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Biochemistry

Pregrowth hormone: Product of the translation *in vitro* of messenger RNA coding for growth hormone

[precursor/wheat germ extract/tryptic peptides/L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK)/secretory proteins]

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ABSTRACT **Membrane fraction RNA isolated from rat pituitary tumor (GC) cells has been translated in a wheat germ extract. A product was synthesized which was immunologically related to growth hormone, but which migrated more slowly than growth hormone upon sodium dodecyl sulfate-acrylamide gel electrophoresis. The mobility of the cell-free product on gels of this type was unchanged by treatment with either KOH or RNase. The mobilities during paper electrophoresis of the methionine-containing tryptic peptides obtained from the cell-free product were identical to those obtained from growth hormone synthesized and secreted by the GC cells. Molecular weights for growth hormone and the cell-free product of 19,500 and 24,000, respectively, were determined by gel electrophoresis of these proteins together with marker proteins of known molecular weights. No protein with the properties of the cell-free product was detected after a 2 min incubation of the GC cells with [^{35}S]methionine. However, treatment of the GC cells with a protease inhibitor, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), led to the appearance of a new polypeptide, immunologically related to growth hormone, and with a mobility on gels identical to that of the cell-free product. These results strongly imply that the cell-free product represents a growth hormone precursor (pregrowth hormone) which is rapidly converted to growth hormone in pituitary cells.**

The synthesis of eukaryotic secretory proteins (including polypeptide hormones) in the form of precursor molecules appears to be quite common (1–11). In many cases the function of the precursor molecule is not yet known. The possibility that growth hormone (GH), a single polypeptide chain protein, may also be synthesized as a precursor has been repeatedly discussed (8, 12–15). However, short pulses with radioactive amino acids of either rat pituitary organ cultures (15) or rat pituitary tumor cells (GC) (16) have failed to reveal a GH precursor. Furthermore, translation in mouse Krebs II ascites tumor cell extracts of RNA obtained from the GC cells yielded a product immunologically related to GH which comigrated exactly with authentic GH on a sodium dodecyl sulfate ($NaDodSO_4$)-acrylamide gel (17).

The latter observation probably represented the best negative evidence then available concerning the existence of a GH precursor. However, it seemed possible that the results observed could be explained by the presence in mouse tumor cells of an enzyme(s) capable of processing a rat secretory protein precursor to its mature form. We therefore initiated studies of the translation of GH messenger RNA (mRNA) in a cell-free system derived from an organism evolutionarily far removed from the rat; i.e., wheat germ. We report here evidence that GH mRNA is translated in wheat germ extracts into a product which is related to but larger than GH. We suggest that this product, designated preGH, represents a precursor of GH.

Abbreviations: GH, growth hormone; PL, prolactin; ICFP, anti-GH immunoprecipitated cell-free product; $NaDodSO_4$, sodium dodecyl sulfate; mRNA, messenger RNA; TPCK, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone.

METHODS

Growth and Labeling of Cells, and Preparation of RNA. The GC cells are a cloned variant of the GH_3 strain of rat pituitary tumor cells (16). GH represents about 8% of the total protein synthesis by the GC cells (16), which, unlike the GH_3 cells, produce little or no prolactin (PL) (Sussman and Bancroft, in preparation). Growth of the GC cells in suspension culture, labeling with radioactive amino acids, and preparation of cytoplasm were as described (16). Preparation of RNA from the membrane fraction of the GC cells was as described (17).

