Biosynthesis of Adrenocorticotropic Hormone in Mouse Pituitary Tumor Cells

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A double antibody immunoprecipitation technique using affinity-purified adrenocorticotropic hormone (ACTH) antiserum was employed to investigate the biosynthesis of ACTH in a mouse pituitary tumor cell line. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cell extracts resolved four forms of ACTH with apparent molecular weights of 4,500, 13,000, 21,000, and 31,000. These four forms of ACTH can be detected by radioimmunooassay of cell extracts or by immunoprecipitation of cell extracts following incubation of cultures in [3H]tryptophan, [3H]lysine, or [3H]tyrosine. The double antibody immunoprecipitation scheme developed is specific, quantitative, and reproducible. ACTH biosynthesis was examined in both steady and pulse-labeling experiments using [3H]lysine or [3H]tyrosine. The results of these experiments are consistent with the proposal that $M_1 = 31,000$ ACTH is the biosynthetic precursor for all three smaller forms of ACTH and that $M_t = 23,000$ ACTH is a biosynthetic intermediate. Both $M_t = 13,000$ ACTH and $M_t = 4,500$ ACTH are derived from the intracellular processing of $M_t = 31,000$ ACTH.

High molecular weight forms of adrenocorticotropic hormone have been observed in pituitary extracts and pituitary tumor extracts from several different species (1–9). The ACTH-secreting mouse pituitary tumor cell line, AtT-20/D-10v, contains several high molecular weight forms of ACTH (1) and provides a convenient experimental system in which to investigate the function of these forms in the biosynthesis of ACTH. Tumor cells were incubated with [3H]-amino-acids and a double antibody immunoprecipitation scheme was used to separate [3H]-labeled ACTH from other [3H]-labeled cell proteins.

METHODS

Production and Purification of Antibodies—AtT-20/D-10v cells were incubated in medium containing no serum for consecutive 24-hour periods (1). The incubation medium was desalted by dialysis against 10% (v/v) acetic acid (at 4°C), lyophilized, dissolved in sterile saline (0.9% NaCl solution), and mixed with complete Freund’s adjuvant (Grand Island Biological Co.). A female New Zealand white rabbit was injected with this mixture, containing an amount of extract equivalent to approximately 1.0 mg of intact ACTH (ACTH activity determined by radioimmunoassay) in the thigh muscles; three booster injections followed at monthly intervals; additional booster injections were given at 2- to 3-month intervals by multiple subcutaneous injections. Radioimmunoassays were performed as described previously (1), except that antiserum Bertha (at a final dilution of 1:10,000 to 1:15,000) was used in this work. Antiserum Bertha reacts on an equimolar basis with $\alpha(1-39)$ and synthetic $\alpha(1-24)$ (kindly provided by Organon). Synthetic $\alpha(1-24)$ and $\alpha(1-39)$ are at least 10-fold more potent at inhibiting binding of $^{125}\text{I}-\alpha(1-39)$ to this antiserum than $\alpha(1-10), \alpha$-melanocyte-stimulating hormone, $\alpha(1-18)\text{NH}_2$, or $\alpha(25-39)$ (fragments of ACTH were provided by Dr. W. Rittel, CIBA-GEIGY Ltd., Basel). With antiserum Bertha, $M_t = 31,000$ ACTH gives competition curves that are slightly steeper than the competition curves for $\alpha(1-39)$ and the other three forms of tumor cell ACTH; the apparent RIA-ACTH (Bertha) value for $M_t = 31,000$ ACTH is determined at the midpoint of the assay.

For use in immunoprecipitation of ACTH, crude antiserum Bertha was purified by affinity chromatography. Synthetic $\alpha(1-24)$ was covalently linked to cyanogen bromide-activated Sepharose 4B (11); the affinity resin contained 0.8 mg of protein/ml of resin. In order to purify up to 4 ml of ACTH antiserum, a 0.5-ml column of $\alpha(1-24)$-Sepharose 4B was prewashed immediately before use with 4 ml of 6 M guanidine HCl, 6 ml of Buffer A (see Table I for definitions of buffers), 4 ml of Buffer B, 10 to 15 ml of Buffer A, and 0.5 ml of nonimmune rabbit serum. Antiserum diluted 3-fold with Buffer A was applied to the column at room temperature at a flow rate of 10 ml/hour. The column was washed with 5 ml of Buffer A, 10 ml of 1 M guanidine HCl, 10 ml of Buffer A, and eluted with 5 ml of Buffer B. The eluate was dialyzed briefly against 500 ml of Solution A (2 x 2 hours) and then dialyzed repeatedly into Buffer C for 2 days. This purified $\alpha(1-24)$ antiserum was treated for 30 min at room temperature with 1% of the binding capacity for $^{125}\text{I}-\alpha(1-39)$ in crude antiserum Bertha was

