonomously functioning thyroid adenoma. These findings suggest that human tumors may harbor oncogenic mutations in various G protein α chain genes.

MANY PROTO-ONCOGENES encode proteins that transmit signals that regulate normal cell growth. Specific mutations convert these genes into oncogenes, whose mutant protein products are responsible for the abnormal growth of malignant cells. In many human tumors, for example, point mutations convert *ras* genes into oncogenes (*1*). These mutations render $p21^{ras}$ oncogenic by inhibiting its ability to hydrolyze bound guanosine triphosphate (GTP), thus trapping the protein in its active, GTP-bound state (*1*). We reported (*2*) functionally similar GTPase inhibiting mutations that activate the α chain (α_s) of a heterotrimeric G protein, G_s, in human growth hormone (GH)–secreting pituitary tumors; the mutations convert the α_s gene into a putative oncogene, termed *gsp* (*2*).

These findings, combined with conserved stretches of amino acid sequence in a large number of G protein α chains, suggested a novel approach for finding oncogenes. Here we report success of this approach in its first application, to the gene encoding the α chain (α_{i2}) of G_{i2}.

This approach is based on two inferences regarding structure and function of G proteins. First, we infer that the two conserved amino acids whose mutational replacements inhibit GTPase of α_s have similar functional roles in α chains of other G proteins. This inference in turn implies that cognate mutations in other α chains will activate the corresponding G proteins. One set of *gsp* mutations (*2*) substitutes arginine for Gln^{227} of α_s, which is equivalent to Gln^{61} of $p21^{ras}$, a frequent site of GTPase-inhibiting mutations in the *ras* proteins. Other *gsp* mutations (*2*) replace Arg^{201} of α_s with cysteine or histidine. This arginine is the target for cholera toxin-catalyzed adenosine diphosphate (ADP)–ribosylation of α_s and the α chain of retinal transducin, a covalent modification that inhibits GTPase activity of both proteins (*3*). The amino acid sequence in regions surrounding both these codons (Table 1) is conserved from unicellular eukaryotes to mammals, further supporting the inference that the two amino acids play conserved GTPase-catalyzing roles in all G proteins.

The second inference is that α chains of other G proteins, like α_s, are protooncogene proteins—that is, that they mediate signaling pathways coupling external stimuli to stimulation of proliferation. This inference is based on evidence that certain hormones and neurotransmitters promote proliferation via receptors that interact with G proteins and thereby trigger signaling pathways that promote proliferation, including activation of phospholipases C and A_2 (*4*).

This second inference dictates a broad-based search for α chain mutations, because current information does not point to specific cells or tissues in which a specific G protein (other than G_s) is known to mediate a proliferative stimulus. This contrasts with the initial search for *gsp* mutations, which was prompted by abnormally elevated adenylyl cyclase activity and GH secretion in a subset of GH-secreting pituitary tumors (*5*). In normal pituitary somatotrophs, G_s cou-

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ples the receptor for GH-releasing hormone (GHRH) to stimulation of adenylyl cyclase, cyclic AMP (adenosine-3′,5′-monophosphates) production, GH secretion, and cell proliferation (*6*). Thus in the case of *gsp*, the cell type, the G protein, and the normal proliferative signals were known before the oncogenic mutations were discovered.

Conversely, identification of tumors carrying oncogenic mutations in other α chains point to specific types of normal cells in which a particular G protein mediates a signaling pathway leading to proliferation. We therefore initiated a program aimed at identifying presumptive GTPase-inhibiting mutations in genes for α chains of the three known G_i proteins, as well as G_o, G_z (*7*), and four other α chains identified from cDNAs (*8*). Because mutations in each gene must be sought in a large number of different human tumors, we surveyed tumors for one α chain gene at a time and used a strategy based on previous searches for point mutations in *ras* genes (*9*). The polymerase chain reaction (PCR) (*10*) was used to amplify a specific region of genomic DNA, and allele-specific oligonucleotides were hybridized at high stringency to detect point mutations in the amplified product (Fig. 1). Allele-specific hybridization can detect point mutations only in samples in which substantial expansion of a mutant clone has occurred. Because clonal expansion relative to normal cells is a clear indication of neoplastic behavior, this method is particularly well suited for detection of mutant genes that are responsible for abnormal proliferation.