Protein Synthesis Assay. Preparation of wheat germ extracts was as described by Roberts and Patterson (18) except that the preincubation step was omitted. Preliminary experiments were performed to optimize stimulation of protein synthesis by GC cell membrane fraction RNA as a function of the concentrations of wheat germ extracts, magnesium acetate, KCl, and [^{35}S]methionine, and of the time of incubation. The final assay system contained 30% (vol/vol) wheat germ S-30 fraction (18), 16 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.6), 24 mM dithiothreitol, 1.4 mM ATP, 0.28 mM GTP, 16.8 mM creatine phosphate, 19 units/ml of creatine kinase, 120 μM of 19 unlabeled amino acids, 1 mCi/ml of [^{35}S]methionine (New England Nuclear, 234 Ci/mmol) or 0.1 mCi/ml of [^{3}H]leucine (Schwarz/Mann, 59 Ci/mmol), 56 mM KCl, 3.5 mM magnesium acetate, and 160–200 μg/ml of GC cell membrane fraction RNA. Reactions of 50–600 μl were incubated at 30° for 90 min. Small aliquots were treated with KOH and Cl_3CCOOH as described (17), and total acid-insoluble radioactivity was determined as described (16). The remainder of the reaction mixture was subjected to immune precipitation and $NaDodSO_4$-acrylamide gel analysis, as described below. In some experiments, (Fig. 2B), post-incubation reaction mixtures were treated with either RNase or cycloheximide. In these experiments, a post-ribosomal supernatant was first prepared by centrifugation at 100,000 × *g* for 1 hr, since RNase treatment of the unfractionated reaction mixture precipitated about 50% of the total acid-insoluble radioactivity. A 25 μl volume of the supernatant received 2 μl of a solution containing 90 mM Na ethylenediaminetetraacetate (EDTA), 10 mM sodium acetate, pH 8, and either 1 mg/ml of RNase or 2.5 mg/ml of cycloheximide, and was incubated at 37° for 20 min. Immune precipitation and gel analysis were then performed as described below.

Immune Precipitation. Samples were either diluted 10 times with phosphate-buffered saline (PBS) (16), or dialyzed

against PBS. After addition of deoxycholate and NP-40 to final concentrations of 1.0% and 0.5%, respectively, the samples were centrifuged at 15,000 × *g* for 2 min. Immune precipitation with baboon antiserum to rat GH was performed as described previously (16), except that GH-containing carrier medium was replaced with 20 μg of rat GH. Immune precipitates were then dissolved in sample buffer for analysis on gels, as described below. In some experiments (Fig. 2A), immune precipitates were dissolved in 0.1 N KOH, incubated at 37° for 20 min, precipitated with 10% Cl_3CCOOH, washed four times with acetone and once with ethanol, air-dried, and dissolved in sample buffer for gel analysis. Gel markers ([^{3}H]GH or [^{3}H]PL) were prepared by precipitation with antiserum to either protein (ref. 16, and Sussman and Bancroft, in preparation) of cytoplasm from cells labeled with [^{3}H]leucine.

$NaDodSO_4$-Acrylamide Gel Electrophoresis. (*1*) *Cylindrical gels.* Samples were boiled for 2 min in sample buffer (10% mercaptoethanol, 2.5% $NaDodSO_4$, 10% glycerol, 0.002 M EDTA, 0.6% saturated bromophenol blue, 0.05 M Tris·HCl, pH 6.8). Electrophoresis was then performed on 13 cm, 15% "disc" $NaDodSO_4$-acrylamide gels as described by Laemmli (19). Gels were then fractionated in a Gilson Aliquogel Fractionator into Triton X-100:scintillation fluid: water (0.3:0.61:0.09), and radioactivity was assayed by scintillation counting. (*2*) *Slab gels.* Gels of this type were employed for the molecular weight determinations (Fig. 4). $NaDodSO_4$ (BDH Chemicals, Ltd., >99% pure) was employed because its use yielded a markedly better straight line fit for the marker proteins than did less pure material. Samples were prepared in sample buffer as above and subjected to electrophoresis in a 14 cm × 1.5 mm 15% acrylamide gel as described (20). The gels were then stained, destained, and dried as described (21) to reveal the marker proteins and GH and PL, and exposed to x-ray film to reveal the cell-free product.

Isolation of GH and the Product of the Cell-Free System. To prepare [^{3}H]methionine-labeled GH, medium from GC cells grown in the presence of [^{3}H]methionine (Schwarz/Mann) was precipitated with anti-GH antiserum as described above. To prepare the [^{35}S]methionine-labeled product of the cell-free system, the reaction mixture (280 μl) was precipitated with anti-GH antiserum as described above. After electrophoresis of the immune precipitate on a cylindrical gel, as described above, either GH or the cell-free product was extracted as follows. Gel fractions (0.2 mm) were incubated overnight at 37° in 0.4 ml of 0.03% $NaDodSO_4$. Small aliquots were removed and assayed for radioactivity as described above to locate the peak fractions, which were pooled. Gel particles were removed from solubilized radioactive material by vacuum filtration through nitrocellulose filters.

