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Effect of Growth Hormone on Protein Phosphorylation in Isolated Rat Hepatocytes

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ABSTRACT: Hepatocytes from male rats were incubated with [^{32}P]P_i for 40 min at 37 °C, thereby equilibrating the cellular ATP pool with ^{32}P. Subsequent exposure to bovine growth hormone for 10 additional min did not change the specific activity of cellular [γ-^{32}P]ATP. Two-dimensional gel electrophoresis or chromatofocusing followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to fractionate phosphoproteins solubilized from control or hormone-stimulated cells. Stimulation of hepatocytes with 5 nM growth hormone for 10 min at 37 °C affected the phosphorylation of a number of proteins including an M_r 46 000 species of pI 4.7 whose phosphorylation was augmented (2.65 ± 0.50)-fold. A significant fraction of the maximal effect of growth hormone on phosphorylation of the M_r 46 000 species was elicited by 1–5% receptor occupancy. Bovine growth hormone, which binds to somatogenic receptors with great specificity, or recombinant human growth hormone, which is not contaminated with other hormones, affected phosphorylation of hepatic proteins similarly. The M_r 46 000 phosphoprotein was isolated in a fraction enriched in cytosol after centrifugation of cellular homogenates. Phosphorylation of the M_r 46 000 phosphoprotein was also increased (1.75 ± 0.35)-fold and (2.15 ± 0.50)-fold by insulin and glucagon, respectively. These observations are consistent with the possibility that selective changes in the phosphorylation state of cellular proteins may mediate growth hormone actions in cells.

The first step leading to growth hormone action is binding to receptors, which have been identified in a number of different cells and membranes (Kelly et al., 1974; Lesniak et al., 1974; Posner, 1976; Fagin et al., 1980), including hepatocytes isolated from rats (Ranke et al., 1976; Donner et al., 1978a–c, 1980; Donner, 1980, 1983; Yamada & Donner, 1984). The growth hormone receptor has been affinity labeled (Donner, 1983; Hughes et al., 1983; Yamada & Donner, 1984; Carter-Su et al., 1984; Gorin & Goodman, 1984) and, in the hepatocyte, is a moiety of 300 000 daltons which contains a binding subunit of M_r 100 000 (Donner, 1983; Yamada &

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Donner, 1984). The somatogenic receptor has been separated from lactogenic sites and purified (Waters & Friesen, 1979). Thus, considerable progress has been made toward biochemical characterization of the site which initiates the actions of growth hormone.

Growth hormone produces diverse changes in cells (Talwar et al., 1975; Wallis, 1980). In liver, growth hormone binding induces the production of somatomedin C (McDonaghey & Sledge, 1970) which mediates many of the effects of growth hormone (Daughaday, 1983). However, the intermediary mechanisms by which growth hormone binding is transduced into biological responses remain largely unknown. One mechanism that may be involved in promoting the actions of many peptide hormones and growth factors is the selective phosphorylation or dephosphorylation of specific phosphoproteins (Greengard, 1978; Rosen & Krebs, 1981).

The isolated rat hepatocyte has been a valuable model in which to study the regulation of protein phosphorylation by peptide hormones, such as glucagon and insulin (Avruch et al., 1978; Garrison, 1978; Le Cam, 1982; Garrison & Wagner, 1982). Since the hepatocyte is also a recognized target for growth hormone action, the goal of this study was to determine if growth hormone affects protein phosphorylation in this cell.

Experimental Procedures

Materials

Male Sprague-Dawley rats (130–190 g) were purchased from Charles River Breeding Laboratories and fed Purina Laboratory Chow ad libitum. Biosynthetic methionyl human growth hormone (Met-hGH)[1] was a generous gift from Kabi Vitrum. Human growth hormone and bovine growth hormone were from Dr. Martin Sonenberg (Sloan–Kettering Institute). Porcine glucagon (0.6 unit/mg) was a gift from Eli Lilly. Other materials were the following: zinc porcine insulin (25.5 USP units/mg) (Eli Lilly); ^{32}P (carrier free) and Aquasol-2 (New England Nuclear); ^{125}I (carrier free) (Amersham); CHAPS, Nonidet P-40, soybean trypsin inhibitor, bacitracin, and luciferase–luciferin reagent (Sigma); bovine serum albumin, crystalline (Miles Pentex); gentamicin (Shering); PBE 94 and polybuffer 74 for chromatofocusing (Pharmacia); collagenase type I from *Clostridium histolyticum* for isolation of hepatocytes, micrococcal nuclease, deoxyribonuclease 1, and ribonuclease A (Worthington); Ampholines (LKB); 2-mercaptoethanol and p*I* markers (Bio-Rad).

