Carboxypeptidase H
A REGULATORY PEPTIDE-PROCESSING ENZYME PRODUCED BY HUMAN HEPATOMA Hep G2 CELLS*

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Human hepatoma (Hep G2) cells have been shown to secrete nanogram quantities of carboxypeptidase N (Grimwood, B. G., Plummer, T. H., Jr., and Tarentino, A. (1988) J. Biol. Chem. 263, 14397–14401). A second carboxypeptidase with an acidic pH optimum (pH 5.3) is also secreted at levels 2–3-fold greater than carboxypeptidase N. This enzyme was partially purified from the conditioned medium and compared with pure bovine pituitary carboxypeptidase H. The two enzymes behaved in a similar fashion in DE52 ion-exchange chromatography and on gel filtration, with the Hep G2 enzyme being slightly larger than the bovine pituitary enzyme (52–54 versus 50–52 kDa). Both enzymes hydrolyzed COOH-terminal basic amino acids from typical synthetic substrates as well as from natural leu-enkephalin peptides and were identical based on pH activity profiles, inhibition by EDTA or guanidinoethylmaleonitrile, and stimulation by CO2+ ions. Inhibition of enzyme secretion from Hep G2 cells by tunicamycin indicated that the Hep G2 enzyme was glycosylated. This finding was confirmed by a parallel deglycosylation of the Hep G2 and bovine pituitary carboxypeptidase H enzymes with peptide-N4-(N-acetylatedglucosaminyl)asparagine amide F. Immunoblots using mouse antiserum to bovine pituitary carboxypeptidase H revealed that the Hep G2 enzyme was immunologically identical to the known plasma carboxypeptidase H enzyme. The two enzymes were immunoreactive with the bovine enzyme but was slightly larger in size (54 versus 52 kDa). Continuous [35S]methionine labeling and purification to near homogeneity using an affinity matrix corroborated the observations that the secreted Hep G2 carboxypeptidase H was slightly larger than bovine pituitary carboxypeptidase H. The Hep G2-secreted enzyme in pulse-chase experiments was initially detected intracellularly after a 15-min pulse as a single protein of about 54 kDa and was present in the 30-min chase medium with no evidence for pre- or postsecretion proteolytic processing. The human adrenergic cell line IMR-32 continuously labeled with [35S]methionine also secreted carboxypeptidase H of the same size as the Hep G2 enzyme.

Carboxypeptidase H (3.4.17.10) is a carboxypeptidase B-type metalloproteinase involved in the removal of COOH-terminal basic amino acids from peptide precursors. It has been isolated from bovine adrenal and pituitary tissues (1–4) and rat insuloma cells (5, 6) and is involved in enkephalin precursor and proinsulin processing. Regulatory peptides (hormones, neurotransmitters) (7) undergo post-translational proteolytic processing events, including removal of signal sequences as well as endoproteolytic and exoproteolytic cleavages (8, 9). In many instances, specific peptides undergo further modifications (10) including amidation (11). High levels of carboxypeptidase H activity are present in many neuroendocrine tissues (12), with lower levels in peripheral tissues for which neuroendocrine functions have not yet been described (13). Carboxypeptidase H-like activity has also been reported in rat parotid (14) and Aplysia atrial glands (15), both of which are exocrine in function, as well as in bovine corpus luteum (16). Recently, relatively high levels of carboxypeptidase H-like activity have been observed in a human pheochromocytoma (15) and ileal carcinoid (17). Activity has been reported in medium conditioned by the mouse adrenocorticotropic cell line AtT-20 (18) and in primary cultures of bovine chromaffin cells (19).

We have demonstrated recently that a human hepatocarcinoma-derived cell line (Hep G2) secretes a neutral carboxypeptidase identical to the known plasma carboxypeptidase N (20). During these studies, another carboxypeptidase with a lower pH optimum and molecular weight consistent with that of carboxypeptidase H was detected in even larger quantities in the conditioned medium. The presence of carboxypeptidase H was unexpected since liver cells normally secrete only carboxypeptidase N into plasma. Nevertheless, in this report we show that the Hep G2 cell line indeed secretes a carboxypeptidase H that is very similar to purified bovine pituitary carboxypeptidase H with respect to its enzymatic, physical, and immunological properties.

