High Molecular Weight Forms of Adrenocorticotropic Hormone Are Glycoproteins*

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Mouse pituitary tumor cells (AtT-20/D-16v) were incubated in medium containing [3H]glucosamine or [3H]mannose. By analyzing immunoprecipitates of cell extracts and culture medium it was shown that [3H]glucosamine and [3H]mannose were incorporated into all three high molecular weight forms of ACTH; label was not incorporated into Mw = 4,500 ACTH (which is thought to be similar to the 39 amino acid polypeptide form of ACTH, α(1–39)). Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis the apparent molecular weights of these glycoprotein forms of ACTH were 31,000, 23,000, and 13,000. Gel filtration in 6 M guanidine hydrochloride indicated that the molecular weights of the forms of ACTH were substantially lower; sodium dodecyl sulfate-polyacrylamide gel electrophoresis has often been found to underestimate the molecular weight of glycoproteins. A significant fraction of the high molecular weight ACTH in tumor cell extracts binds to columns of concanavalin A-agarose and can be eluted with 0.2 M α-methyl-d-mannopyranoside; porcine α(1–39) does not bind to concanavalin A-agarose. High molecular weight glycoprotein ACTH II can be detected in extracts of mouse and bovine pituitary by using concanavalin A affinity chromatography.

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METHODS

Incubation of AtT-20 Cells with Radioactive Precursors

AtT-20 cells were maintained as described previously (1). Incubations with [N-4,5-3H]lysine (New England Nuclear; 100 μCi of medium per well with a final specific activity of 3 Ci/mmol) were performed as described (5). N-[N-6-3H]Glucosamine HCl (New England Nuclear; 10.1 Ci/mmol) was added to normal growth medium to a final concentration of 50 or 100 μM. N-[N-1-3H]Mannose (New England Nuclear, 13.2 Ci/mmol) was used at a final concentration of 134 μM. When cells were incubated in culture medium containing 25 mM glucose and 76 μM [3H]mannose, there was not sufficient incorporation of [3H]mannose into immunoprecipitable material for further analysis. For this reason, during incubations with [3H]mannose the glucose concentration in the culture medium was decreased to 5.6 mM and the nonessential amino acids proline, aspartic acid, glutamic acid, and alanine were each added to the medium at a final concentration of 20 mM each. Immunoprecipitates of cell extracts and culture medium were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described in the previous paper (5).

After incubations with [3H]glucosamine or [3H]mannose, the identity of the [3H] label incorporated into immunoprecipitable material was determined as described below.

[3H]Glucosamine—Culture medium from an 8-hour incubation of AtT-20 cells in [3H]glucosamine-containing medium was immunoprecipitated as described (5); the precipitate was washed two additional times in deionized water. The immunoprecipitate was hydrolyzed in 4 N HCl at 100° in an evacuated tube for 4 hours (6), rotary-evaporated, lyophilized, and dissolved in a small volume of 2 N HCl. Unlabeled glucosamine (10 μg per analysis) was added and the...
sample was analyzed by paper electrophoresis at pH 6.5 (7) and by ascending paper chromatography in isopropanol alcohol/HCl/H₂O (68/16/6.2/14.8) (8); glucosamine was visualized with a cadmium ninhydrin spray (9).

**[H]Mannose—Culture medium from an 8-hour incubation of AtT-20 cells in [H]mannose containing medium was immunoprecipitated and the pellet was washed as described above. The immunoprecipitate was hydrolyzed in 4 M HCl at 100°C in an evacuated tube (10). Unlabeled mannose (50 µg) was added and the sample was rotary-evaporated five times with water, dissolved in 50% ethanol, and aliquots were frozen under nitrogen. The sample was analyzed by repeated ascending paper chromatography in butanol/ethanol/H₂O (10/1/2) (6) and by paper electrophoresis in 0.1% sodium borate, pH 9.5 (11); sugars were visualized with an aniline-phosphoric acid spray (12).