Methods

Hepatocytes prepared by the method of Berry and Friend (1969) were greater than 90% viable, possessed a functional amino acid transport system, synthesized protein, were hormonally responsive (Donner et al., 1978b), and contained specific receptors for hGH (Donner et al., 1978a–c, 1980; Donner, 1980), bGH (Donner et al., 1978a), and insulin (Donner & Corin, 1980). bGH was iodinated by the method of Greenwood et al. (1963) and Lesniak et al. (1973) and separated from free ^{125}I by elution from a column (0.9 × 57 cm) of Sephadex G-75. The binding of ^{125}I-bGH to hepatocytes was assayed by centrifugation as previously described (Donner et al., 1978a). Protein was assayed by staining with Coomassie Blue G-250 using a kit from Bio-Rad.

Labeling Hepatocytes with ^{32}P. Freshly isolated hepatocytes were washed once with phosphate-free Krebs–Ringer bicarbonate medium and then twice with phosphate-free medium supplemented with bovine serum albumin (10 mg/mL), bacitracin (0.8 mg/mL), and gentamicin (50 μg/mL) (buffer A) (Le Cam, 1982). The hepatocytes were diluted to a density of 2×10^6 cells/mL in buffer A containing 15 mM glucose and incubated with [^{32}P]P_i (0.05–0.2 mCi/mL) for 40 min at 37 °C under an atmosphere of 95% O_2–5% CO_2. The cell suspensions were then transferred to tubes containing the concentrations of hormone described in the figure legends and incubated for 10 additional min at 37 °C. Incubation was terminated by adding excess ice-cold Krebs–Ringer bicarbonate medium containing 2 mM phosphate and by centrifugation.

Specific Activity of [γ-^{32}P]ATP. The specific activity of the γ-phosphate of cellular ATP was measured as a function of labeling time to ensure that a steady state had been reached prior to hormone stimulation and that growth hormone did not alter the specific activity of the ATP pool (Garrison, 1983). To accomplish this, hepatocytes were incubated with $^{32}PO_4^{3-}$ as described above. At various times before or after addition of bGH, duplicate aliquots from the incubates were centrifuged (10000*g*, 30 s) and resuspended in 0.6 M perchloric acid (750 μL) to extract nucleotides. The supernatants, containing extracted nucleotides, were separated from residual cellular matter by centrifugation (2 min) and then neutralized with 3 M potassium bicarbonate (75 μL). The precipitates produced by neutralization were removed by centrifugation (2 min) and filtration through Millipore type GV (0.22-μm) filters. An aliquot of each supernatant (100 μL) was injected into a Vydac nucleotide analysis column in a Varian Model 5020 high-performance liquid chromatograph equipped with a Model 2050 variable-wavelength detector set at 269 nm and a Model 4290 integrator. A linear gradient of 45 mM ammonium formate (adjusted to pH 4.6 with phosphoric acid) to 0.5 M sodium phosphate (adjusted to pH 2.7 with formic acid) run over 12 min (2 mL/min) eluted nucleotides from the column. Fractions (0.5 mL) containing ATP, which eluted between 6 and 6.5 min, were pooled and neutralized with 5 M potassium hydroxide (200 μL).

The concentration of ATP isolated from hepatocytes was measured with a luciferase assay (Stanley & Williams, 1969). Standard ATP solutions or ATP from cells (30 μL) and luciferase–luciferin reagent (20 μL, 40 mg/mL) were rapidly mixed in buffer [freshly prepared 0.1 M sodium arsenate (1 mL, adjusted to pH 7.4 with sulfuric acid) containing 40 mM magnesium sulfate, water (1 mL), and 950 μL of 10 mM potassium phosphate and 4 mM magnesium sulfate, pH 7.4] and assayed (0.1 min) for emitted photons in a Packard Model 3255 liquid scintillation counter set to an out-of-coincidence mode. Pulse height spectra measured at several ATP concentrations displayed a sharp maximum between 220 and 230 discriminator units (100% gain). At these settings, the assay was linear from 10^{-10} to 10^{-9} M ATP.

To measure the specific activity of the [γ-^{32}P]ATP, hexokinase was used to transfer the γ-phosphate of the isolated ATP to glucose (Leiter et al., 1978). Additional ATP (0.1 mM final concentration), magnesium chloride (10 mM), and glucose (10 mM) were added to aliquots (950 μL) of the isolated cellular ATP. After addition of hexokinase (3 units/mL), the mixtures were incubated for 30 min at 30 °C and

[1] Abbreviations: HBSS, Hank's balanced salt solution; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Met-hGH, recombinant methionyl human growth hormone; bGH, bovine growth hormone; hGH, human growth hormone; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IEF, isoelectric focusing; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid; DTT, dithiothreitol.

applied to columns of Dowex 1X10 (1 mL). Glucose 6-phosphate was eluted from the columns with water (2 mL) and assayed for ^{32}P in a scintillation counter. The specific activity of the γ-phosphate of cellular ATP was calculated from the amount of isolated ATP used for formation of glucose 6-[^{32}P]phosphate.

