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Structural evidence that endothelial cell growth factor β is the precursor of both endothelial cell growth factor α and acidic fibroblast growth factor

(protein sequence/mass spectrometry/heparin)

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ABSTRACT **Two endothelial cell growth factors (ECGF) have been purified from bovine brain and termed α- and β-ECGF [Burgess, W. H., Mehlman, T., Friesel, R., Johnson, W. V. & Maciag, T. (1985) *J. Biol. Chem.* 260, 11389–11392]. Amino acid sequence analysis indicates that β-ECGF represents a 20 amino acid amino-terminal extension of α-ECGF and a 14 amino acid amino-terminal extension of acidic fibroblast growth factor. These data indicate that both α-ECGF and acidic fibroblast growth factor may be derived from β-ECGF by posttranslational processing. Analysis of the amino-terminal 14 residues of β-ECGF by fast-atom-bombardment mass spectrometry established the amino acid sequence of this region and the identity of the blocking group at the amino terminus (acetyl).**

The endothelium is made up of a monolayer of quiescent cells that form the inner lining of blood vessels *in vivo* (1). The endothelial cell participates in a variety of unique physiological functions that include (*i*) the formation of a selective barrier for the translocation of blood constituents and macromolecules to underlying tissues, (*ii*) the maintenance of a non-thrombogenic interface between blood and tissue, and (*iii*) the maintenance of function as a metabolic organ responsible for the control of hemostasis (1–3). In addition, endothelial cells are an important component for the development of new capillaries and blood vessels (1–4). Although the endothelial cell has a relatively low mitotic index *in vivo* (5), it is modulated during the process of angiogenesis (1–5), which involves the organized migration and proliferation of the endothelial cell at an accelerated rate. The process of neovascularization, which occurs during the development of the vasculature system (3, 4), also occurs postdevelopmentally as part of the pathophysiology of a variety of disease states that include psoriasis, arthritis, diabetic retinopathy, chronic inflammatory conditions, and tumor development (1–4). Thus, the identification and characterization of factors that modulate the migration and proliferation of the endothelial cells is fundamental to the elucidation of the biochemical mechanisms responsible for neovascularization *in vivo*.

Two forms of endothelial cell growth factor (ECGF) have been purified from bovine brain and termed α- and β-ECGF (6). These polypeptides are members of a family of anionic endothelial cell polypeptide mitogens that includes acidic fibroblast growth factor (aFGF) and eye-derived growth factor II (7). It has been shown that (*i*) the polypeptides are potent endothelial cell mitogens, (*ii*) their mitogenic activities are potentiated by heparin, (*iii*) the polypeptides share certain immunoreactivities, and (*iv*) the polypeptides compete for occupancy of the same endothelial cell surface receptor (7). In addition, these and other polypeptides (8–14) are similar with respect to (*i*) their apparent affinity for immobilized heparin, (*ii*) their apparent molecular weights as determined by $NaDodSO_4$/PAGE, (*iii*) their elution from reversed-phase supports, and (*iv*) their amino acid compositions.

The complete amino acid sequence of aFGF derived from bovine brain has been reported (15), and the homology between it and basic fibroblast growth factor (bFGF) has been established (15–17). In this report we provide evidence that both α-ECGF and aFGF may be derived from a common precursor, β-ECGF.

MATERIALS AND METHODS

Acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, and sodium dodecyl sulfate were from BDH. Heparin-Sepharose and molecular weight standards were obtained from Pharmacia. The Vydac C_4 column was from Rainin Instruments (Ridgefield, NJ). The Synchropak C_{18} column was from SynChrome (Linden, IN). Amino acid standards, HPLC-grade water, acetonitrile, CNBr, and constant-boiling HCl were from Pierce. Trypsin and thermolysin were obtained from Calbiochem. All reagents for protein sequence analysis were from Applied Biosystems (Foster City, CA). All other chemicals were reagent grade.