Analysis of Tryptic Peptides. Aliquots of [^{3}H]methionine-labeled GH and the major [^{35}S]methionine-labeled immunoprecipitated cell-free product, isolated as described above, were combined. After addition of carrier bovine serum albumin (30 μg/ml), the combined aliquots (15.3 ml) were made 10% in Cl_3CCOOH. After centrifugation at 21,500 × *g* for 10 min, the resulting precipitate was washed successively with 1% Cl_3CCOOH, acetone–ether (1:1) (twice), and ether to remove $NaDodSO_4$, and air-dried. The precipitated proteins were then subjected to performic acid oxidation (22). The resulting dried residue was dissolved in 20 μl of ammonium bicarbonate (pH 8.5), and trypsin treated with L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) was added [trypsin:serum albumin = 1/20 (weight/weight)] at the beginning and after 2 hr incubation at 37°. After incubation overnight at 37°, 20 μl of running buffer (see below) was added and any precipitate was pelleted by centrifugation. The supernatant was spotted onto Whatman no. 3 filter paper and electrophoresed for 2 hr at 56 V/cm in running buffer [10% (vol/vol) acetic acid–1% (vol/vol) pyridine, pH 3.5]. After drying, radioactive peptides were eluted from 1-cm fractions of the paper by soaking overnight in 1 ml of 0.2% $NaDodSO_4$, and were counted in Triton X–toluene scintillant as described above.

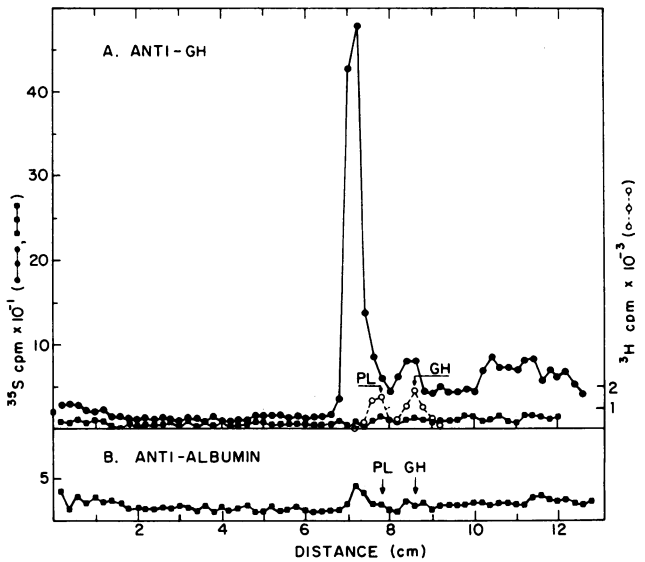

FIG. 1. $NaDodSO_4$-acrylamide gel electrophoresis of immune precipitates of material synthesized in the cell-free system. (A) Anti-GH antiserum precipitates of 25 μl aliquots of [^{35}S]methionine-labeled material synthesized in the reaction mixture in the presence (●) or absence (■) of GC cell membrane fraction RNA were prepared as described in *Methods*. (B) A rabbit anti-rat serum albumin precipitate of a 25 μl aliquot of the same reaction mixture containing membrane fraction RNA as in A was prepared using 20 μg of carrier albumin and antiserum in slight antibody excess. Plots of internal markers have been omitted in B for clarity. The three separate 15% $NaDodSO_4$-acrylamide gels (see *Methods*) depicted in A and B have been aligned on the basis of the positions of the internal [^{3}H]PL and [^{3}H]GH markers added to each gel. (●, ■), [^{35}S]methionine; (o), [^{3}H]leucine.

