A Mutation Affecting the Catalytic Subunit of Cyclic AMP-dependent Protein Kinase in CHO Cells*

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A mutant of Chinese hamster ovary (CHO) cells has been selected which is unresponsive to the growth inhibitory effects of choleratoxin or other agents which elevate intracellular cyclic AMP levels. Crude extracts of the mutant CHO cells exhibit altered cyclic AMP-dependent protein kinase (ATP protein phosphotransferase; EC 2.7.1.37) activity when compared with the parent. With histone as substrate, the cyclic AMP-dependent protein kinase of mutant cells exhibits an approximate $K_a$ for activation by cyclic AMP 100-fold greater than that of the enzyme from wild type cells, while cyclic AMP binding activity is not appreciably altered. The DE52-cellulose elution profile of the mutant protein kinase is markedly different from that observed for the wild type. As determined by DE52-cellulose chromatography, the mutant has more type I protein kinase activity which is stimulated only by high levels of cyclic AMP, whereas type II kinase activity is very low or nonexistent. The approximate $K_a$ for cyclic AMP activation of mutant type I protein kinase ($K_a \sim 1 \mu M$) is significantly altered relative to the parent type I protein kinase ($K_a \sim 2 nM$). The basis of these changes appears to be in the catalytic subunit since the mutant type I protein kinase and the mutant-free catalytic subunit show a decreased affinity for the ATP substrate when compared to the wild type. Furthermore, the mutant-free catalytic subunit has a different protein substrate specificity from the wild type. We conclude that the mutant cells have an altered catalytic subunit of cyclic AMP-dependent protein kinase.

The molecular basis for the action of cyclic AMP in mammalian tissues is not completely understood. Kuo and Greenberg (1) and others have suggested that all of the effects of cyclic AMP are mediated by cyclic AMP-dependent protein kinases, but direct effects of cyclic AMP on transcription as occur in prokaryotes (for review see Ref. 2) and other still undefined mechanisms have not been excluded. We have begun a genetic analysis of somatic cell mutants unable to respond to cyclic AMP in an effort to understand the mechanism of cyclic AMP action in cultured mammalian cells.

Many aspects of the behavior of cultured Chinese hamster ovary cells (CHO) and other fibroblastic cells are controlled by the intracellular concentration of cyclic AMP. This nucleotide regulates cell shape, causing cells to become elongated and flattened. It also slows cell growth (3, 4), decreases agglutinability by plant lectins (5, 6), increases collagen synthesis (7), and in CHO cells induces hamster endogenous virus (8).

When CHO cells are exposed to choleratoxin, which stimulates adenylate cyclase and, thereby, increases cyclic AMP levels, the same cell shape change and growth inhibition occurs as previously reported with cyclic AMP treatment (9). This increase in cyclic AMP level is correlated with activation of cyclic AMP-dependent protein kinase (10, 11), suggesting that the activity of cyclic AMP may be mediated by phosphorylation of cellular components.

In this paper, we report the biochemical characterization of a CHO mutant which is resistant to the growth inhibitory effects of choleratoxin. This is one of a series of independent mutants isolated by a variety of means, which do not show any growth inhibition or morphological change when exposed to 8-bromo-cyclic AMP and are relatively resistant to a variety of other agents which raise intracellular cyclic AMP levels. In order to elucidate the defect in the mutant, cyclic AMP-dependent protein kinase in wild type and mutant cells have been compared. We find the mutant CHO cells have a defective cyclic AMP-dependent protein kinase and that the defect resides in the catalytic subunit of this enzyme. The existence of this mutant provides strong direct evidence that the growth inhibitory and morphological effects of cyclic AMP in CHO cells are mediated by cyclic AMP-dependent protein kinase.

