Enhanced Casein Kinase II Activity in COS-1 Cells upon Overexpression of Either Its Catalytic or Noncatalytic Subunit*

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Casein kinase II consists of catalytic (α) and regulatory (β) subunits complexed into a heterotetrameric α₂β₂ structure. Full-length cDNAs encoding the α and β subunits of human casein kinase II were subcloned into an expression vector containing the cytomegalovirus promoter, and no yielding the expression constructs pCMV-α and pCMV-β. Northern analyses of total cellular RNA prepared from COS-1 fibroblasts 65 h after transfection with pCMV-α or pCMV-β or with both expression constructs showed marked increases in corresponding α and β subunit RNAs. Immunoblot analysis utilizing anti-casein kinase II antiserum of cytosolic extracts prepared from COS-1 cells cc-transfected with pCMV-α and pCMV-β showed 2- and 4-fold increases in immunoreactive α and β subunit protein, respectively, relative to vector-transfected cells. These same cytosolic fractions exhibited an average 5-fold increase in casein kinase II catalytic activity. COS-1 cells transfected with pCMV-α alone exhibited a 3-fold increase in immunoreactive α subunit and a nearly 2-fold increase in cytosolic casein kinase II catalytic activity. Transfection with the cDNA coding for the noncatalytic β subunit alone also caused a near doubling of cytosolic casein kinase II catalytic activity. No increase in immunoreactive α subunit protein was observed in pCMV-β-transfected cells, and no increase in immunoreactive β subunit protein was observed in pCMV-α-transfected cells. These results indicate that a portion of the endogenous cellular casein kinase II protein is not fully active and that raising the concentration of the α or β subunit stimulates this latent activity.

α subunit as the catalytic subunit (8, 9). The native heterotetramer has been shown to undergo autophosphorylation on the β subunit in vitro (10), but the site(s) and the physiological role for this autophosphorylation have yet to be determined. In addition, evidence has been recently presented that the β subunit of casein kinase II is phosphorylated at site(s) other than the autophosphorylation site with concomitant increases in casein kinase II catalytic activity (11-13).

Renaturation studies by Cochet and Chambaz (14) have shown that optimal casein kinase II activity is achieved when α and β subunits are present in a 1:1 molar ratio. Lin et al. (15) and Hu and Rubin (16) have both recently presented data which compare structural and functional properties of purified casein kinase II holoenzyme with purified casein kinase II α subunit expressed in Escherichia coli. Their data independently demonstrate that the isolated α subunit exhibits a low level of catalytic activity relative to native casein kinase II, and their data strongly suggest that the β subunit plays a positive regulatory role in the expression of casein kinase II activity. The aim of the present study was to assess the effects of independently increasing the concentration of either α or β casein kinase II subunits on casein kinase II catalytic activity in intact cells. In this report, we demonstrate the successful transfection of human casein kinase II α and β subunit cDNAs into COS-1 fibroblasts. We find that the effect of overexpressing either of these individual subunits in COS-1 cells is to double total cytosolic casein kinase II catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

β-Glycerophosphate, EGTA,1 aproeinin, phenylmethylsulfonyl fluoride, bovine serum albumin, Triton X-100, and salmon testes DNA were from Sigma. Low specific activity [32P]-labeled protein A (8.31 μCi/μg), [32P]-labeled deoxynucleotides (800 Ci/mmol), and [γ-32P]ATP (3200 Ci/mmol) were obtained from Du Pont-New England Nuclear. Enzyme grade HEPES was purchased from Fischer Biotech, Fairlawn, NJ. P81 paper was purchased from Whatman, Hillsboro, OR. The rabbit anti-Drosophila antisemur was generously provided by Dr. Claiborne Glover, University of Georgia, Athens, GA, and casein kinase II purified from bovine thymus was a generous gift of Drs. Lynn Kozma and Lynn Wolfe, Program in Molecular Medicine and the Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA. The vector pCMV was obtained from Dr. David Russell, Southwestern Medical Center, University of Texas, Dallas, TX (17).

