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Biochemistry

Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity

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ABSTRACT **Mutations were identified in the catalytic subunit (C) of the cAMP-dependent protein kinase (EC 2.7.1.37) that block inactivation by regulatory subunit (R) without compromising catalytic activity. Randomly mutagenized mouse C expression vectors were screened functionally for clones that stimulated gene induction in the presence of excess R. Point mutations in the C coding sequence were identified that result in a His → Gln substitution at amino acid 87 (His87Gln) and a Trp → Arg change at amino acid 196 (Trp196Arg). In contrast to wild-type C, both mutants retained partial activity in the presence of excess R isoform RIα, although only Trp196Arg retained partial activity in the presence of excess R isoform RIIα. A C expression vector that included both mutations was fully active in promoting gene induction and was virtually unaffected by an 80-fold excess of either RIα or RIIα. These results demonstrate that mutations at His-87 and Trp-196 alter R interactions with C at a site that is not involved in substrate recognition or enzymatic activity. In contrast to these randomly generated mutations, a site-specific alteration of the autophosphorylated Thr-197 to an Ala resulted in an 80% loss of biological activity and partial resistance to R inhibition. The location and proximity of His-87 and Trp-196 in the crystal structure of C suggest a surface domain that may interact with a region of R that is outside of the substrate/pseudosubstrate site.**

The intracellular second messenger cAMP mediates most of its effects by regulating the activity of cAMP-dependent protein kinases (cAPK) (1). The cAPK holoenzymes are inactive tetramers that, in the absence of cAMP, are comprised of two regulatory subunits (Rs) and two catalytic subunits (Cs). Activation occurs when two cAMP molecules bind to each R, causing the release of active Cs (1). In the mouse, four R isoforms (RIα, RIβ, RIIα, and RIIβ) and two C isoforms (Cα and Cβ) have been characterized biochemically and genetically and the tissue-specific expression and assembly of these kinase isoforms are thought to play a role in the diverse cellular responses to cAMP (2).

The structural components of R and C that are required to maintain the inactive tetrameric holoenzyme have not been fully resolved. Each RI and RII isoform contains a "hinge" region with consensus pseudosubstrate and substrate sequences, respectively, for cAPK-mediated phosphorylation (3). Interaction between C and the substrate/pseudosubstrate site in R has been described in several reports (for review, see ref. 4) but was first confirmed by Corbin *et al.* (5) who showed that modification of the substrate site Arg-Arg-Val-Ser in RII blocked autophosphorylation and resulted in kinase activation. The recent report defining the crystal structure of C (6) identified the substrate recognition sequence of C and this is clearly one region of R–C contact. However, the substrate binding site is unlikely to be solely responsible for the nanomolar affinity that exists between C and R since most substrates for C have micromolar affinity (7, 8).

The suggestion that other sites of interaction occur between R and C is supported by the following evidence. (*i*) Although denatured RII is still a substrate for C, it cannot inhibit catalytic activity and does not bind cAMP (9). (*ii*) A mutant RII in which the two substrate-site arginines were replaced with alanines is not a substrate for C but binds to C in a cAMP-dependent manner without inhibiting enzymatic activity (4). These reports suggest that additional sites of interaction exist in regions of C and R that are distinct from the substrate binding site and the hinge region, respectively. We used random mutagenesis and a functional screen to identify a specific C domain that regulates interaction with R but that does not participate in catalysis.

EXPERIMENTAL PROCEDURES

Cell Culture. Human JEG-3 choriocarcinoma cells were grown as described (10) on 24-well plates.

Transient Cotransfection of JEG-3 Cells and Luciferase Assay. Luciferase activity was used to assess C activation of a cAMP-responsive reporter gene, α168-luciferase, as described (11), with the following exceptions. Medium (250 μl) was replaced on cells 4–6 hr prior to the addition of 25 μl of calcium phosphate precipitate (pH 6.95) containing 2.5 ng of α168-luciferase, 50 ng of the internal control plasmid RSV-βgal (12), and carrier plasmid pBluescript KS+ (Stratagene) to equal a total of 250 ng of DNA per 12-mm well. Zinc-inducible C and R expression vectors were included as described below. All experiments were performed in duplicate. Precipitates remained on cells for 22–24 hr in a 3% CO_2/97% air incubator and were then replaced with medium containing 2.5% (vol/vol) newborn calf serum and 80 μM $ZnSO_4$. Eighteen to 22 hr later, cells were washed twice with 1 ml of ice-cold phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.6) and then lysed in 100 μl of 0.1% Triton X-100/1 mM dithiothreitol/6 mM $MgSO_4$/4 mM ATP/100 mM potassium phosphate, pH 7.8.

