Decreased transcript expression coincident with impaired glycosylation in the $\beta_2$-adrenergic receptor gene does not result from differences in the primary sequence

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Abstract

Variants of the S49 mouse lymphoma cell line exhibit multiple lesions along the pathway of cyclic AMP generation in response to $\beta_2$-adrenergic stimulation. Two such variants, $\beta^p$ and $\beta^d$, are characterized by decreased receptor binding and mRNA expression, 50% and 25% of wild-type receptor expression, respectively. The rate of $\beta_2$-adrenergic receptor synthesis was measured and found to be decreased in the $\beta^d$ cells vis-à-vis the rate in wild type cells. The molecular mass of the $\beta_2$-adrenergic receptor in the S49 wild-type, $\beta^p$ and $\beta^d$ variant cells was estimated by labeling the receptor with the photoaffinity probe [125I]iodocyanopindololdiazirine. Receptor size was found to be 67 000 and 47 000 Da in the wild-type and 60 000 and 42 000 in the two variant cells. This 6 kDa discrepancy in mass was abolished upon treatment of labeled cell extracts with N-glycosidase F, suggesting the possibility of either N-terminal truncation or altered glycosylation of the receptor in the variant cells. To distinguish between these possibilities, we sequenced the $\beta_2$-adrenergic receptor gene and two kilobases of the 5'-non-coding region. No differences were found in the coding region of the gene from wild-type, $\beta^p$ and $\beta^d$ S49 cells suggesting that both the diminished expression and the decreased size of $\beta_2$-adrenergic receptor in the $\beta^p$ and $\beta^d$ S49 variants are related to impaired glycosylation of the receptor. This hypothesis was substantiated by the reduced retention of the variant cells’ $\beta_2$-adrenergic receptor on immobilized WGA. Furthermore, growth of the S49 cells in the presence of the &-mannosidase II inhibitor, swainsonine, preferentially impaired the ability of the receptors derived from the variant cells to bind to WGA. These results imply that altered expression and glycosylation of G-protein-linked receptors occur as a consequence of one or more mutations outside the receptor’s open reading frame. ©1997 Elsevier Science B.V.

Keywords: $\beta_2$-Adrenergic receptor; cDNA; Cloning; Cyanopindolol; Glycosylation; Iodocyanopindololdiazirine; Pfu; Photoaffinity labeling; PCR; S49; Sequence; Vent; (Murine)

Abbreviations: $\beta$ARK, $\beta$-adrenergic receptor kinase; BIM, N\(^8\)-(bromoacetyl)-N-[3-(4-indolyoxy)-2-hydroxypropyl]-(2)-1.8-diamino-p-methane; DME, Dulbecco’s modified Eagle’s medium; PKA, cyclic AMP-dependent protein kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

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1. Introduction

The $\beta_2$-adrenergic receptor gene has been cloned from several species [1–3]. In each, it is encoded by a single intronless gene, 1.3 kb in length. Transcription-regulating elements are located upstream of the open reading frame [2,4]. The receptor contains seven clusters of hydrophobic amino acids, each cluster being of sufficient size to span the plasma membrane. By homology with bacterial rhodopsin [5], these clusters are postulated to span the membrane. The N-terminal of the $\beta_2$-adrenergic receptor contains two consensus sequences, Asn-X-Ser, for N-glycosylation [6,7]. That such glycosylation actually occurs has been suggested by experiment [8–11]. The core polypeptide has a molecular weight of some 47 000 Da. The C-terminal of the receptor contains consensus sequences for phosphorylation by both $\beta$-adrenergic receptor kinase ( $\beta$ARK) [5], on Ser and Thr residues, and by cyclic AMP-dependent protein kinase (PKA), on the Arg-Arg-Ser-Ser motif [12].

The $\beta_2$-adrenergic receptor, like many other hormone receptors, is dynamically regulated [13]. Regulation of responsiveness to $\beta_2$-adrenergic agonists occurs at many levels, from gene transcription to regulation of the receptor at the cell surface and coupling to the G protein [14,15].