RESULTS

It has been observed previously that GH mRNA is located predominantly or entirely in the membrane fraction of the GC cells (17). Hence RNA from this fraction was incubated in the presence of wheat germ extract, yielding an average stimulation of protein synthesis of 2.7-fold. The products synthesized in the presence of membrane fraction RNA were immunoprecipitated with anti-GH antiserum. $NaDodSO_4$-acrylamide gel electrophoresis of this material (Fig. 1A) revealed a major immunoprecipitated cell-free product (ICFP) which migrated considerably more slowly than GH (19,500 daltons), and slightly more slowly than PL (22,500 daltons). This result suggested that a polypeptide immunologically related to, but larger than, GH was being synthesized in wheat germ extract. The conclusion that the major ICFP is immunologically related to GH was strengthened by the observations that this product was undetectable in an anti-GH immune precipitate of a cell-free extract incubated in the absence of exogenous RNA (Fig. 1A), and that only 6%

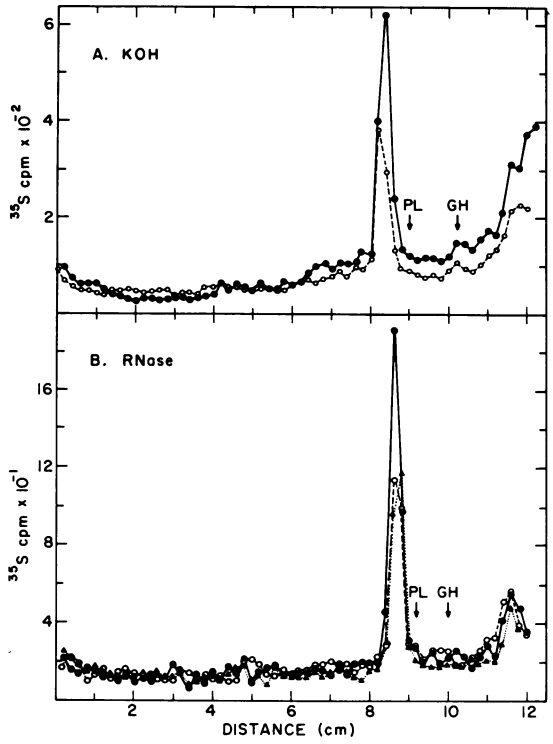

FIG. 2. Effect of KOH or RNase treatment on the mobility of the major ICFP. (A) Aliquots (25 μl) of a reaction mixture incubated with membrane fraction RNA were immunoprecipitated with anti-GH antiserum and were either untreated (●) or treated with KOH (○), as described in *Methods*. (B) The post-ribosomal supernatant of a reaction mixture incubated with membrane fraction RNA was prepared, and 25 μl aliquots were either untreated (●), treated with RNase (○), or treated with cycloheximide (▲), as described in *Methods*. They were then immunoprecipitated with anti-GH antiserum. Gel electrophoresis of the immune precipitates and alignment of gels was as in Fig. 1.

of the major ICFP was coprecipitated during formation of a serum albumin-antiserum albumin immune precipitate (Fig. 1B).

The possibility that the large size of the major ICFP arose from association with tRNA or a tRNA fragment was investigated by incubation of the cell-free product under conditions designed to degrade RNA. The mobility of the major ICFP was not altered by incubation with either KOH or RNase (Fig. 2). The 35% loss of the major ICFP after KOH treatment of the immune precipitate (Fig. 2A) probably occurred during the extensive washing procedure involved in preparation of the sample for gel electrophoresis (see *Methods*). That the 25% loss of the major ICFP during post-incubation treatment of the reaction mixture with RNase (Fig. 2B) is due to proteolytic activity is suggested by the observation that replacement of RNase with cycloheximide during the treatment also led to a 25% loss of the major ICFP (Fig. 2B).

The structural relationship between the major ICFP and GH was further investigated by a comparison of their methionine-containing tryptic peptides. The major ICFP and GH both yielded four major methionine-containing tryptic peptides (Fig. 3). The amino-acid sequence of rat GH has not been determined, so the expected tryptic peptide pattern is not known. However, the mobility of the four major peptides obtained from the major ICFP were identical to those obtained from GH, and the relative amount of each peptide was similar for the two proteins (Fig. 3). This result provides strong evidence that the major ICFP and GH have amino-acid sequences in common.

The sizes of the major ICFP, and of the GH and PL used for internal calibration of gels, were investigated by comparison of their mobilities on a $NaDodSO_4$-acrylamide slab gel with the mobilities of proteins of known molecular weights. Molecular weights for GH, PL, and the major ICFP of 19,500, 22,500, and 24,000, respectively, were obtained (Fig. 4).