Lysates (30 μl) were assayed for luciferase activity as described (13), except that 6 mM $MgSO_4$ and 4 mM ATP were used. β-Galactosidase assays were performed essentially as described (12), using 96-well microtiter plates. Luciferase activity was divided by β-galactosidase activity to normalize for differences in transfection efficiency.

Chemical Mutagenesis of DNA and Construction of Expression Vectors. pMC (4 μg), a plasmid containing the entire mouse Cα cDNA (14), was boiled and then treated with 1 M sodium nitrite/250 mM sodium acetate, pH 4.3, in 20 μl for 43 min at room temperature. The same amount of pMC was treated with 11 M formic acid for 5 min at room temperature.

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Abbreviations: cAPK, cAMP-dependent protein kinase; C, catalytic subunit; R, regulatory subunit; PKI, protein kinase inhibitor.

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The chemically treated plasmids were ethanol-precipitated with 50 μg of chicken oviduct RNA and 167 mM sodium acetate (pH 5.5) prior to PCR amplification of the Cα coding region as described (11). Expression vectors containing amplified Cα cDNA under the control of the metallothionein-I promoter and with the human growth hormone poly(A) region were constructed as described (11) to allow regulation of mRNA expression by zinc concentration. A bacterial library comprised of 1056 clones was constructed (96, 368, and 592 clones from untreated, nitrous acid-treated, and formic acid-treated cDNA, respectively).

Screen of Library Clones. Minipreps comprised of 24 library clones were amplified as 44 pools. Plasmid DNA was prepared as described (11). Each pool (37.5 ng) was cotransfected with and without a 2-fold excess (75 ng) of the RIα expression vector REV_{WT} (10) in the cotransfection assay, and luciferase activity was measured. Minipreps of individual C clones in a positive pool were amplified separately and prepared as described above. Clones (2.5 ng) were included in the cotransfection assay with and without a 30-fold excess of REV_{WT} (75 ng).

Identification of Functional C Mutations. Domain swapping was used to identify positive regions of the mutant C and these regions were then subcloned into pBluescript KS+ and sequenced. Sequencing reactions were performed in both directions with dye-labeled T3 and T7 oligonucleotide primers and *Taq* polymerase according to protocols provided with the sequencing reagents from Applied Biosystems. Samples were analyzed with the Applied Biosystems model 370A DNA sequencer.

Construction of C Expression Vectors Containing Single Amino Acid Mutations. Individual mutations (listed in Fig. 4 and Thr197Ala, ACC → GCC) were introduced into the C coding sequence by PCR mutagenesis using pMC as template. Mutant C expression vectors were mapped and sequenced.

Construction of Double Mutant His87/Trp196. An *Nco* I–*Pst* I fragment from His87Gln and a *Pst* I–*Apa* I fragment from Trp196Arg were ligated into the expression vector described above.

RESULTS

Mutagenesis of C and Screening Strategy. Random mutations were introduced into the C coding sequence by treatment with nitrous or formic acid followed by PCR amplification. The mutant coding region was isolated from the PCR mixture as an 1154-base-pair *Nco* I–*Apa* I fragment and was subcloned into the C expression vector (Fig. 1).

A functional screening strategy was designed to identify mutations that gave constitutive C activity in the presence of excess R by using the transactivation of a cAMP response element-regulated reporter gene, α168-luciferase. The reporter contained the coding region of firefly luciferase preceded by a 168-base-pair sequence isolated from the human chorionic gonadotropin α gene promotor (10, 15). Overexpression of C stimulates transient expression of α168-luciferase and induces luciferase activity 10- to 30-fold in JEG-3 cells (10, 11, 15); however, this induction may be completely inhibited by cotransfection with R expression vectors (11). Therefore, library screening was accomplished by measuring the induction of luciferase activity by mutagenized C in the presence and absence of excess RI.