**MATERIALS AND METHODS**

**Isolation of Cholera Toxin-resistant Mutants**—The CHO cell line 10001 was a subclone of a CHO Pro line kindly sent to us by Dr. L. Siminovitch, and originally derived by Puck et al. (12). After ethylmethanesulfonate mutagenesis in suspension at 37°C (150 µg/ml for 7 h), cells were allowed to grow for 2 weeks under nonselective conditions. At this time, 2.0 x 10^6 cells were washed and suspended at 2 x 10^6 cells/ml in serum-free medium containing 1 µg/ml of choleratoxin (Schwarz/Mann). After 3 h, the medium was made 10% in fetal bovine serum and cells were incubated in suspension for 5 days. Choleratoxin slows CHO cell growth but does not quantitatively kill cells. Growing cells were washed and treated with choleratoxin as described above for 5 more days. At this time, cells were plated on 150-mm dishes in 100 ng/ml of choleratoxin and clones were grown and scored for size and morphology. Strain 10215 which survived this selection was a large clone which did not show the elongated cells characteristic of choleratoxin treatment. In the absence of choleratoxin, growth of these mutant cells was indistinguishable from growth of the parental cells and its modal chromosome number was also unchanged. Full details of this and other selections for protein kinase mutants will be published elsewhere.

**Growth of Cells**—Cells were grown in suspension at 37°C in a

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1 The abbreviations used are: CHO cells, Chinese hamster ovary cells; a-ME medium, o-modification of Eagle's medium; EGTA, ethylene glycol bis(b-aminoethyl)ether-N,N,N',N'-tetracetic acid.

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modification of Eagle's medium with Earle's salts (α-MEM medium) (Flow Laboratories) with 10% fetal bovine serum (Associated Biomedic Systems, Inc.) as described elsewhere (13). The cells were plated on 60- or 100-mm tissue culture dishes (Costar) 3 days prior to each assay in a 5% CO₂ humidified atmosphere. The growth medium was changed 24 h prior to assay.

Adenylate Cyclase Assay—For the determination of adenylate cyclase activity, cells (approximately 4 × 10⁵/100 mm dish) were incubated for 3 h in serum-free α-MEM medium with the indicated concentration of cholera toxin. The cells then were washed twice with phosphate-buffered saline, harvested, and homogenized in a Dounce homogenizer in 50 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, and 0.33 μg esucrose. Adenylate cyclase activity was assayed by measuring the conversion of [α-32P]ATP to cyclic [32P]AMP in crude membranes (12,000 × g pellet) as described previously (14). The standard incubation mixture (final volume, 50 μl) contained 25 mM Tris-HCl (pH 7.8), 10 mM phosphoenolpyruvate, 4 μg of pyruvate kinase, 5 mM MgCl₂, 0.2 mM ATP, 0.5 mM EDTA, 4 × 10⁻⁴ M cyclic AMP, and 50 to 90 μg of membrane protein. The incubation was carried out for 15 min at 37°C.

Cyclic AMP Determination—Cyclic AMP levels were measured by radioimmunoassay in cells grown on 60-mm dishes as previously described (15) except that the samples were acetylated (16) prior to assay.

Protein Kinase Assay—Protein kinase was determined by measuring the transfer of [γ-32P]ATP to histone, as by Corbin et al. (17). Cells (approximately 8 × 10⁵/100 mm dish) were washed twice with cold phosphate-buffered saline and once with cold homogenization buffer (10 mM Tris (pH 7.4), 1 mM dithiothreitol, 4 mM EDTA) harvested by scraping from the dish with a rubber policeman, and disrupted with a Dounce homogenizer (30 strokes) in homogenization buffer. The 50,000 × g supernatant of the homogenate, kept at 4°C was assayed without delay. The reaction was initiated with the addition of cell extract to a mixture containing 25 mM 2-N-morpholinoethanesulfonic acid (pH 7), 0.5 mM EDTA, 2.5 mM NaF, 5 mM magnesium acetate, 600 μg of histone (Type II A, Sigma Chemical), and 0.1 mM [γ-32P]ATP (~300 cpm/pmol, New England Nuclear) in a total volume of 0.1 ml. After incubating at 30°C for 15 min, 25-μl aliquots were spotted onto filter paper strips (Whatman 3MM) and dropped into cold 5% trichloroacetic acid. A no enzyme blank value was subtracted from the total incorporation.

