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Mutation of Glycine 49 to Valine in the α Subunit of G_s Results in the Constitutive Elevation of Cyclic AMP Synthesis†

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ABSTRACT: The G-protein G_s couples hormone-activated receptors with adenylyl cyclase and stimulates increased cyclic AMP synthesis. Transient expression in COS-1 cells of cDNAs coding for the G_s α-subunit (α_s) or α_s cDNAs having single amino acid mutations Gly49 → Val or Gly225 → Thr elevated cyclic AMP levels, resulting in the activation of cyclic AMP dependent protein kinase. Stable expression in Chinese hamster ovary cells of α_sVal49 cDNA resulted in a small constitutive elevation of cyclic AMP that was sufficient to persistently activate cyclic AMP dependent protein kinase activity 1.5–2-fold over basal activity. Stable expression of wild-type α_s or α_sThr225 in Chinese hamster ovary cells was less effective in sustaining elevated cyclic AMP synthesis and kinase activation compared to α_sVal49.

Binding of GTP to the α-subunit of G-proteins regulates the activity of specific enzymes and ion channels (Gilman, 1987; Yatani et al., 1987; Neer & Clapham, 1988). Within the family of G-proteins, G_s is involved in the activation of adenylyl cyclase, resulting in the stimulation of cyclic AMP synthesis (Gilman, 1987). The sequences comprising the guanine nucleotide binding site appear to be highly conserved within the family of G-proteins (Masters et al., 1986), elongation factor Tu, and the *ras* proteins (Halliday, 1984; Leberman & Egner, 1984). Extensive genetic analysis of p21 *ras* has defined several mutations that confer transformation potential to the *ras* protein (Gibbs et al., 1985). The most common transforming mutations such as Gly12 → Val and Ala59 → Thr inhibit the intrinsic GTPase activity (McGrath et al., 1984; Gibbs et al., 1984). Other mutations in *ras* alter the guanine nucleotide binding properties of the protein (Sigal et al., 1986; Walter et al., 1986). In α_s, Gly49 and Gly225 correspond to Gly12 and Ala59, respectively, in the *ras* protein. Mutation of α_sGly49 → Val and transient expression in COS-1 cells resulted in significantly greater stimulation of cyclic AMP synthesis than expression of the wild-type α_s protein. Stable expression in Chinese hamster ovary cells of α_sVal49 resulted in a small but sufficiently elevated cyclic AMP level to constitutively activate cyclic AMP dependent protein kinase activity. The α_sThr225 mutation was significantly less effective than the α_sVal49 mutation in activating adenylyl cyclase in both transient and stable transfection assays.

Experimental Procedures

Cell Culture and DNA-Mediated Gene Transfer. Chinese hamster ovary cells (CHO K1) were maintained in F12 medium supplemented with 10% fetal calf serum. CHO K1 cells were transfected by using the protoplast fusion technique of Sandri-Goldin et al. (1981), and 24 h after transfection, the cells were placed in medium containing 500 μg/mL G418. Approximately 2 weeks after transfection, clones were isolated by using glass cloning rings and expanded and tested for stable expression of plasmid-expressed α_s constructs. Positive clones were subsequently subcloned at least one time. COS-1 cells were maintained in Dulbecco's modified Eagle's medium and 10% fetal calf serum. Expression of α_s constructs in COS-1 cells, which express large T antigen for transient plasmid amplification (Gluzman, 1981), was performed according to the DEAE-dextran procedure described by Ausubel et al. (1987). Transfected cells were screened 65–80 h after transfection.

Construction of Expression Plasmids. Expression vector pCW1-neo is a pUC13 derivative containing the SV40 enhancer, replication origin and early promoter and the SV40 splicing and polyadenylation sequence. Insertion into a unique

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*Hin*dIII cloning site allows expression of the cloned cDNA under the control of the SV40 early promoter. The plasmid also carries the TN5 gene for neomycin resistance under the control of the MoLTR (Spandidos & Wilhie, 1984). In the plasmids pCW1-α_s, pCW1-α_sVal49, and pCW1-α_sThr225, the 1.55-kb *Hin*dIII–*Hin*dIII fragment spanning the 5′-untranslated region, the entire coding region, and 192 bp of the 3′-untranslated region of the rat α_s cDNA (Jones & Reed, 1987) was inserted into pCW1-neo. Orientation of the insert was determined by restriction enzyme analysis.

