Direct Evidence That the Protein Kinase Catalytic Subunit Mediates the Effects of cAMP on Tyrosine Aminotransferase Synthesis*

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The effect of purified beef heart cAMP-dependent protein kinase catalytic subunit on tyrosine aminotransferase activity in intact cultured rat H35 hepatoma cells was directly tested by micro-injection using human red blood cell ghosts as vehicles. Although the micro-injection procedure itself produced temporary fluctuations in protein synthesis and in tyrosine aminotransferase activity in H35 cells, after a recovery period of 8–12 h, these parameters returned to normal in parallel with restoration of full inducibility of the aminotransferase by both 8-Br-cAMP and dexamethasone.

Eight to sixteen hours after fusion of H35 cells with unloaded ghosts, ghosts loaded with bovine serum albumin or mock-loaded with the partially purified protein kinase catalytic subunit, no significant change in the activity of the aminotransferase was detected. In contrast, fusion with ghosts loaded with the catalytic subunit at concentrations between 0.1–2 mg/ml caused reproducible 2–3-fold increases in enzyme activity. Homogeneous preparations of the catalytic subunit exhibited even greater potency as an inducer. The effect was both time- and concentration-dependent and was abolished by inactivation of the catalytic subunit with N-ethylmaleimide prior to loading.

The partially purified inhibitor of protein kinase from beef heart, while not affecting basal tyrosine aminotransferase activity, selectively inhibited the ability of 8-Br-cAMP but not that of dexamethasone to stimulate the activity of this enzyme. In addition, micro-injection of the pure regulatory subunit of the kinase blocked the response of the aminotransferase to low concentrations of 8-Br-cAMP. These results provide strong support for the proposition that the catalytic subunit of protein kinase mediates the effects of cAMP on the synthesis of tyrosine aminotransferase.

Although the role of cAMP-dependent protein kinase is now well established in the control of glycogen metabolism (1, 2), the role of this enzyme as a mediator of the effects of cAMP on selective protein synthesis in eukaryotic cells remains uncertain (3, 4). Considerable indirect, correlative data have been obtained which are consistent with the kinase acting as a mediator of cAMP action on the synthesis of selected proteins (5–6) but no direct, causal evidence has been reported. Studies with variant cells, resistant to the cytotoxic effects of cAMP derivatives as a result of alterations in kinase subunits, have implicated the kinase in the control of cell death, steroidogenesis and phosphodiesterase activity (7–9). The last two processes appear to involve selective increases in protein synthesis although this has not been rigorously established as yet.

Maller and Krebs (10) provided direct evidence that the catalytic subunit of cAMP-dependent protein kinase is responsible for the meiotic arrest of Xenopus laevis oocytes caused by persistently elevated levels of cAMP. Theoretically, these studies provided a means by which the role of protein kinase could be tested in any system but to date such an approach has been limited to mechanical injection of individual cells (11–13) where responses can be readily detected at the single cell level. Such an approach, of course, cannot be used in cases where the response being monitored requires thousands of cells in order to be detected. We have been studying just such a case, in a cultured rat hepatoma cell line, where cAMP derivatives and adrenal steroids selectively stimulate, by apparently different mechanisms, the synthesis of tyrosine aminotransferase (EC 2.6.1.5) (14, 15). Although a sensitive enzyme assay exists (16), at least 50–100 × 10⁶ cells are needed to obtain reliable values for basal aminotransferase activity.

With the advent of a feasible procedure for simultaneously micro-injecting millions of cells with foreign proteins using as vehicles, protein-loaded RBC ghosts, as pioneered by Mekada et al. (17) and Schlegel and Rechsteiner (18), a means was provided to directly test the role of protein kinase as a mediator of the induction of tyrosine aminotransferase by cAMP. We have made use of this approach and have succeeded in micro-injecting protein kinase subunits into rat hepatoma cells. The results obtained provide direct evidence for a mediatory role of the catalytic subunit of cAMP-dependent protein kinase in controlling the synthesis of the aminotransferase.

EXPERIMENTAL PROCEDURES

Materials—8-Br-cAMP was synthesized according to the procedure of Munevaya et al. (19) and purified by chromatography on a Bio-Gel P-2 column. Carrier-free (32P), Na(235I), (35S)methionine and (H)4,5-leucine were obtained from NEN Corp., Boston, MA. All other biochemicals were obtained from Sigma. ATP-(γ-32P) was prepared by the method of Johnson and Wallen (20).

Cell Culture—Reuber H35 hepatoma cells (H4-E-C-III) were

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grown and maintained in monolayer culture using serum-containing Dulbecco's MEM as described previously (5, 14). Cells were used at 70–90% confluency for fusion and are referred to as confluent monolayers in the text. The procedures for harvest of cells and lysis were as previously reported (5).