Cyclic AMP Binding Assay—Cyclic AMP binding activity was determined by the Millipore filtration method (18). The incubation mixture (0.2 ml) containing 50 mM sodium acetate (pH 6.5), 0.2 mg of bovine serum albumin, 1 mM methylisobutylxanthine, 10 mM cyclic [3H]AMP (25,000 cpm/pmol), and the cell extract was incubated for 2 to 8 h at 0°C. The filters were washed with 10 ml of cold 50 mM sodium acetate buffer (pH 6.5), dried in air, and dissolved in 0.6 ml of 5% TCA-NaOH (Amersham). The radioactivity was counted in 15 ml of Hydromix (Yorktown). The blank value (nonspecific binding in presence of 1 μM cyclic AMP) was subtracted from the total binding.

Protein was determined by the method of Lowry et al. (19) with bovine serum albumin as a standard.

DEAE-Cellulose Chromatography—For DEAE-cellulose fractionation, 4.5 × 10⁵ cells were grown on dishes were disrupted in 10 ml of Buffer I (10 mM sodium phosphate, pH 7.6, 0.2 mM EDTA, 4 mM dithiothreitol) with a Dounce homogenizer (30 strokes). The homogenate was centrifuged at 50,000 × g for 15 min and the supernatant was applied to a DE52-cellulose column (0.6 × 13 cm), equilibrated with Buffer I. After sample addition, the column was washed with 25 ml of Buffer I; the column then was eluted with a linear gradient of 35 ml of Buffer II (500 mM NaCl) containing 0.4 M NaCl. The flow rate was 0.25 ml/min and fractions of 1 ml were collected. The fractions containing kinase activity were pooled, dialyzed for 24 h against three changes of Buffer I and concentrated against 70% sucrose (W/V) in 0.2 M NaCl. Free cyclic AMP was removed by dialysis and the concentrated fractions were stored frozen at -70°C.

Isolation of Free Catalytic Subunit—Type I cyclic AMP-dependent protein kinase was isolated from mutant and wild type cells by DE52-cellulose chromatography as described above. Dialyzed, concentrated fractions containing type I protein kinase were incubated with 10 μM cyclic AMP for 60 min at 4°C to obtain binding equilibrium and then for 4 min at 30°C to facilitate the dissociation of catalytic subunit from the cyclic AMP regulatory subunit complex. The dissociation preparation was applied to a DE59 column (0.6 × 4 cm). Free catalytic subunit was eluted with Buffer I containing 1 μM cyclic AMP. Fractions of 0.5 ml were collected; bovine serum albumin (0.1 mg/ml) was added to each fraction tube prior to collection.
Cyclic AMP-dependent Protein Kinase Mutant

Fig. 2. Stimulation by various cyclic AMP concentrations of wild type (●) and mutant (○) protein kinase activity in the supernatant prepared by 50,000 × g centrifugation of homogenates for 15 min. Phosphotransferase activity was measured with 50 µg of protein/assay as described under "Materials and Methods." 

Fig. 3. Cyclic AMP binding activity of the wild type (●) and mutant (○) supernatant prepared by 50,000 × g centrifugation of homogenates for 15 min. Cyclic AMP binding was assayed as described under "Materials and Methods" with 150 to 300 µg of supernatant protein/tube.

dependent protein kinase from wild type CHO cells after DEAE-cellulose chromatography. Two cyclic AMP-dependent protein kinases are eluted from the column with increasing ionic strength as expected from the data of others (20, 21). Type I cyclic AMP-dependent protein kinase elutes between 0.06 and 0.09 M NaCl and type II cyclic AMP-dependent protein kinase elutes between 0.125 and 0.15 M NaCl. The addition of 1 µM cyclic AMP to the assay mix stimulated type I and type II protein kinase activities approximately 2-fold. Both kinase peaks show specific cyclic [3H]AMP binding (Fig. 4B). The cyclic AMP-independent protein kinase activity observed in the fractions prior to the gradient is apparently due to free catalytic subunit since this activity is completely inhibited by the protein kinase inhibitor protein (data not shown).