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed essentially as described by Laemmli (18), using a Mighty Small gel apparatus (Hoefer, San Francisco). Amino acid analysis was performed on samples that were hydrolyzed *in vacuo* in 6 M HCl/0.1% phenol for 24 hr at 115°C. Amino acid compositions were determined based on reversed-phase separation of the phenylthiocarbamoyl derivatives, using the general procedures outlined by Waters Associates. Data collection and reduction was performed using a Waters Associates 840 system.

The purification and digestions of α- and β-ECGF with trypsin and CNBr were done as previously described (6). Trypsin-derived fragments were digested with thermolysin at 37°C in 100 mM ammonium bicarbonate for 3 or 24 hr at an enzyme/substrate ratio of 1:25 (wt/wt). Partial acid hydrolysis was achieved by incubating trypsin-derived fragments in 6 M HCl at 37°C for 12 hr. Automated Edman degradations

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Abbreviations: ECGF, endothelial cell growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; >PhNCS, phenylthiohydantoin.

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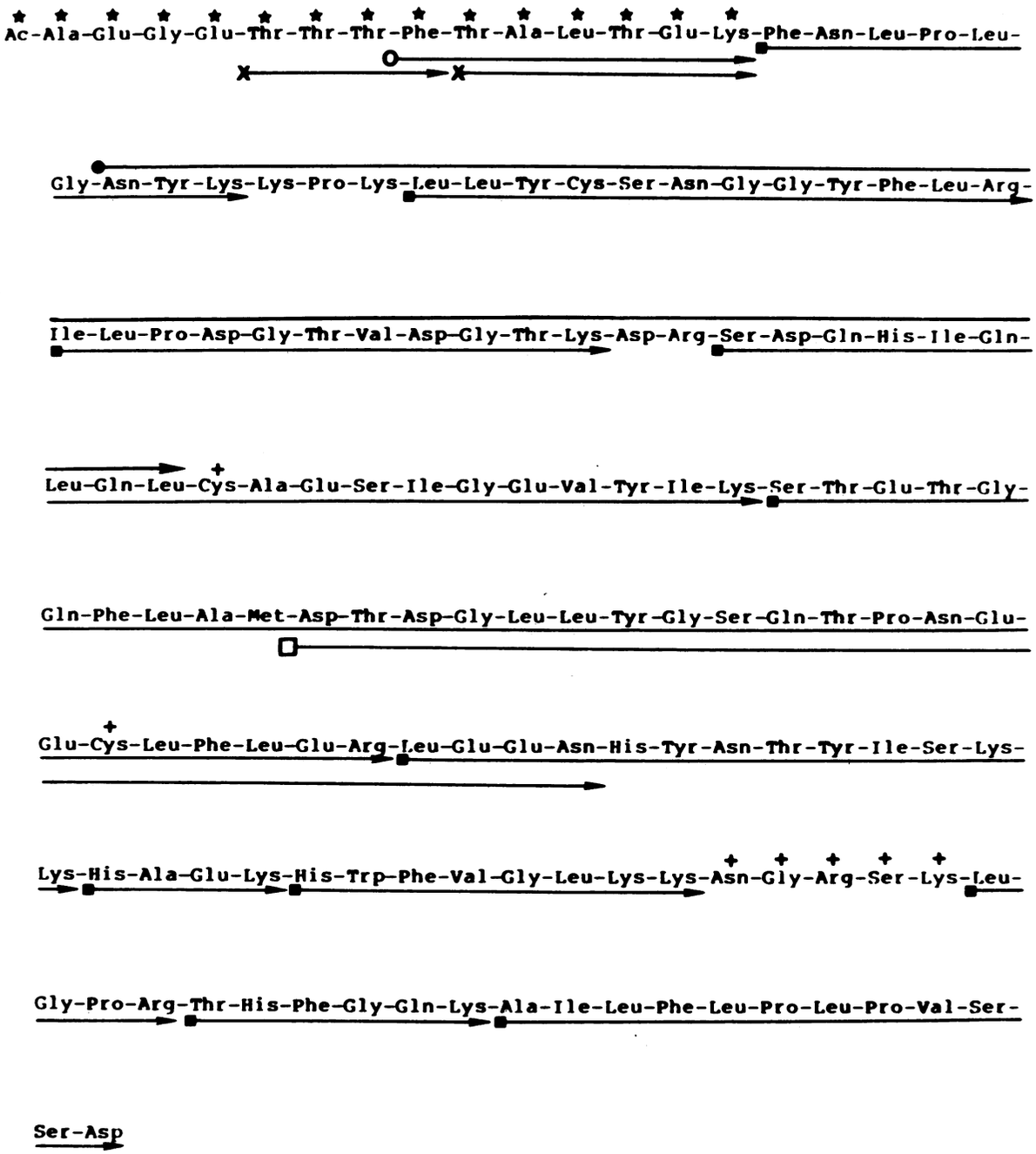