We chose the α chain of G_{i2} (α_{i2}) as the first target of this screening procedure because the coding sequence and intron between the two codons to be tested, that for Arg^{179} and that for Glu^{205} (Table 1), is short enough (*11*) to allow PCR amplification of a single genomic DNA fragment containing both codons. For each tumor, a single PCR reaction was therefore used to generate substrate for hybridization with allele-specific oligonucleotides. We analyzed genomic DNA from 258 human tumors.

Mutations in the Arg^{179} codon of α_{i2} were detected in three different endocrine tumor types—in 3 of 11 tumors of the adrenal cortex and 3 of 10 endocrine tumors of the ovary (2 granulosa tumors and 1 thecoma) (Table 2). No mutations were found in the Gln^{205} codon. The high frequency of mutations in the Arg^{179} codon in tumors of these two cell types suggests that the mutations converted the α_{i2} gene into an oncogene, referred to as *gip*2 (for G_i protein-2). According to this interpretation, Arg^{179} mutations would activate α_{i2} by inhibiting its GTPase activity; this implication has not yet been tested.

The amino acid residues that replaced Arg^{179} in α_{i2} (Table 1)—cysteine and histidine—were the same as those that replaced the cognate Arg^{201} in *gsp* oncogene products found in pituitary tumors (*2*). Of the six possible missense mutations that can result from single-base changes in these codons of α_s and α_{i2}, these mutant proteins may be more biologically active. It may also be significant that both cysteine and histidine mutations are the result of G to A transitions, suggesting that a preferred mechanism of DNA mutation might contribute to the predominance of these two substitutions.

In one tumor of the adrenal cortex, we did not detect a normal allele of α_{i2} (Fig. 1A). Sequence analysis of PCR products revealed a single sequence, corresponding to the Arg^{179} mutation (Fig. 1B). This suggests that the normal allele is absent, because of a deletion event or gene conversion. Loss of the normal allele might imply that its protein product interferes with the oncogenic effect of the mutant protein, so that failure to express normal α_{i2} confers an additional selective advantage on cells carrying an activating α_{i2} mutation in the other allele (*12*).

We also sought *gsp* mutations in human tumors, both to determine their frequency in GH-secreting tumors of the pituitary and to explore the possibility that constitutive activation of α_s may contribute to oncogenesis in other cell types where cyclic AMP may act as a mitogen. A region of the α_s gene including codons 201 and 227 and an intervening intron (*13*) was amplified from tumor genomic DNA. We analyzed genomic DNA from more than 300 tumors.

Mutations in the α_s gene were detected only in GH-secreting tumors of the pituitary gland and in a single thyroid tumor (Table 2). Among 42 GH-secreting pituitary tumors, 18 (43 percent) contained α_s mutations. Of these, 16 mutations were in codon 201 (14 arginine to cysteine, 2 arginine to histidine) and 2 were in codon 227 (both glutamine to arginine). In addition, of 36 thyroid tumors tested, one contained a point mutation in α_s codon 227 (glutamine to histidine); the positive tumor was one of four autonomously functioning adenomas (so-called hot nodules) tested.

