GROWTH-MODULATING HUMAN PLASMA TRIPEPTIDE: RELATIONSHIP BETWEEN MOLECULAR STRUCTURE AND DNA SYNTHESIS IN HEPATOMA CELLS

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1. Introduction

H-glycylhistidyllysine-OH (GHL) is a tripeptide found at approximately 200 ng/ml in human plasma in association with the albumin and α-globulin fractions [1,2]. When added to culture medium at 15–100 ng/ml, synthetic GHL promotes the proliferation of hepatoma cells [2,3], lymphocytes [4] and T-strain mycoplasma [5]; maintains the viability of normal hepatocytes [2,3], eosinophils [6], and macrophages [7]; inhibits the growth of glial cells [8]; and supports the growth and differentiation of neurons [8] and ascaris larvae [9]. At higher concentrations, the tripeptide inhibits L-929 cell growth (500 ng/ml) [10] and maintains the viability of mast cells (20 μg/ml) during degranulation tests [11]. In general, GHL acts to reduce or eliminate the amount of serum required for culture of the various cell types or organisms [2–4,6–9,11]. Although the mechanism of action of GHL is unknown, the tripeptide is co-isolated with copper and iron [12,13], and acts synergistically with these transition metals to stimulate the growth and metabolism of hepatoma cells maintained in growth-limiting amounts of serum [12,13].

In this paper we describe the effects of 9 synthetic analogs of GHL on DNA synthesis in hepatoma cell culture. The results indicate that 2 structural features are involved in the bioactivities of GHL: (1) the histidyllysyl linkage and (2) a glycyl residue in either terminal position.

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2. Materials and methods

Synthetic peptides were obtained from the following suppliers: Gly–His–Lys from Pierce Chemical Co., Rickford, IL; Gly–His–(ε-Gly–His)–Lys from Peninsula Laboratories, San Carlos, CA; Gly–His, Gly–Lys and His–Lys from Sigma Chemical Co., St. Louis, MO; His–Lys–Gly and His–Gly–Lys from Dr C. J. Lote, synthetized by methods detailed elsewhere [14]; Gly–Lys–His, Gly–His–Orn, and Gly–His–Lys–His were prepared in our laboratory by solid phase synthesis [3].

The hepatoma cell line (HTC_4) was obtained from the UCSF Cell Culture Facility; fetal calf serum, Swim's S-77 medium, Eagle's basal medium from GIBCO, Berkeley, CA; [*methyl*-^{3}H]thymidine, 28 Ci/mM, from International Chemical and Nuclear, Irvine, CA; Plastic 25 cm^2 T-flasks from Falcon Plastics, Oxnard, CA.

2.1. *Purification of peptides*

All peptides were repurified by thin-layer silica gel chromatography, using as solvent $CHCl_3$/MeOH/17% NH_4OH : 2/2/1 by volume, and eluted from the plate by methods previously published [2]. The peptide-containing solutions were passed through a Dowex 50-X4 column (1 × 5 cm) at pH 7.0 in 0.1 M sodium phosphate. Double glass distilled water (10 column volumes) was passed through the column and peptide eluted with 5 ml 0.1 NaOH. The basic eluate was immediately neutralized with 1 N HCl, the solution lyophilized, then dissolved in 1 ml of 1% acetic acid. This solution was desalted on a Sephadex G-10 column (1 × 100 cm) eluted with 1% acetic acid, peptide

peaks located by absorbance at 210 nm, and lyophilized. Peptides were converted from acetate salts to chloride salts by relyophilization in 0.02 N HCl. Final peptide concentration and composition were measured by amino acid analysis on a Beckman amino acid analyzer.