EXPERIMENTAL PROCEDURES

Materials

Cell lines, growth media, the affinity matrix (p-aminobenzoyl-L-arginine-Sepharose 6B), GEMSA,1 and peptide-N4-(N-acetylatedglucosaminyl)asparagine amide F were described earlier (20–22). Bovine pituitary glands (tissues) were from Pel-Freeze Biologicals. Methionine-free EMEM (Select-Amine) was from GIBCO and tunicamycin from Sigma. FA-Ala-Arg and FA-Ala-Lys were as described (20). [Leu]enkephalin, [Leu]enkephalin-Arg6, and [Leu]enkephalin-Arg6-Arg were purchased from Bachem. t-Butoxycarbonyl-Leu-

1 The abbreviations used are: GEMSA, guanidinoethyl mercapto-
succinic acid; FA, furylacrylic; EMEM, Eagle’s minimal essential
medium; SDS, sodium dodecyl sulfate; HPLC, high performance
liquid chromatography. Amino acid symbols denote the L-configura-

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Arg-gly-methyl-coumaryl-7-amide and t-butoxycarbonyl-Leu-Lys-Arg-methyl-coumaryl-7-amide were purchased from Peninsula Laboratories. Reagents for immunoblots and autoradiography were as described (20). Aprotinin (Trasylol), 10,000 komberg international units/ml was from FBA Pharmaceuticals.

Methods

Determination of Carboxypeptidase Activity—Carboxypeptidase H activity was quantitated and expressed as milliunits of FA-Ala/ml generated from FA-Ala-Lys or FA-Ala-Arg at pH 5.5 (37 °C). Enzyme activity with the natural substrates [Leu] enkephalin-Arg^2 and [Leu]enkephalin-Arg^2-Arg^2 was determined as described (2). Products were separated from substrate by reverse-phase HPLC (22, 23).

Isolation of Hep G2 Carboxypeptidase—Production of Hep G2-conditioned medium followed the described procedure (20) using a 1:1 mixture of Ham's F-12:Dulbecco's MEM with antibiotics and aprotinin. Unless otherwise noted, all phases of cell culture production and isolation contained aprotinin at 50 komberg international units/ml. The centrifuged (4000 × g, 15 min, 4 °C) conditioned medium was dialyzed overnight against 0.01 M Tris-Cl buffer, pH 7.0 (4 °C), with aprotinin, 20 komberg international units/ml, and chromatographed on a DE52 column (1.5 × 22 cm). Elution was accomplished with a linear gradient (200 ml each side) to 0.25 M NaCl in 0.01 M Tris-Cl buffer, pH 7.0. Fractions containing carboxypeptidase H activity (pH 5.5, EDTA-inhibitable) were identified, pooled, and concentrated by ultrafiltration (Amicon YM-10) and then rechromatographed on an Ultrogel ACA 54 column (1.5 × 196 cm) with 0.01 M Tris-Cl, pH 7.0, 0.25 M NaCl as eluant (5.6 ml/h). Fractions containing carboxypeptidase H activity were pooled and concentrated as above.

Isolation of Bone Carboxypeptidase H—The soluble form of carboxypeptidase H from frozen bovine pituitary glands was purified to homogeneity (3) using affinity chromatography (p-amino-benzoyl-L-arginine-Sepharose 6B) followed by ion-exchange chromatography (DEAE) to concentrate the enzyme.

Preparation of Mouse Immune Serum—Adult mice (BALB/c) were immunized by subcutaneous injection of purified bovine pituitary carboxypeptidase H (0.19 mg of protein/mouse) suspended in Freund's complete adjuvant. After 3 weeks, the mice were given an antigen boost and partially bled (retroorbital plexus) 4 days later followed by two weekly bleedings. Serum was stored with 0.01% NaN3.