We looked for α_s mutations in white blood cells of two patients whose pituitary tumors contained α_s point mutations, and in

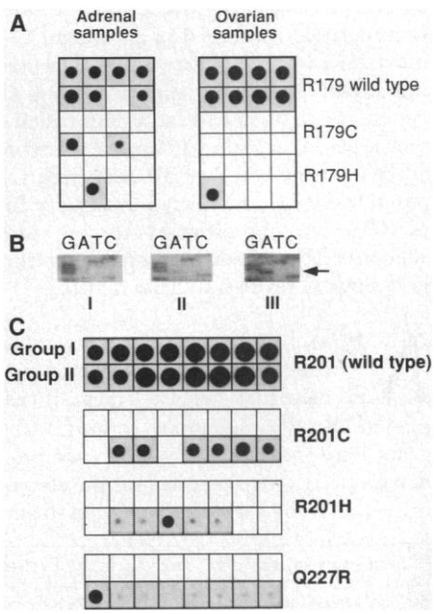

Fig. 1. Point mutations in PCR-amplified DNA encoding α_{i2} (A and B) and α_s (C). (**A**) Autoradiograms of dot blots of α_{i2} DNA amplified from eight human adrenocortical tumors (left) and eight ovarian granulosa cell tumors (right), probed with each of three different allele-specific oligonucleotides differing at codon R179 (*33*); these include wild type (two upper rows), R179C (two middle rows), and R179H (C, Cys; H, His; and R, Arg) (two lower rows). The adrenal tumor lacking a wild-type allele was an adenocarcinoma; the other two adrenal tumors with R179 mutations were adenomas. (**B**) Autoradiograms of gels produced by sequencing the noncoding strands of α_{i2} DNA amplified from three different samples. Each gel shows the noncoding sequence for codon R179, in which the arrow (left) points to the third base of this codon (reading 5′ to 3′ from bottom to top). Sample I shows the noncoding wild-type sequence for the R179 codon (GCG, reading 5′ to 3′, bottom to top); this sample was obtained from histologically normal tissue adjacent to the adrenocortical adenoma used to provide the sequence shown in sample II. Sample II shows both A and G in the position of the third base, thus confirming the presence of both Arg (wild type) and Cys (noncoding sequence GCA) alleles at codon for residue at 179, as the dot blot had indicated. The sequence from sample III confirms the conclusion obtained from a dot blot of DNA from an adrenocortical carcinoma (see above): that is, the presence of a R179C allele (noncoding sequence GCA) without a corresponding wild-type allele (no G in the position corresponding to the third base). (**C**) Autoradiograms of dot blots of α_s DNA from 16 biochemically characterized human GH-secreting pituitary tumors probed with each of four different allele-specific oligonucleotides (wild type, or R201; R201C, R201H, or Q227R) (Q, Gln) (*32*). Group I tumors had low basal adenylyl cyclase activity that responded normally to stimulatory agents; group II tumors had marked elevation of basal adenylyl cyclase activity that responded poorly to stimulatory agents (*5*). A region containing the indicated codons of the α_s or α_{i2} gene was amplified by PCR (*10*) from genomic DNA isolated from either fresh frozen tissue (*9*) or paraffin-embedded tissue (*32*). Point mutations were detected with high-stringency hybridization of allele-specific oligonucleotides to the amplified product (*10, 33*). The DNA sequence was obtained by direct sequencing of the PCR product (*11*).

normal thyroid tissue surrounding the thyroid nodule that contained an α_s mutation. None of these samples contained a mutant α_s gene, whether assessed by sequencing cDNA clones (*2*) or by allele-specific oligonucleotide analysis. These results indicate that the mutations are somatic, and thus are likely to have played a direct causal role in development of the tumors. Furthermore, a normal α_s allele was present in all tumors where a mutant allele was detected, suggesting that mutations that activate G_s are dominant.

Of the 42 GH-secreting pituitary tumors studied, 16 were biochemically characterized on the basis of adenylyl cyclase activity. Eight tumors showing elevated adenylyl cyclase were predicted to harbor an activated α_s; each of these tumors contained a mutation in codon 201 or codon 227 (Fig. 1C). No mutations were detected in eight tumors that showed normal adenylyl cyclase activity. Although α_s mutations in other codons may also inhibit GTPase, the strong concordance between elevated adenylyl cyclase activity and a mutation in codon 201 or codon 227 indicates that activating mutations at other sites are relatively infrequent.

