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Identification of the lesion in the stimulatory GTP-binding protein of the uncoupled S49 lymphoma

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The stimulatory GTP-binding protein (G_s) of the uncoupled mutant of S49 lymphoma cells is deficient in its ability to transduce hormonal signals from ligand-bound β-adrenergic receptors to the catalytic component of adenylate cyclase. In order to define the genetic defect in the G_s of uncoupled S49 cells, a complementary DNA clone encoding the α-subunit of G_s was analyzed and the deduced primary structure of the defective subunit compared to that of the wild-type subunit. A single nucleotide transversion was found that coded for a proline rather than an arginine at residue 389. The results indicate a domain of the α-subunit of G_s that specifically interacts with hormone receptors.

G-protein; Adenylate cyclase; Uncoupled mutant; (S49 lymphoma)

1. INTRODUCTION

Incubation of S49 cells in the presence of terbutaline and cAMP phosphodiesterase inhibitors allows for the selection of mutants of S49 lymphoma cells lacking the stimulatory form of G-proteins [1]. The cyc^- mutant [2] and uncoupled mutant [3] have been invaluable tools for elucidating the functional relationships among G-protein subunits and adenylate cyclase. Reconstitution in S49 cyc^- cell membranes of purified protein components has provided the experimental basis for a model of G-protein-mediated regulation of intracellular cAMP levels [4].

The uncoupled mutant cell has been of biochemical interest because it does not increase production of cAMP in response to β-adrenergic agonists, but is responsive to the presence of cholera toxin, GTP and NaF. This suggests that the α-subunit of G_s is present and capable of stimulating the adenylate cyclase catalyst, but it is no longer capable of transducing the neurohormonal signal via the β-receptor; hence it is uncoupled from the receptor. On two-dimensional isoelectric focusing gels, the G_s-protein of S49 uncoupled cells migrates with an isoelectric point 0.1 pH unit less than wild-type G_s [5]. This charge difference may be the result of a single amino acid change or of a covalent post-translational modification. The latter possibility was eliminated [6] by fusing S49 uncoupled cells to S49 cyc^- cells which do not produce the α-subunit of G_s [7,8]. The resulting heterokaryon behaved phenotypically as the parent S49 uncoupled cell. This result argues against the loss of a post-translational modification mechanism as the lesion in the G_s α-subunit of S49 uncoupled mutant.

To determine if the lesion in the G_s of uncoupled cells is due to an amino acid substitution in its α-subunit, a cDNA library was constructed from mRNA of these cells and a clone having the entire

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coding region of the G_s α-subunit was sequenced. The primary structure of the α-subunit of G_s from uncoupled S49 cells was determined and compared to that of murine, bovine and human α-subunits.

2. MATERIALS AND METHODS

2.1. *RNA analysis*

Total RNA was purified from S49 mutant lymphoma cells using a CsCl cushion [9]. For Northern analysis, 10 μg of total RNA was denatured with glyoxal and resolved through a 1.5% agarose gel prior to transfer to a nitrocellulose membrane [10]. The blot was hybridized to a labeled single-stranded cDNA probe [11] corresponding to an internal *Eco*RI-*Bam*HI fragment of the bovine brain cDNA encoding $G_s\alpha$ [8]. Hybridization was performed in the presence of 50% formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate, 100 μg/ml salmon sperm DNA and 1% SDS. The blot was washed in 2 × SSC with 1% SDS for 30 min at room temperature followed by a 1 h wash in 0.1 × SSC with 1% SDS at 62°C. The hybridization signals were obtained after overnight exposure to Kodak XAR-5 film.

2.2. *DNA analysis*

High-M_r DNA was prepared from S49 cell mutants essentially by the method of Blin and Stafford [12]. Purified DNA was restricted with commercially available restriction endonucleases, resolved through a 0.8% agarose gel and transferred to nitrocellulose [13]. The DNA blot was hybridized to the same probe and conditions described for the Northern blot. The Southern blot was washed in 2 × SSC with 1% SDS for 30 min at room temperature followed by a 3 h wash in 0.5 × SSC with 1% SDS at 60°C. The radiographic images were obtained after a 3 day exposure to DuPont Cronex film.