Purification and Labeling of Proteins—The C subunit of cAMP-dependent protein kinase was purified from beef heart by a modification of the procedures described by Beavo et al. (21) and Corbin et al. (22). This procedure yielded a preparation that exhibited an average purity of 60–80% based upon sodium dodecyl sulfate-gel electrophoresis on slab gels (23). The yield of catalytic subunit was typically 10–30 units/kg assayed against histone HIIA (Sigma) by the procedure of McPherson et al. (24). There was no significant effect of cAMP on the activity of the purified preparations of the catalytic subunit. The average specific activity of the different preparations was 3.5 ± 1.3 units/mg of protein (n = 8). A unit of activity is 1.0 pmol of ³²P transferred to HIIA/min/mg of protein at 30 °C. The catalytic subunit was inactivated (88.4% activity was lost) by incubation in 300 mm N-ethylmaleimide for 4 h at 22 °C (25). Homogeneous catalytic subunit from beef heart and type II regulatory subunit (R subunit) from rabbit skeletal muscle were kindly provided by Dr. Jackie Corbin of Vanderbilt University. Homogeneous catalytic subunit alone was also generously supplied by Dr. Edmund Fischer, University of Washington.

The heat stable inhibitor of protein kinase was partially purified from beef heart by modifications of the procedure of Whitehouse et al. (26). The material used in most experiments was 15–30% pure as judged by sodium dodecyl sulfate-gel electrophoresis. The average yield of the inhibitor was 6,000 units/kg and the average specific activity of the three different preparations was 196,000 units/mg of protein. The activity of the protein kinase inhibitor was assayed with the standard HIIA phosphorylation procedure (24) using highly purified kinase catalytic subunit preparations (average of 4.6 units/mg of protein). Under the conditions used, the suppression of HIIA phosphorylation was proportional to the amount of inhibitor added. The unit of activity is that amount of inhibitor which depresses phosphorylation of HIIA by 15 pmol/min at an activity of the catalytic subunit of 100 pmol/min. Homogeneous inhibitor from rabbit skeletal muscle was kindly provided by Dr. Daniel Friedman of Vanderbilt University.

BSA was labeled with ¹²⁵I (27) and purified by chromatography on Bio Gel P-10. The average specific radioactivity achieved was 1.79 × 10⁶ cpm/mg. BSA was labeled with FITC by the procedure of Mekada et al. (28) and separated from unreacted FITC by chromatography on Sephadex G25. This preparation exhibited an absorbance ratio (495 nm/280 nm) of 2.0 (28).

Tyrosine aminotransferase activity was measured in 20,000 × g supernatant fractions of H35 cell lysates by a modification of the method of Diamondstone (16) as published previously (14). A unit is defined as µg of protein formed in 1 min at 37 °C. Protein was measured in all cases was determined by the method of Lowry et al. (29). Activity is expressed either as units/mg of protein, net increase (value for treated group − value for basal group) or fold increase (value for treated group / value for basal group).

Effects of fusion on protein synthesis were measured by the incorporation of ['H]leucine into hot trichloroacetic acid-insoluble material as described previously (14). The relative rate of synthesis of the aminotransferase was determined by incorporation of ['H]methionine during a 30-min interval. Radioactivity in tyrosine aminotransferase was determined by immunoprecipitation using specific rabbit antibody to the soluble protein measured by precipitation with trichloroacetic acid as described previously (14).

Red Blood Cell Ghost Preparation and Loading of Proteins—Fresh human blood (5–10 ml) was obtained by venipuncture from healthy donors. After centrifugation at 1000 × g at 4 °C and removal of plasma and the buffy coat, the packed RBC were washed 3 times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH₂PO₄, 1.5 mM Na₂HPO₄, pH 7.9). An aliquot (0.15 ml) of the washed, packed RBC were mixed with 1.35 to 2.85 ml of PBS (generally 1.35 ml) containing the protein to be loaded at concentrations up to 30 µg/ml. The procedure used for loading foreign proteins into RBC ghosts was based on the general dialysis method of Mekada et al. (17). The sample was placed inside boiled dialysis tubing (6 mm diameter, Spectrapor) and dialyzed for 45 min at room temperature against 500–750 ml of 6-fold diluted PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH₂PO₄, 1.5 mM Na₂HPO₄, pH 7.2). The bag was then transferred to 500–750 ml of isotonic PBS and dialysis continued for 45 min at room temperature.

The loaded RBC ghosts were collected by centrifugation at 3000 × g for 10 min and washed 4 times with 3 ml of cold isonicitic PBS. The washed RBC ghosts were then suspended in 1.5–2.0 ml of cold glucose-free HBSS and stored at 4 °C until use. The average number of ghosts generated by this procedure was 4 × 10⁷/ml (with 1.5 ml final volume) as determined by a hemacytometer.

Fusion of Loaded Ghosts with H35 Cells—The procedure which has yielded the most consistent micro-injection results was that of Mercer et al. (30). H35 cell monolayers were washed once with glucose-free HBSS at 37 °C. Glucose-free HBSS containing pyrro-hemagglutinin (Sigma) at 50 µg/ml was added to the monolayer (30) and swirled for 2 min. RBC ghosts were then added, usually 200 µl of 50% suspension of ghosts (70–90% confluency + a ratio of 1:10). After swirling, the dishes were incubated for 60 min at 37 °C. The medium was then removed and the monolayer washed once with glucose-free HBSS.