**Gel Filtration in 6 M Gdn·HCl**

Sephadex G-75 was equilibrated and eluted with 6 M Gdn·HCl/0.02% bovine serum albumin; the flow rate of the column (0.9 x 53 cm) was 3 ml/hour. The column was calibrated with blue dextran, carbonic anhydrase, myoglobin, ribonuclease, cytochrome c, lima bean trypsin inhibitor, Trasylol (FBA Pharmaceuticals), α(1-39) glycopeptide, α(1-24), and α-melanocyte-stimulating hormone. Stock solutions of reduced and denatured Trasylol, lima bean trypsin inhibitor, and ribonuclease were prepared by incubation in 7.5% to 10% 2-mercaptoethanol/6 M Gdn·HCl at 70°C for 2 hours (or 3°C for 12 hours). Samples to be analyzed were always incubated in 6 M Gdn·HCl/2.5% 2-mercaptoethanol at 70°C for 1 hour before fractionation.

**Gel Filtration in 1% Acetic Acid**

Sephadex G-75 was equilibrated and eluted with 1% acetic acid/0.02% bovine serum albumin/0.02% NaN₃. The flow rate of the column (1.1 x 55 cm) was 7 ml/hour. When crude extracts of tumor cells or normal pituitary tissue were analyzed, the fractions with a Kᵥ between approximately 0.04 and 0.22 were pooled to give the Mₛ of 20,000 to 30,000 ACTH pool; tumor cell extracts contained a peak of RIA·ACTH activity at Kᵥ = 0.05 to 0.09.

**Affinity Chromatography with Concanavalin A**

Small columns (total volume 200 to 500 µl) of concanavalin A-agarose (Sigma) were prepared in siliconized Pasteur pipettes. Columns were washed immediately before use with 0.10 M acetic acid and then equilibrated with con A buffer (0.01 M Tris·HCl/0.7 mM MgCl₂/1.0 mg/ml bovine serum albumin/1.0 M NaCl/0.1% Triton X-100, pH 7.4). Samples were dissolved in con A buffer and applied to the columns at room temperature (flow rate approximately 3 ml/hour). The columns were washed with about 30 column volumes of con A buffer. Specifically bound material was eluted with 0.2 M α-methyl-d-mannopyranoside (Schwarz/Mann) in con A buffer, followed by 1.0 M α-methyl-β-mannopyranoside in con A buffer. Any material still bound to the column was eluted with 0.1 M acetic acid/1 mg/ml of bovine serum albumin. Recovery of RIA·ACTH activity was always greater than 90%.

**Radioimmunooassay of ACTH**

Two different antisera were used in these studies. Antiserum Bertha has binding specificity for the α(1-24) sequence of α₁(1-39) and was described in detail in the previous paper (5); antiserum NPA (from the National Pituitary Agency; Fred No. 699) has binding specificity for the α(1-18) sequence of α₁(1-39) and its use has been described (1). Radioimmunoassays were performed as described below (1). In experiments using both antisera, RIA·ACTH activity is reported as RIA·ACTH(Bertha) or RIA·ACTH(NPA). Both antisera detect all four forms of ACTH, but antiserum NPA is less sensitive to Mₛ = 23,000 and 31,000 ACTH than antiserum Bertha.