1 The abbreviations used are: EGTA, ethylenebis(oxyethylene-nitritro)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase(s); RI, regulatory subunit of cAMP-dependent protein kinase; III, regulatory subunit of cAMP-dependent protein kinase.
**Methods**

**Subcloning of the Human α and β Casein Kinase II cDNAs into the Expression Vector pCMV**—The vector pCMV contains a unique BglII restriction site located downstream from the cytomegalovirus promoter and upstream from a 3′ human growth hormone DNA sequence and a polyadenylation domain. For subcloning into this site, BamHI linkers were attached to the 1.6-kb human α subunit cDNA (18). The vector was digested with Bglll to remove 109 nucleotides 5′ noncoding sequence, and the cDNA with BglII-BamHI ends was inserted into BglII-digested pCMV. In order to subclone the human β subunit cDNA into pCMV, the 3′ 1.28-kb cDNA (19) was digested with DraI to remove 600 nucleotides of 3′ noncoding sequence including the polyadenylation domain. This 0.68-kb 3′ fragment possessing EcoRI-EcoRI ends was ligated with the 0.16-kb 5′ cDNA fragment possessing EcoRI-EcoRI ends. BamHI linkers were attached to this 0.84-kb cDNA product containing the entire open reading frame. The cDNA, now possessing BamHI ends, was inserted into pCMV at the BglII cloning site. The resultant expression constructs were designated pCMV-α and pCMV-β.

**Transfection of COS-1 Cells**—Transient transfection of pCMV-α and pCMV-β into COS-1 fibroblasts was accomplished by calcium phosphate precipitation essentially as described by Gorman (20). Briefly, COS-1 cells were grown under an atmosphere of 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 50 units/ml penicillin and 50 μg/ml streptomycin sulfate. Eighteen hours prior to DNA treatment, the cells were plated at a density of 5 × 104 cells per 100-mm tissue culture dish. Each dish of cells was transfected with 20 μg of either pCMV vector plasmid DNA, pCMV-α plasmid DNA, pCMV-β plasmid DNA, or co-transfected with pCMV-α and pCMV-β plasmid DNAs in buffer containing HEPES-buffered saline, pH 7.12, 250 mM CaCl2, and 0.04 μg/ml salmon testes DNA as carrier and incubated for 4 h. Cells were washed with complete medium 3 times and glycerol-shocked for 1 min. Cells were then washed 3 times with complete medium and incubated at 37 °C for approximately 65 h prior to harvest.

**RNA Preparation and Analysis**—Total RNA from COS-1 fibroblasts transfected with pCMV-α, or pCMV-β, or pCMV-α or pCMV-β alone or co-transfected with pCMV-α and pCMV-β together, and total RNA from cultured HepG2 cells were prepared according to the method outlined by Davis et al. (21). Northern analyses were performed exactly as described by Heller-Harrison et al. (19).

**Preparation of Cellular Extracts**—100-mm dishes of transfected COS-1 fibroblasts prepared as described above were assayed at 30 °C in a total reaction volume of 25 μl in assay buffer containing 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 50 mM KCl, 20 mM MgCl2, 50 mM NaF, 100 μM NaVO3, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, pH 7.3, and then carefully drawn through a 22-gauge needle 20 times. Lysates were centrifuged for 20 min at 22,000 × g, and supernatants were transferred to a new tube and immediately frozen at −70 °C. Protein was quantitated by the method of Bradford (22).

**Protein Kinase Assays**—3–10 μg of protein from transfected COS-1 fibroblasts prepared as described above were assayed at 30 °C in a total reaction volume of 25 μl in assay buffer containing 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% bovine serum albumin, 0.1% Triton X-100 and was then incubated with gentle agitation for 2–4 h at room temperature in rabbit anti-Drosophila casein kinase II antisera diluted 1/100 in blocking buffer. The nitrocellulose was washed once in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl for 5 min at room temperature and then 4 times in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100 for 5 min at room temperature. The nitrocellulose was incubated with gentle agitation in 125I-protein A in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100 for 45 min at room temperature and then washed once in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl for 5 min at room temperature and then 4 times in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100 for 5 min at room temperature. Immunoreactive proteins were visualized by autoradiography using Kodak XAR film.