FIG. 1. Amino acid sequence of α- and β-ECGF. Sequence data obtained from intact α-ECGF (●), cyanogen bromide fragments (□), trypsin fragments (■), thermolysin fragments of β-ECGF (○), and acid-cleavage fragments of β-ECGF (x) are indicated. The residues marked with plus signs were assigned on the basis of homology with aFGF (15). The residues of β-ECGF marked with asterisks were assigned on the basis of fast-atom-bombardment mass spectrometry. The sequence of the trypsin fragments common to α-ECGF and β-ECGF was obtained using fragments from both polypeptides.

were done with an Applied Biosystems model 470A protein sequencer. Phenylthiohydantoin (>PhNCS) amino acid derivatives were identified by HPLC using a Zorbax ODS (DuPont) silica column (4.1 × 250 mm) and mobile phases of 50 mM sodium acetate (pH 5.0) and acetonitrile, essentially as described (19).

Fast-atom-bombardment mass spectrometry analysis was as follows. An aliquot (1 μl) of the amino-terminal tryptic fragment of β-ECGF (500 pmol) was delivered to 1 μl of liquid matrix (5:1, wt/wt) mixture of dithiothreitol and dithioerythritol on a gold probe for analysis from m/z 65 to m/z 2696. The sample was introduced directly into the mass spectrometer ion source and bombarded with a beam of xenon atoms. Six fast-atom-bombardment spectra were recorded with a Kratos MS-50 mass spectrometer fitted with a high-field magnet and equipped with a Kratos DS-55 data system previously calibrated against cesium iodide/glycerol over the mass range of interest. The mass spectrometer was operated in the positive-ion mode at an accelerating voltage of 8 kV. All other methods of mass spectral analysis were as previously described (20, 21). Samples from two separate preparations of β-ECGF were examined and showed similar results.

RESULTS

Amino-Terminal Sequence Analysis. Undigested, HPLC-purified α-ECGF (≈500 pmol) was subjected to automated Edman degradation. Sequential release of >PhNCS amino

acid derivatives was observed for 45 cycles, with an initial yield of ≈75% and subsequent repetitive yields averaging 92% (Fig. 1). When undigested β-ECGF was subjected to automated Edman degradation, no sequential release of >PhNCS derivatives was observed. Similar results were obtained with three different preparations of α- and β-ECGF.

Amino Acid Sequence Analysis of CNBr-Derived Fragments. We reported previously that digestion of both α- and β-ECGF with CNBr resulted in two distinct fragments as judged by $NaDodSO_4$/PAGE (6). The electrophoretic mobility of one of the fragments was the same for both polypeptides, whereas that of the others differed. When unfractionated, CNBr-digested α-ECGF was subjected to automated Edman degradation, a "double" sequence was observed. The amino acid sequence carboxyl-terminal to the single methionine residue of α-ECGF was derived by subtracting the sequence obtained from undigested α-ECGF at each cycle. When unfractionated, CNBr-digested β-ECGF was subjected to automated Edman degradation, a single sequential release of >PhNCS amino acid derivatives was observed for 25 cycles (Fig. 1). The first 17 residues were identical to the 17 residues of sequence derived from the α-ECGF CNBr digest after subtracting the amino-terminal sequence of intact α-ECGF.

