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Phosphorylation of human growth hormone by the epidermal growth factor-stimulated tyrosine kinase

(epidermal growth factor receptor/phosphotyrosine/somatotropin)

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ABSTRACT **In the present study, we have demonstrated that human growth hormone (hGH) can be phosphorylated by the epidermal growth factor (EGF)-stimulated tyrosine kinase of A431 cell membranes. Phosphotyrosine was the predominant phosphoamino acid released from phosphorylated hGH on partial acid hydrolysis. All five tyrosine-containing tryptic peptides of hGH are also phosphorylated by the EGF-stimulated tyrosine kinase. The highest phosphate incorporation was found for peptide T4 (residues 20–38), which is distinguished by a high frequency of acidic amino acids. The phosphorylated peptides have been characterized by HPLC and two-dimensional mapping on paper. Comparison with the labeled peptides obtained on tryptic digestion of phosphorylated hGH suggests that tyrosine phosphorylation is restricted to two tryptic peptides, T4 (tyrosine-28 or -35) and T6 (tyrosine-42). It is suggested that the absence of early insulin-like activity in the naturally occurring M_r 20,000 variant of hGH, which has an internal deletion spanning residues 32–46, may be a consequence of the loss of the tyrosine phosphorylation sites at residues 35 and 42.**

Considerable interest has recently been shown in the possible role of tyrosine phosphorylation in normal and malignant cellular growth. Although phosphorylation accounts for <0.1% of the phosphoamino acid pool in normal cells (1), levels of phosphotyrosine increase 10-fold after retroviral infection as a result of the tyrosine kinase activity that is an intrinsic property of the transforming proteins of several RNA tumor viruses (2, 3). Stimulation of cellular growth by polypeptides such as epidermal growth factor (EGF) (4, 5), platelet-derived growth factor (6), and insulin (7) may also be related to the tyrosine kinase activities associated with the membrane receptors for these three polypeptides.

Human growth hormone (hGH), somatotropin, is a single-chain polypeptide hormone of M_r 22,000 (8). The amino acid sequence of hGH between residues 30 and 40 is reminiscent of the tyrosine phosphorylation site in the middle T protein of polyoma virus (9, 10), other viral oncoproteins (2, 11–13), and gastrin (14) in that it contains a tyrosine residue preceded by several adjacent glutamic residues (Fig. 1). Therefore, we have tested hGH as a substrate for the tyrosine kinase associated with the EGF receptor (4, 5). A useful control occurs naturally in the M_r 20,000 structural variant of hGH (18), which is a significant component (10%) of all hGH preparations from pituitary extracts. The amino acid sequence of the M_r 20,000 variant hGH is identical to that of normal ("M_r 22,000") hGH apart from a 15-amino-acid-residue internal deletion (residues 32–46) (18–20), which arises from alternative mRNA splicing (Fig. 2). The putative phosphorylation site (tyrosine-35) of normal hGH is thus absent from the M_r 20,000 variant hGH.

hGH	T Y Q E F E E A Y I P K
Gastrin	W L E E E E E A Ⓨ G W M
Polyoma	P L E E E E E E Ⓨ M P M
RSV	A R L I E D N E Ⓨ T A R

FIG. 1. Comparison of amino acid sequences of a region (residues 27–38) of hGH with known tyrosine phosphorylation sites. The amino acid sequences of hGH (residues 27–38) (8), human gastrin (residues 21–32) (15, 16), polyoma middle T protein (residues 318–329) (9, 10), and the oncoprotein of Rous sarcoma virus (RSV) (residues 408–419) (2) were aligned about their modified tyrosine residue. A one-letter notation for abbreviating amino acid residues has been used (17); Ⓨ, modified tyrosine residue.

In this communication, we report that hGH can be specifically phosphorylated by the EGF-stimulated tyrosine kinase of A431 human epidermoid carcinoma cells. The phosphotyrosine-containing peptides obtained by tryptic digestion of phosphorylated hGH were compared by HPLC and two-dimensional peptide mapping with the individually phosphorylated tyrosine-containing tryptic peptides of hGH. The results suggest that EGF-stimulated phosphorylation of hGH is restricted to three of the eight tyrosine residues in hGH.