The elution pattern of the mutant kinase activity is markedly different from that observed for the wild type (Fig. 5). The first peak of kinase activity (type I) elutes at about 0.075 M NaCl. This activity is stimulated approximately 3-fold by 10 µM cyclic AMP. In the region where type II protein kinase was expected only a very small amount of cyclic AMP-independent protein kinase was detected. Two distinct peaks of cyclic AMP binding were observed with Kd values similar to that of type I and type II cyclic AMP-dependent protein kinases from the wild type (Kd ≈ 50 nM).

Characterization of DEAE-fractionated Protein Kinase Activities—Type I protein kinase of the mutant is dissociated by incubation with histone (data not shown). This property is similar to that of the type I protein kinase of the wild type cells and that of numerous other cell types (22, 23). However its apparent Kd for cyclic AMP activation (Kd ~ 1 µM) is dramatically altered from the cyclic AMP activation curve for the parent type I protein kinase (Kd ~ 2 nM). Further, the
Cyclic AMP-dependent Protein Kinase Mutant

The mutant type I protein kinase exhibits a markedly altered ATP concentration curve as compared to the type I protein kinase of the wild type. This is apparently due to a decreased affinity for the ATP substrate.

Characterization of the Free Catalytic Subunit—The abnormal affinity for ATP, as noted with crude extracts and with DE52-purified type I cyclic AMP-dependent protein kinase, suggests that the catalytic subunit of the mutant is defective. To test this hypothesis the catalytic subunit from both wild type and mutant cells was obtained as described under "Materials and Methods." The catalytic subunit isolated from the mutant shows the same alteration in its ATP dependence as noted with type I protein kinase (Fig. 6B). One possible explanation for this finding is that the altered ATP requirement results from increased ATPase activity present in the mutant preparation. Studies carried out to assess this possibility show that neither catalytic subunit preparation significantly hydrolyzed 10 μM [γ-32P]ATP.

The mutant free catalytic subunit also exhibits an altered protein substrate specificity when compared to the wild type free catalytic subunits. When assayed with 0.5 mM [γ-32P]ATP mutant and wild type free catalytic subunits catalyze the phosphorylation of histone f2b to the same extent (Fig. 7A). However, as shown in Fig. 7B, the mutant catalytic subunit is only 40% as effective in phosphorylating casein and does not phosphorylate phosvitin to any detectable extent. The mutant do not differ in the optimal pH (6.5) or the optimal temperature (37°C) of the phosphotransferase reaction with histone II A as substrate. Both catalytic subunits are completely inhibited by the protein kinase inhibitor protein (Sigma Chemical) (data not shown).

Sucrose Density Gradient Sedimentation of DE52-fractionated Protein Kinase Activities—To determine whether any catalytic subunit was bound to the R subunit in the mutant, type I and type II cyclic AMP-dependent protein kinase activities isolated by elution from DE52 columns were sedimented through a 5 to 20% W/V sucrose gradient. As shown in Fig. 8A type I protein kinase of the wild type sediments as a peak of holoenzyme showing cyclic AMP-dependent protein kinase activity and cyclic AMP binding activity with a sedimentation coefficient (s20,w) of 6. The peak of cyclic AMP-independent kinase activity with an s20,w of 3.2 corresponds to the free catalytic subunit apparently dissociated during preparation. Type II protein kinase of the wild type sediments as a peak of holoenzyme with an s20,w of 6.9. A second peak of cyclic AMP binding activity with an s20,w of 5 corresponds to the presence of regulatory subunit not bound to catalytic subunit (Fig. 8B). When type I and type II protein kinases of the wild type are dissociated by incubation for 2 h with 10 μM cyclic AMP, single peaks of protein kinase activity from type I and type II were observed corresponding to free catalytic subunit with an s20,w of 3.2 (Fig. 8, C and D).

