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Bacterial expression of Chinese hamster regulatory type-I and catalytic subunits of cyclic AMP-dependent protein kinase and mutational analysis of the type-I regulatory subunit

Marilyn E. GOSSE,* Robert FLEISCHMANN,* Mallika MARSHALL,* Nan WANG,* Susan GARGES† and Michael M. GOTTESMAN*‡

*Laboratory of Cell Biology and †Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

The type-I regulatory subunit (RI) of the cyclic AMP-dependent protein kinase (PKA) from Chinese hamster ovary (CHO) cells has been cloned and expressed in a strain of BL21(DE3) *Escherichia coli* lacking adenylate cyclase [BL21(DE3)/Δcya]. RI expressed in this bacterial system free of cyclic AMP is soluble and can reconstitute functional PKA. Recombinant CHO Cα is predominantly insoluble with some active soluble protein. Cβ is entirely insoluble and inactive. Soluble recombinant RI and soluble recombinant Cα can associate *in vitro* and be activated by cyclic AMP. Six site-directed mutations of RI were generated to study the interaction of cyclic AMP with RI and RI–Cα subunit interactions. Four cyclic AMP-binding-site point mutants were generated [W261R (tryptophan to arginine at position 261), a novel mutation in site A; V376G, a novel mutation in site B; G200E (site A), and Y370F (site B), previously described in bovine RI were introduced into the CHO RI for comparison purposes]. Mutants W261R, Y370F, and G200E demonstrated decreased 8-N_3-[^{3}H]cyclic AMP binding as well as 5-fold reduced affinity for [^{3}H]cyclic AMP, with threefold increased EC_{50} values for cyclic AMP activation of kinase activity from reconstituted mutant holoenzymes. The mutation at V376G did not alter cyclic AMP binding or activation by cyclic AMP of mutant holoenzyme. A truncation mutant, G200Stop, which lacks both cyclic AMP-binding sites, did not bind cyclic AMP but can inhibit Cα subunit activity. A novel mutation outside the cyclic AMP-binding regions of RI (V89A) weakened the interaction with Cα indicated by a 7-fold lower EC_{50} for mutant holoenzyme activation by cyclic AMP.

INTRODUCTION

Cyclic AMP-dependent protein kinase (PKA) is composed of two regulatory subunits (RI or RII) and two catalytic (C) subunits [1,2]. Multiple forms of RI, RII and C have been described. The inactive holoenzyme consists of two regulatory subunits associated with two catalytic subunits at the kinase active site. Cyclic AMP binds to two separate sites on each of the regulatory subunits, site A and site B, dissociating a dimeric regulatory subunit from the catalytic subunits [3], thereby freeing the catalytic subunits to phosphorylate serine or threonine residues of cellular proteins. The two major forms of PKA, type I and type II, determined by whether RI or RII is associated with C, can be distinguished by DEAE chromatography, cyclic AMP analogue specificity and their ability to undergo autophosphorylation [1]. The differences between the two R subunit proteins have been defined by protein sequencing [4,5], cloning and deduced nucleotide sequences [6–8] of the RI and RII subunits.

PKA appears to be the mediator of essentially all responses to cyclic AMP in mammalian cells [1,2]. This conclusion is based on both biochemical [1,2,9] and genetic analysis [10,11] of cultured cells. In the past, a genetic analysis of Chinese hamster ovary (CHO) cells has been used to study effects of cyclic AMP on growth [12], morphology [12,13], metabolism [14–16] and transcription [17].

The PKA mutants that we and others have isolated that are most defective in cyclic AMP responses appear to be dominant negative mutants of RI in which mutations affecting the cyclic AMP-binding sites block the ability of cyclic AMP to release RI from the holoenzyme [10]. These mutations may potentially be included in expression vectors which can be used as movable genetic elements to inactivate PKA in recipient cells. Such vectors could be used to explore the role of PKA in a variety of cellular regulatory events in which cyclic AMP has been implicated. However, none of the previously existing dominating negative mutants of RI can completely inactivate PKA in recipient cells.

Recently a full-length cDNA for the wild-type RI subunit from CHO cells was cloned and sequenced. The deduced amino acid sequence is highly conserved as compared with bovine and mouse RI subunits (R. D. Fleischmann, M. E. Gosse, I. Abraham and M. M. Gottesman, unpublished work). The goals of the present study are to determine whether the cloned Chinese hamster RI subunit is functionally similar to or different from bovine or mouse RI protein, to generate dysfunctional mutants of the RI protein, to characterize their defects by *in vitro* biochemical analysis so as to define regions of RI that interact with C, and to identify which mutants might be useful as dominant negative mutants for use as movable genetic elements to probe the function of PKA in mediating cyclic AMP effects in cells and animals. Here we present the generation and *in vitro* biochemical analysis of three novel Chinese hamster RI mutations affecting the cyclic AMP-binding sites and a comparison of these mutations with known RI mutants with altered cyclic AMP binding from other sources. In addition, a novel mutation outside the cyclic AMP-binding site which affects interaction of RI with Cα is described. These studies add to our knowledge of the molecular basis of cyclic AMP binding and C subunit

Abbreviations used: PKA, cyclic AMP-dependent catalytic subunit protein kinase; RI, regulatory subunit type I; RII, regulatory subunit type II; C, catalytic; CHO, Chinese hamster ovary; IPTG, isopropyl β-thiogalactoside.