**RESULTS**

**Comparison of Results Using Gel Filtration in Gdn·HCl and Gel Electrophoresis in SDS**—As shown in the previous paper the ACTH activity in AtT-20 cell extracts can be resolved into four discrete forms by polyacrylamide gel electrophoresis in SDS; the apparent molecular weights of the forms are Mₛ = 4,500, 13,000, 23,000, and 31,000 (5). Earlier studies, based on gel filtration in Gdn·HCl, had resolved the ACTH activity in tumor cell extracts into three size classes: Mₛ = 4,000 to 5,500, 6,500 to 9,000, and 20,000 to 30,000 ACTH (1). In order to determine directly the relationship of these different classification schemes, [H]lysine-labeled Mₛ = 13,000, 23,000, and 31,000 ACTH were prepared as described in the legend to Fig. 1. These 4 labeled molecules, separated on the basis of mobility during SDS-polyacrylamide gel electrophoresis, were then analyzed by gel filtration in Gdn·HCl. [H]Lysine-labeled Mₛ = 13,000 ACTH co-migrated with Trasylol (Mₛ = 6,520) upon reanalysis in 6 M Gdn·HCl (Fig. 1B). Similar experiments indicated that Mₛ = 23,000 ACTH eluted at approximately the same position as sperm whale myoglobin (Mₛ = 17,200) during gel filtration in Gdn·HCl; 31,000 ACTH eluted at a slightly larger volume than carbonic anhydrase (Mₛ = 29,000). The Mₛ = 31,000 ACTH and 23,000 ACTH are not well enough resolved during gel filtration in Gdn·HCl to give rise to two separate peaks when analyzed together; both would have been classified as belonging to the Mₛ = 20,000 to 30,000 ACTH pool. The molecular weights of glycoproteins are often overestimated by SDS-polyacrylamide gel electrophoresis and different gel electrophoresis systems can give different molecular weight estimates (15, 16). However, since at present SDS-polyacrylamide gel electrophoresis provides the best means of separating the forms of ACTH, apparent molecular weights determined by gel electrophoresis (as described under “Methods”) will be used to identify the forms of ACTH. In order to determine the true molecular weights of these forms of ACTH it will be necessary to purify them.

**Incubation of Tumor Cells with [3H]Glucosamine or [3H]Mannose—Act-20 cells incorporate significant amounts of [3H]glucosamine into immunoprecipitable material. Analysis of immunoprecipitates of both culture medium and cell extracts indicated that label had been incorporated into each of the three higher molecular weight forms of ACTH (Fig. 2). As seen in Fig. 2A, [3H]glucosamine was not incorporated into immunoprecipitable Mₛ = 4,500 ACTH, although [3H]amino-acids can be incorporated into this fraction (5); Mₛ = 4,500 ACTH is thought to be similar to α₁(1-39) (a simple polypeptide which does not contain carbohydrate) and therefore would not be expected to incorporate [3H]glucosamine. Greater than 95% of the [3H]-labeled material in immunoprecipitates of the culture medium was identified as [3H]glucosamine following acid hydrolysis and either paper electrophoresis or paper chromatography (described under “Methods”).

Similar experiments using [3H]mannose instead of [3H]glucosamine indicated that each of the three high molecular weight forms of ACTH in both cell extracts and culture medium incorporated [3H]mannose (data not shown). Again, as expected, no [3H]mannose was incorporated into immunoprecipitable Mₛ = 4,500 ACTH. Analysis of hydrolysates of the [3H]-labeled immunoprecipitable material in the culture medium indicated that at least 60% of the label incorporated was [3H]mannose.

**Affinity Chromatography with Concanavalin A-Agarose—** The labeling experiments with [3H]glucosamine and [3H]mannose indicated that the high molecular weight forms of ACTH were glycoproteins. The presence of carbohydrate moieties on high molecular weight ACTH might enable some

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High Molecular Weight Forms of ACTH Are Glycoproteins