MATERIALS AND METHODS

Materials. Purified pituitary-derived hGH was provided by the Human Pituitary Advisory Committee, Department of Health, Australia; the M_r 20,000 structural variant hGH was a gift to one of us (M.T.W.H.) from G. E. Chapman (University of Auckland, New Zealand). EGF-α was prepared as described (21). Phosphoserine and phosphothreonine were purchased from Sigma. Phosphotyrosine was a gift from I. J. Stanley (Ludwig Institute, Melbourne, Australia). Trypsin that had been treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington. HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI); 2-propanol was from Ajax (Sydney, Australia).

Phosphorylation Reactions. Protein and peptide phosphorylations were carried out as described (14) by using the EGF-stimulated tyrosine kinase derived from membrane preparations (22, 23) from the human epidermoid carcinoma cell line A431 (4). Proteins and their phosphorylated derivatives were

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Abbreviations: EGF, epidermal growth factor; hGH, human growth hormone; d_p, particle diameter.

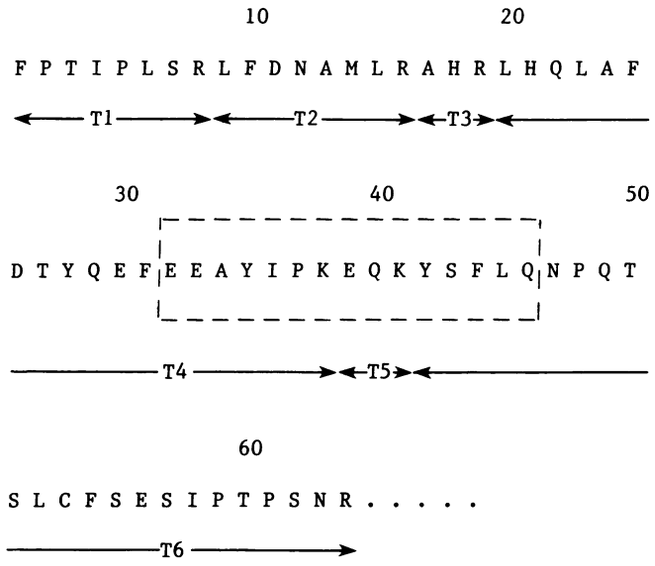

FIG. 2. NH_2-terminal amino acid sequence of hGH (8). The boxed sequence corresponds to residues 32–46 of hGH that are deleted from the M_r 20,000 variant hGH (18–20). The component tryptic peptides (T1–T6), indicated by double-headed arrows and residue numbers shown, are those of hGH.

recovered from the reaction mixture by reversed-phase HPLC on a column (15 × 0.46 cm) packed with butylsilica [Merck LiChrospher, particle diameter (d_p) 10 μm, 50-nm pore diameter bonded with *n*-butyl groups]. Solvent A was 0.1 M ammonium bicarbonate; solvent B was acetonitrile/2-propanol/H_2O (35:35:30)/0.1 M ammonium bicarbonate. After injection of the sample, the column was washed for 15 min with 15% solvent B to elute the [^{32}P]ATP and $^{32}PO_4$ and then developed with a linear gradient from 15 to 100% solvent B in 20 min at a flow rate of 1.2 ml/min. Tryptic peptides and their phosphorylated derivatives (with the exception of peptide T14) were recovered from the reaction mixture by selective adsorption onto cartridges of octadecylsilica (Sep-pak; Waters Associates) by the procedure described for phosphorylated gastrin-17 (14) but with 0.2% heptafluorobutyric acid as primary buffer. The peptides were further purified by reversed-phase HPLC on a column (15 × 0.46 cm) of octadecylsilica (Beckman Ultrasphere, d_p 3 μm) developed with a linear gradient from 0 to 100% solvent C (50% acetonitrile in 0.2% heptafluorobutyric acid) in 50 min at a flow rate of 1 ml/min. ^{32}P-Labeled peptide T14 that did not adsorb to Sep-pak by using the conditions described above was purified by passage through a column (1 × 4 cm) of Dowex AG-1X8 equilibrated with 30% acetic acid as described elsewhere (24).