Two-Dimensional Gel Electrophoresis. After equilibration with ^{32}P and incubation with ligand, 2×10^6 cells were suspended in 180 μL of a buffer containing micrococcal nuclease (7.6 μg), deoxyribonuclease 1 (7.5 μg), ribonuclease A (15 μg), $MgCl_2$ (4 mM), $CaCl_2$ (2 mM), SDS (0.25%), and 2-mercaptoethanol (0.83%) and lysed (Garrels, 1979; Kohno, 1985) by several passages through a 26-gauge needle. Solid urea, Nonidet P-40, and Ampholines (pH 5–7) were added to final concentrations of 9.5 M, 3% w/v, and 2% w/v, respectively. No significant differences in protein staining or phosphorylation were observed if samples were fractionated immediately or frozen before gel electrophoresis.

Two-dimensional gel electrophoresis was conducted as described by O'Farrell (1975) with modifications (Garrison & Wagner, 1982; Kohno, 1985). The first-dimension IEF tube gel (11.5 × 2.2 mm) was made of 4% acrylamide [acrylamide:bis(acrylamide) ratio of 30:1.5] and contained 2% Ampholines (pH 3.5–10:pH 5–7 ratio of 1:4), 2% Nonidet P-40, and 9.2 M urea. Gels were prefocused and run for 7200 V h. IEF standards and soybean trypsin inhibitor were run in parallel tubes that were stained with Coomassie Brilliant Blue R to assay the pH across the first dimension. The second dimension was run on 10% SDS–acrylamide slab gels [30:0.8 acrylamide:bis(acrylamide) ratio, 0.75 mm thick, without a stack]. Electrophoresis was carried out at a constant current of 15 mA for 4–5 h.

Chromatofocusing. Frozen cell pellets (1.6×10^6 cells) were suspended in 2.5 mL of an extraction medium consisting of 10 mM imidazole, pH 7.4, 10 mM NaF, 2 mM EDTA, 2 mM sodium phosphate, and 0.5% CHAPS. The suspension was drawn through a 22-gauge needle 10 times and then through a 26-gauge needle 3 times, resulting in complete cell disruption. The suspension was centrifuged at 105000*g* for 60 min, and an aliquot (1.8 mL) of the supernatant was applied to a PBE 94 column (0.7 × 6 cm) equilibrated at 4 °C with 25 mM imidazole, pH 7.4, and 0.2% CHAPS buffer. The column was eluted with polybuffer 74 (diluted 9-fold) and 0.2% CHAPS, pH 4.0. The absorbance at 280 nm, the ^{32}P content, and the pH of each fraction (1 mL) were measured. Fractions of interest were combined, and trichloroacetic acid was added to a final concentration of 5%. After 10 min at 0 °C, the samples were centrifuged (1500*g*, 15 min), and the resultant protein pellet was neutralized with 0.5 N sodium hydroxide and solubilized by boiling in 3% sodium dodecyl sulfate, 10% glycerol, 62.5 mM Tris-HCl buffer, pH 6.8, and 10 mM sodium phosphate (3 min). Samples from chromatofocusing columns were fractionated on 5–15% gradient gels [37.5:1 acrylamide/bis(acrylamide)] that were 1.5 mm thick by using the discontinuous buffer system described by Laemmli (1970). Electrophoresis was carried out at a constant current of 25 mA for 4–5 h.

Processing of Gels and Autoradiography. Gels were stained for 1 h in 0.5% Coomassie Brilliant Blue R dissolved in isopropyl alcohol/acetic acid/water (25:7:68, by volume) and dried before autoradiography. The standards used to estimate the molecular weights (in parentheses) of phosphorylated species were as follows: myosin (200000), β-galactosidase (116250), phosphorylase *b* (92500), bovine serum albumin (66200), and ovalbumin (45000). The positions and molecular weight values of these standards are shown to the left of each autoradiograph. The apparent molecular weight of phosphorylated species are to the right of each autoradiograph. Autoradiography was carried out in cassettes with Kodak X-O-mat XAR-5 film using a Dupont Cronex Lightning Plus enhancing screen. Films were exposed from 3 to 7 days at −80 °C before being developed. Autoradiograms were scanned with a Beckman DU 8 spectrophotometer or with a Helena Laboratories Quick-Scan Jr. densitometer. On two-dimensional gels, the effects of hormones are quantitated relative to an insensitive protein of M_r 55000, p*I* 5.3.