2.3. *Cloning and sequence analysis*

A complementary DNA library was constructed from S49 uncoupled cell mRNA using the eukaryotic expression vector pcDV [14]. The cDNA library was prepared for hybridization using standard procedures [15] and screened with a uniformly labeled single-stranded probe prepared from a restriction fragment corresponding to the 5′-end of a human cDNA clone encoding $G_s\alpha$. One clone was obtained (pUNC-6) that produced a restriction endonuclease pattern characteristic of $G_s\alpha$ cDNA clones of human and bovine origin. Fragments of the pUNC-6 cDNA were subcloned into the sequencing vectors M13mp18 and M13mp19 and sequenced on both DNA strands with a commercially available universal primer by the dideoxynucleotide terminator method [16]. DNA sequence data were compiled and analyzed with the computer program of Queen and Korn [17].

3. RESULTS

3.1. *Expression of $G_s\alpha$ message in S49 cells*

Northern analysis of total RNA from S49 mutant and wild-type cells is shown in fig.1. There is a single species of mRNA observed in both wild-type RNA and in the RNA from the uncoupled mutant that hybridized to the probe with equal intensity. This result indicates two important characteristics of the S49 uncoupled mutant. First, the presence of an mRNA in an amount equivalent to that observed in wild-type cells suggests that a mutational defect has not altered the rate of transcription of the $G_s\alpha$ gene in uncoupled cells, thus the promoter and regulatory elements of the gene are probably unaltered. Analysis of the G_s-protein in extracts of wild-type and uncoupled S49 cells indicates a protein of identical size that is present in approximately equivalent amounts in membranes [5] suggesting that the translational start and stop codons in the gene are not mutated. The second informative aspect is that the $G_s\alpha$ mRNA that accumulates in uncoupled cells is essentially the same size (1900 bases) as that of wild-type cells. Because there is no species of mRNA that accumulates as a larger or smaller messenger, it is probable that mRNA-processing enzymes and the splice junctions of the $G_s\alpha$ gene are intact in the uncoupled mutant.

The RNA of S49 cyc^- cells indicated by the Northern analysis in fig.1 is quite different from S49 uncoupled cells. The absence of a detectable hybridization signal [8] suggests that the gene encoding $G_s\alpha$ is not transcribed in these cells or that the mRNA is unable to accumulate to levels detectable in hybridization to total RNA. Like the uncoupled cell, $G_s\alpha$ mRNA does not accumulate in

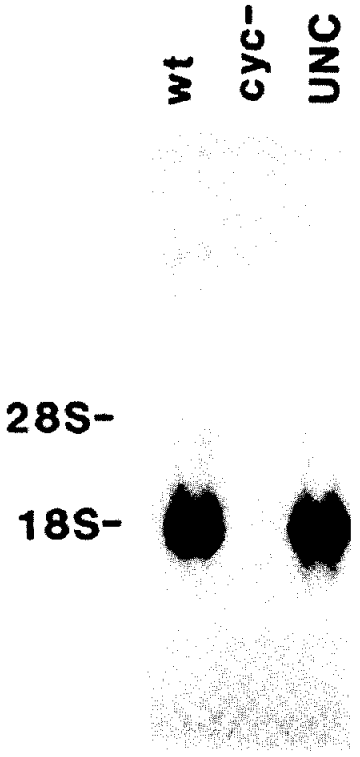

Fig.1. Analysis of $G_s\alpha$ mRNA expression in S49 lymphoma cells. Total RNAs (10 μg) from wild-type (wt), G_s-deficient (cyc^-) and G_s-uncoupled (UNC) S49 cells were hybridized to a $G_s\alpha$ cDNA probe from bovine brain [8].

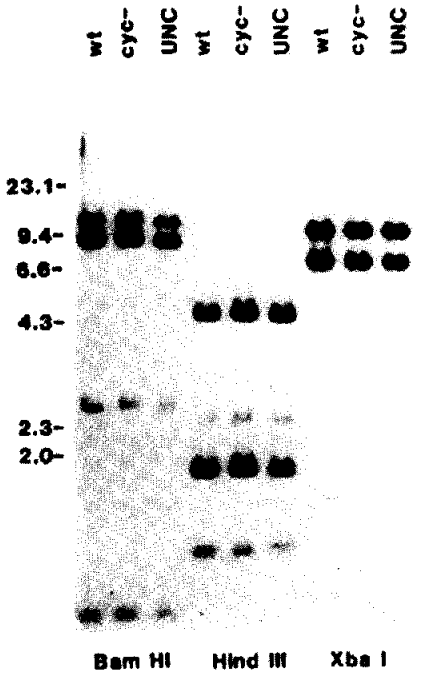

Fig.2. Organization of the $G_s\alpha$ gene in S49 lymphoma cells. Genomic DNAs (5 μg) from wild-type (wt), G_s-deficient (cyc^-) and G_s-uncoupled (UNC) S49 cells were restricted with the indicated restriction endonuclease. After the DNAs were resolved through a 0.8% agarose gel and blotted, they were hybridized to a $G_s\alpha$ cDNA probe from bovine brain [8]. Molecular mass markers (kilobase pairs) are a *Hin*dIII restriction digest of bacteriophage λ DNA.