Amino Acid Analysis. Amino acid analysis was routinely performed on a Dionex D-502 amino acid analyzer after hydrolysis in 6 M HCl at 110°C for 22 hr. Phosphoamino acid analysis of samples after partial acid hydrolysis (6 M HCl at 110°C for 2 hr) was performed essentially as described (14) by using high-voltage electrophoresis at pH 1.9 on Whatman 3 MM paper. The positions of markers (phosphoserine, phosphothreonine, and phosphotyrosine) were visualized by ninhydrin spraying and of ^{32}P-containing spots by autoradiography.

Phosphopeptide Analysis. Before enzymatic digestion with trypsin, hGH was *S*-carboxymethylated (25) in 6 M guanidine·HCl. The reaction mixture was desalted by reversed-phase HPLC on the butylsilica column. The column was developed with a linear gradient from 0 to 100% solvent B in 30 min at a flow rate of 1 ml/min. *S*-Carboxymethyl-hGH was digested with trypsin by using standard methods as described (25). Tryptic peptides from unlabeled hGH were fractionated by reversed-phase HPLC on a column (30 × 0.4 cm) of octadecylsilica (μBondapak C_{18}, d_p 10 μm, Waters Associates). Solvent A was 0.1 M ammonium bicarbonate; solvent B was 50% acetonitrile/50% H_2O/0.1 M ammonium bicarbonate. The column was developed with a linear gradient from 0 to 100% solvent B in 2 hr at a flow rate of 1 ml/min. Identification of tryptic peptides was performed by amino acid analysis (26). ^{32}P-Labeled peptides from a tryptic digest of ^{32}P-labeled *S*-carboxymethyl-hGH were fractionated by reversed-phase HPLC as described above and detected by Cerenkov counting of 1-ml fractions of the column eluent. Alternatively, ^{32}P-labeled tryptic peptides were analyzed by two-dimensional peptide mapping on Whatman 3 MM paper (20 × 20 cm). Electrophoresis was performed in the first direction at 1 kV for 3 hr in acetic acid/pyridine/H_2O (10:1:89), pH 3.5. After drying, chromatography was performed in the second dimension in 1-butanol/pyridine/acetic acid/H_2O (15:10:3:12). The plates were dried and autoradiographed at 22°C by using intensifying screens (DuPont Lightning Plus) and Kodak X-RP5 x-ray film.

RESULTS

Phosphorylation of hGH. In initial experiments, the phosphorylation of hGH by membrane preparations from A431 epidermoid carcinoma cells was investigated (Fig. 3). The results demonstrated that hGH was phosphorylated but that the level of ^{32}P incorporation was low (0.16 mol%). The stimulation (≈3-fold) observed in the presence of 3 μg of EGF per ml (Fig. 4) and the fact that acid hydrolysates of phosphorylated growth hormone contained phosphotyrosine only (Fig. 3 *Inset*) together suggested that the tyrosine kinase associated with the EGF receptor was responsible for phosphorylation.

The sites of phosphorylation on hGH were localized by mapping tryptic digests of ^{32}P-labeled hGH. Three separation techniques (reversed-phase HPLC, electrophoresis, and chromatography) based on different peptide properties were employed to maximize resolution of the phosphorylated tryptic peptides. Six radioactive peaks were observed after reversed-phase HPLC of the tryptic digest. Only two of these (phosphopeptides X and Y, Fig. 4) were in positions expected for individually phosphorylated tryptic peptides and were significantly larger in the trypic digest of hGH phosphorylated in the presence of EGF. When the total tryptic digests were subjected to two-dimensional peptide mapping, autoradiography revealed three major peptides (Fig. 5). The material with an electrophoretic mobility of zero, which corresponded to the peak in the HPLC profile with a retention time of 89 min (Fig. 4), was not phosphorylated to a greater extent in the presence of EGF than in its absence and did not correspond to any of the individually phosphorylated tryptic peptides. The amino acid composition of this material suggested that it was incompletely digested phosphorylated hGH; however, this material was not significantly affected by prolonged digestion with elevated concentrations of trypsin (data not shown). Therefore, we concentrated on the other two labeled peptides (X and Y), both of which were phosphorylated to a greater extent by the membrane preparation in the presence of EGF (Fig. 4).