To induce fusion, 0.5 ml of freshly prepared PEG-8000 (Sigma) at 20 or 44% (w/v) in 150 mm NaCl, 5 mm MgCl₂, 20 mm Tris-HCl, pH 7.4 was rapidly added dropwise around the dish. After incubation for 1 min, the PEG was diluted by addition of 8 ml of serum-free MEM and incubation was continued for 30 min at room temperature. The medium was removed and the monolayer washed once with serum-free MEM, 3 times with MEM containing serum (5% fetal calf and 5% newborn calf serum), and incubated in serum-containing MEM with 5 µg/ml gentamycin at 37 °C prior to harvest and assays. In some cases, 8-Br-cAMP (2 mm) or detemamethasone (1 µM) was added 3 or 4 h prior to harvest. Untreated cells refer to dishes containing cells at comparable levels of confluency which have been kept in serum-containing MEM throughout the period that companion dishes were subjected to, and recovering from the process of fusion.

RESULTS

Loading of RBC ghosts—Using (¹²⁵I)BSA as a model protein to test the effectiveness of loading of RBC ghosts, we obtained results very similar to those reported by Yamaizumi et al. (31). Linear loading of human RBC ghosts could be achieved by the procedure described under "Experimental Procedures" at concentrations of (¹²⁵I)BSA in the dialysis bag up to at least 1 mg/ml (data not shown). Given the specific radioactivity of the labeled BSA, the radioactivity observed in the supernatant after lysis of the loaded ghosts with H₂O and the number of ghosts in the sample, one can calculate that ~10⁸ molecules of BSA were loaded per ghost on the average at 1 mg/ml in the dialysis bag. The radioactivity present in the supernatant fraction after the fourth wash of the loaded ghosts with isotonic PBS was <2% of that found in the supernatant fraction from lysis with H₂O. As shown in Fig. 1, essentially all the RBC ghosts (>99% of all the original RBC) exhibit some degree of fluorescence when loaded with FITC-BSA. There is noticeable variation in the amount of BSA loaded and the morphology of the ghosts as has been seen by others (17). If ghosts were mock-loaded with FITC-BSA (dialysis only against isotonic PBS), no detectable fluorescence was found associated with the ghosts after the usual washing procedure.

Storage of ghosts loaded with (¹²⁵I)BSA at 4 °C for up to 24 h produced less than 5–10% loss of labeled BSA. Exposure of loaded ghosts to trypsin did not lead to loss of (¹²⁵I)-BSA until obvious hemolysis was observed. All of these observations are consistent with the conclusion that the gradual dialysis procedure resulted in the entrapment of substantial amounts of an exogenous protein such as BSA in the RBC ghosts. Based on results with (¹²⁵I)BSA, the overall efficiency of loading averaged over lab-to-lab was 3–4% (3 of 13) which represents somewhat less than 50% of the expected value for complete equilibration (150 µg BSA and 1.35 ml of protein-containing solution).

Fusion Procedure—Using RBC ghosts loaded with (¹²⁵I)BSA, only modest micro-injection of the (¹²⁵I)BSA into H35 cells was observed in the absence of pyrrohemagglutinin (data not shown). When the plant lectin was present at concentrations between 10 and 100 µg/ml, the amount of...
with FITC-BSA. RBC ghosts were loaded with FITC-labeled BSA at 1 mg/ml as described under "Experimental Procedures." After four washes with isotonic PBS, an aliquot of the loaded ghosts was placed on a glass slide and examined in a fluorescence microscope (Leitz MPV 2, 315x magnification). The field was illuminated first with visible light (VIS) to visualize the ghosts and then with ultraviolet light (UV) mercury lamp) to reveal ghosts containing FITC-BSA. Mock-loaded ghosts did not show any detectable fluorescence under the same conditions.

![Loading of RBC Ghosts with FITC-BSA](image)

**FIG. 1. Loading of RBC ghosts with FITC-BSA.** RBC ghosts were loaded with FITC-labeled BSA at 1 mg/ml as described under "Experimental Procedures." After four washes with isotonic PBS, an aliquot of the loaded ghosts was placed on a glass slide and examined in a fluorescence microscope (Leitz MPV 2, 315x magnification). The field was illuminated first with visible light (VIS) to visualize the ghosts and then with ultraviolet light (UV) mercury lamp) to reveal ghosts containing FITC-BSA. Mock-loaded ghosts did not show any detectable fluorescence under the same conditions.

(125I)BSA introduced was increased 5-7-fold in agreement with Mercer et al. (30). The maximum response was achieved between 50 and 100 μg/ml and, since lectins have been reported to affect tyrosine aminotransferase activity (32), we chose to use the lower concentration.

Various concentrations of PEG of different average chain lengths were tested for their efficiency as fusogens, as monitored by trichloroacetic acid-insoluble (125I) present in H35 cell lysates at 12 h after fusion with (125I)BSA-loaded ghosts. To summarize several different experiments, 44% PEG of chain length 8000 gave the greatest yield of micro-injected (125I)BSA. Higher concentrations were not used for fusion because of obvious severe cytotoxic effects as detected by cell detachment from the substrate. By diluting the PEG 16-fold after 1 min, the cytotoxic effects of 44% could be reduced such that cell detachment did not occur. However, as shown later, the response of H35 cells to inducers was reduced for several h after fusion even with these modifications of the original procedure of Mercer et al. (30). In later experiments, lower concentrations of fusogen were used and, although recovery of responsiveness was more rapid, the maximal response of tyrosine aminotransferase to micro-injected catalytic subunit was comparable down to 22% PEG.