The results presented thus far suggest that translation of GH mRNA in wheat germ extracts yields a GH precursor (the major ICFP) which is about 23% larger than GH. Evidence for an intracellular polypeptide corresponding to the major ICFP was obtained by treating the GC cells with TPCK, an inhibitor of chymotrypsin (23). Gel electrophoresis of an immune precipitate of cytoplasm prepared from

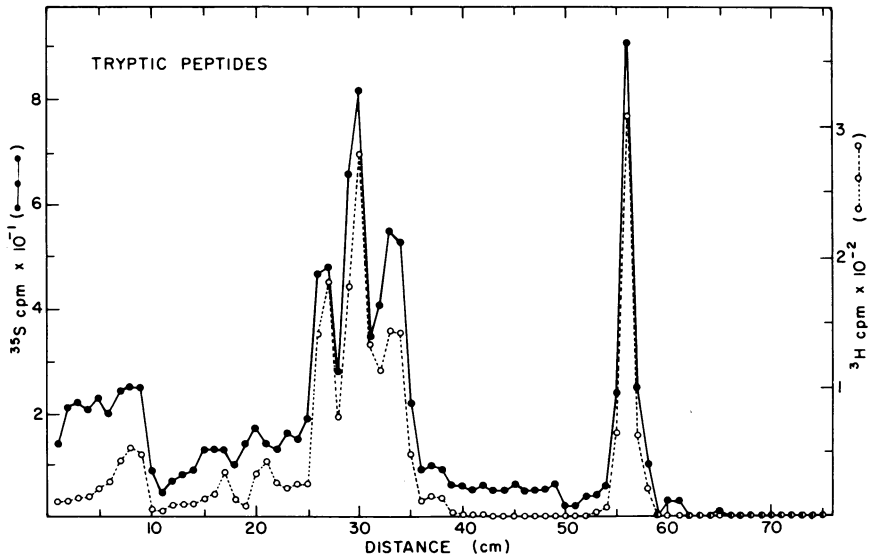

FIG. 3. Methionine-containing tryptic peptides of the major ICFP and GH. The major ICFP labeled with [^{35}S]methionine and GH labeled with [^{3}H]methionine were isolated from $NaDodSO_4$-acrylamide gels, oxidized with performic acid, digested with trypsin, and subjected to paper electrophoresis (pH 3.5), as described in *Methods*. (●), [^{35}S]methionine; (○), [^{3}H]methionine.

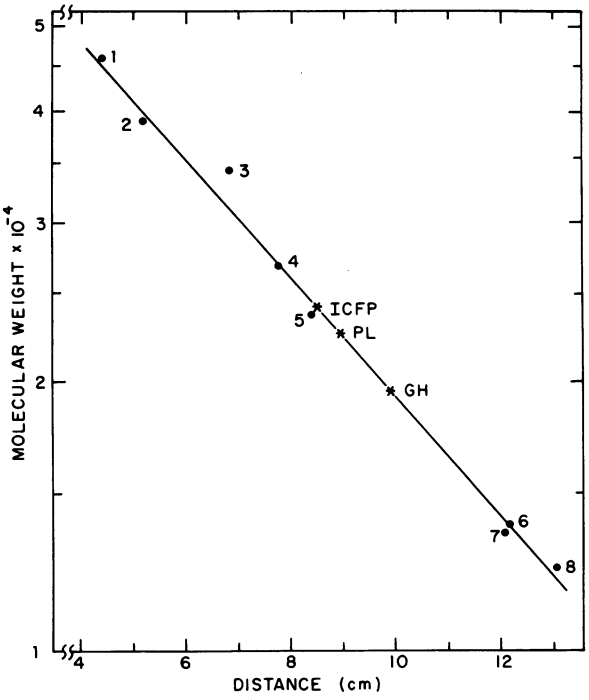

FIG. 4. Determination of molecular weights by $NaDodSO_4$-acrylamide gel electrophoresis. [^{35}S]Methionine-labeled major immunoprecipitated cell-free product (ICFP), unlabeled GH and PL, and eight proteins of known molecular weight were subjected to electrophoresis on a slab gel as described in *Methods*. Each protein was applied to a separate slot in the stacking gel. The marker proteins employed and their molecular weights are: 1, ovalbumin, 46,000; 2, aldolase, 39,000; 3, carboxypeptidase A, 34,400; 4, chymotrypsinogen A, 25,700; 5, trypsin, 23,800; 6, chymotrypsin, 13,900; 7, RNase, 13,700; 8, cytochrome *c*, 12,400. *, ICFP, GH, or PL; ●, marker proteins.