The S49 murine T lymphoma cell line, along with variants that lack critical functions necessary for hormonal stimulation of cellular response, has provided much insight into the mechanism by which $\beta_2$-adrenergic receptors activate adenyl cyclase via $G_\alpha$ [16,17]. The S49 cell has played a seminal role in furthering research into the adrenergic-responsive adenyl cyclase system due to the isolation of multiple variants along the pathway of cyclic AMP generation and response. Two such S49 variants, $\beta^p$ and $\beta^d$, were initially characterized as expressing decreased numbers of $\beta_2$-adrenergic receptors [18]. The $\beta^p$ variant expresses approx. 50% and the $\beta^d$ variant expresses approx. 25% of the wild-type complement of $\beta_2$-adrenergic receptors [18]. This decrease in cell surface $\beta_2$-adrenergic receptor expression has been established by us to correspond to decreased receptor-specific mRNA expression in these variant cells [19].

In the current studies, we have investigated additional biochemical properties of the $\beta_2$-adrenergic receptor in S49 wild-type, $\beta^p$ and $\beta^d$ variant cells, including determination of the primary sequence of the receptor gene from the S49 wild type, $\beta^p$ and $\beta^d$ variant cells. We have documented that receptors are synthesized more slowly in the $\beta^d$ variant cells than in the wild type S49 cells and, using photoaffinity labeling, we have been able to demonstrate a decrease in the molecular mass of the $\beta_2$-adrenergic receptor in the $\beta^p$ and $\beta^d$ variant cells. This difference in mass disappears upon treatment of the labeled cell membranes with N-glycosidase F (glycopeptidase F), suggesting either truncation of the N-terminus of the $\beta_2$-adrenergic receptor in the variant cells, mutation and/or impaired glycosylation at the glycosylation motifs. By sequencing the $\beta_2$-adrenergic receptor gene plus an additional two kilobases of the 5′ non-coding region from the S49 wild-type, $\beta^p$ and $\beta^d$ variant cells we are able to demonstrate that neither of the first two possibilities occurs. Therefore, one or more mutations in other than the receptor coding sequence must be responsible both for the decreased receptor expression and the decreased receptor size.

2. Methods

2.1. Materials

$\left(-\right)^{125}_I$iodocyanopindolol and $\left(-\right)^{125}_I$iodocyanopindololdiazirine was purchased from DuPont NEN and $\left(-\right)^{125}_I$iodocyanopindololdiazirine from Amersham. BIM was a generous gift from Dr. Josef Pitha (National Institute of Aging, Baltimore, MD). Sequenase II dideoxy sequencing kit was purchased from United States and GELase from Epicenter Technologies. NuSieveGTG low melting temperature agarose was purchased from FMC BioProducts. XL1-Blue MRF’ competent bacteria and Epiˇ cement AR film was purchased from Kodak and the UV irradiation chamber from CBS Scientific. (+)-Propranolol was a gift from Ayerst, New York. Endoglycosidase H and N-glycosidase F were purchased from Boehringer Mannheim, swainsonine from Calbiochem, N-acetyl-D-glucosamine from Fluka and immobilized wheat germ agglutinin (WGA) from EY Biochimica et Biophysica Acta 1356 (1997) 281–291.
Laboratories. Oligonucleotides were purchased from Genosys and all other chemicals from Sigma.

2.2. Cell culture

S49 wild type, $\beta^p$ and $\beta^d$ cells were grown in Dulbecco’s modified Eagle’s (DME) medium supplemented with 10% horse serum in a 37°C incubator in 10% CO$_2$ in air. For some experiments, swainsonine (0.2 $\mu$g/ml) was added to the growth medium three days prior to harvesting the cells.