Thyrotropin (TSH) requires cyclic AMP as a second messenger to stimulate proliferation of thyroid cells (*14*). Thus, the α_s mutation found in a thyroid tumor (Table 2) conforms with the possibility that *gsp* mutations would be found not only in tumors derived from pituitary somatotrophs, but also in tumors from other cell types in which cyclic AMP acts as a mitogen. No *gsp* mutations, however, were found in 16 melanomas, six ovarian granulosa cell tumors and nine tumors of the adrenal cortex, despite the ability of cyclic AMP and hormones that activate adenylyl cyclase to stimulate proliferation of melanocytes (*14*), and (under certain circumstances) of steroid-producing endocrine cells (*15*).

The restricted distribution of *gsp* and *gip*2 oncogenes among specific endocrine target cells raises several questions. Why do we find *gip*2, but not *gsp*, mutations in tumors of the adrenal cortex and ovarian granulosa cells, even though peptide hormones capable of stimulating adenylyl cyclase can under certain circumstances promote proliferation of the cells from which these tumors arose? Do these trophic hormones exert their proliferative effects via α_{i2}? Do other hormones promote proliferation of these cells via α_{i2}?

Data on the proliferative behavior of cultured cells from adrenal cortex or ovarian granulosa cells do not yet provide firm answers. Thus, corticotropin (ACTH) stimulates cortisol secretion via G_s, adenylyl cyclase, and cyclic AMP; nonetheless, ACTH and cyclic AMP analogs induce G_1 arrest of adrenal cortical cells in primary cultures derived from bovine adrenals (*16*). Similarly, although lutropin (LH) utilizes G_s and cyclic AMP to promote secretion of sex steroids from ovarian granulosa cells, it inhibits proliferation of these cells in culture (*17*). Taken together, these results suggest that ACTH and LH do not utilize G_s and cyclic AMP to stimulate proliferation of their respective target cells; consequently tumors derived from these cells should not harbor the *gsp* oncogene (Table 2).

G_i proteins inhibit adenylyl cyclase (*18*), mediate muscarinic regulation of K^+ channels (*19*), and have been implicated in several pathways that promote cell proliferation. For example, pertussis toxin treatment—which uncouples G_i proteins from interacting with receptors (*20*)—blocks mitogenic effects of lysophosphatides and serotonin (*21*). However, no direct evidence specifically implicates G_{i2} or any other G_i protein as a regulator of proliferation in any specific cell type or tumor.

Table 1. Conservation of sequences among G protein α chains. Stretches of α_s sequence surrounding the Arg^{201} and Glu^{227} codons are highly conserved in G protein α chains of vertebrates, yeast, and slime mold. Sequences include rat α_s, α_{i1}, α_{i2}, α_{i3}, and α_o, and human α_z (*7*), α_t of bovine retinal rod cells (*29*), the α chain of the G protein (called GPA1 or SCG1) that mediates pheromone signaling in *Saccharomyces cerevisiae* (*30*), and an α chain (Gα1) from *Dictyostelium discoideum* (*31*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

	201										227										
	D	**L**	**L**	**R**	**C**	*R*	**V**	**L**	**T**	**S (205)**	**F**	**D**	**V**	**G**	**G**	**Q**	**R**	**D**	**E**	**R**	**R (232)**
α_s																					
α_{i1}	—	V	—	—	T	—	—	K	—	T (182)	—	—	—	—	—	—	—	S	—	—	K (209)
α_{i2}	—	V	—	—	T	—	—	K	—	T (183)	—	—	—	—	—	—	—	S	—	—	K (210)
α_{i3}	—	V	—	—	T	—	—	K	—	T (182)	—	—	—	—	—	—	—	S	—	—	K (209)
α_o	—	I	—	—	T	—	—	K	—	T (183)	—	—	—	—	—	—	—	S	—	—	K (210)
α_z	—	I	—	—	S	—	D	M	—	T (183)	V	—	—	—	—	—	—	S	—	—	K (210)
α_t	—	V	—	—	S	—	—	K	—	T (177)	—	—	—	—	—	—	—	S	—	—	K (205)
GPA1/SCG1	—	I	—	K	G	—	I	K	—	T (301)	L	—	A	—	—	—	—	S	—	—	K (328)
Gα1	—	V	—	—	S	—	T	K	—	T (184)	V	—	—	—	—	—	—	S	—	—	K (211)