2.2. *Assay of peptide activities*

Changes in rates of DNA synthesis in a line of hepatoma cells (HTC_4) [2] were used to estimate the bioactivity of purified peptides. Prior to assay, cellular growth rate was stabilized to baseline values by reduction of the concentration of fetal calf serum in medium from 10 to 0.7% and subculture in spinner flasks for 3 days. Under these conditions, the cells were quiescent but viable. Cells were removed from medium by centrifugation (500 × *g* for 10 min), and 10^6 cells plated in 25 cm^2 T-flasks (Falcon 3012) in a medium consisting of 90.3% Eagle's basal medium, 10% Swim's S-77 medium, and 0.7% fetal calf serum. Peptides to be tested were added to concentrations of 2, 20, 200 and 2000 ng/ml.

Comparisons of peptide activities were made at 48 hours of incubation, to minimize variability due to differences in initial response of cultures to peptides. Cells were pulsed with 0.5 μCi [^{3}H]thymidine after 44 h in culture, and DNA synthesis was determined following an additional 4 h of incubation, as previously described [2].

3. Results

The activity of analog peptides was compared to activity induced by GHL. Under the conditions employed, GHL was optimally active at 20–200 ng/ml. GHL increased DNA synthesis 1.5-fold at 2 ng/ml, 5.1-fold at 20 ng/ml, 4.4-fold at 200 ng/ml and 1.9-fold at 2000 ng/ml. Among the 9 analogs, Gly–His–Lys–His, His–Lys–Gly and Gly–His–Orn displayed activities comparable with GHL (table 1). At the optimal concentration for GHL (20 ng/ml), Gly–Lys–His and His–Gly–Lys possessed 36% and 13% of GHL activity respectively, while the activity of the pentapeptide Gly–His–(ε-Gly–His)–Lys was intermediate at 20 ng/ml, and slightly more active at higher concentrations in comparison with Gly–Lys–His and His–Gly–Lys. There was no evidence of inhibition at higher concentrations of this pentapeptide, possibly reflecting slow generation of GHL and Gly–His during incubation by proteolytic enzymes. Of the dipeptides, His–Lys had minimal activity equivalent to 3% of GHL activity at 20 ng/ml, and 10% at 200 ng/ml. Gly–His and Gly–Lys were inactive.

Table 1
Effects of Gly–His–Lys and analogs on DNA synthesis in hepatoma cells

Peptide concentration (ng/ml)	2	20	200	2000
Gly–His–Lys	50	408	339	91
Gly–His–Lys–His	82	604	387	84
His–Lys–Gly	31	368	353	56
Gly–His–Orn	16	220	219	56
Gly–Lys–His	2	36	65	31
Gly–His–(ε-Gly-His)–Lys	0	24	68	88
His–Gly–Lys	2	13	81	29
His–Lys	0	8	30	26
Gly–His	0	0	6	3
Gly–Lys	0	0	0	0

Numbers represent % increase in [^{3}H]thymidine incorporation above control values. Average value for DNA synthesis in 12 control cultures without addition of synthetic peptide was 358 ± 30 c.p.m. (± SD). Peptides are listed after Gly–His–Lys in order of activity at a concentration of 20 ng/ml. Within each experimental group, the standard deviation of ^{3}H-incorporation averaged 8% and did not exceed 17% of the mean

4. Discussion

The results indicate that several synthetic analogs approach GHL in ability to stimulate DNA synthesis. Optimal activity appears to be associated with the histidyl-lysyl linkage, as indicated by the effects obtained with Gly–His–Lys–His, His–Lys–Gly, and GHL itself. Activity was decreased when lysine was replaced with ornithine, a closely related amino acid. Deletion of the terminal glycine from GHL essentially eliminated activity. These findings suggest that, in addition to the His–Lys sequence, glycine in either terminal position appears to be essential for expression of activity. The diversity of cell types and organisms which are responsive to GHL is consistent with a general metabolic function for the tripeptide. Recent

evidence suggests that GHL may mediate the transport of transition metals such as copper. GHL functions synergistically with copper and iron to stimulate DNA synthesis and cell growth in hepatoma cultures [12], forms complexes with these metals in human plasma [13], and facilitates uptake of copper [11] by hepatoma cells maintained in growth-limiting amounts (0.7%) of serum [15].