Site-Directed Mutagenesis of the α_s cDNA. The method of Taylor et al. (1985) was used for site-directed mutagenesis with a kit supplied by Amersham, Ltd. Mutagenesis was performed by using a 1554-bp *Hin*dIII–*Hin*dIII fragment of the rat α_s cDNA in pGEM2, which was cloned into M13Mp18. For mutation of Gly49 (GGA) to Val49 (GTA) the oligonucleotide 5′-CCAGACTCTACAGCACC-3′ complementary to bases 325–341 of the coding strand was used as primer. For mutagenesis of Gly225 (GGC) to Thr225 (ACC) the oligonucleotide 5′-AAGCTACACTGGCCGGTCGCG-3′ was used. The mutated recombinant M13Mp18 DNA was transformed into *Escherichia coli* TG1; DNA was prepared from the transformants and screened by oligonucleotide hybridization at 55 °C in 6X SSC. Mutants were picked and verified by DNA sequencing according to the dideoxy procedure (Sanger et al., 1977). For the Gly49 → Val mutation, the 5′ 369-bp *Hin*dIII–*Bam*H1 fragment was recloned into the α_s cDNA by ligation. The Gly225 → Thr mutation was recloned by ligating the mutant 268-bp *Eco*RI–*Bam*H1 fragment into the α_s cDNA.

Other Procedures. Intracellular cyclic AMP levels were determined with the cyclic AMP ^{125}I assay system from Amersham, Ltd., according to the manufacturer's protocol. Cyclic AMP dependent protein kinase activity was measured in digitonin-permeabilized cells by using kemptide as described previously (Heasley & Johnson, 1989). Briefly 5×10^3 cells/well in 96-well dishes were washed and placed in 40 μL of buffer containing 75 μg/mL digitonin, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 100 μM [γ-^{32}P]ATP (5000 cpm/pmol), and 100 μM kemptide (LRRASLG). Kinase assays were allowed to proceed for 10 min at 30 °C and stopped with 10 μL of 25% TCA. Phosphorylated peptide was quantified on phosphocellulose strips as described by Roskoski (1983). Total cyclic AMP dependent protein kinase activity was defined as the difference between reactions containing 3 μM cyclic AMP to maximally activate cyclic AMP dependent protein kinase and reactions containing 25 μg/mL IP-20 to completely inhibit cyclic AMP dependent protein kinase. The synthetic peptide IP-20 corresponds to residues 5–24 of the heat-stable inhibitor of cyclic AMP dependent protein kinase (Cheng et al., 1986). Basal kinase activity was defined as the kemptide kinase activity inhibited by 25 μg/mL IP-20, and this activity is presented in the text as the kinase activity in the absence of cyclic AMP. Cholera toxin labeling of α_s was performed with [^{32}P]NAD as described (Johnson et al., 1978). Northern analysis was performed on total RNA (Maniatis et al., 1982), and immunoblotting was essentially as described (Weiss et al., 1988), using an anti-peptide antibody kindly provided by Henry R. Bourne raised against the peptide PEDATPEPGEDPRVTR, residues 323–338 of the 52-kDa α_s polypeptide.

Results and Discussion

Figure 1 shows the expression of wild-type α_s, α_sVal49, and α_sThr225 in COS-1 cells. The expressed cDNAs code for the 52-kDa form of α_s (Jones & Reed, 1987). As determined by immunoblotting (panel A) and cholera toxin catalyzed ADP-ribosylation (panel B), the 52-kDa α_s polypeptides were increased in expression approximately 3-fold as determined by densitometry of autoradiographs of the immunoblots when COS-1 cells were transfected with pCW1α_s plasmids containing the wild-type or mutant α_s cDNAs. The expressed 52-kDa α_s polypeptides were clearly functional in their ability to serve as substrates for cholera toxin, indicating that they had assumed a native tertiary structure.