Using these conditions with 44% PEG and ghosts loaded at a single concentration of the protein (2 mg/ml) in the dialysis bag, the amount of (125I)BSA micro-injected into H35 cells varied linearly with the ratio of ghosts to cells (data not shown). At the highest number of ghosts added (200 μl/dish, ≈20 ghosts/H35 cell), we calculated from the acid-insoluble radioactivity in cell lysates that over 2 × 10⁶ molecules of (125I)BSA were micro-injected into each H35 cell on the average. From the amount of BSA loaded, it was calculated that the efficiency of micro-injection in this experiment was about 4-5%. This is similar to values reported by others using the same or a different fusogen with different types of cells (18, 31).

Electrophoresis of H35 cell lysates prepared 6 h after fusion of H35 cells with ghosts containing (125I)BSA revealed only a single radioactive component which co-migrated with the (125I)BSA used for loading (data not shown). These results are consistent with intracellular delivery of the labeled BSA and suggest that significant quantities of the protein are still present in an undegraded form several h after fusion. Similar conclusions have been drawn by Neff et al. (33). Use of ghosts loaded with FITC-BSA has shown that 96% or more H35 cells exhibit significant fluorescence as seen in a fluorescence microscope (data not shown).

**Recovery Period**—Both PEG and Sendai virus are known to exert cytotoxic effects on cultured cells (18, 30). We chose to use PEG since it is easier to obtain and because it has been reported to be superior to Sendai virus in fusing RBC ghosts to monolayer cultures (30). One unexpected manifestation of the use of PEG, however, was rapid elevation of tyrosine aminotransferase activity shortly after fusion (Table I). Some increase was seen with all concentrations of PEG tested and was independent of the presence of RBC ghosts whether unloaded or loaded with BSA. Thus, the effect was not due to the injection of either BSA or hemoglobin. After 8-12 h, the aminotransferase activity returned to values typical for H35 cells not subjected to the fusion process (generally 15-30 units/mg of protein). The precise basis for this increase in enzyme activity has not been investigated but it appears to be the result of exposure to PEG, the process of fusion itself, or both, since phytopenagglutinin alone had no reproducible effect on enzyme activity in our hands.

The ability of CAMP derivatives to induce tyrosine aminotransferase was correspondingly diminished during the period when basal enzyme activities were elevated after fusion (data not shown). A similar period of refractoriness to the effects of dexamethasone was also observed (see below). Full responsiveness to these external inducers was restored after the 8-12 h period when basal tyrosine aminotransferase activities had been re-established. A likely explanation for the sharply reduced response to inducers was the significant inhibition of protein synthesis caused by the process of fusion (see below).

Although the inhibition of protein synthesis was less severe with lower percentages of PEG, the degree of fusion was also reduced (data not shown). The best compromise of fusion versus toxicity appeared to be offered by 22% PEG-8000 and

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2 Some hemoglobin remains in the loaded ghosts since they are distinctly red.
most of the experiments were performed with this concentration of fusogen. However, during the early phases of the work, 44% PEG was used to ensure maximum micro-injection of protein kinase subunits.

Effects of Protein Kinase Catalytic Subunit—The C subunit of the cAMP-dependent protein kinase was purified to 60–80% homogeneity from beef heart and loaded into human RBC ghosts under the same conditions as described above for BSA. The activity of the C subunit in the dialysis bag was assayed after each loading process and recovery averaged 66% with a range from 25 to 122%.

As shown in Table II, fusion of H35 cells with RBC ghosts loaded with the C subunit caused a significant increase in aminotransferase activity over that observed with ghosts subjected to mock-loading with this kinase preparation. Since mock-loaded RBC ghosts did not show any increase in enzyme activity, as was true for ghosts loaded only with BSA, the latter was used in all subsequent experiments to conserve purified C subunit.

At 13 h after fusion with the C subunit, the activity of tyrosine aminotransferase was elevated nearly 3-fold and the increase was identical to that achieved by 2 mM 8-Br-cAMP added to cells micro-injected with BSA. It should be noted that the C subunit did not provoke exactly the same degree of response as cAMP derivatives in every experiment, but it has generated 2-4-fold increases in aminotransferase activity in all experiments in which it has been used except one. In that experiment, the C subunit inexplicably lost all inducibility of the aminotransferase by cold trichloroacetic acid-insoluble material showed that the depression in protein synthesis is likely to be responsible for the reduced responsiveness to steroids, cAMP.

Addition of free C subunit (5-25 μg) to cells subjected to fusion conditions without RBC ghosts produced occasional increases in aminotransferase activity. However, the response was erratic, correlated poorly with the amount of subunit added, and was less effective than that observed when the subunit was introduced by way of RBC ghosts (data not shown).

Once reproducible effects of the C subunit could be obtained using 44% PEG, lower concentrations of the fusogen were tested. The response to the micro-injected kinase subunit appears to be somewhat more rapid with 22% PEG and was greater at both time points than with 44% PEG. In contrast to 44% PEG, with 22% PEG, the increase in aminotransferase activity at 12 h was almost as great as at 24 h. The response to the C subunit at 20-24 h after fusion is similar to that at 24 h but does diminish past this time (data not shown).

Concentrations of PEG below 22% were less toxic to H35 cells and the ability of the C subunit to increase the activity of tyrosine aminotransferase in cells subjected to fusion at this concentration was slightly greater at 4 and 8 h after fusion. However, the maximum response was only about 70% of that obtained with 22% PEG and it diminished after 12 h in contrast to that observed with higher concentrations of the fusogen which showed additional increases. These results suggest that the efficiency of fusion at concentrations of PEG below 22% was too low to be practical with the concentrations of protein available to load ghosts.