2.3. Photoaffinity labeling of receptors

S49 cells were harvested and washed twice in phosphate-buffered saline prior to resuspension in Dulbecco’s modified Eagle’s medium supplemented with 0.1% (w/v) bovine serum albumin and 20 mM Heps, pH 7.4. Approx. 5 x $10^7$ cells were incubated in a vol of 1 ml together with 100 pM (--)-[125]iodocyanopindololdiazirine and 10 $\mu$M phentolamine (to reduce non-specific binding) in the presence or absence of 1 $\mu$M propranolol (to define non-specific binding) for 45 minutes at 37°C in a shaking water bath. The cells were diluted with 5 ml ice-cold buffer (137 mM NaCl, 5.4 mM KCl, 1.1 mM KH$_2$PO$_4$, 1.1 mM Na$_2$PO$_4$, pH 7.2), washed at 4°C and resuspended in 2 ml of this buffer. Each batch of resuspended cells was placed in one of the 3.5 cm diameter wells of a 6 well culture plate and the plate was subjected to UV-irradiation for 10 min on ice. The cells were washed twice more in ice-cold buffer (150 mM NaCl, 20 mM Heps, 5 mM EDTA, pH 7.4) and subjected to N$_2$ cavitation in a Parr bomb. The disrupted cells were centrifuged at 200 x $g$ to remove intact cells and nuclei. The resulting supernatant was centrifuged at 35000 x $g$. The membrane pellet was resuspended in a Dounce homogenizer at a protein concentration of 1.5 mg/ml in 150 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, pH 7.4, 1.2% (w/v) digitonin, made from a 1.5% (w/v) digitonin solution prepared as described [21]. After 30 min incubation on ice, the suspension was again centrifuged at 35000 x $g$ and the supernatant retained.

The protein content of the solubilized membrane preparations was determined and equivalent amounts of protein (0.4–1 mg) were passed over columns containing 1 ml immobilized WGA. The eluent was passed over the columns two additional times to maximize $\beta_2$-adrenergic receptor binding and the columns were subsequently washed with 7 ml digitonin buffer (100 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.4, 0.05% (w/v) digitonin). Bound receptor was eluted with 5 ml digitonin buffer containing 300 mM N-acetyl-d-glucosamine.

2.4. Receptor solubilization and WGA column chromatography

Approx. 2 x $10^8$ S49 cells were collected by centrifugation, washed twice in phosphate-buffered saline, twice in 150 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, pH 7.4 then subjected to N$_2$ cavitation in a Parr bomb. The disrupted cells were centrifuged at 200 x $g$ to remove intact cells and nuclei. The resulting supernatant was centrifuged at 35000 x $g$. The membrane pellet was resuspended in a Dounce homogenizer at a protein concentration of 1.5 mg/ml in 150 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, pH 7.4, 1.2% (w/v) digitonin, made from a 1.5% (w/v) digitonin solution prepared as described [21]. After 30 min incubation on ice, the suspension was again centrifuged at 35000 x $g$ and the supernatant retained.

The protein content of the solubilized membrane preparations was determined and equivalent amounts of protein (0.4–1 mg) were passed over columns containing 1 ml immobilized WGA. The eluent was passed over the columns two additional times to maximize $\beta_2$-adrenergic receptor binding and the columns were subsequently washed with 7 ml digitonin buffer (100 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.4, 0.05% (w/v) digitonin). Bound receptor was eluted with 5 ml digitonin buffer containing 300 mM N-acetyl-d-glucosamine.

2.5. $\beta_2$-Adrenergic receptor inactivation protocol

A stock solution of BIM was prepared by diluting 20 mM BIM dissolved in 100% methanol to 10$^{-5}$ M in 100% ethanol. Cells, at a density of 5 x $10^7$/ml, were treated with 10$^{-8}$ M BIM. Following a 2-h incubation at 37°C, the cells were washed, under sterile conditions, by three successive centrifugations.
in 100 ml growth medium at room temperature. Following removal of the BIM, the cells were returned to the incubator in normal growth medium. Aliquots were withdrawn at intervals thereafter for assessment of receptor density as described below.

2.6. β2-Adrenergic receptor binding assay

Measurement of β2-adrenergic receptor was made using intact S49 cells as described previously [19]. Cells were harvested and washed twice in phosphate-buffered saline prior to resuspension in Dulbecco’s modified Eagle’s medium supplemented with 0.1% (w/v) bovine serum albumin and 20 mM Hepes, pH 7.4. Approx. 10⁶ cells were incubated with (−)-[¹²⁵I]iodocyanopindolol for 1 h in a volume of 0.25 ml at 37°C in a shaking water bath. Measurement of solubilized receptor was made in digitonin buffer in a volume of 0.5 ml for 20 h at 4°C. Non-specific binding was defined as that not competed by addition of 1 μM (±)-propranolol. Membranes were trapped on Whatman GF/C filter paper using a Brandel cell harvester. Solubilized receptor was trapped on the same filter paper pre-soaked in 10% (w/v) ac. polyethyleneimine.