Identification of Phosphorylation Sites. The identities of ^{32}P-labeled tryptic peptides X and Y from phosphorylated intact hGH were established by comparing their chromatographic properties with the chromatographic properties determined for the individually phosphorylated tyrosine-containing tryptic peptides of hGH (T4, T6, T10, T14, and T16) listed in Table 1. Phosphopeptide X has been provisionally identified as phosphopeptide T4. The retention time in reversed-phase HPLC of

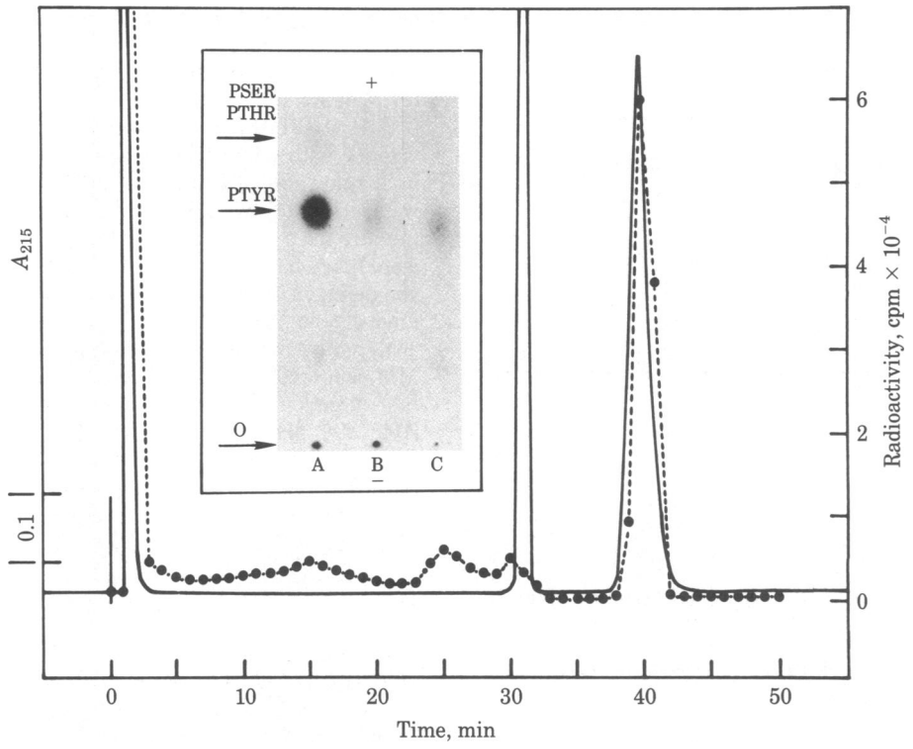

FIG. 3. HPLC separation of ^{32}P-labeled hGH. hGH (200 μg) was phosphorylated for 10 min at 30°C in the presence of 7 μg of A431 cell membrane protein and EGF-α (3 μg/ml) as described elsewhere (14). ^{32}P-Labeled hGH was recovered from the phosphorylation reaction mixture by reversed-phase HPLC on the butylsilica column. The column eluate was monitored for absorbance at 215 nm (——) and radioactivity (●——●), by Cerenkov counting of 1-ml fractions. (*Inset*) Analysis of phosphoamino acids. ^{32}P-Labeled hGH (≈2,000 cpm/2.3 pmol) isolated as described above was hydrolyzed for 2 hr with HCl and analyzed by high-voltage paper electrophoresis at pH 1.9. PTYR, phosphotyrosine; PTHR, phosphothreonine; PSER, phosphoserine; O, origin. A, hGH + EGF; B, hGH − EGF; C, M_r 20,000 variant hGH + EGF.