**RESULTS AND DISCUSSION**

Complementary cDNAs encoding the α and β subunits of human casein kinase II (18, 19) were subcloned into a mammalian expression vector, pCMV (17), containing cytomegalovirus promoter sequences. The resultant expression constructs, pCMV-α and pCMV-β, were transfected individually or were co-transfected into subconfluent COS-1 fibroblasts. Cells were grown to confluence and total cellular RNA was prepared as described under “Experimental Procedures.” Fig. 1 presents comparative Northern analyses of identical quantities (20 μg/lane) of RNAs performed at high stringency utilizing α and β cDNA probes containing the entire open reading frame. Following a 1-h exposure of the autoradiogram, three hybridizing species of RNA migrating at 1.8, 4, and >6.5 kb were observed with RNA prepared from cells transfected with pCMV-α and cells co-transfected with pCMV-α and pCMV-β when probed with the 1.5-kb α subunit cDNA (Fig. 1, lanes 1 and 2). In contrast, no such hybridization signal was observed with RNA prepared from cells transfected with vector or with pCMV-β alone (Fig. 1, lanes 1 and 3). After

**Experimental Procedures**—Western blots were performed as described under “Methods.”

**Fig. 1. Northern blot of total RNA from COS-1 fibroblasts overexpressing casein kinase II subunits.** Total cellular RNA was prepared from cells transfected with pCMV vector or with pCMV-α and pCMV-β constructs. RNAs (20 μg/lane) were electrophoresed on a formaldehyde gel, transferred to nitrocellulose, and the nitrocellulose was hybridized at 42 °C in 50% formamide to the 1.5-kb HepG2 α subunit cDNA (lanes 1–5) or the 0.87-kb HepG2 β subunit cDNA (lanes 6–10). Blots were washed at 62 °C in 0.1 X SSC and autoradiographed for either 1 or for 42 h. Lanes autoradiographed for 1 h: 1, RNA prepared from cells transfected with pCMV vector; 2, RNA prepared from cells transfected with pCMV-α; 3, RNA prepared from cells transfected with pCMV-β; 4, RNA prepared from cells co-transfected with pCMV-α and pCMV-β; 5, RNA prepared from cells transfected with pCMV vector; 6, RNA prepared from cells transfected with pCMV-α; 7, RNA prepared from cells transfected with pCMV-β; 8, RNA prepared from cells co-transfected with pCMV-α and pCMV-β. Lanes autoradiographed for 42 h: HepG2 cell RNA hybridized with α and β subunit cDNA probes as indicated.
approximately 42 h, two major RNA species migrating at approximately 4.6 and 2.7 kb, which resulted from hybridization of the α probe with endogenous casein kinase II RNA, could be seen in these lanes (data not shown). Similarly, when RNAs prepared from transfected COS-1 cells were hybridized with the β subunit cDNA probe, only RNA from cells transfected with pCMV-β or co-transfected with both pCMV-α and pCMV-β demonstrated a strong hybridization signal following a 1-h exposure (Fig. 1, lanes 7 and 8). These RNAs migrated at 1.2–1.5, 3.7, and >6.0 kb. After approximately 42 h, a single band migrating at 4.2 kb, arising from hybridization of host cell casein kinase II RNA with the β cDNA probe, was observed in lanes containing RNA prepared from cells transfected with vector or pCMV-α. It is unclear why multiple higher molecular weight RNA species which react with our α and β subunit-specific probes are so abundant in COS-1 cells transfected with pCMV-α or pCMV-β or with both expression constructs. Our data, presented in Figs. 2 and 3 and Table I in this paper, indicate that correctly processed human α and β subunits are being expressed in COS-1 cells. Hence we have not pursued the identification of these higher molecular weight species further.