Type I cyclic AMP-dependent protein kinase from the mutant sediments essentially as a single peak with cyclic AMP-dependent protein kinase activity and cyclic AMP binding activity with an s20,w of 6 which is similar to that of wild

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Footnote:

1. D. Evain, M. Gottesman, I. Pastan, and W. B. Anderson, unpublished data.
Fig. 8. Sucrose density gradient centrifugation of wild type cyclic AMP-dependent protein kinases. The 5 to 20% w/v sucrose gradients (13 ml) were run for 18 h at 39,900 rpm in an SW 40 Ti rotor and were collected in fractions of 0.35 ml. Aliquots were taken of these fractions and analyzed for protein kinase activity in the presence (A—A) or absence (C—C) of 10 μM cyclic AMP and for cyclic AMP binding capacity (■—■). Phosphotransferase activity is expressed as picomoles of 32P transferred/min/40 μl and cyclic AMP binding is given as femtomoles of cyclic AMP bound/90 μl. Ph. b, phosphorylase b; Hb, hemoglobin. A, PKI initially obtained by elution from a DE52 column (pool of Fractions 36 to 41) was applied to the gradient and sedimented as described. B, PKII, which was isolated by elution from a DE52 column (pool of Fractions 49 to 60), was applied to the gradient and centrifuged as described. C, PKI was incubated for 2 h with 10 μM cyclic AMP and then sedimented through a sucrose gradient containing 10 μM cyclic AMP. D, PKII was dissociated by incubation for 2 h with 10 μM cyclic AMP and then centrifuged through a sucrose gradient containing 10 μM cyclic AMP.

Fig. 9. Sucrose density gradient centrifugation of mutant cyclic AMP-dependent protein kinases. The legend is the same as that given for Fig. 8. Ph. b, phosphorylase b; Hb, hemoglobin.

Fig. 10. Sucrose density gradient centrifugation of the wild type (Panel A) and mutant (Panel B) free catalytic subunits. The free catalytic subunits were isolated as described under "Materials and Methods," and sedimented through a 5 to 20% w/v sucrose gradient as described in the legend to Fig. 8. Protein kinase activity was determined in the absence of cyclic AMP and is expressed as picomoles of 32P transferred/min/40-μl aliquot.
of 4.

The altered sedimentation coefficient noted for the catalytic moiety of mutant type I protein kinase (Fig. 9C) suggested that either the mutant catalytic subunits exist as a dimer or that the mutant type I cyclic AMP-dependent protein kinase may not be completely dissociated by cyclic AMP. When free catalytic subunits isolated from either wild type or mutant type I protein kinase were sedimented through a similar 5 to 20% w/v sucrose gradient, both exhibited an s20,w of 4 (Fig. 10). This result indicates that the altered s20,w of the protein kinase activity presented in Fig. 9C is due to the incomplete dissociation of mutant type I cyclic AMP-dependent protein kinase by cyclic AMP.

**DISCUSSION**

In animal cells increasing cyclic AMP levels by stimulation of adenylylcyclase is thought to activate cyclic AMP-dependent protein kinase, which in turn phosphorylates specific substrates and mediates the biological activity of the stimulating agent (24). Selection of mutants which are defective at various steps in cyclic AMP action provides a powerful tool to evaluate critically the mechanism by which this nucleotide regulates cell behavior.

Cyclic AMP-dependent protein kinase is normally activated by binding of cyclic AMP to a regulatory subunit. This results in dissociation of the regulatory and catalytic subunits (25, 26) and activates the catalytic subunit. It has been suggested for the protein kinases of a number of mammalian tissues that the catalytic subunits are identical but the regulatory subunits differ (27).

Protein kinase mutants have been isolated in mouse lymphoma cells (28, 29) and in neuroblastoma cells (30) on the basis of their resistance to killing by cyclic AMP. Most of these mutants have abnormal regulatory subunits which cannot bind cyclic AMP normally and, hence, the catalytic subunits cannot be activated. Borman et al. (31) have reported the isolation of a CHO variant which does not respond morphologically to dibutyryl cyclic AMP but the biochemical nature of the defect in this cell line has not been reported.

We have recently described the isolation of 11 independent mutants of CHO cells resistant to the growth inhibitory effect of cAMP. In this work, we describe the detailed characterization of one of these mutants (10215) selected on the basis of resistance to cholera toxin. The growth and morphology phenotype of this mutant is reviewed in Table I.