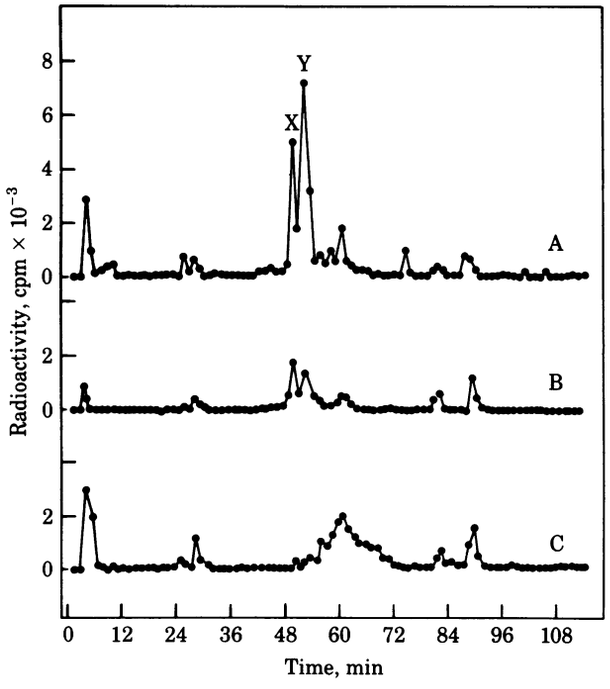

FIG. 4. HPLC separation of tryptic fragments from ^{32}P-labeled *S*-carboxymethyl-hGH. ^{32}P-Labeled hGH (200 μg) was prepared as described in the legend to Fig. 3. ^{32}P-Labeled hGH was *S*-carboxymethylated and digested with trypsin, and the tryptic fragments were separated by reversed-phase HPLC on a μBondapak column. The column eluent was monitored for radioactivity (●——●) by Cerenkov counting of 1-ml fractions. A, hGH + EGF; B, hGH − EGF; C, M_r 20,000 variant hGH + EGF. X and Y, phosphopeptides X and Y.

phosphopeptide X (R_T, 49 min) was in good agreement with that determined for individually phosphorylated peptide T4 (see Table 1). Although phosphopeptide X was identified as phosphopeptide T4 by HPLC, the chromatographic properties of phosphopeptide X derived by two-dimensional mapping (electrophoretic mobility, 0.7 cm; migration rate, R_f, 0.21) are not in complete agreement with those determined for individually phosphorylated peptide T4 (see Table 1). However, the only other neutral or basic phosphorylated tyrosine-containing tryptic peptide in hGH is T16, which has a significantly different retention time on HPLC (see Table 1). Phosphopeptide Y has been assigned as phosphopeptide T6. The chromatographic properties of phosphopeptide Y (R_T, 52 min; electrophoretic mobility, −3.3 cm; R_f, 0.44) were in excellent agreement with those determined for individually phosphorylated peptide T6 (see Table 1) and suggest that tyrosine-42 was labeled in the intact hGH molecule. No evidence was found for the existence of ^{32}P-labeled T10, T14, or T16, indicating that tyrosines-103, -111, -143, -160, and -164 are not phosphorylated by the EGF-stimulated tyrosine kinase.

Phosphorylation of M_r 20,000 Variant hGH. Further evidence that tyrosines-103, -111, -143, -160, and -164 occurring in peptides T10, T14, and T16 were not phosphorylated was obtained by phosphorylation of the naturally occurring M_r 20,000 variant hGH, which lacks part of T4 and T6 including tyrosines-35 and -42 (Fig. 2). Digestion with trypsin and mapping of the resultant peptides as before (Figs. 4 and 5) revealed a general reduction in total radioactivity incorporated and the absence of significant peaks of radioactivity in positions expected for the corresponding phosphopeptides (T10, T14, and T16). This observation is consistent with peptides T4 and T6 containing the major phosphorylation sites in hGH.