‡ To whom correspondence should be addressed.

interactions of the RI subunit as well as providing potentially useful RI subunit mutants for future *in vivo* transfection studies.

MATERIALS AND METHODS

Materials

Histone type IIA, ATP and cyclic AMP (Sigma Chemical Co., St. Louis, MO, U.S.A.), 8-N_3-[^{3}H]cyclic AMP and [^{3}H]cyclic AMP (NEN-DuPont, Wilmington, DE, U.S.A.), [γ-^{32}P]ATP and ^{125}I-Protein A (Amersham Corp., Arlington Heights, IL, U.S.A.), phosphocellulose paper (Whatman, Hillsboro, OR, U.S.A.), DEAE-Sephacel (Pharmacia, Piscataway, NJ, U.S.A.) and Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH, U.S.A.) were used.

Strains and vectors

The BL21(DE3) strain of bacteria was a generous gift from W. Studier (State University of New York at Stony Brook, Stony Brook, NY, U.S.A.) [18]. The bacterial expression vector pVEX-11 [19] was a generous gift from V. J. Chaudhary (National Cancer Institute, Bethesda, MD, U.S.A.).

Preparation of the BL21(DE3)/Δcya strain for bacterial expression

The Δ*cya* 854 allele [20] was introduced in BL21(DE3) by P1 transduction [21] from strain SA2759 (F^- *his rpsL relA ilv*::Tn10 Δcya854), selecting for tetracycline resistance. Δ*cya* transductants were scored by their inability to utilize sorbitol, using MacConkey agar plates with 1% sorbitol [22].

Site-directed mutagenesis of the RI subunit of CHO PKA

The wild-type RI cDNA from CHO cells was cloned, sequenced and found to be 98.2% identical at the amino acid level with the mouse RI subunit (R. Fleischmann, M E. Gosse, I. Abraham and M. M. Gottesman, unpublished work). When compared with mouse RI, there are seven amino acid differences: at position no. 5, Met for Thr; no. 6, Ala for Thr; no. 19, Leu for Ile; no. 20, Thr for Ala; no. 62, Arg for Lys; no. 70, Thr for Ser; and no. 72, Ile for Thr.

Wild-type CHO RI cDNA was subcloned into the *Sal*I site of M13mp18 and oligonucleotide-directed mutagenesis was performed by the method of Kunkel et al. [23]. The following mutations were generated: base no. 784 TGG was changed to CGG to generate W261R; base no. 1118 TAC was changed to TTC to generate Y370F [24]; base no. 601 GGA was changed to GAA to generate G200E [18,25]; and base no. 601 GGA was changed to TGA to generate G200Stop. These mutations were confirmed by single-strand dideoxy DNA sequencing on the DuPont Genesis 2000 automatic sequencer [26]. The *Sal*I fragment was used in PCR [27] with 5′ *Nde*I and 3′ *Hin*dIII primers to generate sites for cloning into the pVEX-11 bacterial expression vector. By cloning into the *Nde*I site of the pVEX-11 vector, the ATG start site is 12 bp from the Shine–Dalgarno sequence yielding efficient translation of the RI cDNA [19]. Final pVEX-11 constructs were tested by dideoxy sequencing. In one pVEX-11 construct, two mutations in the same RI cDNA were evidently introduced by *Taq* polymerase infidelity; at base no. 269 a GTG was changed to a GCG generating V89A, and at base no. 1130 GTG was changed to GGG generating V376G. To study the effects of these two mutations independently, the V376G mutation was excised as a *Pst*I–*Hin*dIII 356 bp fragment and replaced with a wild-type *Pst*I–*Hin*dIII 356 bp fragment; conversely, the *Pst*I–*Hin*dIII 356 bp fragment containing the V376G mutation was cloned into wild-type RI cDNA. Verification of these constructs was obtained by dideoxy sequencing using Sequenase 2.0 [28]. All of the above constructs were used for bacterial expression in the BL21(DE3)/Δcya strain.