A SDS Gel of Immunoprecipitate

B Re-run of 13K ACTH on Gdn.HCl Column

FIG. 1. Comparison of molecular weight determinations by gel electrophoresis and gel filtration. A, SDS-polyacrylamide gel electrophoresis. AtT-20 cells were incubated in [3H]lysine for 12 hours; a 90-μl sample of the culture medium was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis as described in the preceding paper (5), except that protease inhibitors were omitted. Dansylated aldolase and myoglobin were included in the sample; 12sI-CyP(l-39) was analyzed on a separate gel. The gel slices were eluted into 0.5 ml of 50 mM sodium phosphate/0.05% SDS/0.2 mg/ml of bovine serum albumin, pH 7.6, and peaks of immunoprecipitated material were localized by liquid scintillation counting. Aliquots of the eluate. The indicated fractions were pooled, lyophilized, extracted twice with acetone, and blown dry under nitrogen. The peak of [3H]labeled immunoprecipitable material smaller than CyP(l-39) co-migrates with glucagon (29 residues) when analyzed by gel filtration in Gdn.HCl (see B); when protease inhibitors are included during the immunoprecipitation very little of this 29-residue [3H]-labeled material is observed. B, gel filtration in Gdn HCl. The [3H]lysine-labeled M, = 13,000 ACTH pool from A above was dissolved in 6 M Gdn.HCl/2.5% 2-mercaptoethanol; aliquots of blue dextran, Trasylol, and glucagon were added to the sample (final volume 280 μl), which was then incubated at 70°C for 1 hour and applied to the column. The whole of each fraction was combined with 0.4 ml of 6 M urea and 18 ml of scintillation fluid (14), and counted at 38% efficiency. The other standards indicated on the plot were analyzed in similar runs on the same column. The recovery of radioactivity was 93%. (O-O) absorbance at 280 nm; (O—O) [3H]-cpm/fraction × 10⁻².

forms of high molecular weight ACTH to bind to affinity columns containing immobilized lectins (17). The structural requirements for binding of glycoproteins to concanavalin A affinity columns have been studied in great detail; α-methyl-D-mannopyranoside binds to concanavalin A and can be used to elute substances specifically bound to concanavalin A-agarose (18-20). As shown in Fig. 3A, approximately 60% of the M, = 20,000 to 30,000 ACTH pool from tumor cell extracts bound to concanavalin A-agarose and was eluted with α-methyl-D-mannopyranoside; the remainder of the RIA-ACTH activity loaded onto the column did not bind to the concanavalin A-agarose. When α(l-39) was applied to a column of concanavalin A-agarose under identical conditions, less than 2% of the RIA-ACTH activity applied bound to the column and was eluted with α-methyl-D-mannopyranoside (Fig. 3D).

Mouse pituitary extracts also contain high molecular weight forms of ACTH (2), and it was of considerable interest to determine whether the high molecular weight ACTH in normal mouse pituitary was also a glycoprotein. A crude acid extract of mouse anterior pituitary was fractionated by gel filtration and the M, = 20,000 to 30,000 ACTH pool from mouse anterior pituitary was fractionated by gel filtration and the M, = 20,000 to 30,000 ACTH pool was applied to concanavalin A-agarose (Fig. 3C); approximately 40% of the RIA-ACTH activity in the M, = 20,000 to 30,000 ACTH pool bound to concanavalin A-agarose and was eluted with α-methyl-D-mannopyranoside. This result suggests that some of the high molecular weight ACTH in normal mouse pituitary is also a glycoprotein.