Cell Lines Compared for Carboxypeptidase H Activity—Cells were cultured to confluence (20) in 60-cm2 dishes. The medium containing serum was removed, and the cells were washed twice with 5 ml of serum-free Ham's F-12:Dulbecco's modified essential medium. The cultures were then maintained in 2 ml of fresh serum-free Ham's F-12:Dulbecco's modified essential medium for 48 h at 37 °C in a humidified incubator. The conditioned medium could be fractionated on Ultrogel ACA 54 columns (1.5 × 196 cm) with 0.01 M Tris-Cl buffer, pH 7.0, 0.25 M NaCl as eluant (5.6 ml/h). Fractions containing carboxypeptidase H activity were pooled and concentrated as above.

Metabolic Labeling of Hep G2-synthesized Products— Cultures of near confluent Hep G2 cells (20) in 60-cm2 dishes were incubated in 5 ml of methionine- and serum-free EMEM (Select-Amine) for 1 h, 37 °C. The cells were rinsed twice with fresh methionine-, serum-free EMEM, then incubated at 37 °C in 1 ml of methionine-, serum-free EMEM containing Trans-35S-labeled methionine (200 µCi), supplemented with 10 µM unlabeled methionine for 24 h. Pulselabeled cells were methionine-deprived as above and labeled with methionine (400 µCi) Trans-35S-label/dish for 15 min in methionine-, serum-free EMEM and chased with 1 ml/dish serum-free EMEM supplemented with unlabeled methionine to 150 mg/ml.

Affinity Purification of 35S-Metabolically Labeled Carboxypeptidase H—The affinity matrix p-amino-benzoyl-L-arginine-Sepharose 6B was used in a batch-mixing process to purify cell culture-synthesized carboxypeptidase H. To optimize the efficiency of specific carboxypeptidase H binding to affinity matrix at pH 7.5, the cell lysates and medium samples were first mixed with affinity matrix equilibrated at pH 7.5. Since it was established that carboxypeptidase H did not bind in detectable amounts to affinity matrix at pH 7.5 but carboxypeptidase N did, this step was included to reduce possible low level nonspecific carboxypeptidase N and nonrelated proteins binding at pH 5.5.

Monoclonal antibodies were performed in low protein-binding plastic 1.5 ml microcentrifuge tubes ("fuge-tubes," Marsh Biomedical Products, Rochester, NY) or 12 × 75-mm "mini-sorp" tubes (A/S Nunc, Denmark) in the presence, unless otherwise noted, of 150 µg of unlabeled methionine/ml, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride and 100 komberg international units of aprotinin/mL. At the end of the labeling period, the medium was centrifuged 30 s in a microcentrifuge and the supernatant kept on ice until processed. The cells were rinsed twice with ice-cold Dulbecco's phosphate-buffered saline, then 1 ml of ice-cold 0.1 M Tris-Cl buffer, pH 8.0, was added to each dish. Adhering cells were scraped and the suspensions transferred to a glass tissue homogenizer. After 15 up-down passes, the homogenates were transferred to 12 × 75 mm tubes and centrifuged for 15 min at 100,000 × g, 4 °C. The cell lysate supernatants were transferred to microcentrifuge tubes and kept on ice until further processing.

The binding/wash procedures were as follows. Affinity matrix (50 µl of packed pellet) was washed with 1 ml of serum-free EMEM supplemented with unlabeled methionine, detergent, and inhibitors as above. The [35S]methionine-labeled medium and cell lysates (1 ml) were mixed separately with the pH 7.5 equilibrated affinity matrix at room temperature for 15 min (roller mixer). The tubes were centrifuged and the supernatants transferred to a glass tissue homogenizer. After 15 up-down passes, the homogenates were transferred to 12 × 75 mm tubes and centrifuged for 15 min at 100,000 × g, 4 °C. The cell lysate supernatants were transferred to microcentrifuge tubes and kept on ice until further processing.