In the above study, the 1-h exposure of the autoradiogram is shown to demonstrate the marked increase in expression of α and β subunit-specific RNAs in the respective transfected cells as compared with control transfected cells. To further emphasize the magnitude of increased expression of the α and β subunit RNAs, a Northern blot of identical quantities (20 µg/lane) of RNA prepared from HepG2 cells and probed with the same α and β subunit cDNAs is also shown (Fig. 1). The approximate sizes of the two major RNA species present in total HepG2 cell RNA which react with the α subunit cDNA probe are 2.7 and 4.6 kb. These sizes correlate well with the sizes of endogenous α subunit RNAs present in COS-1 cells.

![Fig. 2. Immunoblot of protein fractions from COS-1 fibroblasts overexpressing casein kinase II subunits.](image)

COS-1 fibroblasts were transfected with pCMV vector or with pCMV-α and pCMV-β constructs. Cell fractions were prepared for SDS-PAGE as described under “Experimental Procedures.” 75 µg of protein per lane were boiled in SDS-PAGE sample buffer containing 2-mercaptoethanol and resolved by SDS-PAGE on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose which was subsequently blocked, incubated with rabbit anti-Drosophila casein kinase II antisera, and finally incubated with 125I-protein A. Immunoreactive proteins were visualized by autoradiography. Molecular mass standards in kilodaltons are as follows: myosin heavy chain, 196.0; phosphorylase b, 105.7; bovine serum albumin, 71.0; ovalbumin, 44.2; carbonic anhydrase, 27.8; β-lactoglobulin, 18.3. Lanes: std, vector α β α+β.

![Fig. 3. Casein kinase II activity assay of cytosolic fractions prepared from COS-1 fibroblasts overexpressing casein kinase II subunits.](image)

COS-1 fibroblasts overexpressing casein kinase II subunits. COS-1 fibroblasts were transfected with pCMV vector or with pCMV-α and pCMV-β constructs as described under “Experimental Procedures” and in the legend of Fig. 2. 5 µg of total protein were assayed for 10 min at 30 °C in a total reaction volume of 25 µl in assay buffer containing the synthetic peptide substrate RRREETEEE and 0.2 mM ATP ([γ-32P]ATP). Reactions were terminated by adding an equal volume of 0.01 m ATP and 0.4 N HCl. Reactions were subsequently trichloroacetic acid-precipitated, microcentrifuged for 5 min, and 12.5 µl were spotted onto 1.5-cm² pieces of Whatman P81 paper and counted in a liquid scintillation counter. As indicated, heparin at a final concentration of 1 µg/ml was added to the assay buffer.

**Table I**

Summary of quantitative immunoblot and casein kinase II catalytic activity data from COS-1 fibroblasts overexpressing casein kinase II subunits

Immunoblot analysis as described under “Experimental Procedures” and in the legend for Fig. 2 and catalytic activity measurements as described under “Experimental Procedures” and in the legend for Fig. 3 were performed for cells transfected with either pCMV-α or pCMV-β or both pCMV-α and pCMV-β. Data from immunoblot analyses were quantitated by densitometric scanning of autoradiograms and are expressed as -fold densitometric units over vector-transfected COS-1 fibroblasts. Catalytic activity is expressed as -fold activity over vector-transfected COS-1 fibroblasts. Statistical analysis utilizing the Student’s t test with a confidence interval of 95% was performed.

| Transfected cell line | Protein | Casein kinase II catalytic activity |
|-----------------------|---------|-----------------------------------|
| α                     | 2.8 ± 0.3 | 1.0 ± 0.1 | 1.7 ± 0.26 |
| β                     | 1.0 ± 0.1 | 9.7 ± 4.2 | 18.3 ± 0.18 |
| α + β                 | 2.0 ± 0.1 | 4.1 ± 1.4 | 5.0 ± 0.72 |

Values calculated from four independent experiments.

Values calculated from five independent experiments.