Amino Acid Sequence Analysis of Trypsin-Derived Fragments. When α- and β-ECGF were digested with trypsin and the resulting fragments were purified by reversed-phase HPLC, two major peptides that are unique to β-ECGF were obtained. The remainder of the peptides are common to both the α-ECGF and the β-ECGF digests (Fig. 2). The amino acid sequences of the majority of the trypsin-derived fragments have been determined, and their positions have been established by either direct overlap or by homology to aFGF (15). The amino acid compositions of one of the peptides common to both the α- and β-ECGF digests did not contain lysine or arginine. Considering the specificity of trypsin for peptide bonds following lysine or arginine, this peptide was assigned to the carboxyl terminus of both α- and β-ECGF. The amino acid sequences of these peptides were identical, providing

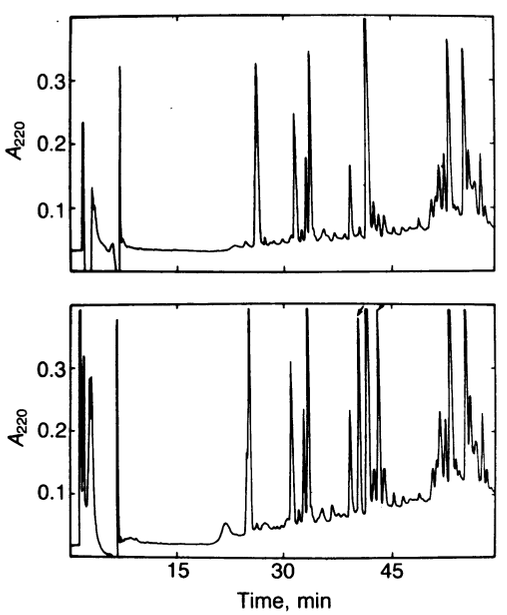

FIG. 2. Preparation of tryptic fragments of α- and β-ECGF. Tryptic digests of α-ECGF (*Upper*) and β-ECGF (*Lower*) were obtained as described (6). The digests were applied to a Synchropak C_{18} column and purified as described (6). The resulting profiles indicated that two tryptic fragments are unique to β-ECGF (arrows). The earlier-eluted of the two is the blocked amino-terminal tryptic peptide of β-ECGF. The recoveries of the peptides ranged from 65 to 90%.