2.7. Isolation of DNA

High molecular weight genomic DNA was isolated from S49 cells by the method of Herrmann and Frischhauf [22] as described previously [19].

2.8. PCR amplification of the β2-adrenergic receptor

The β2-adrenergic receptor coding sequence was amplified in overlapping segments using the thermostable DNA polymerase, Pfu. One of each primer pair was synthesized with an artificial EcoRI recognition site (denoted in italics) on its 5'-end to facilitate cloning; 5’1F GGAATTCGTAACAGCCTACGCAGTAGCC (−2115 to −2096), 5’1R AGCAGCTACAATGACACCCC (−861 to −879), 5’2F TTACTGCTTGTGCGGTCTCT (−1026 to −1006), 5’2R GGAATTCCTCGCATGCCCTCACAGCC (−415 to −430), 5’3F GGAATTCGGGTGCCGCGACGAC (−472 to −458), 5’3R AGCAAGAAGTCTGCAGTGCGT (16 to 35), 1F GGAATTCACCTGCTACCGCCA (−19 to −4), 1F’ GGTGCACGTACCCTGCTAAGCTGCCT (−28 to −6), 1R CAGAATCTCGACCCAGAAGTT (307 to 326), 2F CTGATCTAGTCTAGCTAGC (233 to 252), 2R GGAATTCGTCGATGTGCTCGGTCA (1305 to 1289). Numbering of the primers excludes the EcoRI sites and is based on the sequence of the S49 β2AR gene beginning at +1 with the A in the ATG start codon.

The PCR products were recovered by gel purification on a 1% NuSieve agarose gel, recovered using GELase, digested with EcoRI and cloned into M13mp18 for sequencing. Blue/white selection was applied to the M13 clones. Positive clones were sequenced using the Sequenase II kit. The β2-adrenergic receptor fragments were sequenced using both the Universal M13 −40 sequencing primer and sequence-specific primers.

2.9. Construction of the β2-adrenergic receptor - pBluescript construct

The two overlapping PCR products encompassing the entire β2-adrenergic receptor coding sequence were recombined by PCR. Out of several strategies that we employed, this proved to be the most successful for recombining the coding sequence fragments. 0.1 ng of each fragment, amplified using the 1F-1R and 2F-2R primers, were purified on a NuSieve 1% agarose gel, combined in a final volume of 100 ml and subjected to a single round of PCR without primers followed by an additional 24 cycles of PCR in the presence of the 1F’ and 2R primers. The following temperature parameters were employed, 1 min 95°C melt, 1 min 55°C soak, 1 min 72°C extend. The terminal extension cycle was carried out for 5 min at 72°C. The 1F’ primer, which lacks the artificial EcoRI site present in primer 1F, was used both to prevent primer dimer formation and to allow directional cloning. The S49 β2-adrenergic receptor coding sequence was ligated into pBluescript II SK +, the 5′-end into the Smal site and the 3′-end into the EcoRI site.

2.10. Quantitation

Protein was quantitated by the method of Peterson using bovine serum albumin as standard [23]. DNA and RNA were quantitated by E₂₆₀.
3. Results and discussion

The two $\beta_2$-adrenergic receptor-deficient S49 cell variants, $\beta^p$ and $\beta^d$, were first reported in 1979 [18]. We have previously extended this observation by demonstrating that the $\beta^p$ and $\beta^d$ variants also express decreased receptor mRNA levels. The decreases in receptor mRNA levels in the variant cells correlate with the decreases in receptor expression. There is no change in gene expression [19].

To determine whether the decrease in receptor mRNA level is also correlated with a decrease in the rate of receptor synthesis, wild type and $\beta^d$ cells were treated with a $\beta_2$-adrenergic receptor-specific alkylating agent, BIM (Fig. 1). Following removal of the alkylating agent, the rate of receptor recovery was measured. The initial rate of receptor recovery was slowed in the $\beta^d$ cells vis-à-vis the rate observed with the wild type cells.