*The nomenclature proposed by Li (10) is used to refer to the various fragments of ACTH (denoted by $\alpha(1-39)$).
Acids—AtT-BO/Digv cells were cultured in Dulbecco-Vogt medium containing 2.5% horse serum (1). Equal aliquots of AtT-20 cells were plated into Falcon Microtest wells (No. 3040) and grown to confluency; for tryptophan incubations, the medium contained 8 M urea/1.5% sodium dodecyl sulfate/5% 2-mercaptoethanol/50 mM sodium acetate/50 mM H3BO3; titrated to pH 8.5 with NaOH.

D. 30 mg/ml of phenylmethylsulfonyl fluoride/30 mg/ml of iodoacetamide; in absolute ethanol, made fresh within hours of its use.

E. 10 mM NaH2PO4/1 mM Na2EDTA/0.1% Triton X-100; titrated to pH 7.6 with NaOH.

F. 20 mg/ml of bovine serum albumin/50 μg/ml of poly(lys-lysine) (average molecular weight 15,500; Schwarz/Mann); dissolved in Buffer F.

G. 0.5 mg/ml of synthetic α(1-24) (Organon); dissolved in Buffer F.

H. 200 mM KCl/50 mM NaH2PO4/5 mM Na2EDTA/0.25% Triton X-100; titrated to pH 7.6 with NaOH.

I. 10 mM NaH2PO4/15 mM NaCl; titrated to pH 7.2 with NaOH.

J. 8 M urea/15% sodium dodecyl sulfate/5% 2-mercaptoethanol/50 mM sodium acetate/50 mM H3BO3; titrated to pH 8.5 with NaOH.

recovered in the purified α(1-24) antiserum. In the immunoprecipitation procedure described below, purified α(1-24) antiserum must be utilized; crude antiserum Bertha cannot be used for immunoprecipitation.

A crude ammonium sulfate fraction of nonimmune rabbit γ-globulin (12) was dialyzed against physiological saline (0.9% NaCl solution) and made up to its original volume. Both the rabbit γ-globulin and commercial goat anti-rabbit γ-globulin (Miles Laboratories or Pacific Biological Co.) were treated with protease inhibitors (Solution D) as described above for affinity purified α(1-24) antiserum.

Incubation of AtT-20 Cells with Radioactive Amino Acids—AtT-20/D-16v cells were cultured in Dulbecco-Vogt medium containing 2.5% horse serum (1). Equal aliquots of AtT-20 cells were plated into Falcon Microtest wells (No. 3040) and grown to confluency; each well contained 50 + 5 mg of RIA-ACTH (mean ± S.D.). Incubation medium contained L-[5,5-3H]tyrosine or L-[4,5,6-3H]tyrosine (Amersham-Searle or New England Nuclear) at a final specific activity of 1 to 7 Ci/mmol; the final concentrations of tyrosine and lysine were 0.4 and 0.8 mM, respectively, as in normal growth medium. For tryptophan incubations, the medium contained L-[5,5-3H]tyrosine or L-[4,5,6-3H]tyrosine (Amersham-Searle or New England Nuclear) at a final specific activity of 1 to 7 Ci/mmol; the final concentrations of tyrosine and lysine were 0.4 and 0.8 mM, respectively, as in normal growth medium. For pulse-labeling experiments, medium containing H-lysinoic acid was replaced with 200 μCi of normal growth medium after the desired labeling period; the specific activity of the H-lysinoic acid was diluted more than 100-fold by this procedure. At the end of the incubation, medium was removed and mixed with an equal volume of unlabeled medium containing 2% Solution D; the medium was centrifuged and the supernatant was stored frozen. Cells were extracted into 150 μl of 5 N acetic acid containing 5 mg/ml of bovine serum albumin, 5 mM glycine, and 1% Solution D; samples were frozen and thawed three times on dry ice and extracted at 4°C for 12 to 16 hours. Extracts were diluted with 300 μl of water, centrifuged, and the supernatant was lyophilized in aliquots; for each sample, a single lyophilized aliquot was dissolved in Solution E containing 1% Solution D immediately before immunoprecipitation.