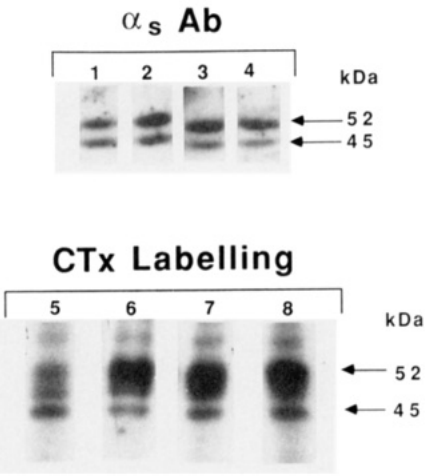

FIGURE 1: Expression of recombinant α-subunit polypeptides in COS-1 cells. The expression plasmid pCW1 is a pUC13 derivative that has a unique *Hin*dIII restriction site for insertion of a cDNA for expression under the control of the SV40 early promoter. COS-1 cells were transfected at 60% confluency in 60-mm dishes by using 2 μg of plasmid DNA according to the DEAE-dextran procedure described by Ausubel et al. (1987). Membranes used for immunoblots and cholera toxin labeling were prepared 65 h after transfection. Lanes 1–4 show immunoblots, and lanes 5–8 show the cholera toxin catalyzed ADP-ribosylation of α_s polypeptides of transfected cells. For immunoblots 25 μg of membrane protein from transfected COS-1 cells was loaded on a 10% polyacrylamide–SDS gel, electrophoresed, transferred to nitrocellulose, and blotted with an antisera raised against the α_s synthetic peptide (PEDATPEPGEDPRVTR, residues 323–338), a gift from Dr. Henry R. Bourne. Cells from the same transfection were also used for the cholera toxin catalyzed ADP-ribosylation of α_s polypeptides with [^{32}P]NAD as substrate. Twenty-five micrograms of cholera toxin labeled membrane protein was electrophoresed on SDS gels and autoradiographed. The α_s cDNA used for transfection encodes for the 52-kDa form of α_s so that autoradiographs from both immunoblots and cholera toxin labeling could be quantitated for plasmid expression of the 52-kDa α_s polypeptide by densitometry and the relative ratios of 52- and 45-kDa α_s bands could be compared. Average expression of the plasmid-encoded 52-kDa α_s polypeptide was 2–3 times that of the wild-type 52-kDa band. Lanes 1 and 5 represent COS-1 cells transfected with pCW1 without a cDNA insert; lanes 2 and 6 represent pCW1-α_s, lanes 3 and 7 represent pCW1-α_sVal49, and lanes 4 and 8 represent pCW1-α_sThr225.

Transfection of COS-1 cells with cDNAs coding for wild-type α_s or the single amino acid substitution Gly49 to Val resulted in the enhanced synthesis of cyclic AMP relative to control transfections with plasmid that did not contain an α_s cDNA (Table I). From our experience, transient expression in COS-1 cells is an excellent measure of functional α_s activity. Expression of α_{i2} by transfection in COS-1 cells slightly diminishes cyclic AMP levels relative to controls (Table I), and α_s reproducibly elevates cyclic AMP levels. The G-protein α-subunit expressed from cDNAs is generally 2.0–3.0-fold greater in abundance than the corresponding endogenous wild-type 52-kDa α-subunit with no detectable change in the 45-kDa α_s-subunit or the βγ-subunit as determined by immunoblotting and densitometry (not shown). The stimulation of cyclic AMP synthesis upon expression of the α_s polypeptide using the transient COS-1 cell assay provides a simple, rapid