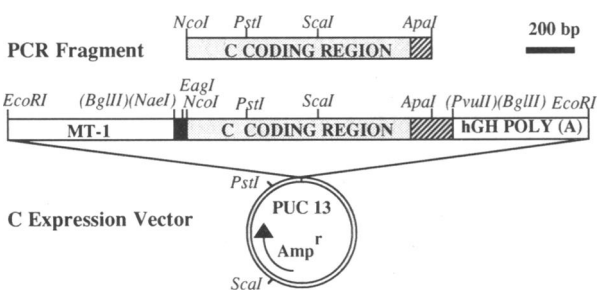

FIG. 1. Construction of mutant C expression vectors. Mutagenized C DNA was PCR-amplified and inserted into a zinc-inducible expression vector. Restriction sites used to construct vectors are indicated. Sites in parentheses are not reconstituted. MT-I, metallothionein-I promoter; hGH, human growth hormone; bp, base pair(s); Amp^r, ampicillin resistance.

Screen of Mutagenized C Vectors. Individual clones were pooled in groups of 24 and plasmid minipreps were tested for activity in the cotransfection assay. Most were completely inhibited in the presence of RI; however, one nitrous acid-treated pool (C7) and one formic acid-treated pool (C18) led to constitutive gene induction. As an example, identification of the positive clone C7.3 from pool C7 is shown in Fig. 2. The C7 pool expressed about 7% constitutive C activity (Fig. 2*A*). The 24 individual clones in this pool were then screened for activity in the presence of RI and, as shown in Fig. 2*B*, 9 clones were clearly inactive, 14 were active and completely inhibited by RI, and one clone (C7.3) was active but partially unregulated. The distribution of active and inactive clones was representative of that in the remaining positive pool C18 (data not shown). Clones C7.3 and C18.8 were chosen for

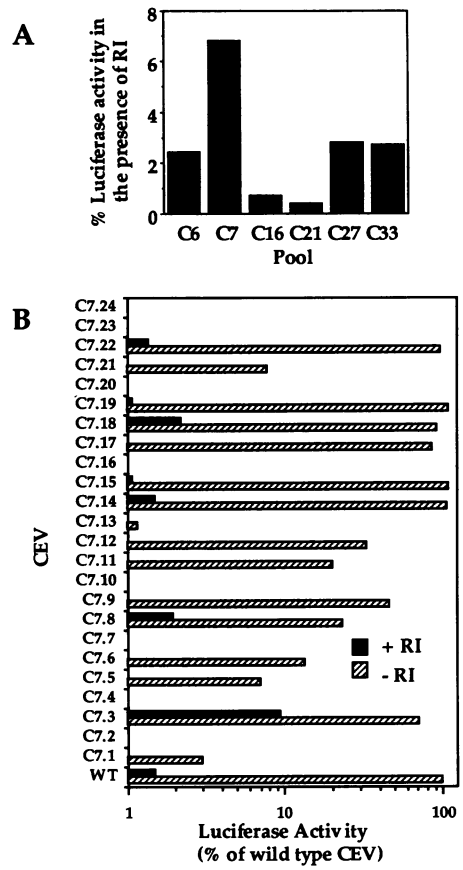

FIG. 2. Mutant C clones activate gene induction in the presence of R. (*A*) Pooled clones were cotransfected with the luciferase reporter gene, in the presence and absence of REV_{WT} (RI). Luciferase activity reported is equal to the percentage of C activity that remains in the presence of R, after subtracting activity contributed by endogenous kinase (<5% of total activity). Activity in the absence of R was comparable for all pools. (*B*) Individual clones from pool C7 were cotransfected with REV_{WT} (RI). Luciferase activity for all clones is expressed as a percentage of the activity induced by wild-type C (WT) minus activity contributed by endogenous kinase. CEV, C expression vector.

further analysis. As a control, pool C27 (Fig. 2*A*) was analyzed individually and contained no positive clones (data not shown).