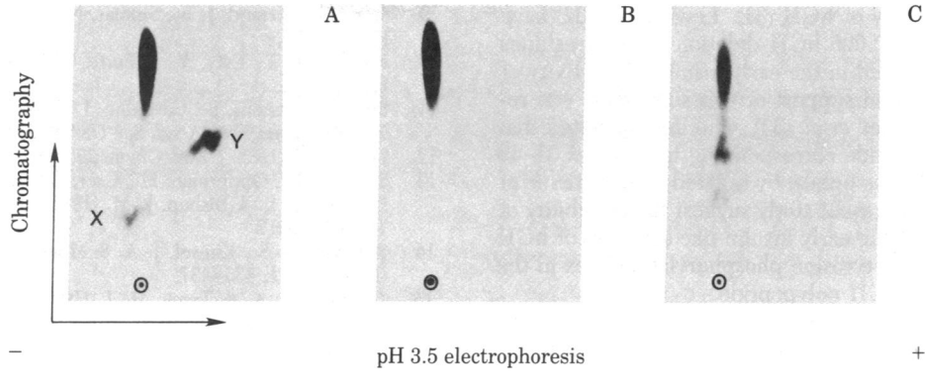

FIG. 5. Two-dimensional analysis of tryptic fragments from ^{32}P-labeled *S*-carboxymethyl-hGH. Tryptic digests of ^{32}P-labeled *S*-carboxymethyl-hGH were applied to Whatman 3 MM paper and electrophoresed in the first dimension toward the anode at 1 kV for 3 hr at pH 3.5. The paper was dried and chromatography was performed in the second dimension in butan-1-ol/acetic acid/H_2O/pyridine. ^{32}P-Labeled peptides were located by autoradiography. *A*, hGH + EGF; *B*, hGH − EGF; *C*, M_r 20,000 variant hGH + EGF.

DISCUSSION

The present study provides evidence for the specific phosphorylation of hGH by the EGF-stimulated tyrosine kinase of A431 cell membranes. Comparison of the chromatographic properties of the labeled peptides isolated from tryptic digests of phosphorylated growth hormone with the properties of individually phosphorylated tyrosine-containing tryptic peptides of hGH indicates that peptide T6 (tyrosine-42) is one major phosphorylation site. The other major phosphorylation site is provisionally assigned as peptide T4 (either tyrosine-28 or -35) by comparison of the tryptic digests of phosphorylated M_r 20,000 variant hGH and phosphorylated normal hGH. It is interesting to note that two of these three tyrosine residues (tyrosine-35 and tyrosine-42) are readily nitrated *in vitro* (27) and are therefore presumably situated on the surface of the molecule. When intact hGH is phosphorylated in the presence of EGF, twice as much label is incorporated into phosphopeptide X (T4) as into phosphopeptide Y (T6) (Fig. 4). In contrast, when the individual tryptic peptides T4 and T6 are phosphorylated the incorporation ratio is 40:1 in favor of phosphopeptide T4 (Table 1). This difference is probably a result of the fact that tyrosine-42 is the NH_2-terminal residue of phosphopeptide Y (T6); peptides containing NH_2-terminal tyrosines are known to be poor substrates for tyrosine kinase (28).

The effect of phosphorylation on growth hormone activity is not known. The fact that the M_r 20,000 variant hGH, which has lost both tyrosines-35 and -42, retains full activity in the rat tibia test (29) suggests that tyrosine phosphorylation may not be involved in the growth-promoting activity of growth hormone. However, there are physiological differences between the major form of hGH (M_r 22,000 form) found in pituitary extracts and the M_r 20,000 variant hGH. The best-documented difference between these two forms of hGH is the lack of early insulin-like activities in the M_r 20,000 hGH form—for example, the ability of hGH to lower blood levels of glucose and free fatty acids within 1 hr (30). This insulin-like activity is consid-