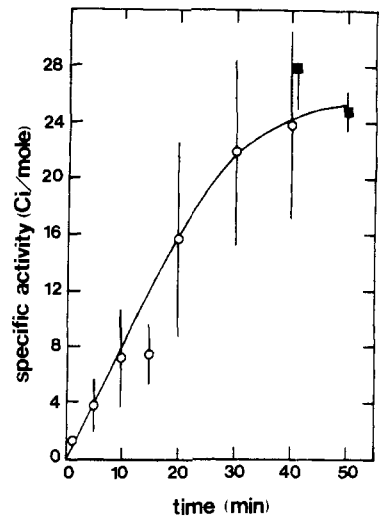

FIGURE 1: Specific activity of hepatic [γ-^{32}P]ATP. Hepatocytes (4×10^6 cells/mL) were incubated with $^{32}PO_4^{3-}$ (0.1 mCi/mL) under an atmosphere of 95% O_2–5% CO_2. At various times prior to (○) or after (■) addition of bGH (5.6 nM), duplicate 1-mL aliquots were removed for extraction of cellular nucleotides. ATP was purified by HPLC, and the specific activity of the γ-phosphate was measured by hexokinase-mediated phosphorylation of glucose as described under Methods. Data points represent the average of duplicate measurements ± SEM.

RESULTS

The specific activity of [γ-^{32}P]ATP was measured as a function of labeling time after addition of ^{32}P to hepatocytes as described under Methods. The specific activity approached a steady-state level within 40 min of initiating the labeling procedure (Figure 1). Addition of bovine growth hormone to ^{32}P-labeled hepatocytes did not alter the specific activity of the ATP pool. Thus, conditions were established in which the effects of growth hormone on the phosphorylation state of various target proteins could be assayed.

Two-dimensional gel electrophoresis was used to fractionate phosphoproteins solubilized from control (Figure 2, top) or methionyl human growth hormone stimulated hepatocytes (middle). This analytical method showed that hormone stimulation affected many protein targets, the phosphorylation of some being enhanced and others diminished. An M_r 46000 species, whose phosphorylation was augmented, was among the major phosphoproteins that were sensitive to growth hormone. The inability of Coomassie staining to visualize a protein corresponding, for example, to the hormone-sensitive M_r 46000 species suggests that targets for growth hormone are not necessarily highly abundant proteins (Figure 2, bottom).

The M_r 46000 protein was sensitive to insulin, glucagon, and bovine growth hormone as well as to Met-hGH (Figure 3). In experiments identical with the one illustrated by Figure 2, Met-hGH, bGH, insulin, and glucagon augmented phos-

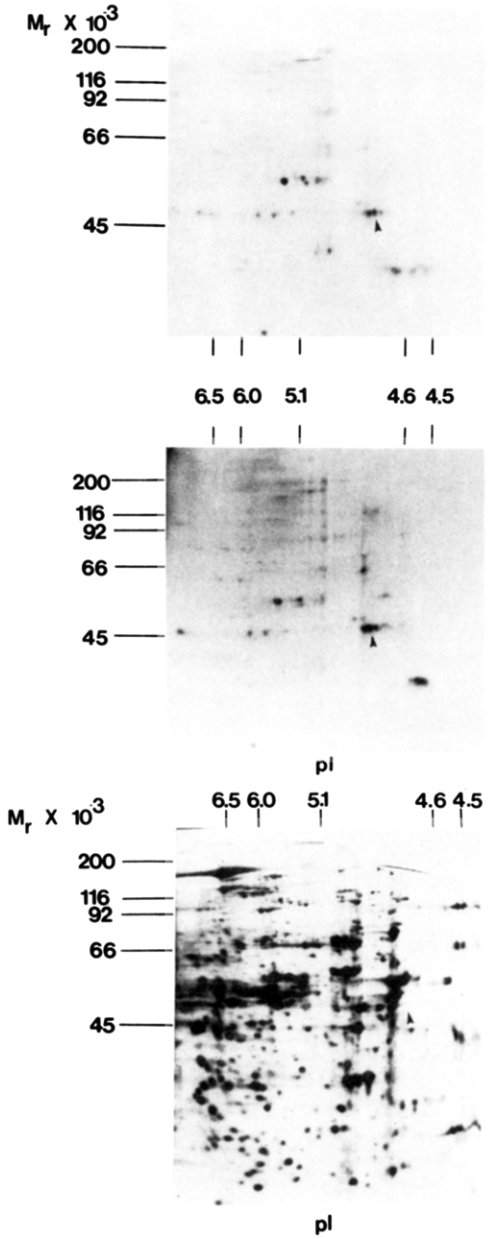

FIGURE 2: Two-dimensional gel electrophoresis of phosphoproteins. Hepatocytes equilibrated with ^{32}P were transferred to tubes with or without 5 nM methionyl growth hormone and incubated for 10 min before being processed for two-dimensional gel electrophoresis. (Top) Phosphorylation in control cells; (middle) phosphorylation in cells incubated with hormone; (bottom) Coomassie staining of proteins from 2.7×10^5 cells. Arrows point to the 46 000-dalton growth hormone target (top and middle) or to the position to which this phosphoprotein fractionates relative to other more abundant proteins.