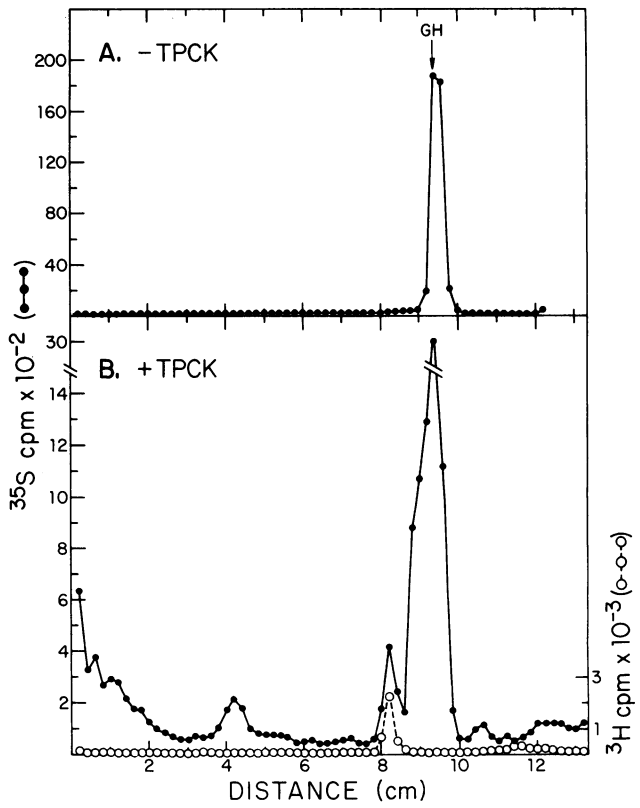

FIG. 5. $NaDodSO_4$-acrylamide gel electrophoresis of material synthesized by GC cells in the presence of TPCK and then precipitated by antiserum to GH. GC cells were concentrated to 10^7/ml and incubated in medium containing 1/100 the normal methionine concentration, in the absence (A) or presence (B) of 100 μg/ml TPCK for 2 min. [^{35}S]Methionine (final concentration 50 μCi/ml) was then added, and incubation was continued for 30 min. Cytoplasm was then prepared as described (16). Incorporation of [^{35}S]methionine into acid-insoluble material in the TPCK-treated cells was 3% of that in the untreated cells. (A) Cytoplasm was immune precipitated as described in *Methods*. (B) Cytoplasm was combined with a wheat germ reaction mixture which had been incubated in the presence of GC cell membrane RNA plus [^{3}H]leucine as described in *Methods*. Immune precipitation was then performed as described in *Methods*. Gel electrophoresis of the immune precipitates was as in Fig. 1. (●), [^{35}S]methionine-labeled product of the GC cells; (o), [^{3}H]leucine-labeled cell-free product.

cells incubated with TPCK plus [^{35}S]methionine revealed the synthesis of a small but reproducible amount of a protein which comigrated exactly with the major ICFP (Fig. 5B). No such protein was observed in cells not treated with TPCK (Fig. 5A). The peak observed at 4.2 cm in Fig. 5B has not been consistently observed and its identity is not yet known.

DISCUSSION

In the present studies we have observed that translation in a wheat germ extract of RNA from the membrane fraction of cultured rat pituitary tumor (GC) cells yields a protein (the major ICFP) which is related to GH. The structural relationship between the proteins has been demonstrated both immunologically and by a comparison of their tryptic peptides. The major ICFP differs from GH, however, in that it is about 23% larger than GH. For reasons discussed below, we suggest that the major ICFP is a precursor of GH, which we have designated preGH.