Expression of the wild-type and mutant RI and C subunits in *Escherichia coli*

The pVEX-11 RI constructs were used to transform the BL21(DE3)/Δcya strain, and colonies were selected on Luria-broth agar plates supplemented with 50 μg/ml ampicillin. For each protein preparation, a new transformation of the BL21(DE3)/Δcya strain was performed. Expression of the RI subunits was induced with isopropyl β-thiogalactoside (IPTG) as described [29]. After induction, the bacteria were harvested and resuspended in buffer A (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 15 mg/l phenylmethanesulphonyl fluoride, 5 mM 2-mercaptoethanol). The bacteria were frozen and thawed three times on solid CO_2, sonicated (Tekmer Sonic Disrupter) and subjected to centrifugation at 12000 *g* for 20 min at 4 °C. The supernatant fraction was either concentrated using Centricon microconcentrators (Amicon, Beverly, MA, U.S.A.) and stored at −20 °C or purified on a DEAE-Sephacel column (1.5 cm × 3 cm) pre-equilibrated with buffer A. Elution of the full-length RI subunits was at 0.15 mM NaCl in buffer A. The truncated mutant RI G200Stop was eluted at 0.5 mM NaCl in buffer A. All of the proteins eluted from the DEAE-Sephacel column were dialysed overnight in buffer A to remove NaCl, and concentrated using Centricon microconcentrators with a 10 kDa molecular-mass cutoff. Protein was detected in the partially purified preparations by the Bio-Rad assay using BSA standard [30]. Typically, approx. 30–40 μg of DEAE-purified RI subunits were recovered from a 500 ml culture.

Bacterial expression vectors containing the catalytic subunits, Cα and Cβ, were a generous gift from R. Maurer (University of Iowa, Iowa City, IA, U.S.A.) [31]. The C subunit expression in bacteria was performed as for the RI subunit except that the IPTG induction of C subunit expression was for 1 h. DEAE-Sephacel purification of the Cα subunits was essentially as described for the RI subunits, except that the Cα subunits were eluted in the void volume. All of the proteins eluted from the DEAE-Sephacel column were concentrated as described for the RI protein. Typically, approx. 15–20 μg of DEAE-Sephacel-purified Cα subunits were recovered from a 500 ml culture.

Cyclic AMP-binding assays

Binding of [^{3}H]cyclic AMP to the recombinant RI subunit was performed essentially as previously described [32]. Scatchard analysis to determine the affinity constant (K_d) was as described [33]. The 8-N_3-[^{3}H]cyclic AMP covalent labelling of the RI protein was as described [32]. In all binding experiments, non-specific binding was determined in the presence of 40 μM cyclic AMP.

Preparation of antisera and Western-blot analysis of the C subunits

The insoluble fraction (inclusion body) containing the Cβ subunit expression was partially purified by the method of Bruggemann et al. [34]. The inclusion body preparation (1 μg/ml protein) was

used to inject rabbits using a standard protocol [34]. Western-blot analysis using the rabbit polyclonal anti-(C subunit) serum 4448 was as described [35].

For Western-blot analysis of CHO cell protein, cells were harvested (40000 or 120000 cells) and resuspended in boiling SDS/PAGE loading buffer (final volume 40 μl). Samples were then loaded on to the prepared gel.

Kinase assays

Equal amounts (30 μg) of the recombinant Cα subunit and wild-type or mutant RI subunit were added together in the presence of 50 mM Mops, pH 7.0, and 10 mM $MgCl_2$ (buffer B). In some experiments, bovine heart C subunit, purified to homogeneity as previously described [36] and stored at −70 °C in 50% glycerol, was used for reconstitution. RI and Cα were allowed to interact for 1 h at 4 °C, and the RI/Cα holoenzyme was purified on DEAE-Sephacel pre-equilibrated with buffer B. The holoenzyme was eluted with buffer B containing 0.5 mM NaCl and stored at −20 °C until ready for assay. The partially purified holoenzyme was used in an *in vitro* kinase assay as described [37]. Briefly, the PKA holoenzyme (1.6 μg), buffer B and 1 μg/ml histone type IIA were combined in a final reaction volume of 100 μl, and the reaction was initiated by the addition of 100 μM [γ-^{32}P]ATP (100 c.p.m./pmol). The samples were allowed to incubate in the absence or presence of the indicated concentrations of cyclic AMP for 10 min at 37 °C. Then 25 μl was spotted on to phosphocellulose paper and washed with 75 mM phosphoric acid, and the samples were analysed for ^{32}P content by liquid-scintillation counting.

Measurement of RI inhibition of Cα subunit kinase activity

DEAE-Sephacel-purified bacterially expressed Cα (0.8 μg) was incubated with the indicated concentrations of DEAE-Sephacel-purified wild-type or mutant RI subunits in the absence or presence of the indicated concentrations of cyclic AMP in buffer B for 1 h at 4 °C. The reaction was initiated by the addition of 100 μM ATP (100 c.p.m./pmol [γ-^{32}P]ATP) and 1 mg/ml histone type IIA. After 10 min at 37 °C, the reactions were terminated as described above.