The rapid equilibrium of intracellular pools of amino acids with amino acids in the culture medium has been seen in many systems (13-16) and was verified directly with the AtT-20 cells. Cultures were incubated with H-lysinoic acid for 10 min, rinsed twice with isotonic saline (0.9% NaCl solution), and extracted with ice-cold 1 N HCl. The specific activity of the acid-soluble pools was determined using amino acid analysis to measure the amount of each amino acid, and paper electrophoresis at pH 1.9 and pH 6.5 (17) to determine the radioactivity associated with each amino acid. The pools of free lysine and tyrosine inside the cells were found to be 95 ± 8% (mean ± S.D.) equilibrated with lysine and tyrosine in the culture medium in 10 min.

Immunoprecipitation—A double antibody immunoprecipitation scheme was used. Each sample to be analyzed was dissolved in Solution E and then split into two equal aliquots; one aliquot received a large excess of α(1-24) (Solution G) and served as a measure of any noncompetitive binding. Purified α(1-24) antiserum (20 μl), 20 μl of either Buffers F or G, and 10 μl of sample (containing approximately 5 ng of RIA-ACTH) were incubated at 4°C for 12 to 16 hours in 0.5 ml plastic centrifuge tubes (Brinkmann). Samples were diluted with 175 μl of Buffer E and any precipitate present was removed by centrifugation. Nonimmune rabbit γ-globulin, goat anti-rabbit γ-globulin, and 75 μl of Buffer H were added to 200 μl of this supernatant; the final volume was brought to 575 μl by addition of Buffer E. Samples were further incubated for 4 hours at 4°C; immunoprecipitates of cell extracts were washed once in 500 μl of Buffer H and once in 500 μl of Buffer I. Immunoprecipitates of tissue culture medium were washed twice in Buffer H and twice in Buffer I. The washed immunoprecipitates were dissolved in Buffer J and incubated in boiling H2O for 5 min; aliquots were counted and the remainder was analyzed by SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis—SDS-gel electrophoresis (10% acrylamide/0.5% N,N,N,N′-methylenebisacrylamide) was performed as described by Davies and Stark (18). Molecular weight markers used were: bovine serum albumin, heavy and light chains of rabbit γ-globulin, aldolase, yeast alcohol dehydrogenase, carbamoyl anhydrate, myoglobin, ribonuclease, cytochrome c, and α1-(39). All samples contained dansylated aldolase and dansylated myoglobin (19) as internal standards; mobilities were calculated with respect to the myoglobin marker. After electrophoresis, gels were cut into 2-mm discs. For radioimmunoassays, gel slices were smashed and eluted into 0.05 M sodium phosphate/0.06% SDS/0.2 mg/ml of bovine serum albumin pH 7.6 at 57°C for 12 hours. For analysis of H-labeled immunoprecipitates, gel slices were eluted into 1 ml of 0.1% SDS/0.5 mM uracil/0.5 mM NaHCO3 at 37°C for 10 hours (20). 10 μl of scintillation fluid (25% Triton X-114/75% xylene; Ref. 21) were added. The recovery of radioactivity after gel electrophoresis was always greater than 90%; tritium was counted at 38% efficiency.