Table I: Cyclic AMP Levels and Cyclic AMP Dependent Protein Kinase Activity in COS-1 Cells Transfected with Wild-Type and Mutant α_s cDNAs[a]

plasmid	cyclic AMP (pmol/mg of protein)		cyclic AMP dependent protein kinase (pmol mg^{-1} min^{-1})		
	−MIX	+MIX	−cAMP	+cAMP	act. ratio
pCW1	22	48	229	2089	0.11
pCW1-α_s	26	150	441	2340	0.19
pCW1-Thr225	32	56	433	2280	0.19
pCW1-Val49	50	310	466	2070	0.22
pCW1-α_{i2}	19	34	173	2976	0.07
none	21	48	187	1688	0.11

[a] Transient expression in COS-1 cells was performed by transfection of 4 μg of plasmid DNA per 10-cm dish with DEAE-dextran (Ausubel et al., 1987). After transfection cells were incubated for 41 h and then split into wells of 96-well plates and assayed for cyclic AMP content and cyclic AMP dependent protein kinase activity as described under Experimental Procedures. The activity ratio is the cyclic AMP dependent protein kinase activity measured in the absence of added cyclic AMP that was inhibited by the IP-20 peptide divided by the maximal kinase activity determined in the presence of 3 μM cyclic AMP. Cells from the same transfections were used for immunoblotting and cholera toxin catalyzed ADP-ribosylation. Cyclic AMP measurements are the mean of replicate wells that were within 5% of one another. All cells were incubated in the absence or presence of 500 μM methylisobutylxanthine (MIX) for 10 min prior to fixation.

measurement of functional α-subunit activity that does not apparently involve changes in βγ expression.

In the presence of a phosphodiesterase inhibitor (methylisobutylxanthine, MIX), transient expression of wild-type α_s increased cyclic AMP levels approximately 3-fold over controls. The α_sVal49 mutation increased cyclic AMP synthesis nearly 7-fold over control transfections and 2-fold greater than the cyclic AMP levels achieved with the wild-type α_s cDNA. On the basis of immunoblots (Figure 1), the expression of the α_s and α_sVal49 polypeptides was similar, indicating that the increased activity observed with the Val49 mutant was due to an increased α_s activity resulting from the mutation.

The relative changes in cyclic AMP synthesis in α_s and α_sVal49 transfected COS-1 cells were also reflected in an increased cyclic AMP dependent protein kinase activity (Table I). The protein kinase activity measurements were performed in the absence of a phosphodiesterase inhibitor and thus were a direct functional measure of the relative effectiveness of each construct in regulating cyclic AMP synthesis in intact cells.

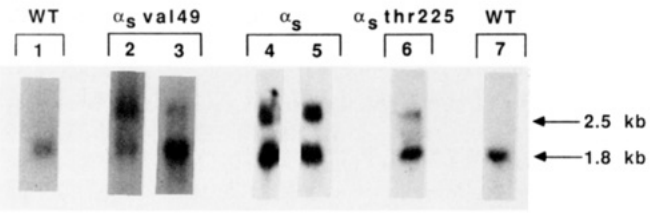

FIGURE 2: Northern blot analysis of wild-type and mutant α_s transcripts in Chinese hamster ovary cells. RNA (10 μg) from wild type (lanes 1 and 7), α_sVal49 clones 96 and 88, respectively (lanes 2 and 3), α_s clones 5 and 14 (lanes 4 and 5), and $\alpha_s$thr225 (lane 6) was analyzed following electrophoresis on 0.8% denaturing formaldehyde agarose gels and blotting on nitrocellulose filters. The blots were probed with nick-translated 1.55-kb α_s cDNA and autoradiographed at −80 °C for 12 h. The endogenous α_s mRNA is 1.8 kb in CHO cells whereas the pCW1 α_s transcripts are 2.5 kb, allowing the resolution and detection of the plasmid-expressed transcript from the endogenous α-subunit mRNA.

The mutation Gly225 to Thr resulted in an α_s molecule that elevated basal cyclic AMP levels and cyclic AMP dependent protein kinase activity, but whose effect on cyclic AMP synthesis in the presence of MIX was significantly less than that observed with either wild-type α_s or α_sVal49. Given similar levels of expression as determined by immunoblotting, this observation suggests that α_sThr225 is a functional α_s molecule, but whose maximal activity is probably less than the wild-type polypeptide.