Other than a decrease in the number of receptors and a corresponding decreased maximal ability of agonists to promote altered cyclic AMP generation, cellular regulation of cyclic AMP by both $\beta_2$-adrenergic and PGE$_1$ receptors expressed by the $\beta^p$ and $\beta^d$ variants cells appears to be normal [18]. However, to corroborate this conclusion from a biochemical standpoint, the receptors were radiolabeled in situ using $^{125}$Iodocyanopindololdiazirine, the photo-affinity analogue of $^{125}$Iodocyanopindolol [24]. Following UV-irradiation of the intact cells, a crude membrane preparation that was essentially devoid of nuclear material, was prepared. The labeled membranes were electrophoresed on a discontinuous SDS-PAGE system and the covalently radiolabeled $\beta_2$-adrenergic receptors were visualized by autoradiography (Fig. 2). Two radiolabeled bands were seen with membranes prepared from wild-type S49 cells with molecular mass of 67 000 Da and 47 000 Da. The larger molecular mass band was the major component; the smaller fragment was presumably a product of proteolytic degradation. This pattern of $\beta_2$-adrenergic receptor labeling is similar to that reported previously in wild-type S49 cells [8].

The labeling pattern observed with the receptor-deficient variant cells was weaker than that obtained with wild-type S49 cells, as expected due to the lower receptor abundance in the variants. The characteristic phenotype of the $\beta^p$ and $\beta^d$ S49 cells, that of decreased $\beta_2$-adrenergic receptor expression [18],
Fig. 3. Glycosidase sensitivity of the β2-adrenergic receptor. β2-Adrenergic receptors on intact were labeled with [125I]iodocyanopindolol and the membrane proteins were electrophoresed on SDS-PAGE following treatment with endoglycosidase H or endoglycosidase F, as indicated. The radiolabeled receptors were visualized by autoradiography.

was confirmed several times throughout these studies by [125I]iodocyanopindolol binding, performed as described previously [19].

In addition to the decreased intensity of labeling observed with the variant cells, we observed a shift of the labeled bands toward smaller molecular mass, 60 000 and 42 000 Da in the variants vis-à-vis 67 000 and 47 000 Da in the wild-type (Fig. 2). The degree of this size shift was equivalent in the two variant cells. One possible explanation for the decrease in apparent molecular mass of the β2-adrenergic is aberrant receptor glycosylation since the mass of the core protein is 47 000 Da based on its deduced amino acid sequence [25]. Furthermore, glycosylation appears to be unimportant for β2-adrenergic receptor function in S49 cells [8].

To determine whether altered glycosylation was indeed responsible for the decreased molecular mass, membranes prepared from photoaffinity labeled cells were treated with endoglycosidase H and N-glycosidase F prior to fractionation on SDS-PAGE (Fig. 3). Treatment with endoglycosidase H was without effect, but N-glycosidase F normalized the sizes of the specifically radiolabeled species from the wild-type, βp and βd S49 cells. A single but diffuse band centered around 47 000 Da was observed following N-glycosidase F treatment. These results are in accord with previous reports suggesting that the β2-adrenergic receptor is glycosylated with complex rather than high-mannose carbohydrate and that N-glycosidase F, which cleaves complex carbohydrates, has previously been shown to decrease the molecular mass of the β2-adrenergic receptor [26].

To test the hypothesis that the βp and β2-adrenergic receptor is glycosylated aberrantly, we mea-

Fig. 4. WGA binding of solubilized β2-adrenergic receptors. Crude membranes were prepared from S49 wild-type, βp and βd S49 cells and solubilized with digitonin. The solubilized material was passed repetitively over columns containing immobilized WGA, the flow through and wash collected and termed the ‘non-retained’ fraction. The bound material was eluted with 300 mM N-acetyl-D-glucosamine and this was termed the ‘sugar-eluted’ fraction. Protein and β2-adrenergic receptor content of each of the fraction was determined. Recovery of protein and β2-adrenergic receptor was equal to 88 ± 6% and 104 ± 13% (n = 6) respectively, and 9.4 ± 3.2-fold receptor purification was achieved in the sugar-eluted fractions. The asterisks indicate achievement of statistically significant difference from wild type by two-tailed T-test at the P < 0.01 level (n = 2).
Fig. 5. The S49 wild-type β2-adrenergic receptor sequence. The nucleotide sequence of the S49 wild-type β2-adrenergic receptor gene sequence is illustrated with the open reading frame denoted by upper case letters, whereas 5' and 3' non-coding sequence is denoted by lower case letters. Numbering starts at +1 with the A in the ATG start codon. The locations of two single base differences between the wild-type and variant cells’ sequences are noted by arrows. These correspond to loss of a T from a run of T centered around -1660 and change of a T to G at position -1292 in both variants’ 5' non-coding sequence.