When HepG2 cell RNA was hybridized with the β subunit cDNA probe, a major band migrating at 1.2 kb and a minor band migrating at 4.2 kb were revealed. The differences in relative abundance of casein kinase II α subunit RNAs observed in COS-1 cells and HepG2 cells may be due to transcriptional processing. It has been previously suggested (19) that in certain cell types, e.g. HepG2 cells, casein kinase II β subunit-specific RNAs undergo transcriptional processing resulting in a deletion of a portion of the 3'-untranslated sequence flanking the termination codon and generating lower molecular weight RNA species such as the 1.2-kb RNA. As indicated, an exposure time of 42 h was required to achieve a hybridization signal that was approximately equal in intensity to that of the 1-h exposure of the autoradiogram in which RNAs were prepared from overexpressing cells. Hence, these results demonstrate that transcription of the human casein kinase II cDNAs is being driven by the cytomegalovirus promoter contained within the expression vector and that
RNA levels of both the α and β subunits are increased approximately 28-fold relative to controls. In order to assess the presence of human casein kinase II α and β subunit protein in the COS-1 cells transfected with pCMV-α and pCMV-β, immunoblot analysis was performed. Transfected cells were harvested and cytosolic fractions were prepared as described under “Experimental Procedures.” Fig. 2 shows the results of a representative immunoblot utilizing a rabbit antiserum generated against Drosophila casein kinase II which cross-reacts with both the α and β subunits of casein kinase II α from a variety of species. Quantitation of immunoreactive α and β protein was determined by densitometric scanning of autoradiograms from several experiments (Table I). Cytosolic fractions prepared from cells transfected with pCMV-α or co-transfected with pCMV-α and pCMV-β showed approximately 2.8- and 2.0-fold respective increases in immunoreactive α subunit protein relative to α subunit protein present in cytosolic fractions prepared from vector-transfected cells. No increase in immunoreactive α subunit protein was observed in the cytosolic fraction prepared from cells transfected with pCMV-β alone (Table I). As depicted in Fig. 2, the expressed human α subunit has a mobility identical to that of the COS-1 cell monkey α subunit. The mobility of both the human and monkey α subunits is decreased with respect to purified casein kinase II α subunit from bovine thymus (Fig. 2, std lane).

Cytosolic fractions prepared from cells transfected with pCMV-β or co-transfected with pCMV-α and pCMV-β showed 9.7- and 4.1-fold increases in total immunoreactive β subunit protein, respectively, relative to β subunit protein present in cytosolic fractions prepared from vector-transfected cells (Table I). No increased expression in immunoreactive β subunit protein was detected in samples prepared from cells transfected with pCMV-α alone. Interestingly, the expressed human β subunit observed from cells either co-transfected with both pCMV-α and pCMV-β or transfected with pCMV-β alone migrated as a closely spaced doublet of molecular mass ~24–26 kDa (Fig. 2). The lower band of this doublet co-migrates with the β subunit of casein kinase II α subunit from bovine thymus and with the COS-1 cell monkey β subunit. As the predicted amino acid sequence of the human casein kinase II β subunit contains two potential N-linked oligosaccharide consensus sequences, the variation in the mobility of the β subunit, resulting in the upper band of the doublet, might be due to the glycosylation of a population of the expressed β subunits. However, digestion of samples identical to those shown in Fig. 2 with endoglycosidase F did not alter the mobility of either band in the doublet (data not shown).

The functionality of the expressed casein kinase II α and β subunit proteins was examined by assaying fractions prepared from the transfected cells for phosphotransferase activity. Table I presents such results where casein kinase II catalytic activity was measured, utilizing the synthetic peptide substrate RRREETEE (23), in cytosolic fractions from cells transfected with pCMV vector, pCMV-α, pCMV-β, or co-transfected with pCMV-α and pCMV-β. Samples prepared from several experiments in which COS-1 cells were transfected with pCMV-α or pCMV-β showed average 1.7- and 1.8-fold increases, respectively, in casein kinase II catalytic activity relative to that in vector-transfected cells. The cytosolic fraction prepared from cells co-transfected with pCMV-α and pCMV-β showed a 5-fold increase in casein kinase II catalytic activity relative to vector-transfected controls (Table I). Heparin has been shown to be a potent negative effector of casein kinase II activity in vitro (26). At a concentration of 1 μg/ml, heparin virtually abolished the casein kinase II activity present in the cytosol of cells transfected with either pCMV-α or pCMV-β or with both expression constructs, as shown in a representative experiment (Fig. 3).