A minor cell-free product the same size as GH and precipitated by anti-GH antiserum has been occasionally observed (Fig. 1A). Lacking sufficient material for tryptic peptide analysis, its relation to GH remains unclear. In earlier experiments with a different wheat germ extract, little or no material other than the major ICFP was observed (data not shown), suggesting that the fast-moving components observed in the present experiments might reflect occasional premature chain termination in the particular extract employed. A comparison of the sizes of the products of translation of GH mRNA in Krebs ascites cell extracts (17) and in wheat germ extracts suggests that Krebs cells may contain, and wheat germ lack, an enzyme(s) capable of cleaving preGH to GH. A similar comparison of the products of the translation in two different cell-free systems of gamma-globulin L chain mRNA (3, 24) and placental lactogen mRNA (25) has suggested that Krebs cells contain enzymic activity capable of converting other precursors to their mature forms.

PreGH is clearly different from the "large GH" observed in rat pituitary organ cultures. "Large GH" consists of GH which is apparently either noncovalently bound to another protein (13) and/or bound to RNA (14). Thus it is probably not a GH precursor in the usual sense, i.e., a single polypeptide chain which is cleaved to yield the mature protein.

The possibility that the large size of preGH is an artifact of the wheat germ system must be considered. Evidence has been presented that this product is not simply GH with residual RNA attached. Furthermore, tryptic peptide analysis has demonstrated both the structural relationship of preGH to GH and the fact that wheat germ extracts translate GH

mRNA in phase (and apparently also remove the NH_2-terminal methionine from the preGH product). Finally, previous studies have demonstrated the ability of wheat germ extracts to translate correctly a number of mRNAs from animals or animal viruses (18, 26–30).

The concept that preGH is a true precursor of GH has gained additional support from the results of experiments with intact GC cells. In previous experiments, no product with the properties of preGH was observed after a 2 min pulse of the GC cells with [^{35}S]methionine (31), suggesting that preGH normally is extremely short-lived. In the present studies, treatment of the GC cells with TPCK, an inhibitor of chymotrypsin, revealed the synthesis of a polypeptide which has the same immunological properties and is the same size as preGH (Fig. 5B). The small amount of radioactivity in this polypeptide relative to the amount in GH suggests that under the conditions presently employed TPCK is relatively inefficient in preventing the cleavage (presumably by a chymotrypsin-like enzyme) of preGH to GH. Experiments similar to those reported here have permitted the detection of a proposed precursor of gamma-globulin L chain in intact mouse myeloma cells (32).

Either of the types of experiments reported in the present studies is subject to possible artifacts. In spite of the evidence to the contrary cited above, it is conceivable that preGH might be the result of initiation or termination (or both) of the synthesis of GH at a unique incorrect site on the GH mRNA molecule. Although as a protease inhibitor TPCK exhibits specificity for chymotrypsin (23), this reagent has also been observed to inhibit initiation of protein synthesis in animal cells (ref. 33, and Tushinski and Bancroft, unpublished observations), and to exhibit effects on *Escherichia coli* which are reversible by glutathione (34). Hence either type of experiment, taken alone, could only suggest the existence of a GH precursor. However, the observations that the preGH molecules observed *in vitro* and *in vivo* are both precipitated by anti-GH antiserum and have identical mobilities on a $NaDodSO_4$-acrylamide gel provides strong evidence that preGH is a biosynthetic precursor of GH.

PreGH has properties in common with two other putative precursors of mammalian proteins which have been observed. The proposed precursors of gamma-globulin L chain (3, 32), proparathryoid hormone (10), and now GH have all first been observed as the product of the translation of mRNA in heterologous cell-free systems. Attempts to detect the precursors during short pulses of intact normal cells or gland slices with radioactive amino acids have been unsuccessful, suggesting a rapid cleavage of the precursors, perhaps even before synthesis is completed. Since all three are secretory proteins, it is possible that the extra peptides in precursors of this particular type serve some early function in the segregation by secretory cells of proteins destined for export. We suggest the use of prefix *pre* to designate very short-lived precursors of this type, to distinguish them from the more long-lived precursors designated by *pro* (proinsulin, proparathyroid hormone, etc.).

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