Concanavalin A Affinity Chromatography of Whole Tumor Cell Extracts—The observation that a large percentage of the immunoreactivity in isolated M, = 20,000 to 30,000 ACTH pools bound to columns of concanavalin A-agarose and eluted with α-methyl-D-mannopyranoside suggested that affinity chromatography might provide a useful first step in purifying high molecular weight ACTH from crude tumor cell extracts and crude pituitary extracts. An extract of AtT-20 cells was applied to a column of concanavalin A-agarose. The ACTH activity...
and 40% of the RIA-ACTH(NPA) activity applied to the column was in 5 N acetic acid (21). The lyophilized extract was dissolved in 1% acetic acid/5% 2-mercaptoethanol, and fractionated by gel filtration on Sephadex G-75 in 1% acetic acid (“Methods”). The peak of the $M_r = 20,000$ to 30,000 ACTH pool was located at $K_a = 0.05$ by radioimmunoassay and fractions with a $K_a$ of 0 to 0.21 were pooled, lyophilized, and dissolved in 0.5 ml of con A buffer. A 200-µl concanavalin A-agarose column was loaded as described under “Methods”; the 0.5-ml sample was followed with 0.5 ml of con A buffer and collected in one fraction (labeled flow thru). The column was washed with six 1-ml aliquots of con A buffer and eluted with four 1-ml aliquots of 0.2 M α-methyl-D-mannopyranoside (αMM) in con A buffer followed by two 1-ml aliquots of 1.0 M α-methyl-D-mannopyranoside in con A buffer. The column was then washed with three 1-ml aliquots of 0.1 M acetic acid (HAc). A background value of 0.5 pg/µl has been subtracted from the RIA-ACTH(NPA) values plotted. Recovery of RIA-ACTH(NPA) activity was 104%; 61% of the RIA-ACTH(NPA) activity applied to the column was eluted with α-methyl-D-mannopyranoside. When an unfractionated extract of bovine anterior pituitary was analyzed on columns of concanavalin A-agarose (data not shown). Thus high molecular weight ACTH which can be specifically bound to concanavalin A-agarose was analyzed by gel filtration, it was clear that concanavalin A affinity chromatography had selectively removed RIA-ACTH activity eluting in the highest molecular weight region of the $M_r = 20,000$ to 30,000 ACTH pool (Fig. 5B). Extracts of dog and rat pituitaries also contain high molecular weight ACTH which can be specifically bound to concanavalin A-agarose (data not shown). Thus high molecular weight glycoproteins with ACTH activity appear to be a common component of ACTH-secreting systems.

**DISCUSSION**

By incubating AtT-20 cells with $^3H$]glucosamine or $^3H$]mannose it can be shown that $M_r = 13,000$, 23,000, and $M_r = 13,000$ ACTH was present in a relatively decreased amount compared to the amount of $M_r = 13,000$ ACTH present in whole cell extracts (5). As expected, there was no peak of $M_r = 4,500$ ACTH. The $M_r = 31,000$ ACTH represents about 30% of the RIA ACTH(Bertha) activity in crude cell extracts. Concana
al ven A-agarose affinity chromatography selects out approximately 30% of the RIA-ACTH(Bertha) activity in whole tumor cell extracts and approximately 65% of this ACTH activity is $M_r = 31,000$ ACTH. Thus it appears that most of the $M_r = 31,000$ ACTH in cell extracts is capable of binding to concanavalin A-agarose. Similar results were obtained when RIA-ACTH(NPA) was determined.

**High Molecular Weight Glycoproteins with ACTH Activity in Other Species**—High molecular weight ACTH has been observed in pituitary tissue of many different species (22-28; our own observations). It was thought that affinity chromatography on concanavalin A-agarose might provide a simple means of determining whether these high molecular weight forms of ACTH were glycoproteins in species other than mouse. The major form of ACTH in bovine anterior pituitary extract is $M_r = 4,500$ ACTH (Fig. 5A), but approximately 5% of the input RIA-ACTH(NPA) activity elutes in the region of the $M_r = 20,000$ to 30,000 ACTH pool. When a pool of $M_r = 20,000$ to 30,000 ACTH from bovine pituitary was analyzed on columns of concanavalin A-agarose, 11% of the RIA-ACTH activity bound to the column and eluted with α-methyl-D-mannopyranoside. When an unfractionated extract of bovine pituitary powder was applied to a column of concanavalin A-agarose, only 0.3% of the RIA-ACTH(NPA) activity applied to the column bound and eluted with α-methyl-D-mannopyranoside (see legend to Fig. 5B). However, when the small amount of RIA-ACTH activity specifically bound to concanavalin A-agarose was analyzed by gel filtration, it was clear that concanavalin A affinity chromatography had selectively removed RIA-ACTH activity eluting in the highest molecular weight region of the $M_r = 20,000$ to 30,000 ACTH pool (Fig. 5B). Extracts of dog and rat pituitaries also contain high molecular weight ACTH which can be specifically bound to concanavalin A-agarose (data not shown). Thus high molecular weight glycoproteins with ACTH activity appear to be a common component of ACTH-secreting systems.
31,000 ACTH all incorporate both H-labeled precursors. Affinity chromatography of whole tumor cell extracts on columns of concanavalin A-agarose indicates that a significant percentage of the \( M_r = 31,000 \) ACTH can bind to the column and be eluted with \( \alpha \)-methyl-\( \beta \)-mannopyranoside. Mouse pituitary and bovine pituitary extracts also have high molecular weight ACTH activity that binds specifically to concanavalin A-agarose. Thus the high molecular weight forms of ACTH appear to be glycoproteins while \( M_r = 4,500 \) ACTH is a simple polypeptide.

