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Calcium Binding and Putative Activity of the Epidermal Growth Factor Domain of Blood Coagulation Factor IX

Linda H. Huang[*], Xiao-Hong Ke[*], William Sweeney[#] and James P. Tam[*$]

[#]Hunter College, The City University of New York, New York, NY 10022

[*]The Rockefeller University, 1230 York Ave., New York, NY 10021

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ABSTRACT The first epidermal growth factor (EGF)-like domain of human Factor IX and two chimeric analogs of this domain and EGF were synthesized unambiguously and purified to homogeneity. The synthetic EGF-like domain and its analogs showed the correct mass ions by the fission ionization mass spectrometry and similar disulfide pairings as those found in EGF, but failed to exhibit any putative EGF activity in the receptor and mitogenic assays. However, in NMR titration experiments, the EGF-like domain and one of its analogs were found to bind Ca^{2+} but not Mg^{2+}. Our results therefore show that the EGF-like domain of Factor IX has the ability to bind calcium ion, shares the structural motif of EGF but does not retain the active determinants responsible for the EGF activity.

The tandemly repeating epidermal growth factor (EGF)-like domain is a striking motif with undetermined functions in the blood clotting factor family (1). Except in protein C which has four, Factor IX, X, protein S or protein Z has two repeating units each containing about 40 amino acids with the characteristic alignment of disulfide bonds found in EGF (2). Similar to the blood clotting family, EGF-like sequences are also found as repeating domains embedded in large proteins such as receptors, developmental tissues and extracellular matrix (1). Like the EGF-like domain in blood clotting factors, little is known about their structure or functional roles and none regarding their EGF-like activities.

Recently, our laboratory has explored the biological functions of these EGF-like homologous sequences by chemically synthesizing these domains as discrete molecules to test their biological functions *in vitro*. One of such proteins that we have examined is Factor IX. Factor IX (Christmas Factor) is the precursor of a serine protease required for

[$]To whom all correspondence should be addressed.

Abbreviations: Boc, tertbutoxycarbonyl; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformomide; DMS, dimethylsulfide; mEGF, murine epidermal growth factor; EGF, epidermal growth factor; TFA, trifluoroacetic acid; GFD-FIX, EGF-like domain of human blood coagulation factor IX (45-87).

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blood clotting by the intrinsic clotting pathway (3). Individuals lacking this protein may show a congenital bleeding disorder known as christmas disease or hemophilia B. Factor IX is a single- chain glycoprotein (Mr 55,000-57,000). Sandwiched between the γ-glutamic acid and the protease domain is the first growth factor domain (GFD-FIX, residues 45-87) which has 43 amino acids. In this paper, we describe the chemical synthesis, the determination of the disulfide linkages, and the biological activities of this GFD of Factor IX and two of its chimeric EGF analogs.

EXPERIMENTAL PROCEDURES

Solid-phase Peptide Synthesis. The GFD-FIX and its analogs were synthesized manually by the stepwise solid phase approach (4) using N^{α}-tert-Boc-Thr(Bzl)-OCH_2Pam resin (0.76 mmol/g of resin). Boc-benzyl strategy was used and the following protection for side chains were as follows: Asp(OBzl), Cys(4-MeBzl), Glu(OBzl), His(Dnp), Lys(2-ClZ), Ser(Bzl), Thr(Bzl), and Tyr(2-BrZ). Synthesis adhered to the procedure we have described recently (5).

Prior to the HF cleavage, the peptide-Pam resins containing His(Dnp) residues were treated 5 - 6 times, each with about 15 ml of 1 M thiophenol in DMF for 8 h to remove N^{im}-Dnp protecting group from His and then with 10 ml TFA in CH_2Cl_2 for 20 min to deblock the Boc group. After drying the peptide resin, the low-high HF cleavage procedure was carried out to remove the peptide from the resin. In the low HF step, DMS, p-cresol and p-thiocresol were transferred into the HF reaction vessel to premix with the peptide resin with 20 mg cysteine to minimize the side reactions with thiol moiety of the peptide during the workup. After the HF reaction vessel was cooled down to -78 ^{o}C in an acetone-dry ice bath for 30 min, HF was transferred to reach the final volume of 10 ml to give a reaction mixture of: HF:DMS:p-cresol:p-thiocresol, 25:65:8:2 (vol/vol) and a 2 hr-reaction proceeded at 0° C. After the removal of HF and DMS, the high HF step was initiated by charging liquid HF to 15 ml to give HF:p-cresol:p-thiocresol, 94:5:1 (vol/vol). After 1 hr at 0 ° C, HF was removed. The resulting resin and crude peptide containing aromatic scavengers and organic byproducts were extracted twice with cold ethyl ether/mercaptoethanol (98:2; vol/vol) to remove p-cresol and p-thiocresol. The crude peptide was then extracted with 100 ml of 8 M urea/0.2 M dithiothreitol in a 0.1 M Tris-HCl buffer, pH 8.4.