Analysis of the incorporation of (3H)leucine into hot and cold trichloroacetic acid-insoluble material showed that the process of fusion with 22% PEG caused substantial inhibition of protein synthesis, as suspected (Fig. 2). The rate of recovery of protein synthesis after fusion correlated reasonably well with the return of inducibility of the aminotransferase by dexamethasone and with the response to the micro-injected C subunit. These results, coupled with the fact that the maximum effect of the kinase subunit on the aminotransferase is seen at about 16 h after fusion with 22% PEG (Fig. 4), strongly suggest that the depression in protein synthesis is likely to be responsible for the reduced responsiveness to steroids, cAMP.

### TABLE I

| RBC ghost preparation | Recovery after fusion | Tyrosine aminotransferase units/mg protein |
|-----------------------|----------------------|----------------------------------------|
| Experiment 1          |                      |                                        |
| None                  | 3                    | 50.8 ± 3.0 (4)                         |
| Empty                 | 3                    | 42.7 ± 1.0 (12)                        |
| Mock-loaded           | 3                    | 39.0 ± 1.5 (12)                        |
| BSA loaded            | 3                    | 42.1 ± 1.3 (12)                        |
| Not subjected to fusion | 3                | 14.4 ± 1.3 (4)                         |
| Experiment 2          |                      |                                        |
| None                  | 13                   | 29.9 ± 1.6 (2)                         |
| Empty                 | 15                   | 34.8 ± 2.4 (2)                         |
| Mock-loaded           | 17                   | 26.8 ± 0.4 (2)                         |
| BSA loaded            | 19                   | 24.2 ± 1.1 (2)                         |
| Not subjected to fusion | 17                | 25.0 ± 3.0 (2)                         |

### TABLE II

| RBC ghost preparation | Recovery after fusion | Tyrosine aminotransferase units/mg protein | -fold |
|-----------------------|----------------------|----------------------------------------|-------|
| Experiment 1          |                      |                                        |       |
| Mock-loaded with C subunit | 8                 | 28.3 ± 2.5 (4)                        | 1.77  |
| Loaded with C subunit | 8                    | 48.6 ± 2.2 (8)                        |       |
| Experiment 2          |                      |                                        |       |
| Mock-loaded with C subunit | 8                 | 12.4 ± 0.7 (6)                        | 1.90  |
| Loaded with C subunit | 8                    | 23.5 ± 0.9 (6)                        |       |
| Experiment 3          |                      |                                        |       |
| Loaded with BSA       | 13                   | 20.8 ± 1.3 (3)                        |       |
| Loaded with BSA + 8-Br-cAMP | 13           | 86.7 ± 11.7 (9)                      | 2.81  |

*Significance of observed difference, p < 0.001.
**Significance of observed difference, p < 0.005.

8-Br-CAMP was added to cells micro-injected with BSA 4 h prior to harvest. Values shown are mean ± S.E. with number of observations in parentheses.
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Inactivation of C subunit with N-ethylmaleimide abolishes its ability to affect tyrosine aminotransferase activity

Confluent monolayers of H35 cells were fused with human RBC ghosts loaded with BSA (1 mg/ml) or highly purified (~60–80%) C subunit from beef heart (1 mg/ml) before and after treatment with 300 μM N-ethylmaleimide at room temperature which caused >80% loss of catalytic activity. Fusion was achieved with 22% PEG and the recovery period was 15 h. Values shown are mean ± S.E. with number of observations in parentheses.

| Protein micro-injected | Tyrosine aminotransferase |
|------------------------|---------------------------|
| BSA                    | 19.5 ± 2.9 (4)            |
| C subunit              | 45.2 ± 1.5 (4)*           |
| NEM-treated C subunit  | 20.1 ± 2.8 (4)*           |

*Significance of observed difference, p < 0.001.

**Significance of observed difference, p > 0.85.

In order to rule out the possibility that some minor contaminant in the partially purified C subunit preparations was responsible for the effects on the aminotransferase, it was necessary to use a micro-injected pure C subunit. As shown in Fig. 4, the pure C subunit evoked a response virtually identical to that produced by 8-Br-cAMP. The maximum effect was achieved at a ~20:1 ratio of ghosts to cells using ghosts loaded at 0.5 mg/ml which represents somewhat less C subunit than was needed with preparations which were only 60–80% pure. The response at 200 μl of ghosts was at, or very close to the maximum effect expected since 2 mM 8-Br-cAMP is known to produce a maximum response in these cells (5, 34). These results strongly suggest that the response of the aminotransferase to the partially purified preparations of the kinase must be due to the C subunit itself and not to some contaminant.