RESULTS AND DISCUSSION

In the previous report, we demonstrated that Hep G2-conditioned medium could be fractionated on Ultrogel AcA 34 to...
obtain two distinct carboxypeptidases: 1) a 280-kDa enzyme with a neutral pH optimum which was identical to human plasma carboxypeptidase N, and 2) a smaller carboxypeptidase (54 kDa) with an acidic pH optimum tentatively designated, based on its properties, as a carboxypeptidase H (20). Although carboxypeptidase N is a normal constituent of human plasma and could reasonably be expected to be secreted by Hep G2 cells (20), carboxypeptidase H is not present in plasma and is generally associated with regulatory peptide-processing neuroendocrine systems.

To investigate this anomaly further, our earlier fractionation protocol was modified to provide a more highly purified Hep G2 carboxypeptidase H, suitable for comparison with authentic bovine pituitary carboxypeptidase H. The Hep G2 carboxypeptidases were both absorbed to DE52 (Fig. 1A). Elution with a linear salt gradient resulted in a nearly complete separation of carboxypeptidase N, which eluted at 0.20 M NaCl, from carboxypeptidase H (shaded area, Fig. 1A), which eluted at 0.25 M NaCl. The carboxypeptidase H activity was always greater than that of carboxypeptidase N, and the enzyme profiles were independent of cell passage number (data not shown). The active fractions shown in Fig. 1A (shaded area) were pooled, concentrated, and applied to an Ultrogel AcA 54 column (Fig. 1B). Carboxypeptidase H activity (shaded area) eluted as a single sharp peak with an overall recovery of 86%; and unlike earlier chromatography on AcA 34, it was well separated from most nonenzyme protein. The Hep G2 carboxypeptidase H and pure bovine pituitary carboxypeptidase H were similar in size based on their elution from Ultrogel AcA 54; the Hep G2 enzyme appeared slightly larger (M in between 52,000 and 54,000) than the bovine enzyme (Fig. 1B), which was estimated at about 50,000. Slight differences in size may be attributed to species and/or tissue source. Supattapone et al. (4) reported a membrane form of bovine carboxypeptidase H with a molecular weight of about 52,000, slightly larger than the soluble form (50,000). Multiple forms of carboxypeptidase H-like enzyme with molecular weights slightly higher than the bovine pituitary soluble and membrane forms have been reported for human liver carcinoma (55–57 kDa) (15) and rat insulin cell [35S]labeled enzyme (54–55 kDa) (26).

The enzymic properties of Hep G2 and bovine pituitary carboxypeptidase H were compared for several substrates. Both enzymes showed identical pH profiles for cleavage of FA-Ala-Arg with 100% enzyme activity at pH 5.5 and 5.0% or less activity at either pH 4.0 or 7.1. FA-Ala-Arg was used as the primary substrate to minimize any interference from carboxypeptidase N activity and because of the reported preference for carboxypeptidase H for arginine substrates (27). However, at identical substrate molarities, bovine pituitary and Hep G2 carboxypeptidase H exhibited an enzyme activity ratio of FA-Ala-Lys to FA-Ala-Arg of 2:1 and 3:1, respectively, or a clear preference for catalysis of the lysyl substrate. A preference for Lys was also noted for the benzoyl substrates. Natural peptides with-Lys as the COOH-terminal amino acid may be more readily processed by carboxypeptidase H than Arg-terminal natural peptides even though it has been reported (27) that enkephalin-Arg binds with greater affinity to the bovine enzyme than enkephalin-Lys.