sured the ability of immobilized WGA to bind solubili-
ized β2-adrenergic receptors from each of the three cells. Fig. 4 shows that immobilized WGA bound the receptor solubilized from the wild type S49 cells significantly better than that from the βd cells (P < 0.01, two-tailed T-test). Retention of the β2-adrenergic receptors solubilized from the βp cells was intermediate between that from the wild type and βd cells, although this difference failed to reach statisti-
cal significance. These results also suggest that the β2-adrenergic receptor on the βp and βd cells is not glycosylated normally.

George et al. reported that swainsonine, presum-
ably as a result of its inhibition of α-mannosidase II, re-
duced the mobility of the S49 β2-adrenergic recep-
tor on SDS-PAGE [8]. We confirmed the previous data that there was little or no effect (≤20% in our hands, data not shown) of swainsonine treatment on receptor affinity for (−)-iodocyanopindolol diazirine and receptor number. In contrast, Cervantes-Olivier et al. found that treatment of A431 human epidermoid carcinoma cells with either tunicamycin or monensin caused a redistribution of β2-
adrenergic receptors away from the cell surface to-
gether with a decreased cyclic AMP response to isoproterenol [11]. These contrasting effects of glyco-
sylation inhibitors on the β2-adrenergic receptor may be the result of other, more general, differences be-
tween the S49 and A431 cells.

Growing S49 cells in the presence of 0.2 μg/ml swainsonine for three days reduced the ability of digitonin-solubilized β2-adrenergic receptors to ad-
here to immobilized WGA. Furthermore, growth of the βp and βd variant cells in the presence of swainsonine caused a greater inhibition of the ability of the digitonin-solubilized β2-adrenergic receptors to bind to immobilized WGA (66 ± 1% and 70 ± 1% inhibition, respectively) than the receptor from the wild type cells (44 ± 3% inhibition) in three experi-
ments. We conclude, therefore, that altered glycosyla-
tion of the β2-adrenergic receptor occurs in the βp and βd variant cells and, furthermore, that this
glycosylation defect must lie upstream of the removal of mannose residues on the α₁₆ arm of the high mannose structure [8].

There are several possible explanations for aberrant glycosylation of the β₂-adrenergic receptor. These include N-terminal truncation of the receptor, mutation of one or both of the two consensus glycosylation sites or a malfunctioning glycosylation mechanism. To determine whether the molecular basis for apparent alteration in β₂-adrenergic receptor glycosylation in the variant cells might lie within the β₂-adrenergic receptor gene, we sequenced the entire coding region and the proximal two kilobases of the 5′ non-coding region of the β₂-adrenergic receptor from the wild-type, βp and βd S49 cells (Fig. 5).

The murine β₂-adrenergic receptor gene, like that from all species thus far examined, is intronless and the 5′ flanking region contains transcription factor binding sites, indicative of regulatory control of transcription [2,25]. We were able to utilize PCR amplification of genomic DNA. Genomic DNA was amplified by PCR using these primer pairs and the products were cloned into M13 mp18. The PCR products thus cloned were sequenced and the sequences verified in at least two clones.

We found no differences in the coding region of the β₂-adrenergic receptor gene from the wild-type and variant cells (Fig. 5). Thus, the decreased β₂-adrenergic receptor mass in the βp and βd cells is not due to a change in the primary sequence of the receptor in these cells. Lack of 5′ truncation of the receptor transcript as a possible cause is also ruled unlikely by the fact that the size of the β₂-adrenergic receptor mRNA is equivalent in the wild-type, βp and βd cells [19].