Localization of Mutant Domains. The large number of inactive clones (35–45%) suggested that multiple mutations might exist in the C coding sequence. Therefore, the phenotypic mutations were localized by swapping portions of mutant and wild-type vectors to generate the chimeric C expression vectors shown in Fig. 3. The 5′ halves of both the C7.3 and C18.8 clones were found to encode the mutant phenotype.

Identification of Specific Mutations. Nucleotide sequences of the positive *Nco* I–*Sca* I fragments of both C7.3 and C18.8 were compared to wild type. Base changes that predict protein coding sequence changes are summarized in Fig. 4*A*. Two codon changes were found in clone C7.3, and three changes were found in clone C18.8. These results confirmed that multiple mutations were randomly created in the cDNA molecules as a result of the original chemical treatment.

Expression of Cs Containing Single Codon Changes. Each codon change was introduced separately into the C sequence to determine which amino acid change altered R interactions. The mutation Ala3Val was not included based on the observation that an expression vector designed to truncate the amino-terminal 22 amino acids of C appeared to be wild type with respect to both activity and inhibition by R as measured by gene induction in the transient luciferase assay (S.A.O., unpublished observation). Fig. 4*B* shows that the single amino acid changes His87Gln and Trp196Arg were responsible for the mutant phenotypes, whereas His131Arg and Glu155Lys had no effect.

The only mutation previously reported to affect R interaction was isolated from a genetic screen in yeast. The mutated yeast C contained the single mutation Thr241Ala that reduced the binding affinity for R without a complete loss of catalytic activity (16). The homologous mutation in the mouse C, Thr197Ala, was tested and the results are included in Fig. 4*B*. This mutant had an 80% reduction in biological activity but remained partially active in the presence of excess R. Similar results were observed using a Thr197Ala vector that contained a different alanine codon (data not shown). By analogy with the yeast Thr241Ala mutation, it appears that the mouse Thr197Ala mutation has resulted in a loss of catalytic activity, although the biological assay described here would also be affected by a decrease in C protein stability. Mutations that affect both catalytic activity and R interactions might be due to changes in recognition of the R pseudosubstrate site.

Titration of Mutant C with R Isoforms. Titrations of C activity with R in the transient cotransfection assay provide indirect information about the efficiency with which various R isoforms can inhibit the mutant C. As shown in Fig. 5*A*, the ability of RIα to inhibit gene induction activated by C was slightly decreased by the presence of the mutation His87Gln, giving a 2.2-fold shift of the IC_{50} from an R/C ratio of 0.9–2. This mutation also prevented RIα from suppressing luciferase activity completely, even at a 30-fold excess, whereas a 10-fold excess of RIα was capable of completely inhibiting wild-type C. In contrast, the His87Gln mutant did not prevent RIIα from suppressing luciferase activity to the same levels reached with wild-type C (Fig. 5*B*).

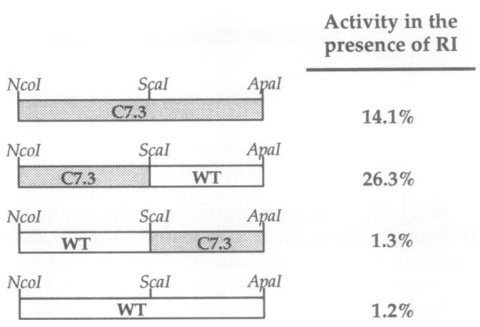

FIG. 3. Functional mutations map to the 5′ half of C. Expression vectors comprised of domains from both mutant and wild-type C were cotransfected with the luciferase reporter gene, with and without REV_{WT} (RI). Activity (mean of four experiments) was calculated as described in Fig. 2*A*.