Table 1. Phosphorylation of tryptic peptides of hGH

Peptide*	Peptide sequence†	Peptide concentration μM‡	^{32}P incorporation, mmol/mol	Phosphopeptide properties: R_T, min§	R_f¶	Electrophoretic mobility, cm‖
T4 (20–38)	L-H-Q-L-A-F-D-T-Y-Q-E-F-E-E-A-Y-I-P-K	84.8	12.4	48 (63)	0.47	+2.2
T6 (42–64)	Y-S-F-L-Q-N-P-Q-T-S-L-C-F-S-E-S-I-P-T-P-S-N-R	87.7	0.3	53 (59)	0.44	−2.9
T10 (95–115)	S-V-F-A-N-S-L-V-Y-G-A-S-D-S-N-V-Y-D-L-L-K	37.8	0.2	42 (65)	0.62	−5.0
			0.6	40	0.52	−1.3
T14 (141–145)	Q-T-Y-S-K	79.3	1.7	7 (11)	0.23	−11.3
T16 (159–167)	N-Y-G-L-L-Y-C-F-R	88.4	0.6	40 (54)	0.55	+1.6
			0.3	32	0.63	−6.6

Tryptic peptides of unlabeled *S*-carboxymethyl-hGH were isolated by reversed-phase HPLC on a 10-μm octadecylsilica column (μBondapak) as described (27) and phosphorylated for 10 min at 30°C in the presence of 7 μg of A431 cell membrane protein and EGF-α (3 μg/μl) as described (14). ^{32}P-Labeled tryptic peptides with the exception of T14 were isolated from the phosphorylation reaction mixture by selective adsorption onto a cartridge of octadecylsilica (Sep-pak), followed by HPLC on a 3-μm column of octadecylsilica (Ultrasphere). ^{32}P-Labeled T14 was purified on a column of Dowex AG-1X8. Two forms of ^{32}P-labeled T10 and T16 were recovered by HPLC. Because these peptides each contain two tyrosines and differ significantly in electrophoretic mobility, the two forms of ^{32}P-labeled peptide presumably represent the possible mono- or di-phosphorylated derivatives.

* Tryptic peptides are numbered in their final order in the sequence (see ref. 8); numbers in parentheses represent positions of amino acid residues in the sequence.

† See ref. 8.

‡ Determined by amino acid analysis.

§ Conditions for determination of retention times (R_T) of ^{32}P-labeled tryptic peptides in reversed-phase HPLC on a 10-μm octadecylsilica column (μBondapak) are given in the *Materials and Methods*; values in parentheses represent retention times for unlabeled tryptic peptides fractionated in the same manner (see ref. 26).

¶ Migration rates (R_f) for ^{32}P-labeled tryptic peptides on 3 MM paper in butan-1-ol/acetic acid/H_2O/pyridine (15:10:3:12) are given with respect to distance traveled by the solvent front.

‖ Electrophoretic mobility of ^{32}P-labeled tryptic peptides is given as the distance traveled (cm) from the origin toward the cathode under standard electrophoretic conditions.

ered an intrinsic property of hGH (31). Lewis *et al.* (32) have suggested that the M_r 20,000 hGH deletion region (residues 32–46) might be implicated in the early insulin-like activity of the hormone. Evidence in support of this suggestion was recently provided by Yudaev *et al.* (33), who demonstrated that a synthetic tetradecapeptide corresponding to residues 31–44 of hGH stimulated glucose uptake by isolated adipose tissue of rats. The results of the present study suggest the possibility of a link between some of the early insulin-like activities of hGH and the presence of two tyrosine phosphorylation sites in the deletion region of the hGH polypeptide.

It is not known whether hGH is a natural substrate for the EGF receptor tyrosine kinase. One possible approach to examine this question would be to test for a synergistic action of EGF or other growth factors on the insulin-like effects of hGH. Another unanswered question concerns the mechanism by which circulating hGH reaches the receptor-associated tyrosine kinase at its presumptive site on the inner surface of the cell membrane (34). One possibility is that hGH is bound and internalized prior to its phosphorylation; alternatively, only active proteolytic fragments of hGH (33) may reach the intracellular kinase. The recent reports of an insulin-stimulated tyrosine kinase activity associated with the insulin receptor (7) raise the intriguing possibility that it is this enzyme that is physiologically important, and thus that the insulin-like effects of hGH may be mediated through phosphorylation of hGH by the insulin receptor.

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