In order to further characterize the phenotype of the α_sVal49 construct, Chinese hamster ovary (CHO) cells were transfected and clones stably expressing the plasmid-encoded α_s transcripts were isolated. The plasmid-expressed α_s transcript is approximately 2.5 kb whereas the endogenous α_s mRNA is 1.8 kb. The northern blot shown in Figure 2 verifies that the G418-resistant clones expressed the plasmid-derived mutant or wild-type α_s transcripts, which are present at levels nearly equal to or somewhat less than the endogenous α_s transcript.

Table II shows cyclic AMP levels and cyclic AMP dependent protein kinase activity ratios in CHO clones stably expressing wild-type α_s, α_sVal49, or α_sThr225 transcripts. In the absence of MIX the α_s and α_sThr225 clones show little change in basal cyclic AMP levels relative to control clones. The four independent α_sVal49 clones show a small elevation in cyclic AMP levels relative to control clones. Use of the highly sensitive cyclic AMP dependent protein kinase activity

Table II: Cyclic AMP Levels and Cyclic AMP Dependent Protein Kinase Activity in CHO Cells Transfected with Wild-Type and Mutant α_s cDNA[a]

clone	cyclic AMP (pmol/mg of protein)		cyclic AMP dependent protein kinase (pmol mg^{-1} min^{-1})		
	−MIX	+MIX	−cAMP	+cAMP	act. ratio
pCW1	6, 4	20, 21	255	2414	0.10
			75	1763	0.04 (0.08)
			255	2414	0.10
α_s-5	6, 5	45, 41	239	1724	0.14 (0.10)
			84	1451	0.06
α_s-14	6, 5	60, 39	223	2147	0.10 (0.10 ± 0.03), N = 6
α_sThr225	7, 4	35, 35	207	1714	0.12 (0.12 ± 0.04), N = 6
α_sVal49					
−85	7, 7	82, 81	307	1737	0.18 (0.16 ± 0.02), N = 4
−88	7, 8	96, 83	272	2042	0.13 (0.14 ± 0.02), N = 4
−96	14, 7	195, 122	439	1738	0.25 (0.22 ± 0.02), N = 3
−90	7, 8	92, 81	400	1847	0.22 (0.18 ± 0.02), N = 4

[a] Chinese hamster ovary (CHO) cells were transfected by protoplast fusion by using the appropriate pCW1 construct. Independent G418-resistant clones were picked, subcloned one time, and characterized for cyclic AMP content and cyclic AMP dependent protein kinase activity as described in the legend to Table I. For cyclic AMP measurements cells were incubated at room temperature for 10 min in the absence or presence of 500 μM methylisobutylxanthine (MIX). Each cyclic AMP value represents replicate measurements that varied by less than 5%. Representative kinase measurements in the absence and presence of 3 μM cyclic AMP are shown for each clone. Values in parentheses show the mean activity ratio ± SEM of the indicated number of independent kinase activity determinations for the various clones.

assay also indicated that cyclic AMP levels were constitutively higher in α_sVal49 clones relative to the other CHO clones analyzed. The α_s and α_sThr225 transfected clones show little difference in kinase activity ratio relative to control CHO clones, whereas α_sVal49 clones show a 1.3–2.5-fold increase in the kinase activity ratio, consistent with elevated cyclic AMP levels in these clones that sustain an activated cyclic AMP dependent protein kinase activity. Thus, the α_sVal49 construct is capable of constitutively activating cyclic AMP synthesis and maintaining a persistent activation of cyclic AMP dependent protein kinase in the absence of a phosphodiesterase inhibitor. The α_sThr225 clone showed a very modest effect on cyclic AMP levels or kinase activity. However, this mutant transcript was also rather inefficiently expressed in CHO cells.