Table 2. Summary of human tumors screened for mutations in codons 179 and 205 for α_{i2} and codons 201 and 227 for α_s. DNA for PCR amplification (*10*) was isolated from either fresh tissue (*9*) or paraffin-embedded tissue (*32*). Three ovarian sex cord stromal tumors (two granulosa cell tumors, one thecoma) and three adrenal cortical tumors (two adenomas and one carcinoma) contained a mutation in α_{i2} codon 179. Eighteen GH-secreting pituitary adenomas contained a mutation in α_s codons 201 or 227.

Tumor type	α_{i2}		α_s	
	Tested	Mutation	Tested	Mutation
Pituitary adenoma				
GH	4	0	42	18 (43%)
Prolactin	7	0	12	0
TSH	1	0	2	0
ACTH	2	0	7	0
Nonsecretor	2	0	3	0
Ovarian sex cord stromal tumors*	10	3 (30%)	6	0
Adrenal cortical tumor†	11	3 (27%)	9	0
Thyroid tumor‡	24	0	25	1 (4%)
Melanoma	16	0	16	0
Glioblastoma	29	0	29	0
Ovarian adenocarcinoma	10	0	8	0
Gastric adenocarcinoma	14	0	13	0
Renal cell carcinoma	17	0	17	0
Breast adenocarcinoma	25	0	30	0
Bladder transitional cell carcinoma	9	0	8	0
Pancreatic adenocarcinoma			12	0
Acute myelogenous leukemia			8	0
Squamous cell carcinoma	33	0	33	0
Colonic adenocarcinoma	20	0		0
Hepatoma	12	0	15	0
Prostatic tumor§	12	0	12	0
Total	258		309	

*Granulosa cell tumors (seven), thecomas (two), and androblastoma (one). †Adenoma (five for α_s, and five for α_{i2}); carcinoma (four for α_s and six for α_{i2}). ‡Papillary carcinoma, four; follicular carcinoma, four; follicular adenoma, two; Hürthle cell carcinoma (three for α_s and one for α_i); Hürthle cell adenoma (six for α_s and none for α_i); multinodular goiter, four; autonomously functioning nodules (four for α_s and none for α_t); and undifferentiated carcinoma, one. §Benign hyperplasia, six; adenocarcinoma, six.

The possibility that one or more physiologically relevant mitogens for adrenal cortical or ovarian granulosa cells does act via α_{i2} is consistent with available evidence. Angiotensin and fibroblast growth factor (FGF) stimulate thymidine incorporation and cell division of adrenal cortical cells in primary culture (*16, 22*). Of these, angiotensin appears more likely to act via α_{i2}, because many of its effects are mediated by G proteins (*23*). This possibility can be easily tested: Inhibition of hormone action by pertussis toxin depends on the toxin's ability to ADP-ribosylate α chains of G_i proteins, including α_{i2} (*18, 20*); if angiotensin's proliferative action on adrenal cortical cells is mediated by α_{i2}, it should be blocked by pertussis toxin. Proliferation of ovarian granulosa cells is stimulated by FGF and epidermal growth factor (EGF) (*24*). Although most mitogenic effects of FGF (*25*) and EGF (*26*) are mediated by receptor tyrosine kinases rather than by G proteins, pertussis toxin has been found in a few instances to block nonmitogenic effects of both hormones (*27*), suggesting the possible existence of G protein–linked receptors for these growth factors—receptors that might deliver a proliferative signal not mediated by phosphorylation of tyrosine residues.