RESULTS AND DISCUSSION

Site-directed mutations in CHO cell RI

The site-directed mutations made in the RI subunit of the CHO type-I PKA are summarized in Figure 1. The aim of this mutational analysis was to disturb either cyclic AMP binding or the interaction of the RI and C subunits. In order to verify the sensitivity of our *in vitro* assay system, known mutations in RI or RII proteins from other sources were generated for comparison. The new mutations W261R and V376G, near binding sites A and B respectively, were compared with known cyclic AMP-binding mutations in site A and site B, G200E [38,39] and Y370F [31] respectively. A truncation mutant, G200Stop, was created to determine if the C subunit could be inhibited in the total absence of cyclic AMP-binding sites. A mutation at position 89 (V89A), near the putative hinge region where RI and C interact, was used to begin to define the extent of the domain on RI that interacts with C. The cDNAs for the wild-type and mutant RI subunits were cloned into a bacterial expression vector as described in the Materials and methods section.

In this study, genetic modification of the BL21(DE3) bacterial expression strain of Studier and Moffatt [18] was performed to delete the adenylate cyclase gene, resulting in the BL21(DE3)/Δcya strain. Thus expression of the RI subunits in this strain has not had to include tedious steps to remove *E. coli* cyclic AMP, and holoenzyme formation would not be affected by residual cyclic AMP in the samples. The lack of adenylate cyclase in the BL21(DE3)/Δcya results in a slower growing strain, which can be easily identified from the parental strain, eliminating the possibility of contamination.

The wild-type and mutant regulatory subunits were all well expressed in BL21(DE3)/Δcya and were recovered as soluble proteins. In Figure 2, a typical example of RI and G200Stop subunit expression is shown after DEAE-Sephacel purification. The wild-type RI protein is a 49 kDa protein (lane 1). In contrast, the truncation mutant, G200Stop, is a 29 kDa protein (lane 4).

The recombinant wild-type and mutant regulatory subunits bind 8-N_3-[^{3}H]cyclic AMP in a specific manner (Figure 3).

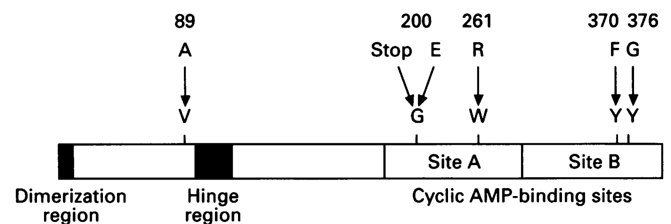

Figure 1 Schematic diagram of the site-directed mutations made in the RI subunit of the CHO type-I cyclic AMP-dependent protein kinase

CHO regulatory subunit type I of the cyclic AMP-dependent protein kinase is schematically shown. The functional regions of the RI protein are shown: the dimerization region, the R/C interaction site or hinge region and the two sites for cyclic AMP binding, site A and site B. Amino acid substitutions (numbers) generated by site-directed mutagenesis are indicated (arrows). Mutations are V89A, G200Stop, G200E [38,39], W261R, Y370F [31] and V376G.

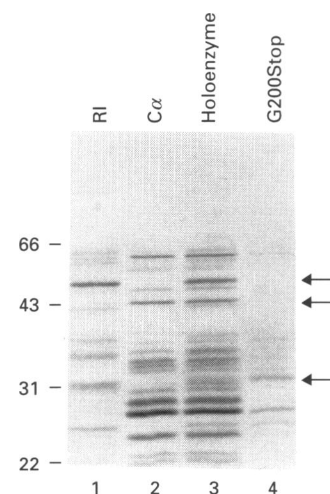

Figure 2 Expression of the wild-type and mutant RI proteins in *E. coli* BL21(DE3)/Δcya

CHO wild-type and mutant RI proteins were expressed in the *E. coli* strain BL21(DE3)/Δcya as described in the Materials and methods section. DEAE-Sephacel-purified bacterial proteins (0.8 μg of RI, Cα or G200Stop; 1.6 μg of RI/Cα holoenzyme) were boiled in 2 × SDS/PAGE loading buffer, loaded on a polyacrylamide gel and electrophoresed as described (see the legend to Figure 4). The gel was stained with Coomassie Blue, destained overnight and dried. Molecular-mass markers (kDa) are shown.