A

Expression Vector	WT Codon	Mutant Codon	Amino Acid Change
C7.3	GAG	AAG	Glu155Lys
	TGG	AGG	Trp196Arg
C18.8	GCC	GTC	Ala3Val
	CAC	CAA	His87Gln
	CAC	CGC	His131Arg

B

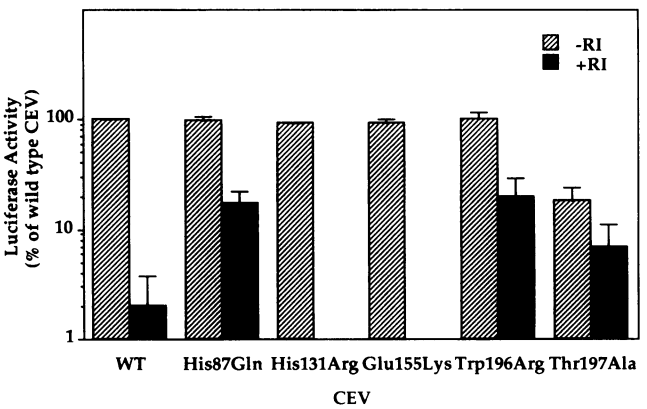

FIG. 4. Point mutations that predict amino acid substitutions are responsible for the mutant phenotype. (*A*) Positive mutant clones were sequenced and the predicted amino acid substitutions are indicated. (*B*) Expression vectors with single codon changes were assayed with and without REV_{WT} (RI). Activity was calculated as described in Fig. 2*B*. CEV, C expression vector. Values are the mean ± SD (n = 3–9).

Fig. 5*C* shows that the ability of RIα to inhibit gene induction activated by C was decreased by the presence of the Trp196Arg mutation, as demonstrated by the 3.8-fold shift of the inhibitory curve. This corresponds to a shift in the IC_{50} from an R/C ratio of 0.8–3. This mutation prevented RIα from suppressing luciferase activity to the same levels reached with wild-type C (Fig. 5*C*), and 25% of the activity remained even if 80 times more RIα than C was included (data not shown). RIIα also inhibited this mutant C less efficiently than wild-type C, as demonstrated by a 10-fold shift in the IC_{50} value from an R/C ratio of 0.8 to 8 (Fig. 5*D*). This mutation allowed retention of 30% activity, even when RIIα was present in 30-fold excess (Fig. 5*D*).

Although these assays represent an indirect measure of cAPK-subunit interactions, it is clear that the C mutation Trp196Arg weakened C interactions with both RIα and RIIα, based on the observed shifts in IC_{50} values and the limited inhibition observed at high R concentrations. The His87Gln mutation had a significant effect only on C interaction with RIα and had a negligible effect on the interaction with RIIα.

Expression of the Double Mutation His87Gln/Trp196Arg. A C that contained both the His87Gln and Trp196Arg mutations was constructed (Fig. 6*A*) and titrated with R in the transient assay. As shown, the double mutant was fully active but almost completely unregulated by RIα (Fig. 6*B*) and RIIα