cyc^- cells to a larger or smaller size than the wild-type $G_s\alpha$ mRNA which again argues against a compromised RNA splicing mechanism in cyc^- cells. $G_s\alpha$ produced in cyc^- cells after gene transfer can revert the insensitivity to isoproterenol indicating that the recombinant protein is processed to an active form [18]. A likely explanation for the absence of a hybridization signal in cyc^- cell RNA is that the promoter governing transcription of the $G_s\alpha$ gene no longer allows transcription to occur in these cells.

3.2. *$G_s\alpha$ gene organization in S49 mutant cells*

Restriction endonuclease fragment patterns of genomic DNA from S49 wild-type, cyc^- and uncoupled cells that hybridized to a bovine cDNA probe for $G_s\alpha$ are shown in fig.2. Regardless of the enzyme used to restrict the DNAs, an identical pattern of hybridizing fragments emerged. This result argues against a gross alteration of DNA organization in either of the S49 mutants analyzed; however, deletions or inversions of DNA within a confined domain of the gene might not be detected by an analysis with the restriction endonucleases used. The lack of restriction fragment length polymorphisms in the gene for $G_s\alpha$ of S49 uncoupled cells is not surprising because intact G_s of apparent normal molecular mass has been observed in membrane extracts of S49 uncoupled cells [5].

3.3. *S49 uncoupled $G_s\alpha$ cDNA clone analysis*

A cDNA clone encoding $G_s\alpha$ was isolated by cross-hybridization to a restriction fragment from