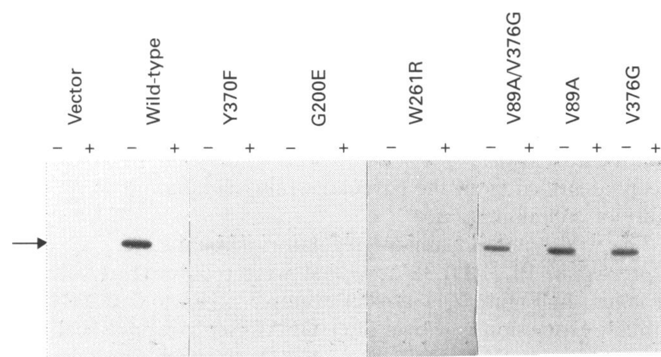

Figure 3 8-N_3-[^{3}H]cyclic AMP binding to the recombinant wild-type and mutant RI proteins

DEAE-Sephacel-purified wild-type or mutant CHO cell RI subunits (1.6 μg) were incubated with 1 μM 8-N_3-[^{3}H]cyclic AMP in the absence or presence of 40 μM cyclic AMP in 50 mM Mes, pH 6.2, containing 10 mM $MgCl_2$, 1 mM isobutyl methylxanthine and 0.1 mM 2-mercaptoethanol (buffer C) for 1 h at 37 °C. The samples were then incubated on ice for 15 min and exposed to u.v. light (General Electric no. F15T8-BLB) for 10 min. The samples were boiled in 2 × SDS/PAGE loading buffer, loaded on a polyacrylamide gel and electrophoresed as described (see the legend to Figure 4). Staining with Coomassie Blue demonstrated that equal amounts of RI protein were loaded (0.8 μg). The gel was soaked for 30 min in a solution of 3% 2,5-diphenyloxazole in acetic acid and rinsed once in distilled water. The dried gel was exposed to Kodak XR-OMAT film for approx. 1 week. –, + indicates absence or presence of 40 μM cyclic AMP.

Table 1 Binding affinities of [^{3}H]cyclic AMP and relative EC_{50} of cyclic AMP activation of holoenzyme from wild-type and mutant RI proteins

Wild-type or mutant RI subunits were expressed in BL21(DE3)/Δcya *E. coli* and partially purified by DEAE-Sephacel as described in the Materials and methods section. These RI subunits (0.8 μg) were incubated with [^{3}H]cyclic AMP in the absence or presence of 40 μM cyclic AMP in buffer C for 1 h at 37 °C. Bound [^{3}H]cyclic AMP was separated from free by rapid filtration on nitrocellulose as described [32]. The amount of [^{3}H]cyclic AMP bound was determined by liquid-scintillation counting. The K_d values were determined by Scatchard analysis [33]. The EC_{50} values were determined from the experiments shown in Figure 5. Values represent means ± S.E.M. ($n = 3$) except where indicated.

RI subunit	K_d (nM)	EC_{50} (nM)
Wild-type	6±3	63±2
G200E	29±3	124±4
W261R	32±3	196±2
Y370F	29±4	121±3
V89A/V376G	7±1 ($n = 2$)	12±2
V376G	5±2 ($n = 2$)	56±1
V89A	6±1 ($n = 2$)	9±0

Binding of 8-N_3-[^{3}H]cyclic AMP is detected as a single band in the autoradiogram after SDS/PAGE. At relatively high 8-N_3-[^{3}H]cyclic AMP concentrations, the intensity of the band corresponds to the maximal binding of 8-N_3-[^{3}H]cyclic AMP by wild-type RI protein. The mutations V89A and V376G do not alter 8-N_3-[^{3}H]cyclic AMP binding, whereas mutations W261R, G200E and Y370F reduce binding of 8-N_3-[^{3}H]cyclic AMP, as seen by the decreased band intensities as compared with the wild-type RI protein band intensity.

The relative affinities of these RI subunits for [^{3}H]cyclic AMP are shown in Table 1. The affinity for cyclic AMP is threefold to fivefold less for the cyclic AMP-binding-site mutants W261R, G200E and Y370F than for wild-type RI. The affinity for cyclic AMP of V376G, near site B, is similar to that of the wild-type protein. The V89A mutation, which changes an amino acid on the N-terminal side of the hinge region, also does not alter affinity for cyclic AMP. As expected, the truncation mutant, G200Stop, was unable to bind either 8-N_3-[^{3}H]cyclic AMP or [^{3}H]cyclic AMP (results not shown).

The CHO cell RI mutations in the cyclic AMP-binding sites provided fundamental data on the function of the two binding sites. The W261R, G200E and Y370F mutations all created proteins that demonstrated decreased affinity for cyclic AMP. The reduced covalent binding of the u.v.-activated 8-N_3-[^{3}H]cyclic AMP ligand is consistent with published data on the G200E and Y370F from bovine and mouse RI mutants respectively [29,31,38,39].