RESULTS

Resolution of Forms of ACTH on SDS Gels—Earlier studies using gel filtration in guanidine HCl resolved the ACTH activity in normal mouse pituitaries and in the AtT-20/D-16v cells into three size classes: M, = 6,500 to 9,000 ACTH, M, = 20,000 to 30,000 ACTH (1, 2). The use of SDS-polyacrylamide gel electrophoresis (18) has made it possible to achieve better resolution of the different forms of ACTH (Fig. 1). Reference to peaks of ACTH immunoactivity will be based on their apparent molecular weight in this gel system. ACTH activity in the M, = 23,000 ACTH and 31,000 ACTH; there was relatively more ACTH = 23,000 ACTH and 31,000 ACTH; there was relatively more ACTH = 93,000 ACTH in the medium than in the cell extracts. The peak of M, = 13,000 ACTH seen in both cell extracts and culture medium corresponds to the M, = 6,500 to 9,000 ACTH previously described; this substantial difference in apparent molecular weight is considered further in the accompanying paper (23). As was seen using gel filtration in guanidine HCl, the predominant form of ACTH in cell extracts comigrated with 125I-α1-(39) (M, = 4,500 ACTH), but there was little if any of this form of ACTH in the culture medium. It has not been determined whether the differences between the ACTH pattern in cell extracts and in culture medium are due to selective secretion of various forms, selective proteolysis, or interconversion after secretion.

Immunoprecipitation of ACTH Activity—To study the kinetics of labeling of the different forms of ACTH, it was necessary to extract all four forms quantitatively and separate them from other labeled cell products. Extraction of cultures into cold acetic acid and subsequent solubilization in low ion...
strength sodium phosphate buffer containing 0.1% Triton X-100 (Solution E) was found to solubilize 95 ± 8% (mean ± S.D.) of the RIA ACTH solubilized by heating cultures in 6 M guanidine HCl plus 5% 2-mercaptoethanol (1). The technique of double antibody immunoprecipitation using purified α(1-24) antiserum was used to separate the four forms of ACTH from other radioactively labeled proteins in a single step. Nonspecific binding was monitored by performing control immunoprecipitations in the presence of sufficient α(1-24) to exceed the binding capacity of the purified α(1-24) antiserum by 100-fold.

The adequacy of the immunoprecipitation procedure was determined by including trace amounts of [3H]-α(1-39) during analysis of AtT-20 cell extracts. Immunoprecipitation and charcoal adsorption (24) both indicated that over 90% of the [3H]-α(1-39) was bound to antibody. At least 95% of the immunoprecipitated [3H]-α(1-39) was retained in the pellet through the washing procedure described under "Methods." The presence of cell extract containing 5 to 10 ng of RIA-ACTH activity depressed the binding of [3H]-α(1-39) by less than 5%; the amount of specific [3H]-labeled immunoprecipitable material was not altered by doubling or halving the standard amount of purified α(1-24) antiserum used. Duplicate immunoprecipitations of a sample performed on different days agreed within ±10%.

The method of immunoprecipitation and separation of the forms of ACTH was tested using [3H]tryptophan as the labeled precursor (Fig. 2). The same four forms of ACTH detected with the ACTH radioimmunoassay (Fig. 1) were found in the immunoprecipitate (Fig. 2, open circles); all four forms of ACTH were displaced from the immunoprecipitates by excess α(1-24) (Fig. 2, closed circles). Again, immunoprecipitates of tissue culture medium did not show the same pattern as immunoprecipitates of cell extracts; there was very little [3H]tryptophan-labeled M, = 4,500 ACTH in the medium and the amount of M, = 23,000 ACTH was relatively enhanced.

Steady Labeling Experiments—In order to observe the kinetics of labeling of the different forms of ACTH in the tumor cells, several identical cultures were incubated with [3H]lysine and harvested after increasing periods of time from 10 min to 6 hours (Fig. 3). Incorporation of [3H]lysine into trichloroacetic acid-precipitable material proceeded linearly for the entire 6-hour incubation. Cell extracts were analyzed by immunoprecipitation and gel electrophoresis. After a 10-min incubation the only form of ACTH labeled with [3H]lysine was M, = 31,000 ACTH. After longer periods of incubation more [3H]lysine was incorporated into M, = 31,000 ACTH, and radioactivity was also incorporated into M, = 23,000, 13,000 and 4,500 ACTH. This pattern of labeling is consistent with, but does not demonstrate, a precursor-product relationship between M, = 31,000 ACTH and the smaller forms of ACTH; the smaller