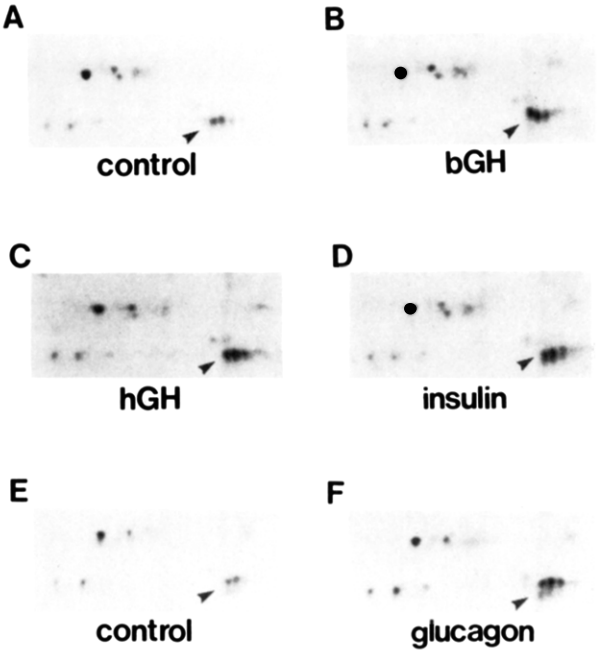

FIGURE 3: Effect of various hormones on phosphorylation of a 46 000-dalton protein. Hepatocytes were equilibrated with ^{32}P and then incubated for 10 min with 5 nM (A) control (no addition); (B) bGH, (C) Met-hGH, (D) insulin, (E) control (for glucagon) (no addition), and (F) glucagon. Phosphoproteins were fractionated by two-dimensional gel electrophoresis. Only the portion of each two-dimensional gel that contained the M_r 46 000 hormone-sensitive complex is shown.

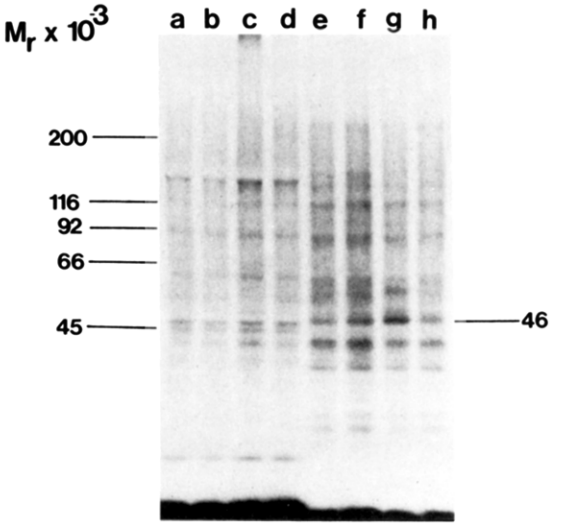

FIGURE 4: Phosphoproteins in membrane and soluble cell fractions. Hepatocytes were equilibrated with ^{32}P and then incubated for 10 min with 5 nM (lanes a and e) control (no addition), (lanes b and f) bGH, (lanes c and g) glucagon, and (lanes d and h) insulin. The hepatocytes were collected by centrifugation, suspended in hypotonic buffer (10 mM imidazole, 2 mM phosphate, 10 mM NaF, and 2 mM EDTA, pH 7.4), and lysed by 10 passages through a 22-gauge and then 3 passages through a 26-gauge needle. A soluble fraction enriched in cytosolic protein (lanes e–h) was separated from cell membranes (lanes a–d) by centrifugation (10000g, 10 min) and then precipitated by addition of an equal volume of 10% TCA at 0 °C. After centrifugation, the protein precipitate was solubilized into SDS–PAGE buffer which was neutralized with sodium hydroxide. The cell membrane fraction was also solubilized into SDS–PAGE buffer. Equal amounts of membrane or cytosolic protein were fractionated on a 5–15% gradient gel [37.5:1 acrylamide:bis(acrylamide) ratio] after reduction with DTT (50 mM).

phorylation of the M_r 46 000 species (2.65 ± 0.50)-fold, (2.03 ± 0.25)-fold, (1.75 ± 0.35)-fold, and (2.15 ± 0.50)-fold, respectively (n = 2) (Figure 3). Since the growth hormone sensitive M_r 46 000 species is also sensitive to insulin and glucagon, we chose to focus this study on that phosphoprotein.