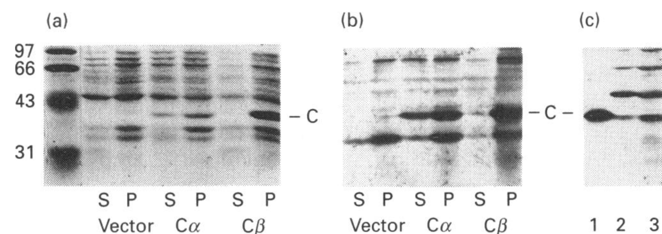

Figure 4 Differential solubilities of CHO Cα and Cβ PKA catalytic subunits when expressed in *E. coli*

The BL21(DE3)/Δcya strain of *E. coli* was transformed with vector (pVEX-11), pVexCα, and pVexCβ, and protein expression was induced by IPTG. The bacteria were harvested, resuspended in buffer B, frozen on solid CO_2 and thawed three times. The bacterial lysates were sonicated and the samples centrifuged at 4 °C in a Microfuge. The pellet fraction was resuspended in buffer B and both supernatant and pellet were analysed for protein as described in the Materials and methods section. Samples (7.5 μg of protein) were boiled in 2 × SDS/PAGE loading buffer (125 mM Tris/HCl, pH 6.8, 4% SDS, 10% glycerol, 0.02% Bromophenol Blue, 4% 2-mercaptoethanol) and loaded on a 3.5% stacking and 10% resolving SDS/polyacrylamide gel. Electrophoresis was at 40 A, constant current. (**a**) Coomassie Blue-stained gel of bacterially expressed CHO cell Cα and Cβ subunits. Molecular-mass standards (kDa) are shown. S and P indicate supernatant and pellet respectively. In (**b**), the proteins from a gel similar to the one shown in (**a**) were transferred to nitrocellulose for 1 h at 60 V at 4 °C. The nitrocellulose was incubated with preimmune or antiserum 4448 (1:500) overnight as described in the Materials and methods section. The immunoreactive bands were detected by use of the Vectastain kit (Vector Laboratories, Burlingame, CA, U.S.A.) utilizing a secondary anti-rabbit goat antibody conjugated to horseradish peroxidase. The position of the C subunits is indicated. The additional immunoreactive bands are proteins in the *E. coli* lysate that are recognized by antiserum 4448. (**c**) Western blot of purified C subunit from bovine heart and CHO cell extracts using antiserum 4448. The conditions for electrophoresis were as for (**a**); protein was transferred to nitrocellulose, and the immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham). Protein was loaded as follows: lane 1, 100 ng of purified bovine C subunit [40]; lane 2, protein from 40000 CHO cells; and lane 3, protein from 120000 CHO cells as described in the Materials and methods section.

Expression of CHO Cα and Cβ in BL21(DE3)/Δcya

In order to test the ability of cyclic AMP to activate the holoenzyme formed using wild-type or mutant RI subunits, we sought to express the CHO C subunits in the same strain of *E. coli*. Expression of the Cα and Cβ subunits of PKA in the BL21(DE3)/Δcya strain of *E. coli* is shown in Figure 4(a). Some Cα subunit protein is found in the supernatant fraction of the bacterial lysate; however, the majority of the Cα protein is inactive insoluble protein. Cβ expression in *E. coli* results in almost completely insoluble inactive protein. Efforts to resolubilize the Cα or Cβ subunit from inclusion bodies by 8M urea or guanidinium chloride followed by slow dialysis in buffer A (see the Materials and methods section) did not result in active enzyme. Furthermore, growing bacterial cultures at lower

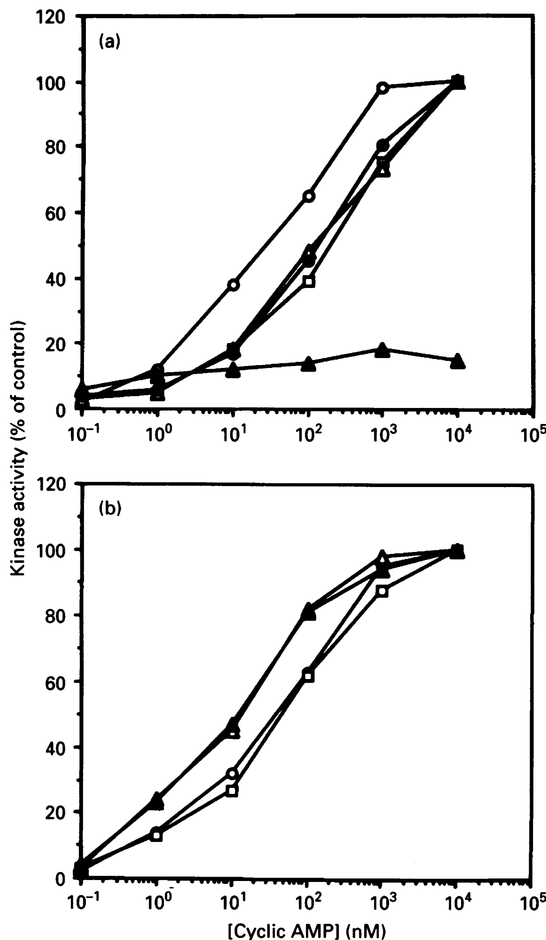

Figure 5 Cyclic AMP activation of wild-type and mutant RI/Cα CHO cell reconstituted holoenzyme