Fig. 1. Cell extract and culture medium analyzed by SDS-polyacrylamide gel electrophoresis: radioimmunoassay. A, a culture was harvested 3 hours after feeding; the sample was processed as described under "Methods" except that the acetic acid used for extraction did not contain bovine serum albumin. Recovery of RIA-ACTH activity was 107%. B, cultures were incubated in zero serum medium for six consecutive 24-hour periods; the pooled medium sample was treated with phenylmethylsulfonyl fluoride (final concentration 0.35 mg/ml), diluted with an equal volume of 48 mM HCl, and ACTH activity was concentrated by ion exchange chromatography on Amberlite CG-50 (22). Recovery of RIA-ACTH activity was 104%. For both gels, A and M mark the position of internal dansylated aldolase and dansylated myoglobin markers. [3H]-α(1-39) was included in each gel and migrated to the position marked 4.5 K.

Fig. 2. Cell extract and culture medium analyzed by SDS-polyacrylamide gel electrophoresis: immunoprecipitation. A culture was incubated in 100 μl of [3H]tryptophan medium for 8 hours and processed as described under "Methods." Approximately 6 ng of RIA-ACTH activity were applied to each gel. For immunoprecipitates, 4.5 K marks the position of [3H]-α(1-39) analyzed on a parallel gel. Immunoprecipitable counts represented 0.7% of the trichloroacetic acid-insoluble radioactivity in the cell extract. O, specific immunoprecipitate; performed using Solution F; ●, α(1-24) competed immunoprecipitate; performed using Solution G.
forms of ACTH might be synthesized by slower pathways not involving $M_r = 31,000$ ACTH.

Similar results were found in steady labeling experiments using $[^{3}H]$tyrosine instead of $[^{3}H]$lysine.

In studying the kinetics of labeling of the four forms of ACTH, it is necessary to take into account ACTH secreted into the culture medium. For example, after the 6 hour incubation in $[^{3}H]$lysine (Fig. 3C), the medium contained approximately the same amount of $[^{3}H]$-labeled immunoprecipitable material as the cell extract (data not shown). However, at the short times used for pulse-labeling experiments (see below), the amount of labeled ACTH secreted is not significant; after 30 min there was less than 1% as much $[^{3}H]$-labeled immunoprecipitable material in the medium as in the cells, and after 2 hours that ratio was about 10%.

**Pulse-labeling Experiments**—In order to expose the cells to a short pulse of $[^{3}H]$-labeled amino acid with minimal disturbance to the system, the concentration of amino acids present in normal growth medium was used throughout the pulse and chase periods. Fig. 4 shows the results of exposing the $\text{AT}-20$ cells to a 20-min pulse of $[^{3}H]$tyrosine and then analyzing the cultures after varying periods in nonradioactive medium.

Immunoprecipitation of a sample exposed to $[^{3}H]$tyrosine for 20 min showed that label had been incorporated only into $M_r = 31,000$ ACTH (Fig. 4A). After a 20-min chase incubation in unlabeled tyrosine (Fig. 4B) the amount of label in $M_r = 31,000$ ACTH had decreased and label had begun to appear in the smaller forms of ACTH. After a 100-min chase incubation (Fig. 4C) there was very little label in $M_r = 31,000$ ACTH or in $M_r = 23,000$ ACTH, but the amount of label in $M_r = 13,000$ ACTH and $4,500$ ACTH continued to increase. Results of a similar pulse chase experiment with $[^{3}H]$lysine are summarized in Fig. 5. A progression of label from $M_r = 31,000$ ACTH to $23,000$ ACTH, the smaller forms of ACTH can be seen. The label in $M_r = 31,000$ ACTH disappeared with a half-time of 15 to 20 min. Label appeared in $M_r = 23,000$ ACTH after a lag period and then disappeared with a half-life of 15 to 20 min. Finally, label appeared in $M_r = 13,000$ ACTH and $4,500$ ACTH more slowly than in the two higher molecular weight forms of ACTH; $M_r = 13,000$ ACTH did not label and chase more rapidly than $M_r = 4,500$ ACTH. The results obtained with $[^{3}H]$lysine and $[^{3}H]$tyrosine were identical.