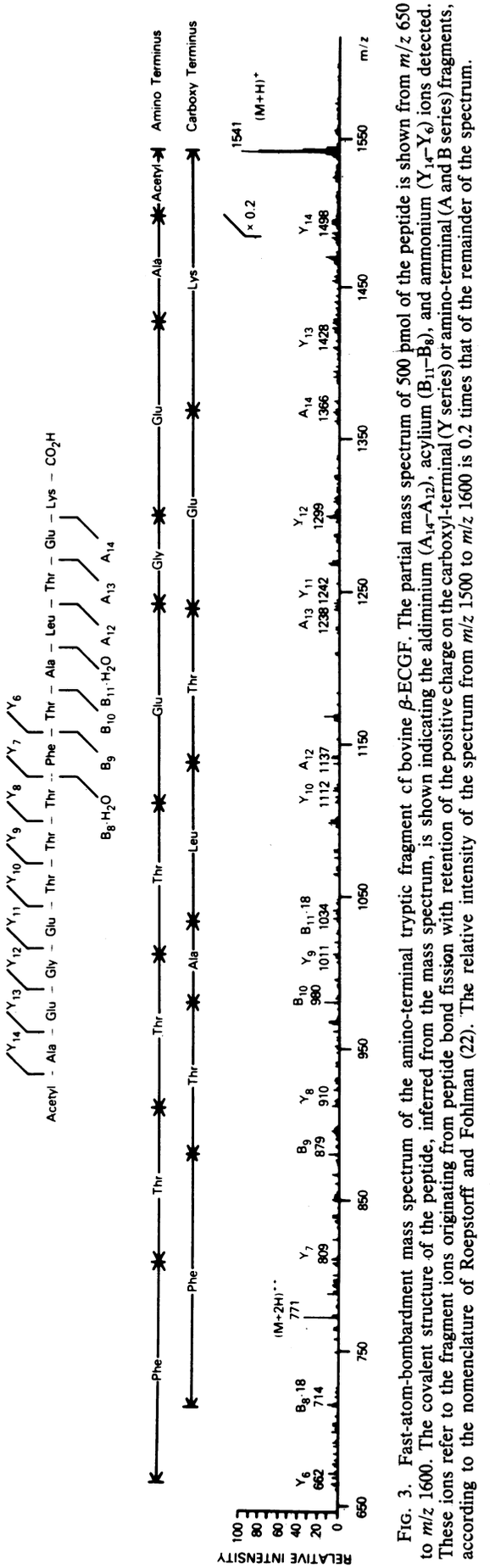

FIG. 3. Fast-atom-bombardment mass spectrum of the amino-terminal tryptic fragment of bovine β-ECGF. The partial mass spectrum of 500 pmol of the peptide is shown from *m/z* 650 to *m/z* 1600. The covalent structure of the peptide, inferred from the mass spectrum, is shown indicating the aldiminium (A_{14}–A_{12}), acylium (B_{11}–B_8), and ammonium (Y_{14}–Y_6) ions detected. These ions refer to the fragment ions originating from peptide bond fission with retention of the positive charge on the carboxyl-terminal (Y series) or amino-terminal (A and B series) fragments, according to the nomenclature of Roepstorff and Fohlman (22). The relative intensity of the spectrum from *m/z* 1500 to *m/z* 1600 is 0.2 times that of the remainder of the spectrum.

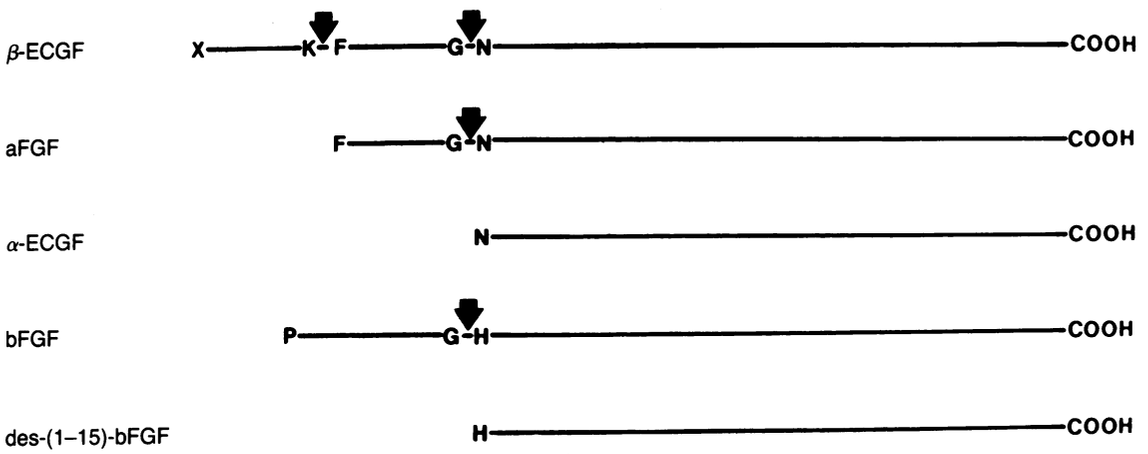

FIG. 4. Schematic representation of potential cleavages involved in the generation of aFGF and α-ECGF from β-ECGF and the generation of the des-(1–15) form of bFGF from bFGF. The Gly-Asn (G-N) and Gly-His (G-H) bonds align perfectly when ECGF or aFGF are aligned with the bFGFs to maximize amino acid sequence homology. X indicates the blocking group of β-ECGF; K, Lys; F, Phe; G, Gly; N, Asn; P, Pro; and H, His.