In contrast to the lack of any sequence differences noted in the open reading frame of the wild-type and variant S49 cells’ β₂-adrenergic receptor gene sequence, two single base mutations were noted in the first two kilobases of the variant cells’ β₂-adrenergic receptor 5′ non-coding sequence compared to that of the wild-type. These were loss of a T from a run of T, centered around −1660, and a T instead of a G at position −1292 in both the variant cells. These regions do not correspond to known regulatory sites and we do not know whether they functionally important in regulating receptor gene transcription. We cannot rule out the possibility that these differences may be the result of either PCR artifacts, allelic differences or of random genetic drift that has taken place in these cultured cells.

The entire S49 β₂-adrenergic receptor coding sequence was reconstituted by performing PCR with the 1F and 2R primers on the two fragments that together comprise the entire coding sequence. This PCR-mediated ligation, or ‘jumping PCR’ as it has also been termed [27] offers a simple way to reassemble larger DNA fragments from overlapping fragments generated by PCR. The fragment corresponding to the entire coding sequence was isolated on a low-melting temperature agarose gel and cloned into the EcoRI site in pBluescript SK(+) +.

The sequence of the murine β₂-adrenergic receptor gene has been determined by two groups from genomic libraries of a C57BL mouse strain [3] and a 3T3-L1 mouse cell [25]. The S49 lymphoma cell line was derived from a tumor induced in a Balb/c mouse [28]. When the coding sequence of the S49 β₂-adrenergic receptor is aligned with that from the two other murine β₂-adrenergic receptor sequences reported, there is good accordance with only ten nucleotide differences that result in a single inferred amino acid change, His or Asp, at residue 21 (Table 1). At least some of the sequence differences might

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Nucleotide sequences differences and the corresponding inferred amino acids are shown for three murine β₂-adrenergic receptors, those obtained from S49 cells, C57 [3] and 3T3 [25] mice.
be ascribed to sequencing errors. Where the sequence of the S49 β2-adrenergic receptor disagrees with one of the other two sequences, it agrees with the other. The S49 sequence is, we believe, the consensus sequence for the murine β2-adrenergic receptor sequence.

The decreased molecular mass of the β2-adrenergic receptor in the βp and βd variants is likely to be the result of aberrant receptor glycosylation in these cells. Thus, we are left with a paradox. How to explain both decreased receptor expression and decreased receptor size in the βp and βd cells?

There is at least one precedent for altered glycosylation causing altered expression of gene transcripts; this is the insulin receptor [29]. Treatment of lymphocytes with the glycosylation inhibitors 2-deoxyglucose or tunicamycin elicits a time-dependent reduction of insulin receptor mRNA levels [29]. Some transcription factors are known to be glycosylated [30,31]. It is conceivable that altered glycosylation, while affecting neither the binding properties nor the functional activity of the β2-adrenergic receptor, affects the activity of a transcription factor(s) necessary for gene transcription. Such a glycosylation defect would have to be specific to the extent that it effects the β2-adrenergic receptor and factor(s) regulating β2-adrenergic receptor mRNA expression.

The prostaglandin E1 receptor is expressed normally in the variant cells in terms of its ability to stimulate cyclic AMP formation [18]. George et al. [8] show that treatment of cells with glycosidase inhibitors elicits a decrease in the number of prostaglandin E1 receptors. Although PGE1 receptor expression appears unaltered in the βp and βd variants, preventing glycosylation through the use of inhibitors is likely to have a far greater impact on the cells than the more specific glycosylation defect that we believe we have uncovered. Our hypothesis is that the glycosylation defect in the βp and βd variant cells is specific in nature, its effect being directed towards the β2-adrenergic receptor and one or more transcription factors. Further studies will be necessary to identify the precise glycosylation defect in these S49 variant cells.

These results provide the first example of a decreased G-protein linked receptor expression that appears to be coincidental with altered receptor sensitivity to glycosidase, consistent with altered receptor glycosylation. Both the decreased receptor expression and impaired glycosylation result from a lesion outside of the receptor’s open reading frame. This observation is particularly intriguing in view of the fact that other reports have demonstrated altered glycosylation with no effect on β2-adrenergic receptor expression and function [8]. Decreased transcript expression coincident with impaired glycosylation may turn out to be a phenomenon of regulatory significance in other settings beside the expression of β2-adrenergic and insulin receptors.

References