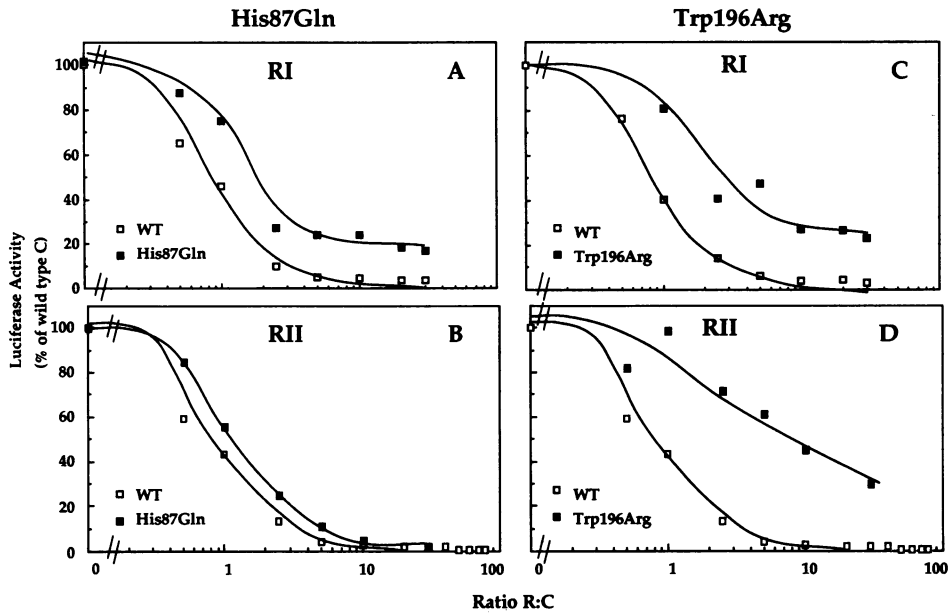

FIG. 5. Inhibition of His87Gln and Trp196Arg mutants by RI and RII. C vectors (2.5 ng) that expressed single codon changes were titrated with increasing amounts of R vector (1.25–195 ng of R) to give R/C ratios of 0.5–78 in the transient assay. Activity was calculated as described in Fig. 2B. (A) His87Gln mutant with REV_{WT} (RI). (B) His87Gln mutant with RIIα expression vector ZR (17). (C) Trp196Arg mutant with REV_{WT} (RI). (D) Trp196Arg with ZR (RII). Wild-type (WT) values are the same as in B. All values are the mean of two to four experiments.

(Fig. 6C). In both cases, the two mutations appeared to act synergistically to prevent inhibition by R.

All of the C mutants described above were tested for biological activity and compared to wild-type C by measuring the induction of luciferase activity with various concentrations of C. With the exception of Thr197Ala, the mutants all appeared to have the same activity as wild type (data not shown).

DISCUSSION

A biological screen of randomly generated mutations in C led to the identification of two amino acid replacements that affected interaction with R without diminishing catalytic activity. Mutation of either His-87 → Gln or Trp-196 → Arg produced a C with constitutive-gene-inducing activity in transfected JEG-3 cells, even in the presence of excess R. A C containing both mutations, His87Gln and Trp196Arg, was completely unregulated by either RI or RII in cotransfected cells.

The proximate positioning of His-87 and Trp-196 in the C protein structure identifies a domain that is at least partially responsible for high-affinity interaction with R. The recently described crystal structure of a C–protein kinase inhibitor (PKI) peptide complex (6) predicts that His-87 is involved in stabilizing the phosphate group on the autophosphorylated Thr-197 and that the neighboring Trp-196 is exposed on the surface of the subunit. It has been postulated that the phosphorylated Thr-197 may play a significant role in conformational stability, perhaps by ensuring that adjacent hydrophobic residues are positioned correctly to recognize the hydrophobic P + 1 site on substrates (18). The role of the hydrophobic Trp-196 on the surface of C is unknown, but this tryptophan or a homologous tyrosine is conserved in all the cyclic nucleotide-regulated protein kinases (19). Perhaps Trp-196 makes a specific contact with one of the hydrophobic residues on the carboxyl-terminal side of the substrate/pseudosubstrate site in R. A Trp → Arg replacement would introduce a strong positive charge at this position and may disrupt potential hydrophobic interactions. His-87 and Trp-196 do not directly contact the PKI peptide in the crystal structure; therefore, the mutations His87Gln and Trp196Arg might not be expected to interfere with the ability of PKI to block C activity. Preliminary results indicate that, as predicted, cotransfection with a PKI expression vector (provided by R. Maurer, University of Iowa) effectively inhibits the activity of the double mutant C (S.A.O., unpublished data).