Hep G2 carboxypeptidase H was compared with the bovine pituitary enzyme in regard to activation by Co2+ and inhibition by EDTA and GEMSA. Both enzymes showed a dramatic enhancement of activity using FA-Ala-Arg after preincubation with Co2+ (a 15-fold enhancement for the bovine enzyme versus a 19-fold enhancement for the Hep G2 enzyme), which was similar to that reported earlier for the bovine enzyme (3). Both enzymes behaved as metalloproteinases and were completely inhibited by pretreatment with 1 mM EDTA for 1 h. Both enzymes were inhibited approximately 60% when assayed with FA-Ala-Arg (1.25 mM) in the presence of 1 μM GEMSA, a specific inhibitor for carboxypeptidase B-like enzymes (22, 27). These experiments demonstrated that the pH 5.5 Hep G2 enzyme could not be an aberrant secretion of a lysosomal carboxypeptidase B-like enzyme since this type is not appreciably activated by Co2+ or inhibited by EDTA or GEMSA (2).

To verify that the Hep G2 carboxypeptidase H demonstrated catalytic properties indicative of the carboxypeptidase H-type enzyme associated with neuroendocrine tissue, we examined by reverse-phase HPLC the ability of the Hep G2 enzyme to hydrolyze the natural substrates [Leu]enkephalin-Arg2-Arg (21) and [Leu]enkephalin-Arg2 (porcine dynorphin A1–7, and A1–6, respectively). As can be seen in Fig. 2A, [Leu]enkephalin-Arg2 was hydrolyzed about 50% to [Leu]enkephalin by the Hep G2 enzyme. The partial conversion of [Leu]enkephalin-Arg2 to [Leu]enkephalin-Arg2 and [Leu]enkephalin is shown in Fig. 2C, where approximately 2 times more hexapeptide was formed than the pentapeptide. The formation of the hexapeptide as an intermediate product was also noted for bovine pituitary carboxypeptidase H (2). With Co2+, the Hep G2 carboxypeptidase H reactions went to completion (Fig. 1, B and D).

In Fig. 3, enzyme from bovine pituitaries and Hep G2-conditioned medium was visualized by immunodetection fol-
Following Western blots. The primary antibody raised in mice against the soluble bovine enzyme cross-reacted with the Hep G2 enzyme. The bovine enzyme is visible as a doublet (Fig. 2, lane 2) of molecular weight 50,000–52,000. In lane 2, the Hep G2 carboxypeptidase H also appears as a doublet of a slightly higher molecular weight. Immunocross-reacting proteins of the lower molecular weight form were typical of most enzyme preparations from 48-h Hep G2-conditioned medium and likely represent partial degradation due to proteolytic activity. A partially purified Hep G2 enzyme preparation (DE52, AcA 54) after a 1-month storage at 4 °C with aprotinin still lost 90% of its original enzyme activity, coincident with disappearance of the 54-kDa enzyme band (data not shown) and the appearance of low molecular weight peptides.

Bovine carboxypeptidase H is a glycoprotein that contains two asparagine-linked glycosylation sites (28). Treatment with peptide-N\(^\text{4}\)-(N-acetyl-\(\beta\)-glucosaminyl)asparagine amidase F resulted in a small increase in electrophoretic mobility (Fig. 3, lane 3), which corresponded to complete deglycosylation, as evidenced by loss of \(^{131}\)I-concanavalin A binding (data not shown). It was suspected that the Hep G2 carboxypeptidase H was also a glycoprotein since its secretion in culture was sensitive to tunicamycin (data not shown). Deglycosylation by peptide-N\(^\text{4}\)-(N-acetyl-\(\beta\)-glucosaminyl)asparagine amidase F (Fig. 3, lane 1) resulted in a proportionally similar increase in electrophoretic mobility, suggesting that the extent of glycosylation of both proteins was similar.