additional evidence that the differences between α- and β-ECGF may be limited to the amino-terminal regions.

When the two tryptic peptides that are unique to β-ECGF were subjected to automated Edman degradation, sequential release of >PhNCS amino acid derivatives was observed for one of the peptides but not the other. The sequence of one of the peptides was identical to the amino-terminal residues of aFGF and extended into the amino-terminal three residues of α-ECGF. The observed sequence was Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys (Fig. 1). The other unique tryptic peptide had an amino acid composition (normalized to one residue of lysine) of $Glu_{2.8}$,$Gly_{1.2}$,$Thr_{4.7}$,$Ala_{1.9}$,$Leu_{1.1}$,$Phe_{1.2}$, Lys_1, yet no release of >PhNCS amino acid derivatives was observed with loads as high as 1 nmol. Thus, this peptide was assigned as the amino-terminal tryptic fragment of β-ECGF.

Amino Acid Sequence Analysis of the Blocked β-ECGF Tryptic Peptide After Subdigestion by Thermolysin or Limited Acid Hydrolysis. Digestion of the blocked tryptic peptide for 3 hr with thermolysin resulted in the appearance of two major fragments as judged by reversed-phase HPLC. When these fragments were subjected to automated Edman degradation, sequential release of >PhNCS amino acid derivatives was observed from one of them (Fig. 1). The observed sequence was Phe-Thr-Ala-Leu-Thr-Glu-Lys.

When the intact tryptic fragment was subjected to partial acid hydrolysis and reversed-phase HPLC, several fragments were obtained that yielded sequential release of >PhNCS amino acid derivatives (Fig. 1). We infer from these observations that the structure of the amino-terminal tryptic fragment of β-ECGF is X-(Ala,Glu,Gly,Glu)-Thr-Thr-Thr-Phe-Thr-Ala-Leu-Thr-Glu-Lys.

Mass Spectrometric Analysis of the Blocked β-ECGF Tryptic Peptide. Fast-atom-bombardment mass spectrometric analysis of the amino-terminal tryptic peptide of β-ECGF was conducted in order to confirm chemical sequence analysis data and to assign the amino-terminal tetrapeptide and blocking group. The strategy for mass spectral analysis of the peptide was similar to that previously described (20). The partial fast-atom-bombardment mass spectrum from m/z 650 to m/z 1600 for this peptide is shown in Fig. 3. The entire mass listing was examined from m/z 356 to m/z 2665. The major ion observed, the $(M+H)^+$, has a nominal mass of 1541. The amino acid composition of this peptide predicts a mass of 1499 excluding the amino-terminal blocking group. The difference of these two values is 42 atomic mass units, consistent with an acetyl moiety in amide linkage with the amino-terminal residue. The predicted mass for the acetylated peptide $(M+H)^+$ is 1540.74, which compares favorably with the measured mass, 1540.85. In addition, the $(M+2H)^{2+}$ ion was observed at m/z 771.