Crude extracts of the mutant cells have normal cyclic AMP binding activity (Kd, 8 x 10^-8 M) (Table I) but decreased cyclic AMP-dependent protein kinase activity. The mutant extracts require about 100-fold higher cyclic AMP concentrations for activation than wild type extracts. When the mutant protein kinase was chromatographed on DEAE-cellulose, normal cyclic AMP binding activity was observed in the type I and type II regions, suggesting that both regulatory subunits are present and normal. However, mutant type I protein kinase had a 100-fold increase in the apparent Kd, for cyclic AMP binding of protein kinase despite normal cyclic AMP binding (Table II). Furthermore, very little kinase activity was found in the type II region despite normal cyclic AMP binding (Fig. 5 and Table II). Sucrose density gradient studies showed that the cyclic AMP binding noted in the PKII region of the mutant was accounted for almost entirely by free regulatory subunit (Fig. 8). The normal cyclic AMP binding, the altered Kd, of type I protein kinase and the absence of type II protein kinase all suggested a defect in the catalytic subunit.

To investigate this hypothesis, the mutant catalytic subunit was dissociated from R1 by a high concentration of cyclic AMP, and isolated free of R1. The mutant catalytic subunit was found to have an altered affinity for ATP (Fig. 6B) and an altered protein substrate specificity (Fig. 7). The substrate specificity results show that the mutant catalytic subunit is as effective as that of the wild type in phosphorylating the basic proteins histone IIA and fβ. However, the acidic protein phosphovitin is not phosphorylated by the mutant catalytic subunit. Casein is a mixture of both acidic and basic proteins, and the mutant subunit is about 40% as effective as the wild type in catalyzing the phosphotransferase reaction with this substrate. Since the apparent affinity of the mutant catalytic subunit is not altered, it is conceivable that certain proteins within the mixture are phosphorylated normally, while other, possibly acidic, proteins within the casein mixture are not phosphorylated at all. These results indicate that the mutant catalytic subunit may be defective in its ability to phosphorylate certain endogenous substrates even under conditions where it might be dissociated from the regulatory subunit R1.

Taken together these data indicate that the catalytic subunit of mutant protein kinases is abnormal. We propose that in the mutant, this altered catalytic subunit binds more tightly than normal to the regulatory subunit R1, but can be dissociated by high cyclic AMP concentrations. In this model, the failure to find type II cyclic AMP-dependent protein kinase could be due either to a reduced affinity of R2 for the mutant catalytic subunit or to the failure of R2 to compete with R1 for
mutant catalytic subunit. Reconstitution experiments with purified R₁, R₂, and C need to be performed to confirm this proposal. These have not yet been performed due to the limited amount of purified enzyme obtainable and the lability of some of the components.

The small amount of cyclic AMP mediated effect seen in the mutant cells (Table I) may be due to residual type I protein kinase activity at very high cyclic AMP concentrations or to a small amount of wild type catalytic subunit still present in the mutant. Since CHO cells have a nearly diploid DNA content and by G binding techniques have been shown to contain essentially all of the euploid chromatin (32), it seems possible that a large number of autosomal gene products will be represented by two alleles.

Mutants in which one gene is altered will have a recognizable phenotype only when the mutant product is dominant. In the case of our protein kinase mutant in which an abnormal catalytic subunit binds tightly to the R₁ regulatory subunit, a possible phenotype only when the mutant product is dominant.

It is possible that a large number of autosomal gene products will be represented by two alleles.

Although it is not possible with a mutant which affects both type I and type II cyclic AMP-dependent protein kinases to attribute specific components of the biological response to cyclic AMP to either of these protein kinases, the existence of this mutant provides strong direct evidence for the hypothesis that the growth inhibitory and morphologic effects of cyclic AMP are mediated through the catalytic activities of either or both protein kinases in CHO cells and that both kinases share a common catalytic subunit. In addition, a mutant such as this can be used to test whether or not the biological effects of a variety of agents are mediated through cyclic AMP-dependent protein kinases.

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