Bovine pituitary and adrenal chromaffin granule carboxypeptidase H has been purified (3) with the affinity matrix p-amino benzoyl-L-arginine-Sepharose 6B, originally used at pH 7.0 for the purification of plasma carboxypeptidase N (21). Fricker and Snyder (3) demonstrated that at pH 6.0, the affinity matrix efficiently bound bovine carboxypeptidase H. We also found at pH 5.5 that 90% of the Hep G2 enzyme could be bound to the affinity resin directly from conditioned serum-free medium using a batch-mixing process. At this pH, no carboxypeptidase N activity (FA-Ala-Arg, pH 7.5) was detected bound to the resin. The efficiency of enzyme binding was determined by following the lowering of activity in the supernatant and recovery of this activity eluted by raising the pH to 7.5, an alternate procedure suggested by L. Fricker.\(^2\) Once the protocols were established, it became possible to purify and concentrate (GEMSA elution) in one step the Hep G2-secreted carboxypeptidase H (Fig. 4, lane 2) from a complex mixture of highly radioactive secreted proteins (lane 3) produced from an overnight \(^{35}\)S)methionine incubation. It is apparent that under these conditions the system is highly selective for carboxypeptidase H, with only a low degree of apparently nonspecific binding evident from the profile of eluted enzyme (lane 2). For comparison, the soluble bovine carboxypeptidase H was run in parallel (lane 1) with the isolated \(^{35}\)S)methionine-labeled Hep G2 carboxypeptidase. The data indicate, as before (Fig. 3), that Hep G2 cells synthesize enzyme that is slightly larger in molecular weight than the bovine enzyme.

The production of carboxypeptidase H by Hep G2 cells was also monitored by pulse-chase experiments in Fig. 5. Nonspecific protein binding to affinity matrix was reduced by modifying the original procedure to include a binding step at pH 7.5 and higher salt/detergent concentrations during pH 5.5 binding and washing. The secreted carboxypeptidase H, pu-

\(^2\) L. Fricker, personal communication.
Fig. 4. A comparison of bovine pituitary carboxypeptidase H and 35S-metabolically labeled Hep G2 carboxypeptidase H. Lane 1, bovine pituitary carboxypeptidase H (11.5 milliunits of enzyme activity, 2.0 µg of protein) stained with Coomassie Blue; lane 2, 35S-Hep G2 carboxypeptidase H isolated by affinity binding at pH 5.5 and desorption at pH 7.5 (see "Results"); 1.5 x 10^6 dpm of 35S applied (7.2 µl) out of 2.2 x 10^6 dpm of 35S eluted (0.1% 35S incorporation). Lane 3, 35S-Hep G2-conditioned medium not bound to the affinity matrix; 7.9 x 10^6 dpm of 35S applied (7.2 µl) out of 2.2 x 10^6 dpm of 35S eluted. Polyacrylamide, 10%. Fluorography was for 2 days, -70 °C.

Fig. 5. Autoradiograms of 35S pulse-labeled affinity-purified secreted and cellular carboxypeptidase H from Hep G2 cells. Fluorography was for 4 days, -70 °C. Coomassie Blue-stained low molecular weight (m.w) protein markers (Bio-Rad). Enzyme source: medium, 15-min pulse (670 dpm of 2,800 dpm eluted), 30-min chase (1,188 dpm applied of 4,970 dpm eluted), 60-min chase (2,080 dpm applied of 14,400 dpm eluted), 90-min chase (2,200 dpm applied of 22,900 eluted), 120-min chase (2,270 dpm applied of 18,900 dpm eluted); cellular, 15-min pulse (2,340 dpm applied of 19,500 dpm eluted), 30-min chase (1,300 dpm applied of 10,870 dpm eluted), 60-min chase (1,490 dpm applied of 12,136 dpm eluted), 90-min chase (1,760 dpm applied of 7,330 dpm eluted), 120-min chase (1,320 dpm applied of 5,550 dpm eluted). Polyacrylamide, 12.5%.

rified to homogeneity by this procedure, was present as a single 54-kDa band as early as 30-min chase. The 35S-labeled carboxypeptidase H that was recovered from the detergent-lysed cellular fraction during a 15-min pulse had the same molecular weight as the enzyme in the medium, indicating that no subsequent proteolytic processing had occurred. In all the cellular fractions, a faint band larger than 110 kDa appeared in the autoradiogram. It is not known if this material is associated with carboxypeptidases H or N or nonrelated material. Quantitatively, it represents a small fraction of the total eluted radioactivity.