**DISCUSSION**

In order to examine the kinetics of synthesis of ACTH, it is necessary to purify all four forms of ACTH away from all other cell products in high yield. The immunoprecipitation procedure described here was shown to be reproducible, specific ($[^{3}H]$-labeled material was displaced by $\alpha(1-24)$) and quantitative. In addition, pituitaries are known to contain enzymes that can destroy ACTH activity (9, 25); therefore the protease inhibitors phenylmethylsulfonyl fluoride and 1,4-diazocetamide were used in these studies to prevent degradation or interconversion during the extraction and immunoprecipitation procedures. Pulse-chase experiments were performed without protein synthesis inhibitors; the rapid equilibration of cellular amino acid pools with the tissue culture medium indicated that a pulse of label could be introduced into the biosynthetic pathway simply by replacing the culture medium. The fact that the amount of label in $M_r = 31,000$ ACTH decreased steadily throughout the incubation in unlabeled amino acid indicates that the chase procedure was effective; in the continued presence of radioactive amino acids, $M_r = 31,000$ ACTH would have continued to label linearly for at least 40 min (Fig. 3).

The studies reported here indicate that synthesis of $M_r = 4,500$ ACTH in $\text{AT}-20$ mouse pituitary tumor cells proceeds through a complex biosynthetic pathway beginning with the

![Fig. 4. Pulse-chase with $[^{3}H]$tyrosine: analysis of immunoprecipitates. Six identical cultures were each incubated in 60 $\mu$l of growth medium containing $[^{3}H]$tyrosine (7.0 Ci/mmol) for 20 min; radioactive medium was removed, cultures were rinsed with 100 $\mu$l of unlabeled growth medium and then incubated in 200 $\mu$l of unlabeled growth medium. Individual cultures were analyzed after 0, 20, 40, 60, 100, or 150 min in unlabeled medium; the results of the 0-, 20-, and 100-min chase periods are shown. Approximately 7 ng of RIA-ACTH activity were applied to each gel. After the 20-min incubation in $[^{3}H]$tyrosine, 1.4% of the trichloroacetic acid-insoluble radioactivity in the cell extracts was immunoprecipitable; trichloroacetic acid-insoluble counts (incorporated during the brief incubation in $[^{3}H]$tyrosine) disappeared during incubation in unlabeled growth medium with a half-life of 3 to 4 hours. O, specific immunoprecipitate; performed using Solution F; $\bullet$, $\alpha(1-24)$ competed immunoprecipitate; performed using Solution G.](http://www.jbc.org/)

![Fig. 3. Steady labeling with $[^{3}H]$lysine: analysis of immunoprecipitates. Six identical cultures were each incubated in 100 $\mu$l of medium containing $[^{3}H]$lysine (1.1 Ci/mmol). Individual cultures were harvested at 10, 20, 40, 60, 120, and 360 min of incubation; the results of the 10-, 20-, and 360-min incubations are shown. Approximately 5 ng of RIA-ACTH activity were applied to each gel. For short incubation times, immunoprecipitable counts represented 0.6% of the trichloroacetic acid-insoluble radioactivity. O, specific immunoprecipitate; performed using Solution F; $\bullet$, $\alpha(1-24)$ competed immunoprecipitate; performed using Solution G.](http://www.jbc.org/)
However, extracts of mouse anterior pituitary also contain high molecular weight forms of ACTH (2), and it is anticipated that a similar biosynthetic pathway will function in the mouse pituitary. It is not yet known whether any of the high molecular weight forms of ACTH are normally secreted by the pituitary and thus might have a functional role in the periphery in addition to their presumptive role as intermediates in the biosynthesis of $M_r = 4,500$ ACTH.

Definitive proof of the biosynthetic pathway for ACTH requires purification and characterization of the different forms of ACTH. Until the amino acid compositions of the different forms are known, it will not be possible to calculate the efficiency of the conversion process (Figs. 4 and 5) accurately. Approximately 40% of the [3H]lysine (or [3H]tyrosine) incorporated into $M_r = 31,000$ ACTH during a 20-min pulse-labeling period appears in $M_r = 13,000$ ACTH plus 4,500 ACTH after a chase period of 45 to 60 min, so the conversion process appears to be quite efficient. The AtT-20 cell line will be useful for studying other aspects of ACTH biosynthesis, such as the structural relationships of the forms of ACTH, the polypeptide fragments created during conversion of 31,000 ACTH to the smaller forms of ACTH, and the enzymes involved in the conversion process.

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