Hormone-stimulated rat hepatocytes were fractionated into membrane and soluble (cytosol) fractions. Among the hormone-sensitive targets, the M_r 46 000 protein sensitive to bGH, glucagon, or insulin was recovered in the cytosol-enriched fraction (Figure 4). In this and in the other experiments shown, phosphoproteins that would correspond to components of the growth hormone (Donner, 1983) or insulin (White et al., 1985b) receptors were not observed. Insulin also stimulates phosphorylation of several endogenous substrates including pp185 and pp120 (White et al., 1985a; Rees-Jones & Taylor, 1985). Observation of the endogenous protein kinase substrates or phosphorylated insulin receptors usually requires enrichment of these proteins, which was not done in this study.

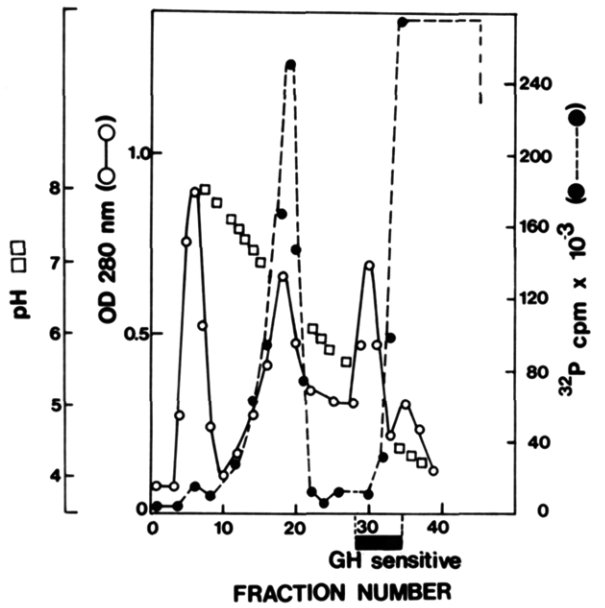

FIGURE 5: Fractionation of hepatic proteins by chromatofocusing. Phosphoproteins from hepatocytes were fractionated on a column of PBE 94. The pH gradient (□) and the elution of protein (A_{280}, ○) and ^{32}P (●) from the column were assayed.

By analogy, it will be necessary to enrich the growth hormone receptor to determine if it is a protein kinase or a substrate for phosphorylation.

Complete quantitation of protein phosphorylation on two-dimensional gels can require sophisticated densitometry and computer analysis (Garrison & Wagner, 1982), and the direct comparison of many samples is difficult. In contrast, phosphoproteins from many experiments can be fractionated on a single one-dimensional gel which can be quantitated simply. In this study, it was found that the large number of phosphoproteins from hepatocytes produced high backgrounds, making it difficult to characterize phosphorylation by a one-dimensional procedure (not shown). For this reason, a method was developed in which phosphoproteins were fractionated first by chromatofocusing (Figure 5) and then by SDS–PAGE. There was no significant difference in the elution of protein (A_{280}) or ^{32}P from chromatofocusing columns separating supernates of control cells or cells stimulated with growth hormone, insulin, or glucagon. Each fraction of control or stimulated cells recovered from chromatofocusing was next fractionated by SDS–PAGE to assay for hormone-sensitive targets. A 46 000-dalton protein of p*I* 4.7 that eluted in fractions 29–34 was sensitive to bovine growth hormone (Figure 6), native human growth hormone, insulin, and glucagon (not shown). The identity of the M_r 46 000 species observed in the experiments illustrated by Figures 2 and 6 was additionally confirmed by fractionating phosphoproteins isolated after chromatofocusing using two-dimensional gel electrophoresis (not shown).

Chromatofocusing/SDS–PAGE permitted quantitation of the effect of growth hormone on phosphorylation of the M_r 46 000 species (Figures 6 and 7). The stimulatory effect of bGH was observed at concentrations as low as 1.7×10^{-11} M; phosphorylation was maximally augmented about 3-fold after treatment of cells with 10 nM bGH. The dose response of the effect of growth hormone on phosphorylation of the M_r 46 000 dalton protein was compared to the fractional occupancy of receptors by various concentrations of ^{125}I-bGH (Figure 7). Binding to only 1–5% of the growth hormone receptors elicited a significant fraction of the maximal stimulation of phosphorylation of the M_r 46 000 species. As the binding capacity