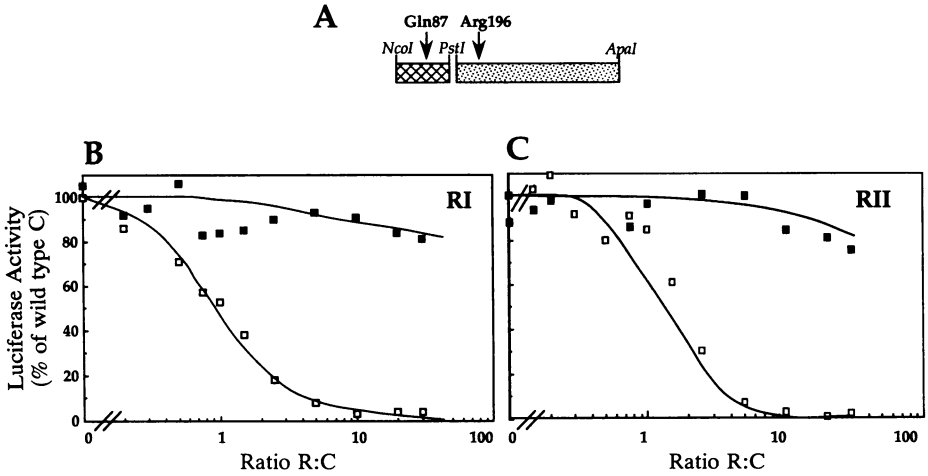

FIG. 6. Double mutant His87Gln/Trp196Arg is unregulated by RI or RII. (A) A vector containing the two mutations, His87Gln and Trp196Arg, was constructed. (B) Double mutant C was titrated with increasing amounts of REV_{WT} (RI) in the transient cotransfection assay. (C) Double mutant His87Gln/Trp196Arg was titrated with ZR (RII). Luciferase activity was calculated as in Fig. 2B. □, Wild type; ■, His87Gln/Trp196Arg.

The assay system described here tests the ability of R to inhibit the biological function of a mutant C under physiological conditions that include the presence of basal concentrations of cAMP. These levels may be too low to activate a wild-type holoenzyme, but the observation that a plateau occurs in the ability of RI to inhibit either the His87Gln or Trp196Arg mutant C (Fig. 5 *A* and *C*) suggests that in the presence of basal cAMP, the mutant holoenzyme may be partially dissociated. The double mutant C may still bind both RI and RII with low affinity, but the titration experiments shown in Fig. 6 suggest that basal cAMP concentrations are sufficient to keep this mutant holoenzyme fully dissociated. This idea is supported by the observation that a mutant RI lacking functional cAMP binding sites (2) is more effective than wild-type RI as an inhibitor of the double mutant C (S.A.O., unpublished data).

Previous studies identified a mutation in the yeast C that resulted in unregulated activity (16). This mutation (Thr-241 → Ala) also produced a decreased affinity for both ATP and the peptide substrate tested (16). We tested the homologous Thr-197 → Ala mutation in the mouse C and the results indicate that this mutant retains only 20% of the activity of wild-type C but is partially resistant to inhibition by R. These results are in good agreement with the yeast mutant but indicate that this mutation, unlike His87Gln and Trp196Arg, may affect C interactions with R by modifying the substrate recognition site. A recent report examining the biological activity of a Thr197Ala mutant C in a transactivation assay using the enkephalin promoter indicated that the Thr197Ala mutant was completely inactive (20). Our results showing partial activity may be due to differences in substrates or assay sensitivity. Results obtained with homologous kinases support the suggestion that a phosphorylated Thr-197 is crucial for protein kinase function. Phosphorylation of cdc2 at Thr-161 in *Xenopus* and at Thr-167 in yeast (both homologous to Thr-197) has been shown to be required for both activation of cdc2 kinase activity and for binding to cyclins (21, 22). Homologous threonine phosphorylation in mitogen-activated protein kinase also appears to be required for function (23). Therefore, it is possible that Thr-197, in a number of homologous protein kinases, may be involved in determining catalytic activity as well as promoting protein–protein interactions.

The transient biological assay described here may prove to be useful in screening for dominant mutations in any of the components of the cAMP system, including receptors, guanine nucleotide binding proteins, adenylate cyclases, or phosphodiesterases. The likelihood that an unregulated mutant C might arise physiologically and result in a disease state is suggested by observations that somatic mutations in another component of the cAMP system, the stimulatory guanine nucleotide binding protein α subunit Gsα, give rise to tumors (24) or a mosaic endocrine disease, McCune–Albright syndrome (25). The constitutively active C we have constructed with His87Gln and Trp196Arg mutations will also be useful in cell regulation studies to determine the role of various kinases in hormonal responses.

By using a random mutagenesis/screening approach, we have identified a domain on C that appears to act as a high-affinity binding site for R. The characterization of more C mutants should further define this domain and perhaps indicate other sites of interaction. Our model for the cAPK holoenzyme suggests an R anchored to C in the vicinity of Trp-196 by high-affinity interactions. This promotes the occupancy and inhibition of the active site on C by the substrate/pseudosubstrate region of R. When cAMP binds to R, it disrupts the high-affinity C–R interaction. We speculate that the region of R that contacts Trp-196 and neighboring amino acids on C is the region that changes its conformation in response to cAMP.

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