Recombinant wild-type and mutant CHO cell RI proteins were reconstituted with recombinant Cα proteins as described in the Materials and methods section. The holoenzyme (1.6 μg) was incubated with the indicated concentrations of cyclic AMP in buffer A. The reaction was initiated with the addition of [γ-^{32}P]ATP and histone type IIA as described in Materials and methods section. In (**a**), cyclic AMP-activated wild-type (○), W261R (●), G200E (□), Y370F (△) and G200Stop (▲) CHO cell RI/Cα holoenzyme kinase activity is shown. The kinase activity is shown as a percentage of Cα activity alone (1482 ± 451 c.p.m./μg; n = 8. In (**b**), wild-type (○), V89A/V376G (▲), V89A (△) and V376G (□) RI/Cα holoenzyme kinase activity is shown. The kinase activity is shown as a percentage of Cα activity alone (1689 ± 19.5 c.p.m./μg of protein; n = 3). Data represent a typical experiment that was reproduced three times.

temperatures did not alter compartmentalization of the Cα or Cβ subunit expression in *E. coli* [25]. The soluble Cα protein was useful; it demonstrated kinase activity similar to purified bovine C subunit (results not shown) and once partially purified (Figure 2, lane 2) could easily be reconstituted with bacterially expressed RI subunit (Figure 2, lane 3). Therefore the soluble Cα subunit was used in all subsequent analyses.

The insoluble Cβ protein was an excellent antigen. The resulting rabbit antiserum 4448 recognizes both bacterially expressed Cα and Cβ (Figure 4b) and purified bovine C subunit. The cross-reactivity of the antiserum with both C subunits is not surprising, as the Cα and Cβ proteins from CHO cells are nearly identical at the amino acid level [24]. Western-blot analysis of C subunit expression in BL21(DE3)/Δcya confirms the insoluble nature of the Cβ protein and the partial solubility of the Cα protein (Figure 4b). Antiserum 4448 is also useful for detecting the presence of C subunit in CHO cells (Figure 4c).

The expression of the CHO catalytic subunits presented a rather unique problem. If the Cα and Cβ proteins are so similar, then why does *E. coli* package one in inclusion bodies as insoluble protein (Cβ) more than the other (Cα)? We followed other protocols to either resolubilize or alter the mechanism of inclusion body formation by growing the cultures at lower temperatures as has been reported [25]. However, these methods did not change the relative solubility of the C subunits. We cannot account for these differences in solubility, but they do suggest that Cα and Cβ may differ structurally from each other in subtle ways. The holoenzyme formed from soluble recombinant RI and Cα did not differ from the holoenzyme formed under other conditions [25], indicating that the wild-type RI and Cα subunit from the Chinese hamster function similarly to cloned subunits from other sources [1].

Interactions of recombinant RI with recombinant Cα

The ability of the recombinant wild-type or mutant RI subunits to interact with and inhibit the Cα subunit is shown in Figure 5. The holoenzyme (RI, Cα) formed *in vitro* was incubated with increasing concentrations of cyclic AMP to activate subunit dissociation. The effective concentration of cyclic AMP that activates the resulting holoenzyme to 50 % of maximal activation (EC_{50}) was measured and is shown in Table 1. In the mutants W261R, G200E and Y370F (Figure 5), the activation curves are shifted to the right, indicating that they required more cyclic AMP to cause subunit dissociation and thereby activate Cα kinase activity. The binding-site mutants, not surprisingly, have altered cyclic AMP-mediated activation of the holoenzyme. The EC_{50} values for this activation do not differ from published activation values [29,31], providing additional evidence that the RI subunit from CHO cells is similar in function and activity to the mouse or bovine subunit.

Unlike previously reported deletion mutants [2], the truncation mutant, G200Stop, lacks both cyclic AMP-binding sites, yet apparently interacts with the Cα subunit and inhibits its activity (Figure 5a). Thus this mutant represents an RI subunit that inhibits the C subunit but this inhibition is not relieved by addition of cyclic AMP. This truncation mutant, G200Stop, may have the greatest potential as a movable genetic element, providing the tightest dominant negative effect in recipient cells, as it cannot bind cyclic AMP but can inhibit C. The potential of this RI mutation to inactivate the catalytic subunit could provide a powerful tool for blocking cyclic AMP responses in cells in which it is expressed.