Addition of 8-Br-cAMP to cells micro-injected with the C subunit did not increase enzyme activity beyond that pro-

![Fig. 2. Time course for recovery of protein synthesis and inducibility of tyrosine aminotransferase by C subunit and steroids. Confluent monolayers of H35 cells were fused with RBC ghosts loaded with partially purified C subunit (1 mg/ml) (PK-C) or with BSA (1 mg/ml) using 22% PEG. All of the dishes were incubated with 25 μCi of (3H)-leucine (130 Ci/mmol) for 60 min before harvest. Dexamethasone (DEX) (1 mM) was added to dishes containing cells micro-injected with BSA 4 h prior to harvest. Cells were harvested at the times indicated for assays as described under "Experimental Procedures." The values for protein synthesis (C--C) represent the percentage of hot trichloroacetic acid-insoluble radioactivity present in 20,000 × g supernatant fractions of both groups of cells subjected to fusion relative to that in companion cells not subjected to fusion. McFarland and Atkinson (11) and/or Hultberg et al. (12) have shown that the subunit micro-injected. This proposition was plausible since steroids and cAMP elevate tyrosine aminotransferase activity by stimulating its rate of synthesis (3, 14, 15).

Inactivation of the C subunit (>98% loss of activity) with N-ethylmaleimide prior to fusion abolished the ability of a C subunit preparation to stimulate aminotransferase activity (Table III). The untreated C subunit in this experiment exhibited the usual recovery after the loading process and produced a typical 2–4-fold increase in the activity of the aminotransferase. These results are consistent with the suggestion that the C subunit is the active component present in the micro-injected preparation.

If this conclusion is correct, then the response to the C subunit should not only be dependent on time, but also on the amount of the subunit micro-injected. This proposition was tested in two different ways: first, by loading aliquots of RBC ghosts with different concentrations of the C subunit while using a fixed (~20:1) ratio of ghosts to H35 cells during fusion, and, second, by increasing the ratio of ghosts to H35 cells during fusion while using two fixed concentrations of the C subunit during loading. As shown in Fig. 3, the response of the aminotransferase proved to be dependent upon the amount of C subunit micro-injected with either approach. The maximum response appears to be reached around 1 mg/ml of the highly purified kinase preparation used during loading and at 200 μl of ghosts/dish (~20:1 ratio to H35 cell).
Confluent H35 cells were fused with human RBC ghosts loaded with BSA (1 mg/ml) or partially purified (60-80%) C subunit (1.7 mg/ml) from beef heart. Fusion was achieved with 22% PEG and the recovery period was 16 h. Medium containing one-fourth the usual concentration of methionine was added to all dishes 4 h prior to harvest and 1 µM dexamethasone was added to some dishes at the same time. Thirty min prior to harvest, 100 µg of 8-methionine (1.13 Ci/µmol) was added to all dishes. Cells were harvested for assays of enzyme activity, soluble protein content and incorporation of 35S into hot trichloroacetic acid-insoluble material (total soluble protein) and into tyrosine aminotransferase by immunoprecipitation as described under "Experimental Procedures." Values shown are mean ± S. E. of four separate observations.

![Graph](image-url)

**FIG. 4.** Comparison of the concentration dependency relationships for pure C subunit and 8-Br-cAMP. Each dish of confluent H35 cells was subjected to fusion at 22% PEG with the indicated volumes of RBC ghosts loaded either with 1 mg/ml pure C subunit (○) or 1 mg/ml BSA (●). The latter group of dishes was incubated for the last 4 h with the indicated concentrations of 8-Br-cAMP. The recovery period was 16 h at which time cells were harvested for assays as described under "Experimental Procedures." Each value is the mean of three (○) or four (●) observations and the S. E. values were within 5%. The specific activity of the aminotransferase was 20.5 ± 0.4 in cells micro-injected with BSA to which no 8-Br-cAMP was added.

As was also expected, an increase in the rate of overall protein synthesis occurred when 8-Br-cAMP was added. The specific activity of the tyrosine aminotransferase was 5.4 ± 0.3 in cells micro-injected with BSA (data not shown). Such results are to be expected if the C subunit is the mediator of cAMP action since these two agents induce the aminotransferase by apparently different mechanisms (14, 15).

Micro-injection of the C subunit significantly stimulated the relative rate of synthesis of tyrosine aminotransferase without causing any notable change in the rate of overall protein synthesis (Table IV). These results are consistent with reports that derivatives of cAMP exert their effects on the aminotransferase by selectively stimulating its synthesis (3, 14, 15). Dexamethasone, added to cells micro-injected with BSA 4 h prior to harvest, stimulated the rate of aminotransferase synthesis to a considerably greater extent than did micro-injected C subunit. This is consistent with the fact that adrenal steroids invariably produce a substantially higher degree of stimulation of enzyme synthesis than do cAMP derivatives (3, 14, 15).

**Effects of the Inhibitor of Protein Kinase—**If the C subunit of protein kinase is in fact responsible for the effects of cAMP on tyrosine aminotransferase, then micro-injection of the heat-stable inhibitor protein should block the ability of 8-Br-cAMP but not that of dexamethasone to induce the aminotransferase. In two different experiments with the inhibitor prepared from beef heart (15-30% pure), 64-82% inhibition of the response of the aminotransferase to 8-Br-cAMP was observed with only a minor reduction in the effects of dexamethasone (Table V). Of equal importance, neither inhibitor preparation altered basal aminotransferase activity. Given the short half-life of this enzyme (14, 15), nonspecific inhibition of protein synthesis would be expected to substantially depress aminotransferase activity. As was the case with the effects of the C subunit, the ability of the inhibitor to block induction by 8-Br-cAMP was dependent on the concentration of protein injected (Fig. 5). Once again, there was no significant effect on basal aminotransferase activity and induction by dexamethasone was virtually unaffected by any concentration of the inhibitor protein. A preparation of the inhibitor protein purified to homogeneity has been tested in one experiment and it also led to virtually complete extinction of the effects of 8-Br-cAMP (data not shown).