Abundant evidence indicates that ACTH, LH, and TSH can trigger the generation of second messengers other than cyclic AMP, including products of the Ca^{2+}-phosphoinositide cascade (*28*). By the same token, these and other cyclic AMP–elevating hormones could stimulate cell proliferation by G_{i2} or another G protein distinct from G_s (and, probably, via a different receptor as well). Such a second pathway could mediate the proliferation of adrenal cortical cells that follows prolonged elevation of ACTH in vivo (*15*).

Although the *gip2* and *gsp* mutations raise many questions, they also provide clear signposts for exploring the diverse mix of signaling pathways that mediate regulation of proliferation and differentiated function in endocrine target cells. The occurrence of *gsp* and *gip2* mutations in a restricted subset of human tumors follows a pattern rather different from that of several well-established oncogenes, such as *ras* and *myc*, that are found in various tumors. This difference presumably reflects the fact that G proteins usually mediate hormonal regulation of cell functions other than proliferation. Other α chain genes may be subject to oncogenic mutation. Each such mutation would presumably appear in tumors derived from a specific subset of cells, presumably those in which the corresponding normal G protein participates in a signaling pathway that promotes proliferation.

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10. Nested amplification primers were used in the PCR to improve specificity and yield [K. Mullis *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **51**, 263 (1986)]. Genomic DNA (100 to 500 ng of DNA from fresh tissue or 10 μl of the DNA solution from paraffin-embedded tissue) was first amplified with 30 pmol of the outer primers (GCGCTGTGAACACCCCACGTGTCT and CGCAGGGGGTGGGCGGTCACTCCA for α_s; CCCCCCATCCCCAGCTACCT and TCTCACCATCTCCTCGTCCTC for α_{i2}) in 100 μl of 0.1 mM dNTP's (N, any nucleoside), 50 mM KCl, 20 mM tris, pH 8.3, 2.5 mM $MgCl_2$, BSA (100 mg/ml), and Taq polymerase (1.5 units) (Perkin-Elmer Cetus). The amplification program was as follows: 5 min at 95°C, 30 cycles of 1 min at 95°C, 2 min at 50°C, and 2 min at 72°C. A second amplification reaction with 30 pmol of the inner primers (GTGATCAGCAGGCTGACTATGTG and GCTGCTGGCCACCACGAAGATGAT for α_s; ATTGCACAGAGTGACTACATCCCC and GGCGCTCAAGGCTACGCAGAA for α_{i2}) was done with 2 μl of the initial amplification mixture under the same dNTP and buffer conditions as above and 0.5 unit of Taq polymerase. The program for the second amplification reaction was: 30 s at 94°C, 40 s at 57°C, and 45 s at 72°C. A portion (5 μl) of the final product was sized on a 2% Nusieve and 1% Seakem agarose gel and visualized by ethidium bromide (EtBr) staining. A 526-bp fragment was obtained for α_s and a 504-bp fragment for α_{i2}.
11. For direct sequencing of PCR product, a single band of appropriate size was excised from EtBr-stained agarose gel under ultraviolet (UV) light. The excised band was placed into a Costar spin-x 0.22-μm cellulose acetate filter unit, frozen at −70°C for 15 min, and centrifuged (Eppendorf microfuge) for 15 min at full speed. The DNA-containing eluate was transferred to a microcentrifuge tube, 20 μg of glycogen was added, and DNA was precipitated with 0.2 volumes of 3 M sodium acetate and 0.3 volumes of isopropanol. DNA was sedimented (microcentrifuge), washed with 70% ethanol, dried at reduced pressure, and resuspended in 20 μl of double-distilled water. The sample was sequenced according to the Sequenase double-stranded protocol with 7.75 μl of DNA solution and 2.5 pmol of sequencing primer.
12. In this context, it may be significant that pituitary tumors containing the putative *gsp* oncogene were reported (*2*) to contain more mRNA encoding the mutant α_s allele than mRNA expressed from the wild-type α_s allele. It is, therefore, not yet clear whether activating mutations in G protein α chains are truly dominant, or whether they require some reduction in expression of the wild-type gene to manifest their effects.
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32. For isolation of genomic DNA from paraffin-embedded tissue [D. K. Wright and M. M. Manos, in *PCR Protocols: A Guide to Methods and Applications*, M. Innis, D. Gelfand, J. Sninsky, T. White, Eds. (Academic Press, San Diego, 1990), pp. 153–158] three to five adjacent 5-μm sections were cut from paraffin blocks and mounted on glass slides. One slide was stained with hematoxylin and eosin and used as a guide to select a region on the other slides composed entirely of tumor. With a razor blade, excess paraffin and unwanted tissue were removed from the unstained slides, and the remaining tumor tissue was scraped into a sterile 1.5-ml microcentrifuge tube. To remove contaminating paraffin, the sample was incubated with 5 μl of octane (anhydrous, Aldrich) or Hemo-De (Fischer) at room temperature for 30 min with shaking. The tissue sample was centrifuged (5 min, 1000*g*), and the supernatant was discarded. The tissue sample was extracted twice with 500 μl of absolute ethanol and dried at reduced pressure. The tissue was treated with proteinase K (0.2 mg/ml) in 100 μl of digestion buffer (50 mM tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20) at 37°C overnight, the digest was