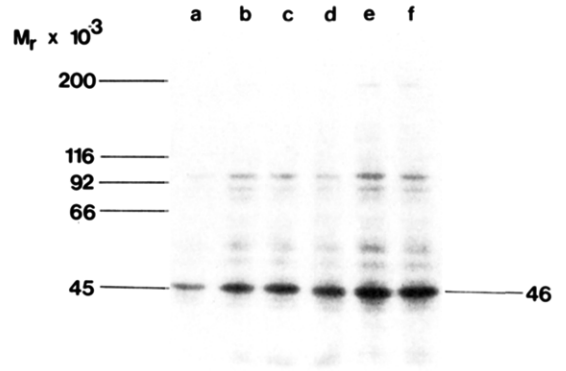

FIGURE 6: Dose response of growth hormone induced phosphorylation. Hepatocytes were equilibrated with ^{32}P and then stimulated with (lane a) control (no additions), (lane b) 0.017 nM bGH, (lane c) 0.04 nM bGH, (lane d) 0.2 nM bGH, (lane e) 1.1 nM bGH, and (lane f) 5 nM bGH. Fractions 29–34 from chromatofocusing columns were combined and processed as described under Methods. Equal volumes of each sample were reduced with 100 mM dithiothreitol and analyzed by SDS–PAGE.

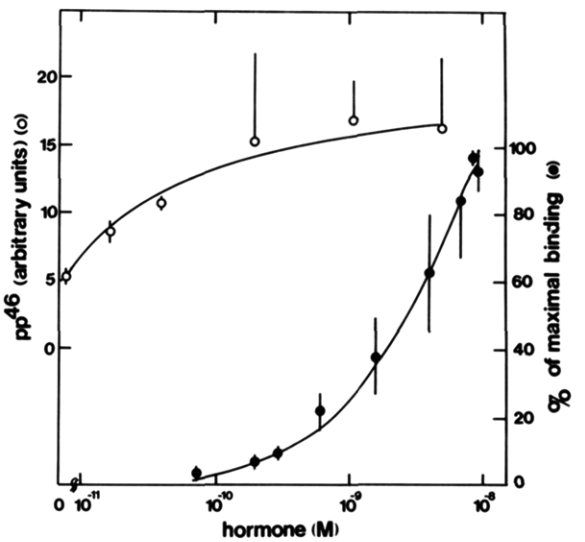

FIGURE 7: Relationship of growth hormone binding and phosphorylation. Each lane in the autoradiogram shown in Figure 6 and in a replicate experiment was quantitated by scanning densitometry. The relative intensity (peak area) of the 46 000-dalton species is plotted against the growth hormone concentration (○). In separate experiments ($n = 2$), various concentrations of ^{125}I-bGH were incubated with hepatocytes for 60 min at 23 °C. The fraction of maximal specific binding is plotted against the free hormone concentration (●). In the presence of 10 nM free hormone, 13.74 fmol of ^{125}I-bGH was specifically bound per 10^6 cells. Data points are the average of the duplicate experiments ± SEM.

of hepatocytes approached saturation (at about 10 nM hormone), the effect of hormone on phosphorylation of the 46 000-dalton species approached a limiting value.

In some gels, growth hormone appeared to affect the phosphorylation of other proteins in fractions 29–34, particularly one of M_r 92 000. Most of this phosphoprotein eluted from chromatofocusing columns in more alkaline fractions than the 46 000-dalton protein. Evaluation of these fractions indicated that the 92 000-dalton protein was not sensitive to growth hormone, a conclusion supported by two-dimensional gel electrophoresis (Figure 2). Therefore, in order to use chromatofocusing/SDS–PAGE, it is necessary to ensure quantitative recovery of a phosphoprotein in the combined fractions being assayed.

Discussion

Phosphoproteins from control and hormone-stimulated hepatocytes were fractionated by two-dimensional gel electrophoresis (IEF/SDS–PAGE). This procedure demonstrates that growth hormone affects the phosphorylation state of numerous targets in the rat hepatocyte, diminishing the phosphorylation of some species and augmenting the phosphorylation of others. Two-dimensional SDS–PAGE and chromatofocusing/SDS–PAGE both showed that various growth hormones increase the phosphorylation of a protein of M_r 46 000 with a pI of about 4.7. Among the major cellular phosphoproteins, this target, which was isolated in a cytosol-enriched cell fraction, was most affected by growth hormone and was also sensitive to insulin and glucagon.