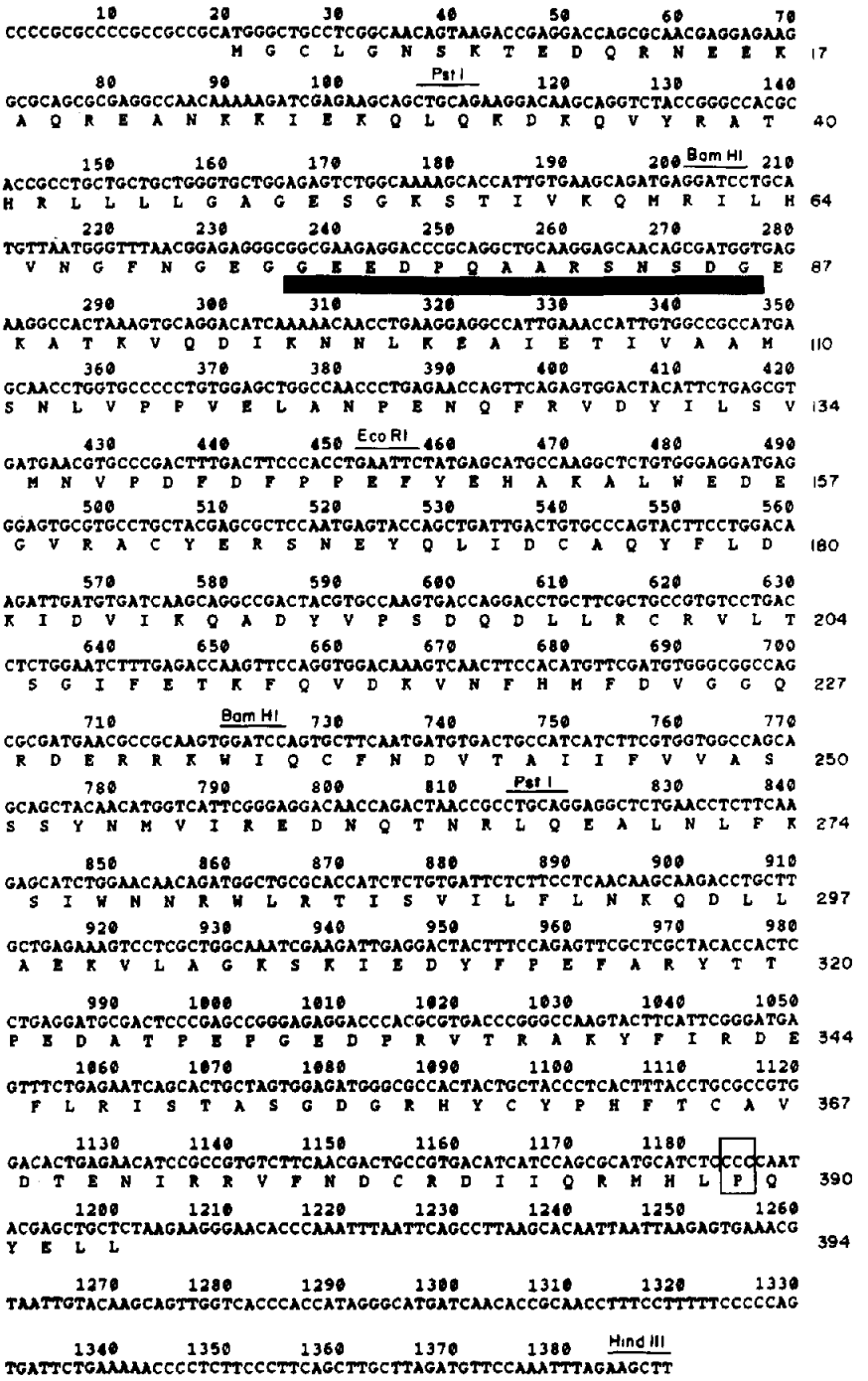

Fig.3. The S49 uncoupled $G_s\alpha$ cDNA sequence. The nucleotide sequence of the cDNA insert of pUNC-6 encoding the α-subunit of G_s is listed in the 5′ to 3′ orientation. The initiating methionine residue is located at nucleotide residue 20; the $G_s\alpha$ sequence is read to nucleotide residue 1201. The underlined sequence indicates the peptide domain that specifies the longer form of $G_s\alpha$. The boxed nucleotides indicate the mutated codon for proline at amino acid residue 389.

a human $G_s\alpha$ cDNA clone (in preparation). This clone, pUNC-6, was sequenced on both DNA strands after subcloning restriction endonuclease fragments into the vectors M13mp18 and M13mp19 [19]. The complete nucleotide sequence of the S49 uncoupled cDNA is presented in fig.3. An open reading frame is found from the initiating methionine at nucleotide residue 20 to the termination codon at residue 1204. The cDNA in pUNC-6 encodes the long form of $G_s\alpha$ due to the presence of the 14 amino acid region from residues 73 to 86 [20].

The nucleotide sequence of the S49 uncoupled cDNA for $G_s\alpha$ has more than 95% identity to bovine [21,22], rat [23], human [24] and is almost exactly identical to that of murine $G_s\alpha$ [25] except for the extreme amino-proximal domain. The first 13 amino acid residues of the published murine sequence of a cDNA encoding $G_s\alpha$ do not correlate with those of the S49 uncoupled sequence shown in fig.3 or with the bovine, rat or human sequences. Conversely, the first 14 amino acid residues of the S49 uncoupled $G_s\alpha$ sequence match exactly with the published bovine and rat sequences and differ only by one amino acid (Asn-5) with the human sequence. It is likely that the published sequence for the murine $G_s\alpha$ cDNA clone is in fact not full-length, having undergone a deletion near the 5′-end.

For our purpose of comparing the primary structures of the uncoupled G_s α-subunit and functional $G_s\alpha$ we will rely on the bovine and rat sequences for the amino-proximal domain. Because these first 14 amino acids are identical to functional forms of $G_s\alpha$, we can eliminate this region as containing the uncoupled lesion. Comparing the remainder of the S49 uncoupled $G_s\alpha$ sequence with the murine form reveals an absolute agreement in