centrifuged to remove undigested debris, and the DNA-containing supernatant fraction was incubated at 95°C for 8 min.

33. Nylon filters (Pall Biodyne-B, 0.45 μm) were briefly rinsed in water and mounted on a Bio-Rad dot-blot apparatus. A sample (4 μl) of each final amplification product was denatured in 0.4 M NaOH and 25 mM EDTA for 5 min and spotted on the filter. The DNA was cross-linked to the filter with a Stratalinker (Stratagene) set at auto cross-link. The filters were prehybridized in 5× SSPE and 0.5% SDS at 50°C for 30 min; a ^{32}P end-labeled oligonucleotide (1 ng) was added to this prehybridization solution, and the whole reaction was incubated at 50°C for 45 to 60 min. The hybridized filters were washed briefly in 2× SSPE and 0.1% SDS at room temperature, and then incubated for 10 min in 3 M tetramethyl ammonium chloride, 0.2% SDS, and 50 mM tris (pH 8.0), at the following temperatures: for α_s codon 201, 64.5°C; α_s codon 227, 67°C; α_{i2} codon 179, 61.5°C; α_{i2} codon 205, 67.5°C. The filters were exposed to Kodak X-AR film for 2 to 6 hours at −70°C with intensifying screens. For subsequent hybridization with different oligonucleotides the Nylon filters were stripped by boiling the filters for 5 min in 2× SSPE and 0.1% SDS and then processed as described above. Oligonucleotide probes used to screen the tumors were specific for wild type and for each missense or nonsense single-base change at the two codons; base changes that would be silent were not tested. The probe sequences (with the wild-type codon in bold type) were as follows: α_s codon 201, TTCGCTGC**CGT**GTCCTGACT (six mutant codons tested); α_s codon 227, GTGGGTGGC**CAG**CGCGATGA (eight mutant codons tested); α_{i2} codon 179, TACGGACC**CG**CGTAAAGACC (six mutant codons tested); α_{i2} codon 205, GTGGGTGGT**CAG**CGGTCTGA (eight mutant codons tested).