Hepatocytes from female animals contain lactogenic and somatogenic receptors (Ranke et al., 1976). Human growth hormone binds to each of these receptor types (Kelly et al., 1974; Ranke et al., 1976) and often is contaminated with traces of prolactin which is also of pituitary origin. Thus, it can be difficult to distinguish effects mediated by lactogenic and somatogenic receptors. The experiments described in this report were conducted with hepatocytes from male animals which contain few, if any, receptors for prolactin (Ranke et al., 1976). The probable absence of lactogenic receptors together with the ability of bGH, which binds to somatogenic receptors exclusively (Kelly et al., 1974; Ranke et al., 1976; Yamada & Donner, 1984), and recombinant growth hormone, which is not contaminated with other hormones, to similarly affect phosphorylation of the M_r 46 000 protein and other proteins suggests that these changes are initiated by hormone binding to somatogenic receptors.

Growth hormone binding to liver results in the production of insulin-like growth factor I (somatomedin C) (McConaghey & Sledge, 1970), a mediator for many biological actions of growth hormone (Daughaday, 1983). It is therefore reasonable to question if the hormone-stimulated phosphorylation, including that of the M_r 46 000 protein, was mediated by IGF-I or was produced by growth hormone directly. Rat liver contains receptors for insulin and IGF-II but not IGF-I (Massague & Czech, 1982). IGF-I binds to receptors for IGF-II but not insulin in rat liver (Massague & Czech, 1982). Therefore, actions of IGF-I in the hepatocyte would probably result from binding to IGF-II receptors. Since low concentrations of IGFs do not activate rapid insulin-like effects in liver (Czech, 1982), it is considered unlikely that growth hormone induces sufficient IGF-I to mediate the phosphorylation responses. Therefore, the properties of the receptors on hepatocytes suggest that growth hormone can directly affect the phosphorylation of an M_r 46 000 protein and other proteins.

Maximal response to a peptide hormone is often elicited by occupancy of a small fraction of the available binding sites (Birnbaumer & Pohl, 1973). For example, binding of insulin to 2–10% of the receptors on adipocytes produces maximal effects on glucose transport (Olefsky, 1975), glucose oxidation (Kono & Barham, 1971), and lipogenesis (Gliemann et al., 1975). Low levels of receptor occupancy by insulin or glucagon also elicit disproportionately large effects on hepatic protein phosphorylation (Le Cam, 1982). The concentration of growth hormone necessary to induce half-maximal conversion of preadipocytes to adipocytes (Nixon & Green, 1983) or to increase lipolysis in adipocytes (Grichting et al., 1983) is sufficient to result in binding to only 10% of the receptors in the cell membrane. The effects of somatogenic hormones on phosphorylation of the M_r 46 000 protein also indicate a nonlinear relationship between binding and response. If phosphorylation of the M_r 46 000 species is considered a measure of signal transmission, most growth hormone receptors are spare and are not necessary for biological response. A consequence of this would be that the hepatocyte is extremely sensitive to low concentrations of growth hormone.

Two-dimensional gel electrophoresis and chromatofocusing/SDS–PAGE identify the same hepatic phosphoprotein as a target for growth hormone. Each method shows that growth hormone induces a 2–3-fold increase in the phosphorylation of an M_r 46 000 species with a pI of about 4.7. These observations validate chromatofocusing/SDS–PAGE as a procedure for characterizing the effects of hormones on cellular protein phosphorylation and therefore lend methodological significance to this study. Chromatofocusing/SDS–PAGE supplements two-dimensional gel electrophoresis by making it possible to simply and conveniently quantitate hormone effects on protein phosphorylation. Side by side comparison of many samples on a one-dimensional gel is possible after chromatofocusing has separated hormone-sensitive targets from many other phosphoproteins. This procedure may prove useful in other systems.

A basis exists for believing that changes of protein phosphorylation may play a role in the cellular response to growth hormone. Recently, it has been shown that the antilipolytic, insulin-like effect of growth hormone in the adipocyte results from decreased phosphorylation of hormone-sensitive lipase (Bjorgell et al., 1984). The time course and magnitude of the effects of growth hormone were similar to those resulting from exposure of cells to insulin, suggesting that the antilipolytic effects of both hormones were exerted through a common mechanism (Bjorgell et al., 1984).

This report demonstrates that in a second target tissue, liver, growth hormone alters protein phosphorylation and can act on targets common to insulin and glucagon. The effects of growth hormone, insulin, and glucagon on the 46 000-dalton phosphoprotein are rapid and of comparable magnitude, maximally increasing phosphorylation about 3-fold. While the identity and specific function of the M_r 46 000 complex is presently unknown, the demonstration that this minor cellular constituent is a common target for the actions of at least three hormones may suggest that it has an important regulatory function. Since hepatocytes may contain numerous phosphoproteins differentially sensitive to growth hormone, insulin, and glucagon, many pathways probably exist through which each hormone can affect the actions of the others.

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Registry No. GH, 9002-72-6; insulin, 9004-10-8; glucagon, 9007-92-5.

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