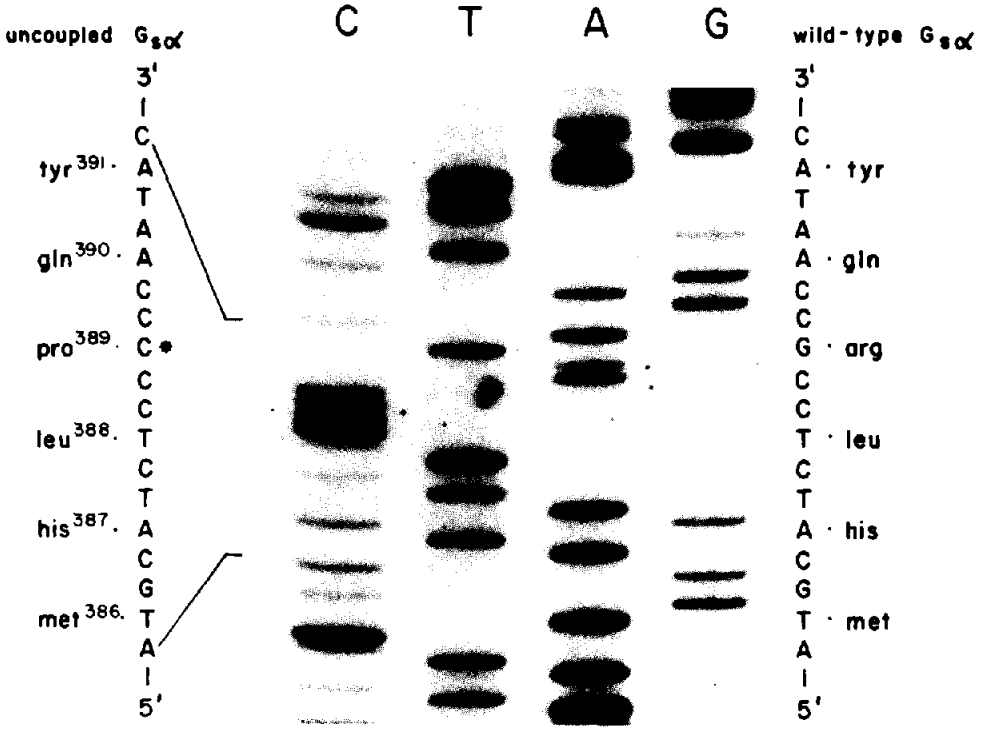

Fig.4. Identification of the G-C transversion in the S49 uncoupled $G_s\alpha$ gene. The *Bam*HI-*Hind*III restriction fragment (nucleotide residues 720–1390 in fig.3) was subcloned into the vector M13mp19. A portion of the sequence ladders obtained from this fragment encoding the carboxy-terminal region of $G_s\alpha$ is shown. On the left are listed the nucleotide sequence and inferred amino acid sequence derived from the uncoupled $G_s\alpha$ cDNA fragment. On the right is listed the murine sequence corresponding to a wild-type $G_s\alpha$ [25]. The transversion is indicated by the cytidine residue with an asterisk in the uncoupled sequence which is a guanine residue in the wild-type sequence.

sequence except for Arg-389 which is a proline in the uncoupled form of $G_s\alpha$.

That the amino acid change at residue 389 is due to a mutational event is shown in fig.4. The nucleotide sequence of the carboxy-terminal domain between Met-386 and Tyr-391 clearly indicates a series of 5 cytosine residues; nucleotides 1184–1186 code for proline. The wild-type (murine) sequence is CGC which encodes arginine.

4. CONCLUSION

The present results address the genetic basis of the nonfunctional α-subunit of the stimulatory GTP-binding protein of S49 uncoupled lymphoma cells. Preliminary evidence suggesting that the lesion in G_s is a mutation is presented in figs 1 and 2. Northern analysis of the $G_s\alpha$ message indicated an apparent normal size and amount while blot analysis of DNA failed to indicate that the gene for $G_s\alpha$ in S49 uncoupled cells was compromised. Following molecular cloning of the cDNA for the α-subunit of G_s and a comparison of the deduced primary structure of the protein with a functional version from rat, bovine and murine sources, a single amino acid substitution was identified in the uncoupled mutant protein. The substitution of an amino acid with a nonpolar side chain (proline) for an amino acid with an ionic side chain (arginine) at residue 389 can explain the isoelectric pH shift of the uncoupled $G_s\alpha$ that was observed previously [5].

The location of an amino acid substitution near the carboxy-terminus of the α-subunit of G_s suggests that this domain of the protein is directly involved in the coupling with the β-adrenergic receptor. It may be informative to determine whether the uncoupling of the mutant $G_s\alpha$ from the β-adrenergic receptor is due to the loss of an ionic interaction involving Arg-389 or the introduction of a turn in the polypeptide domain with the substitution of a proline residue. Identification of this lesion represents a further step towards a greater understanding of the molecular events of neurohormonal information transfer from cell surface receptors to adenylate cyclase.

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