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Metalloantibodies

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A metalloantibody has been constructed with a coordination site for metals in the antigen binding pocket. The Zn(II) binding site from carbonic anhydrase B was used as a model. Three histidine residues have been placed in the light chain complementarity determining regions of a single chain antibody molecule. In contrast to the native protein, the mutant displayed metal-dependent fluorescence-quenching behavior. This response was interpreted as evidence for metal binding in the three-histidine site with relative affinities in the order Cu(II) > Zn(II) > Cd(II). The presence of metal cofactors in immunoglobulins should facilitate antibody catalysis of redox and hydrolytic reactions.

CATALYTIC ANTIBODIES HAVE NOW been induced with haptens that are designed to use a number of different strategies for effecting catalysis [for reviews, see (*1, 2*)]. Distortion, charge stabilization, and proximity have all been used to advantage. Antibody side chains in the binding pocket can participate directly in some of the reactions. Binding sites modified covalently and by site-directed mutagenesis have also yielded increases in catalytic rate. Finally, metal complexes have been enlisted to effect catalysis of simultaneously bound substrates for specific hydrolysis of the peptide bond.

The advent of facile systems for the recombination and expression of the antibody repertoire in bacteriophage λ allows consideration of a new approach for utilizing antibody-bound cofactors (*3–6*). In this system a cofactor binding light chain could be combined with a heavy chain library derived from the polymerase chain reaction (PCR) products of a mouse previously immunized with a substrate or substrate analog of interest. Ideally, significant binding of substrate can be maintained by the heavy chain (*3, 4*), and a few combinations will be produced in which the substrate is bound in a suitable position to facilitate reaction with the cofactor. In this approach the diversity of the antibody repertoire is used to enhance a catalytic design. Successful implementation of this strategy requires the production of antibodies that present cofactors in proximity to bound antigen. We report the production of an antibody that simultaneously binds the reporter antigen fluorescein and directly coordinates metal ions such as Cu(II).

The general design strategies for remodeling antibodies is the subject of a separate report (*7*). Briefly, for the work described here, a computer search was conducted on the known catalytic metal binding sites in metalloenzymes, and the backbone configurations around these sites were compared with those of the complementarity determining regions (CDRs) in antibody light chains. A striking similarity was discovered between the antiparallel β-sheet structure around the three His ligand residues of the zinc binding site of carbonic anhydrase B (*8*) and the β-sheet structure around residues 34, 89, and 91 [numbering of Kabat *et al.* (*9*)], in CDRs L1 and L3 of antibody light chains (Fig. 1, A and B). Substitution of these three antibody residues with His should form a binding site that resembles that of the natural metalloenzyme (Fig. 1B) (*10*). Furthermore, a bound metal will be located deep in the groove of the binding site (see Fig. 1C) with the metal protruding slightly toward the heavy chain. This should make the vacant coordination site on the metal atom highly accessible to substrate. Since the β-sheet structure in this region is rigorously conserved among the antibodies of known structure (*11, 12*), our design should be generally applicable to antibody light chains.

The single chain fluorescein binding molecule (referred to here as native sequence protein) (*13*) with the 212 linker derived from the antibody 4-4-20 was chosen for our metal binding studies for several reasons. First, the three-dimensional structure of the parent antibody combining site with bound fluorescein is known (*14*). Second, the gene has been cloned into a highly efficient expression vector (*13*), which is readily amenable to site-directed mutagenesis. Third, the six Trp residues in the molecule surround the new metal binding site, which suggests that bound metals such as Cu(II) would effectively quench Trp fluorescence (Fig. 1D). Model building indicated metal to nearest atom distances for five Trp residues of 5 to 10 Å and for one Trp residue of 15 Å. Finally, bound fluorescein was expected to be useful as a spectroscopic probe for locating bound metal in the properly folded antibody combining site (Fig. 1D).

Histidine residues were introduced into L1 at Arg^{34} and L3 at Ser^{89} and Ser^{91}. Since Ser^{91} and Arg^{34} form hydrogen bonds to the bound fluorescein (*14*), the fluorescein binding constant should be lower for the mutant protein, but should still be measurable because of the high binding constant for the native sequence protein (*13*). Inspection of

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