Further information about the amino acid sequence of the amino-terminal tryptic peptide of β-ECGF can be gained from the remainder of the mass spectrum (Fig. 3). Fast-atom-bombardment mass spectra of peptides display fragment ions consistent with peptide bond fission followed by various neutral losses. Fission of the peptide bond with retention of the positive charge on the amino-terminal fragment ion yields predominantly the aldiminium ion series, whereas the retention of the positive charge on the carboxyl-terminal fragment ion yields predominantly the ammonium ion series. Other fragment ion series are sometimes present, such as acylium ion, but are generally not as abundant. The ammonium ion series, Y_{14}–Y_6 (22), was consistent with an amino-terminal sequence of Ac-Ala-Glu-Gly-Glu-Thr-Thr-Thr-Phe. The aldiminium ion series, A_{14}–A_{12}, was consistent with a carboxyl-terminal sequence of Thr-Glu-Lys-OH. In addition, four ions of the acylium ion series, B_8–B_{11}, were identified and supported the carboxyl-terminal sequence Phe-Thr-Ala-Leu-Thr-Glu-Lys-OH. Acylium ions B_8 and B_{11} were observed with the loss of 18 atomic mass units, suggesting the loss of H_2O from threonyl side chains. The combination of ammonium, acylium, and aldiminium ions is consistent with the sequence of Ac-Ala-Glu-Gly-Glu-Thr-Thr-Thr-Phe-Thr-Ala-Leu-Thr-Glu-Lys-OH.

DISCUSSION

In this report, we have provided a structural basis for the functional similarities between α- and β-ECGF and aFGF. In addition, we have established that α-ECGF is probably identical to the des-(1–6) form of aFGF reported by Gimenez-Gallego *et al.* (15), which they termed acidic FGF-II. More important, we have established that β-ECGF represents an amino-terminal extension of both α-ECGF (by 20 residues) and aFGF (by 14 residues). The remainder of the sequences of all three polypeptides from bovine brain appear to be identical to one another.

That the amino terminus of β-ECGF is probably acetylated and thus not susceptible to Edman degradation suggests that β-ECGF, unlike α-ECGF or aFGF, could go undetected during amino-terminal sequence analysis of related polypeptides. One indication of the presence of β-ECGF in preparations of other polypeptides would be an observation of unusually low initial yields followed by high repetitive yields during automated Edman degradations (14, 17, 23, 24).

Although the structural relationships of α- and β-ECGF to one another and to aFGF are now clear, the mechanisms responsible for the occurrence of the three polypeptides is not. The predicted amino-terminal modification of β-ECGF that would result in the formation of aFGF involves cleavage of a Lys-Phe bond, a cleavage that may involve serine proteases (Fig. 4). Presumably, this cleavage occurs at a reduced rate during the preparation of α- and β-ECGF, since the neural tissue is extracted at near neutral pH rather than the acidic conditions used in the preparation of aFGF (13). Differences in the relative amounts of α-ECGF or aFGF-II purified from bovine brain may also be due to the different extraction conditions used. However, the generation of α-ECGF or aFGF-II from either β-ECGF or aFGF requires cleavage of a Gly-Asn bond (Fig. 4). We are not aware of an enzymatic or chemical mechanism capable of specific cleavage of this bond, although we have detected a heat-labile activity in crude bovine brain extract that is capable of generating the tripeptide Asn-Tyr-Lys from the synthetic peptide Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys (unpublished observations). Potential processing of a Gly-His bond in bFGF at residues 15–16 is indicated by amino-terminal sequence analysis of an angiogenic factor isolated from corpus luteum (23). The position of this bond is the same as the Gly-Asn bond in ECGF, when the polypeptides are aligned to maximize amino acid sequence identity (Fig. 4). A pre–pro relationship for these polypeptides is suggested that cannot be established unequivocally until the genes encoding them are characterized.

The biological activities of α- and β-ECGF and aFGF are similar to one another and distinct from those of bFGF (7). It will be important to determine the functional significance of the high degree (≈50%) of amino acid sequence identity between the acidic and basic mitogens. In addition, the functional significance of the amino-terminal extensions of α-ECGF and aFGF has not been established. It is possible that the relatively small amino-terminal fragments that may result from cleavage of β-ECGF to form either aFGF or α-ECGF also contribute to the complex mechanism of neovascularization *in vivo*.

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