To determine whether the Hep G2 enzyme is processed in cell culture from larger precursor forms, it may be necessary to shorten the pulse time from 15 min and/or purify the enzyme with immunoaffinity procedures since the affinity matrix-binding procedure would not be expected to resolve a prepro-form in which the active site was not yet formed with access to the ligand. If simultaneous translational/processing events occur in Hep G2 cells for the formation of its carboxypeptidase H, then cell-free translation experiments would be needed to resolve precursor forms. A prepro-form of carboxypeptidase H has been suggested to occur on the basis of sequence analysis of bovine cDNA (27) and cell-free translational products from mRNA of a human carcinoid tumor (17).

Finally, normal and transformed cell lines were compared for the presence of carboxypeptidase H activity in conditioned medium and cellular fractions. The results summarized in Table I show that Hep G2 and human neuroblastoma, IMR-32, produce significant quantities of carboxypeptidase H, whereas rat hepatocytes, rat hepatoma (H35), diploid human embryonic lung, and mouse adrenal tumor (Y-1) cells produce only 1–2% as much activity. The IMR-32 cell line from a human neuroblastoma (29), characterized as adrenocortical (30), produced nearly as much carboxypeptidase H as did the Hep G2 cultures, and 35S-labeled enzyme (24-h incorporation) appeared to be identical in size to the Hep G2-secreted carboxypeptidase H (not shown).

Our observations that H35, diploid human embryonic lung, and Y-1 cell lines and primary hepatocytes express low enzyme activities are supported by the report that carboxypeptidase H-like enzyme is present in nonneuroendocrine tissues (13). Additionally, low levels (compared with pituitary) of proopiomelanocortin-like mRNA and trace amounts of immunoreactive proopiomelanocortin peptides occur in normal nonneuroendocrine tissues (including liver) (31). Certain adrenal-pituitary tumors may produce high levels of processing enzymes as has been for carboxypeptidase H from a pheochromocytoma (15) and another peptidergic tumor, an ileal carcinoid (17). Establishment of cells from normal or malignant tissues in culture and metabolic labeling of the product will be necessary to establish unequivocally the reason for the presence of "site-inappropriate" levels of individual regulatory enzymes.
peptides and/or processing enzymes such as the inappropriate level of carboxypeptidase H enzyme secreted by Hep G2 cells. The Hep G2 cell line is an example in which expression of more than one processing enzyme occurs. A thiol endoprotease that processes proapolipoprotein A-1 to apolipoprotein A-1 has been able to measure low amounts of this enzyme activity by sequence analysis, as has been done with bovine pituitary carboxypeptidase H. The Hep G2 cell line is an example in which expression of the physiological cause for the production of these enzymes is continuing. The presence of active carboxypeptidases H and N secreted into the medium by Hep G2 cells does not appear to be necessary for survival. When Hep G2 cells were cultured in the presence of 100 mg/L GEMSA (EMEM, 10% fetal bovine serum) for three weekly passages (trypsin treatment), no differences from controls were observed in viability, attachment, and spreading. Carboxypeptidases H and N activities in the 2nd week after withdrawal of inhibitor from free medium using the synthetic substrate peptides t-butoxycarbonyl-Leu-Arg-Arg-(Lys)-methyl-coumaryl-7-amide developed by Mizuno and associates (33). Work on establishing the physiological cause for the production of these enzymes is continuing. The presence of active carboxypeptidases H and N secreted into the medium by Hep G2 cells does not appear to be necessary for survival. When Hep G2 cells were cultured in the presence of 100 mg/L GEMSA (EMEM, 10% fetal bovine serum) for three weekly passages (trypsin treatment), no differences from controls were observed in viability, attachment, and spreading. Both carboxypeptidases H and N activity levels in the medium were similar to controls when examined in the 2nd week after withdrawal of inhibitor from the medium. Work is in progress to obtain quantities of affinity-purified carboxypeptidase H synthesized by Hep G2 and IMR-32 cells, sufficient for further study, such as NH2-terminal amino acid sequence analysis, as has been done with bovine pituitary carboxypeptidase H.

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