The difference in cyclic AMP synthesis in α_s-, α_sThr225-, and α_sVal49-transfected CHO clones relative to control clones is most evident when cyclic AMP levels are measured in the presence of a phosphodiesterase inhibitor. CHO clones transfected with wild-type α_s cDNA had 2-fold greater cyclic AMP levels relative to control cells in the presence of MIX. The Thr225 clone had somewhat lower cyclic AMP levels than the α_s-transfected clones; however, as noted above the expression of the α_sThr225 was also low in CHO cells. In contrast, all of the α_sVal49 CHO clones expressing relatively low or high transcript levels for the mutant α_s showed greater cyclic AMP levels relative to either control CHO cells or α_s-transfected clones. Consistent with the COS-1 cell transient expression assays, the α_sVal49-expressing CHO clones demonstrated greater cyclic AMP synthesis than either wild-type or α_s-transfected clones. The phenotype of these clones has been stable now for greater than 1 year and more than 100 cell doublings, indicating the constitutive increase in cyclic AMP synthesis in the α_sVal49 clones results from a stable phenotype due to expression of the mutant α_s cDNA.

Our results demonstrate, particularly with the transient expression in COS-1 cells but also with stable transfectants in CHO cells, that α_sVal49 is more effective in elevating cyclic AMP levels and sustaining cyclic AMP dependent protein kinase activity than expression of either wild-type α_s or α_sThr225. In the p21 *ras* protein, the corresponding mutation of α_sVal49 is Gly12 → Val, and for α_sThr225 it is Ala59 → Thr. Both of these mutations appear to inhibit the intrinsic GTPase activity of p21 *ras* (Gibbs et al., 1985). In COS-1 cells the level of expression of each α_s construct was similar. Thus, the enhanced activity observed with the α_sVal49 mutant is most probably a direct result of GTPase inhibition as a result of the Gly → Val amino acid substitution in the α_s polypeptide. We have not yet measured intrinsic GTPase activity of the various mutants since this will require bacterial expression and isolation of the proteins (Graziano et al., 1987). The level of expression of α_sVal49 polypeptide in CHO cells appears to be low relative to that of the endogenous α_s, but its enhanced activity is sufficient to sustain an elevated cyclic AMP level and activation of cyclic AMP dependent protein kinase.

The diminished activity of the α_sThr225 mutant relative to wild-type α_s expression in COS-1 cells that is so readily apparent when cyclic AMP levels are measured in the presence of MIX is unclear, but suggests the mutation might alter α_s such that its activity is less than that of the wild-type polypeptide. The H21a mutation described in α_s from S49 cells is a mutation of the adjacent amino acid Gly226 to Ala (Tyler et al., 1988). The H21a phenotype is a loss in the ability of G_s to activate adenylyl cyclase even though it is coupled to receptors. The Gly225 to Thr mutation also has a diminished ability to activate adenylyl cyclase and may possibly have a similar structural change, although less dramatic, as the Gly226 to Ala mutation. More experiments are required to resolve the properties of this mutation.

Interestingly, a third mutation, α_sAsp295 → Ala, did not influence the synthesis of cyclic AMP or the activation state of cyclic AMP dependent protein kinase (not shown). Asp295 in α_s corresponds to Asp119 in p21 *ras* and is involved in ionic interactions with the 2-amino group of the guanine ring of GDP and GTP. Mutation of this Asp residue markedly enhanced the transformation potential of p21 *ras*, apparently by influencing the rate of GDP dissociation from the guanine nucleotide binding site (Sigal et al., 1986; Walter et al., 1986). Further studies will be required to determine what, if any, influence the Asp295 → Ala mutation has on guanine nucleotide binding and activation of α_s and if it influences the phenotype of other α_s mutations such as α_sVal49 or α_sThr225.