**TABLE IV**

| Protein micro- | Tyrosine aminotransferase (A) | Total soluble protein (B) | Relative synthetic rate (A/B) |
|---------------|-------------------------------|---------------------------|-----------------------------|
| micro-injected | units/mg protein | cpm | cpm × 10⁻⁶ | |
| BSA           | 43.4 ± 3.4                   | 1,863 ± 86                | 4.65 ± 0.07                 | 0.40 ± 0.06 |
| C Subunit     | 96.8 ± 2.3                   | 2,831 ± 119*              | 4.21 ± 0.17                 | 0.67 ± 0.02 |
| BSA + Dexamethasone | 305.4 ± 7.3   | 13,773 ± 1588*            | 4.79 ± 0.13                 | 2.91 ± 0.41* |

* Significance of observed difference, p < 0.001.

**DISCUSSION**

From the data presented in this report, it is probable that the effects of cAMP on tyrosine aminotransferase synthesis are, in fact, mediated by the catalytic subunit of cAMP-dependent protein kinase. This conclusion is supported by several observations: 1) both partially purified and homogeneous C subunit cause time- and concentration-dependent increases in aminotransferase activity; 2) the maximum response to pure C subunit occurs at about 0.5 mg/ml while that to the partially purified subunit (60-80% pure) required somewhat higher concentrations; 3) the partially purified inhibitor protein from beef heart blocked the ability of 8-Br-cAMP to induce the aminotransferase but did not significantly affect either basal enzyme activity or the ability of dexamethasone
indirect results was essential to establish its validity unequivocally. This is the excess 8-Br-CAMP. All of these results are to be expected if of low concentrations of 8-Br-CAMP to induce the aminotransferase to act as an inducer; after addition of 8-Br-CAMP and varying concentrations of the aminotransferase was under editor protein. Confluent monolayers of H35 ghosts loaded with BSA (1 mg/ml) or partially purified (≈15–30% pure) protein kinase inhibitor (0.15 mg/ml) from beef heart. Fusion was achieved with 22% PEG and the recovery period was 16 h. 8-Br-CAMP (0.1 mm) was added 4 h prior to harvest. Values shown are mean ± S. E. with number of observations in parentheses.

### Table V

| Protein micro-injected | Tyrosine aminotransferase activity after | No additions | 8-Br-CAMP | Dexamethasone |
|------------------------|-----------------------------------------|-------------|-----------|---------------|
| **Experiment 1**       |                                         |             |           |               |
| BSA                    | 18.2 ± 1.0 (3)                          | 40.6 ± 0.6 (3)* |           |               |
| Inhibitor              | 17.9 ± 1.5 (6)                          | 25.9 ± 2.4 (6)* |           |               |
| % inhibition with inhibitor | 2%                                      | 64%         |           |               |
| **Experiment 2**       |                                         |             |           |               |
| BSA                    | 18.6 ± 0.5 (3)                          | 53.2 ± 2.3 (3)* |           |               |
| Inhibitor              | 18.8 ± 1.8 (4)                          | 25.1 ± 1.2 (4)* |           |               |
| % inhibition with inhibitor | 0%                                      | 82%         |           |               |

* Significance of observed difference, p = 0.005.

** Table VI **

| Protein micro-injected | Tyrosine aminotransferase activity after | No additions | 8-Br-CAMP |
|------------------------|-----------------------------------------|-------------|-----------|
| BSA                    | 20.5 ± 0.4 (3)                          | 30.8 ± 1.5 (2)* |           |
| R subunit              | 20.4 ± 1.3 (4)                          | 22.9 ± 1.1 (3)* |           |
| % inhibition with R subunit | 1%                                      | 76%         |           |

* Significance of observed difference, p = <0.01.

** Table VII **

| Tyrosine aminotransferase activity | No additions | 8-Br-CAMP |
|------------------------------------|-------------|-----------|
| Protein micro-injected             |             |
| BSA                                | 20.5 ± 0.4 (3) | 30.8 ± 1.5 (2)* |
| R subunit                          | 20.4 ± 1.3 (4) | 22.9 ± 1.1 (3)* |
| % inhibition with R subunit         | 1%          | 76%       |

* Significance of observed difference, p = <0.01.

** Table VIII **

| Tyrosine aminotransferase activity | No additions | 8-Br-CAMP |
|------------------------------------|-------------|-----------|
| Protein micro-injected             |             |
| BSA                                | 20.5 ± 0.4 (3) | 30.8 ± 1.5 (2)* |
| R subunit                          | 20.4 ± 1.3 (4) | 22.9 ± 1.1 (3)* |
| % inhibition with R subunit         | 1%          | 76%       |

* Significance of observed difference, p = <0.01.

particular true since the effect of cAMP requires de novo protein synthesis in the present instance (14), in contrast to its effect on enzymes controlling glycogen or fatty acid metabolism (1, 2). Given that control of selective protein synthesis in Escherichia coli involves interaction of the cAMP binding protein with genetic elements (3, 4), a direct effect of the R subunit in the case of tyrosine aminotransferase was not a trivial possibility. The fact that the C subunit itself induces the aminotransferase, coupled with the inhibition of the effects of low concentrations of 8-Br-CAMP by the R subunit, provides a compelling argument against the cAMP binding protein as an agent of induction. Low levels of 8-Br-CAMP should be titrated by the R subunit injected, but this effect should be surmountable by excess 8-Br-cAMP as was the case. Even if the R subunit injected contained some noncovalently bound cAMP, a possibility which is difficult to eliminate completely, it is likely to have been degraded during the 12-h recovery period. H35 cells contain low levels of cAMP apparently because they are able to degrade the nucleotide at an exceedingly rapid rate (34). Thus, any bound cAMP would have largely disappeared allowing the R subunit to interact with the 8-Br-cAMP which was present inside the H35 cell.