Among the side chains of amino acids, the imidazole ring of histidine is the most effective chelator of transition metals such as copper and iron [16–18]. Thus, the histidyl residue in GHL and its active analogs may be essential for chelation of these metals. Histidyl residues in peptides or proteins have a higher affinity for copper than free histidine. Compared with histidine, both the dipeptide Gly–His and human albumin have considerably greater affinities for cupric ions [17–19]. Accordingly, GHL would be expected to bind copper with an affinity comparable to that of human albumin at its copper binding site, which contains a histidyl residue at position 3 adjacent to a lysyl residue at position 4 [19]. Since copper transport in human plasma occurs primarily through the albumin-bound copper fraction, GHL may play a role in the exchange of copper between plasma and tissues.

Evidence suggesting mediation of copper transport by low molecular weight compounds present in the circulation has been frequently reported [20–23]. GHL is isolated in highest concentration from the albumin-rich fraction of plasma (Cohn Fraction V), where the tripeptide may form part of a ternary complex with albumin and copper, as does histidine [19]. It is possible, therefore, that a constituent of plasma with an affinity for transition metals may accept copper from the albumin-bound copper fraction in plasma, and may serve as a molecular weight carrier for transport of the metal from plasma to tissues. A hypothetical transport pathway for copper based on these considerations and involving GHL, albumin and histidine is outlined in fig.1.

The lysyl residue of GHL may participate in charge-dependent interactions between the bioactive peptides and the cell surface. Thus, although the dipeptide Gly–His may chelate copper and iron nearly as effectively as GHL, chelation may not be accompanied by cellular binding in the absence of the lysyl residue with its basically charged ϵ-amino group. Similarly, the higher activity of tripeptides with histidyl-lysyl

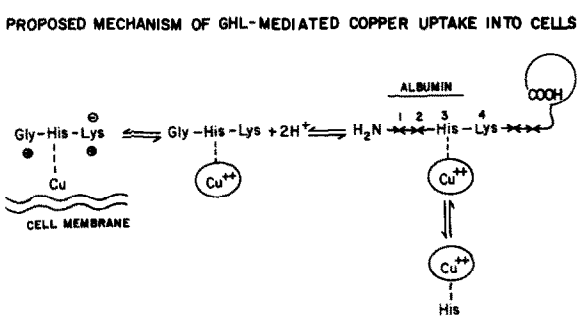

Fig.1. The postulated mechanism of GHL-mediated transport of copper involves exchange of the metal between its binding sites on albumin, free histidine and GHL, with subsequent orientation of the GHL-copper complex on the cell surface in a configuration suitable for interaction with the plasma membrane.

linkage compared with the dipeptide His–Lys, may reflect structural requirements of binding sites at the cell surface.

Although the biological effects of GHL have been demonstrated in diverse systems in vitro [1–11], among peptides studied in this paper only GHL itself and a tripeptide with the tentative structure His–(Gly–Lys) have been isolated from biological sources. GHL has been extracted from plasma of several mammalian species, including man [1]. His–(Gly–Lys) is a component of cat spinal cord, and both His–Gly–Lys and His–Lys–Gly have been reported to suppress the firing of neurons located in the spinal cord and medula oblongata of cats [14]. These results imply that GHL and other oligopeptides composed of histidine, lysine and glycine may exert a variety of tissue-specific regulatory functions. The ability of such peptides to chelate transition metals in human plasma, and the synergistic effects of GHL-transition metal complexes in hepatoma cells [12] suggest a potential role for these complexes in metabolism of essential metal ions. Further studies of the biological properties of synthetic analogs of low molecular peptides found in vivo may clarify the mechanisms of action of these important and poorly understood modulatory factors.

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