The double mutant V89A/V376G had an activation curve shifted to the left, i.e. sixfold less cyclic AMP is required to activate the Cα kinase activity. To determine which mutation was responsible for this result, the experiment in Figure 5(b) was performed. The region of the RI cDNA containing V376G mutation and the region containing the V89A mutation were independently cloned into a wild-type RI cDNA, thereby creating two separate RI mutants, V89A and V376G. These recombinant proteins were then tested for cyclic AMP activation of the holoenzyme as described in the Materials and methods section. The activation of the V376G RI/Cα protein was identical with the wild-type RI/Cα holoenzyme. However, the EC_{50} for PKA activation by cyclic AMP of the V89A RI/Cα holoenzyme was sevenfold less than the EC_{50} for the activation of the wild-type RI/Cα holoenzyme (Table 1). Thus the shift to the left of the activation curve for the double mutant can be attributed to the V89A mutation alone. In addition, the V376G site-B mutation

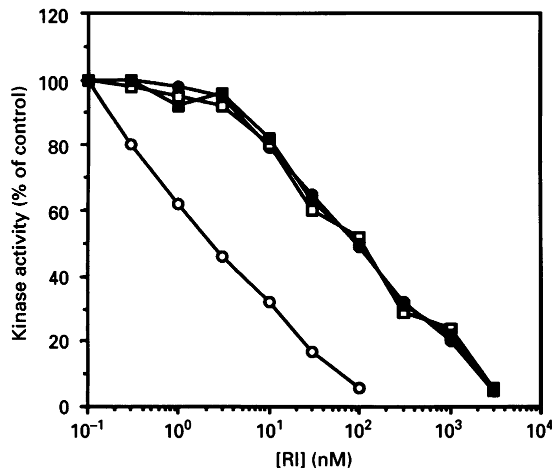

Figure 6 The V89A mutation alters the ability of regulatory subunit to interact with Cα

Bacterial expressed Cα subunits were DEAE-Sephacel purified and incubated with the indicated concentrations of DEAE-Sephacel-purified wild-type or mutant CHO RI subunits for 1 h at 4 °C in buffer A. Values are shown as a percentage of Cα alone (1526 ± 261 c.p.m./μg of protein). The figure shows wild-type (○, ●) and V89A (□, ■) in the absence (○, □) or presence (●, ■) of 60 or 10 nM cyclic AMP for wild-type and V89A RI protein samples respectively. Data represent a typical experiment that was repeated three times.

does not alter cyclic AMP binding, nor does it alter the cyclic AMP activation of the holoenzyme complex (Table 1 and Figure 5b). The V376G mutation in site B served as a negative control. This mutation did not alter cyclic AMP binding, nor did it alter the cyclic AMP-mediated activation of the holoenzyme. This result suggests that residue 376 may not be essential for cyclic AMP binding, or that the Val-Gly mutation is too conservative to alter function.

The decreased EC_{50} of cyclic AMP activation of the V89A/Cα holoenzyme could possibly be attributed to a decreased association or an increased dissociation of this mutant RI protein for Cα. To address this point, increasing amounts of the wild-type or V89A RI protein were added to the Cα protein (Figure 6). The ability of the RI subunit to inhibit the Cα subunit activity was measured in the absence and presence of cyclic AMP at a concentration equal to the EC_{50} value obtained from the experiment in Figure 5. The ability of the wild-type RI subunit to inhibit Cα activity was shifted to the right in the presence of 60 nM cyclic AMP. IC_{50} values were 3 nM in the absence of and 100 nM in the presence of 60 nM cyclic AMP. In contrast, the VA89 mutant RI protein was less efficient at inhibiting the Cα subunit. In the absence of cyclic AMP, its IC_{50} of 97 nM was similar to the wild-type RI in the presence of cyclic AMP. These data taken together suggest that decreased association of the V89A mutant with C was responsible for its phenotype. The V89A mutant RI inhibition of the Cα subunit was not altered in the presence of 10 nM cyclic AMP, its EC_{50} value.

The mutation at V89A does not alter cyclic AMP binding; however, this RI subunit does have a reduced ability to interact with the Cα subunit. The IC_{50} values for wild-type RI subunit inhibition of Cα obtained here are similar to those reported for yeast regulatory subunit (BCY1) [40] and differ approximately tenfold from values reported for wild-type bovine RI [41]. The difference between these previous studies and ours is that we have prepared RI in the absence of cyclic AMP, so it was not necessary to remove bound cyclic AMP from RI before reconstitution with C. Perhaps this apo-RI more clearly represents nascent RI as it interacts with C within the cell. The V89A mutation generates an RI protein that is a hundredfold less inhibitory than wild-type, which is similar to reported BCY1 mutations in the defined hinge region. Therefore the interaction site of the RI subunit previously defined as amino acid positions 94–99 (Arg-Arg-Arg-Gly-Ala-Ile) with the Cα subunit [2] may need to be extended to include the valine at residue 89. Further knowledge of this region will be essential to permit an understanding of why cyclic AMP binding results in dissociation of RI from C, and whether further changes in this region could actually prevent such a dissociation.

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