The marked 5-fold increase in casein kinase II activity observed in extracts of COS-1 cells co-transfected with pCMV-α and pCMV-β (Table I) is associated with increases in the cellular protein concentration of both subunits (Fig. 2). These data suggest that the heterologously expressed human α and β subunits assemble into native casein kinase II heterotetramers. This hypothesis is consistent with previously published results from Cochet and Chambaz (14) who demonstrated that increasing the amount of casein kinase II β subunit in the presence of a constant amount of α subunit enhanced casein kinase II catalytic activity to a maximum of 5-fold relative to the catalytic activity of the α subunit alone. This maximum activity occurred when α and β subunits were present in a 1:1 stoichiometry. Likewise, the consistent increase in casein kinase II activity we observe in cytosolic fractions prepared from cells transfected with pCMV-α alone (1.7-fold greater than vector-transfected cells) is not surprising in light of the fact that the α subunit bears the ATP-binding site. These cells express approximately 3-fold more α subunit than vector-transfected cells (Table I). It has been previously shown by a number of investigators that isolated α subunit exhibits a low level of casein kinase II catalytic activity relative to native casein kinase II. Hu and Rubin (16) and Lin et al. (15) have independently expressed the α subunit of casein kinase II in E. coli, and both have shown the $k_{cat}/K_m$ of the purified, expressed α subunit to be <10% of the $k_{cat}/K_m$ of casein kinase II holoenzyme.

In contrast, the significant 1.8-fold increase in casein kinase II catalytic activity observed in cells transfected with pCMV-β alone (Table I) was unexpected because the β subunit does not contain an ATP-binding site. Furthermore, no increase in α subunit protein could be detected in extracts prepared from pCMV-β-transfected cells (Fig. 2). One possible explanation for the increase in casein kinase II activity when either pCMV-α or pCMV-β alone is transfected into COS-1 cells is that there are excess free α and β subunits present in these cells. Increases in α or β subunit due to overexpression would result in an increase in casein kinase II holoenzyme and a consequent increase in catalytic activity. This hypothesis is consistent with the high ratio of α subunit/β subunit immunoreactivity in control COS-1 cells compared with that ratio observed with purified enzyme (Fig. 2). However, the discrepancy in the immunoreactivity ratio for α and β subunits could also arise from differences in species reactivity with the casein kinase II antisera employed. It would appear from our data that the availability of free α subunits must be limited, since COS-1 cells transfected with pCMV-β alone exhibited an almost 10-fold increase in β subunit protein yet displayed only a 1.8-fold increase in activity. The hypothesis is also consistent with our finding that increasing the expression of the α subunit alone resulted in a greater increase in activity than would have been predicted based on the above mentioned studies by Cochet and Chambaz (14), Hu and Rubin (16), and Lin et al. (15). In either case, our findings contrast with recent observations regarding the subunits of cAMP-dependent protein kinase. Uhler and McKnight (27) demonstrated that stable overexpression of the Ca and Cβ subunits of cAMP-dependent protein kinase in NIH 3T3 cells resulted in increased levels of RI protein but not RII protein in these cells. Furthermore, it was shown that RI-specific mRNA levels were not increased in these cells suggesting that the degradation rate of RI had been altered by the presence of increased C.
subunit. The authors suggested that this was due to a stabilization of RI by its associating with C in the holoenzyme complex. We find no evidence for this phenomenon relating to casein kinase II since no detectable increase in casein kinase II α subunit protein was observed in cells transfected with pCMV-β in the four separate experiments we performed.

An alternative hypothesis relates to the possibility that a significant amount of cellular casein kinase II activity is present in an inhibited form. An endogenous casein kinase II inhibitor has been described (28). Such an inhibitor might normally be associated with β subunit in the holoenzyme complex and could be competitively bound by overexpressed α subunit. Such a mechanism would allow a population of inhibited holoenzyme to become active. Further experiments are required to test this hypothesis. Regardless, the data presented here strongly suggest that some portion of the endogenous COS-1 cell casein kinase II catalytic (α) subunits are less than fully active. This latent enzyme activity is significantly enhanced upon independent expression of β subunits.

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