The differences in cyclic AMP levels observed in the presence and absence of the phosphodiesterase inhibitor MIX indicate that altered expression of G_s is compensated by the cell in an attempt to regulate the stimulation of specific pathways by cyclic AMP. Similar compensatory mechanisms have been observed in other cell types (Insel et al., 1975). We have also observed a compensatory increase in $\beta\gamma$-subunit expression in CHO clones expressing mutant α_s polypeptides (Woon et al., 1989). Thus, CHO cells use multiple mechanisms in an attempt to regulate the cyclic AMP pathway. Nonetheless, the α_sVal49 mutation was capable of sustaining an activated cyclic AMP dependent protein kinase in the absence of a phosphodiesterase inhibitor that is approximately 1.5–2.0-fold greater than control basal kinase activity. Expression of wild-type α_s had much less of an effect on cyclic AMP synthesis in stable CHO clones. Most likely, this is due to the attentuation of α_s by $\beta\gamma$. This contrasts with the ability of α_s to stimulate cyclic AMP synthesis in transient COS-1 expression assays. Our findings indicate that specific mutations such as Val49 in α_s can alter its activity similar to that which has been observed in the *ras* protein. In contrast, however, other mutations such as Thr225 and Ala295 in α_s had little effect in regulating cyclic AMP synthesis, indicating that not all mutations that activate *ras* by influencing either GTPase or GTP-binding activities would similarly activate α_s. Further analysis of mutations in α_s will define differences in *ras* and α_s GTP binding and GTPase regulation as well as the influence of $\beta\gamma$ interaction on α_s function.

Our work also begins to address an important issue regarding the expression of mutant G-proteins in cells that express a background of the wild-type gene product. We have found that the transient COS-1 cell assay is an easy and fast assay for functional analysis of mutant α_s polypeptides. However, the levels of expression of plasmid-derived α_s polypeptides in stable transfectants in CHO cells and other fibroblast lines such as hamster CCL39 cells tend to be low. We have observed this phenomenon using different promoters and plasmid constructions, suggesting the relative levels of the G-proteins are a tightly regulated process. Therefore, the characterization of strong dominant mutations will be required for the manipulation of G-protein-regulated pathways by transfection of mutant α-subunit cDNAs.

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Articles

Synthesis and Determination of the Stereochemistry of 23,25-Dihydroxy-24-oxovitamin D_3, a Major Metabolite of 24(*R*),25-Dihydroxyvitamin D_3

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ABSTRACT: Two C(23) epimers of 23,25-dihydroxy-24-oxovitamin D_3 (**1**), a major metabolite of 24(*R*),-25-dihydroxyvitamin D_3, were chemically synthesized for the first time and their stereochemistries at C(23) are determined on the basis of X-ray analysis. The C(23) stereochemistry of the natural 23,25-dihydroxy-24-oxovitamin D_3 was determined to be *S* by comparing the spectral properties and HPLC behavior of the two synthetic isomers with those of the natural metabolite.

24(*R*),25-Dihydroxyvitamin D_3 [24*R*,25-$(OH)_2D_3$][1] (DeLuca & Schnoes, 1976, 1983) is a major metabolite of vitamin D_3 circulating at a concentration nearly 100 times higher than the metabolically active form (Shepard et al., 1979), 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$]. However, the biological importance of this metabolite is still controversial. It has been claimed that 24*R*,25-$(OH)_2D_3$ has a number of in vivo actions such as stimulation of skeletal mineralization (Ornoy et al., 1978; Endo et al., 1980; Malluche et al., 1980; Lidor et al., 1987), regulation of secretion of parathyroid

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[1] Abbreviations: 24*R*,25-$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 23,25-$(OH)_2$-24-oxo-D_3, 23,25-dihydroxy-24-oxovitamin D_3; UV, ultraviolet; IR, infrared; ^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon-13 nuclear magnetic resonance; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; 25-OH-24-oxo-D_3, 25-hydroxy-24-oxovitamin D_3; HMPA, hexamethylphosphoramide; MCPBA, *m*-chloroperbenzoic acid; DMSO, dimethyl sulfoxide; 23*S*,25-$(OH)_2D_3$, 23(*S*),25-dihydroxyvitamin D_3; 25*R*-$(OH)D_3$ 26,23*S*-lactone, 25(*R*)-hydroxyvitamin D_3 26,23*S*-lactone; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3.

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