When pools of \( M_r = 20,000 \) to 30,000 ACTH from both mouse pituitary tumor cells and mouse anterior pituitary were analyzed by affinity chromatography on concanavalin A-agarose (Fig. 3, A and C) some of the RIA-ACTH activity applied to the column was not capable of binding to the affinity resin. Under the conditions used in these studies, the ability of the high molecular weight forms of ACTH to bind to concanavalin A-agarose and be eluted with \( \alpha \)-methyl-\( \beta \)-mannopyranoside should be determined by the carbohydrate present (17, 19). The presence of mannose residues in a carbohydrate side chain will not necessarily enable that carbohydrate moiety to bind to concanavalin A-agarose; the structure of the carbohydrate chain is also important (16, 20). Upon close examination, the carbohydrate side chains on most glycoproteins exhibit microheterogeneity (29). This microheterogeneity can be a direct result of the biosynthesis of the glycoproteins or a result of alteration of the carbohydrate side chains during purification. Many of the extraction procedures developed for the purification of \( \alpha(1-39) \) involve prolonged incubation in strong acids (21, 30). Such extraction procedures (including some of those used in these studies) may modify the carbohydrate side chains of high molecular weight ACTH (6, 31). Once a satisfactory extraction procedure has been developed, affinity chromatography with concanavalin A and other lectins of known specificity should be of use in comparing the carbohydrate moieties on the different forms of ACTH.
The existence of a high molecular weight glycoprotein precursor to \( M_r = 4,500 \) ACTH (5) may provide an explanation for a number of previously uninterpretable histological observations. When normal pituitaries (human, rat, or amphibian) are examined for the presence of carbohydrate by the periodic acid-Schiff method or the phosphotungstic acid method, the secretory granules of several cell types appear to contain glycoproteins. In addition to the cells thought to produce the glycoprotein hormones (folliculin, lutropin, and thyrotropin), immunologically identifiable ACTH cells stain for carbohydrate (3, 4, 35). The secretory granules of ACTH-secreting tumors also react with carbohydrate stains (37). The secretory granules of hormone and prolactin cells do not stain for carbohydrate (3, 4, 32-36); in contrast, the secretory granules of growth hormone cells contain significant amounts of some of the high molecular weight glycoprotein forms of ACTH or glycopeptides generated by the cleavage of \( M_r = 31,000 \) ACTH to form \( M_r = 4,500 \) ACTH.

At present the role of the carbohydrate moieties on the high molecular weight forms of ACTH is unknown. The sugars could be involved in the intracellular processing of the hormone or in control of its secretion (38). If the glycoprotein forms of ACTH are normally secreted into the blood, they may have different effects on target cells than the well studied \( M_r = 4,500 \) ACTH. The glycoprotein forms of ACTH may have different half-lives in plasma than \( M_r = 4,500 \) ACTH and slight alterations in the carbohydrate moieties may alter their plasma half-lives (39). It is also possible that the glycopeptides created during the conversion of \( M_r = 31,000 \) ACTH to the smaller forms of ACTH could have biologically important functions of their own.

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