Based on the content of the C subunit determined either by its purification to homogeneity from several tissues (35), or by comparison of its activity in H35 cell extracts with that in rabbit skeletal muscle (36), one can calculate that the average H35 cell contains approximately 27% X 10^7 molecules of endogenous C subunit. Assuming that the efficiency of injection (≈5%) and micro-injection (≈5%) of BSA is analogous to that of the C subunit, and given that the maximum response to the kinase subunit occurs at about 0.5 mg/ml in the dialysis bag, one can estimate that at least 1 X 10^7 molecules of the C subunit must be injected on the average into each H35 cell to produce a full response. Since there is substantial variation in the amount of protein loaded into each ghost (see Fig. 1), the fact that an apparent excess of C subunit (i.e. beyond the endogenous level) is required is not surprising since multiple fusion events would be needed to deliver a maximum amount of the subunit into cells which fused with ghosts loaded with below average amounts of this protein. Furthermore, there is some loss of catalytic activity during loading (average = 34% loss) which would reduce the estimate to ~680 X 10^7 molecules of C subunit/cell. Finally, some degradation of the injected C subunit can be expected to occur during the 12-16-h recovery period (33) which should bring the estimate down still further.

Averages of microgram of C subunit added per dish for maximal response, ≈5.6 μg ± 0.05, ≈290 mg/dish injected. Each dish contains ≈4 X 10^7 cells, 70 μg of C subunit/cell. 6.02 X 10^7 molecules C subunit/41,000 g = 1.47 X 10^9 molecules/μg/cell = 1 x 10^7 molecules C subunit/cell.
Although these calculations are only approximate, they provide additional assurance that the observed response can be realistically ascribed to the C subunit injected since the amount required is reasonably close to the level of endogenous C subunit. It is known from results reported previously by us (5, 34) and Liu (36) that all of the endogenous C subunit must be activated to fully induce the aminotransferase (4).

Similar calculations for the inhibitor protein lead to an estimated 115 x 10^3 molecules required to be injected/cell for a maximum response which is less than the amount of C subunit required and even less than the level of endogenous C subunit. However, the estimate of inhibitor injected is less precise since only moderately purified material was used. In addition, smaller proteins are both loaded more efficiently (51) and degraded less rapidly (33) than larger proteins. Thus, it is reasonable to believe that the effective concentration of the inhibitor protein/H35 cell has been substantially underestimated compared to that of the C subunit.

It is of interest to note that the response to the injected C subunit (see Fig. 3) does not appear to go beyond that produced by optimal concentrations of 8-Br-cAMP. Assuming the lack of any substance in the C subunit preparations which artificially restricts the response, this result implies that the degree of response is dictated by the level of the putative site at which cAMP regulates the synthesis of tyrosine aminotransferase.

Thus, there is reason to question any role of phosphorylation of the aminotransferase in regulating its control of aminotransferase synthesis might be. The precise site at which cAMP regulates the synthesis of tyrosine aminotransferase has not been worked out and remains controversial (37). It is possible that one of the kinase substrates previously identified is involved in this system and exhibits multiple functions similar to phosphorylase kinase (1, 2). Alternatively, it could be an as yet unidentified protein similar to one identified by two-dimensional gel electrophoresis (38). The possibility exists that a phosphatase inhibitor like inhibitor 1 (12) could be the substrate and, in this case, a constitutive, cAMP-independent kinase would have to tonically phosphorylate the substrate involved. Tyrosine aminotransferase itself has been reported to be a substrate for the CAMP-dependent kinase in vitro (39), although there is no evidence that such is the case in vivo. Indeed, all three inducers increase phosphorylation of the aminotransferase in vitro (40) even though steroids and insulin do not activate the CAMP-dependent kinase (41). Thus, there is reason to question any role of phosphorylation of the aminotransferase in regulating its synthesis.

The explanations for the inhibition of protein synthesis and the elevation of aminotransferase activity generated by the process of fusion are not known. Whatever the mechanisms prove to be, after 8-12 h, H35 cells return to normal with respect to overall protein synthesis and inducibility of tyrosine aminotransferase. Attempts to fuse ghosts with monolayer cells using Sendai virus have proved to be less effective than with PEG in our hands, and the virus is also known to be cytotoxic (18, 30). In any event, the present methods should have widespread applicability to any cultured cell system where the role of protein kinase in vivo is uncertain and needs to be tested directly. The results presented in this report provide, to our knowledge, the first direct evidence for mediation by the C subunit of an effect of cAMP on protein synthesis in eukaryotic cells.

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