Refolding, Disulfide Formation and Purification. To remove dithiothreitol and the residual cresol, the crude peptide solution was dialyzed (Spectra por 6; molecular weight cutoff,1000) at room temperature for 48 h under decreasing concentrations of urea solution aganist 2 l each of deaerated and N_2-purged 8 M, 5 M, 3 M, and 1.5 M urea, all in 0.1 M Tris-HCl (pH 8.45). During the dialysis, no precipitation was observed. Oxidation and refolding the peptide was performed by adding 1 mM oxidized glutathione and 1 mM reduced glutathione into the peptide solution after dilution to 500 ml with 0.1 M Tris-HCl (pH 8.45). The solution was stirred slowly at 4 ^{o}C , no precipitate was observed during the refolding process. The 500 ml-peptide solution was purified by a preparative HPLC (Waters system; VYDAC C_{18} reverse-phase; 2.5x30 cm; UV at 225 nm; flow rate, 20 ml/min; eluted with a linear gradient of acetonitrile 0.04% TFA.)

Characterization. GFD-FIX was hydrolyzed in 5.7 N HCl and the hydolysate analyzed by amino acid analysis which agreed with the calculated value. The molecular mass was identified by Cf-252 fission ionization mass spectrometry.

Enzymatic Digestion to Detemine Disulfide Linkages. The disulfide linkage of GFD-FIX was determined by enzymatic digestion with recrystalized thermolysin (15 μg) in 0.1 ml of 0.1 M pyridine-acetic acid buffer (pH 6.5) at 45^{o}C for 24 h (15). The digested fragments were separated on C_{18} reverse phase HPLC (0.5 μm, 0.46 x 25cm) eluted with a liner gradient of CH_3CN and 95% of H_2O/0.045% TFA. The peaks were collected and hydrolyzed with 5.7 N HCl at 110 ^{o}C for 24 hr. The sequences were determined by amino acid analysis.

Biological Assays. The ability of to compete with ^{125}I-EGF binding to the EGF receptor was determined on subconfluent monolayer of formalin-fixed A-431 cells after 1 hr incubation at 22 ^{o}C with synthetic GFD-FIX and its analoges (6). Mitogen assay was performed on normal kidney cells, clone 49F (6).

Calcium Binding. The fraction of GFD-FIX with calcium bound was estimated from the chemical shift of a resolved resonance near 5.4ppm which was sensitive to the presence of calcium. Although a small number of resonances were observed to have calcium dependent chemical shifts, only this resonance was well resolved. Sequential additions of a 0.5 M solution of $CaCl_2$ in D_2O were made directly to a 5 mm NMR tube to a maximum concentration of 86 mM, and spectra were recorded. The protein concentration was less than 0.1 mM. Before addition of calcium, the freshly prepared $CaCl_2$ solution and the peptide solution were separately adjusted to approximately pD 5. Similar titrations were performed for human factor IX(45-80,82-87)/ human EGF(41) and human EGF(1-16)/human factor IX(59-87), although the maximum calcium concentrations used were 126 mM and 175 mM, respectively, and the protein concentrations were less than 0.5 mM.

Proton NMR spectra were recorded at room temperature using a JOEL GX-400 FT spectrometer. A pulse repeat time of 2.148 seconds was used. Uncorrected pH meter readings were used to obtain pD values.

The data were analyzed in three different ways, each based on the assumption that the total calcium concentration was approximately equal to the concentration of calcium free in solution. The first method was to plot log[Ca^{2+}] vs. log([GFD-FIX]/[GFD-FIX-Ca^{2+}]). The value of the dissociation constant is given by the intercept of the line on the log[Ca^{2+}] axis. The data were examined by plotting the calcium induced shift in the NMR resonance at 5.4ppm, Δ, vs. Δ/[Ca^{2+}], and by plotting [Ca^{2+}] vs. [Ca^{2+}]/Δ (9).

RESULTS

The synthesis of GFD-FIX (residues 45-87, Fig. 1) was achieved by the stepwise solid-phase method using the Boc-benzyl protecting group strategy. The crude and unprotected peptide after the HF cleavage from the resin support was refolded by an improved condition using a slow refolding process with gradients of denaturants and mixed disulfides (5). Under such a process, the most thermodynamic stable form appeared as a sharp peak in the reverse phase HPLC of the crude and unpurified synthetic product mixture (Fig. 2A). Two sequential purification by preparative HPLC (Fig. 2B and C) gave a homogenous product in 25% overall yield.

Analysis of the purified synthetic GFD-FIX (45-87) showed that it was homogeneous by analytical HPLC under different conditions and by amino acid analysis. The molecular mass was identified by Cf-252 fission ionization mass spectrometry which gave a (M+H)$^{+}$ ion of 4750.6 which agreed well with the calculated value of 4749.9. Furthermore, no

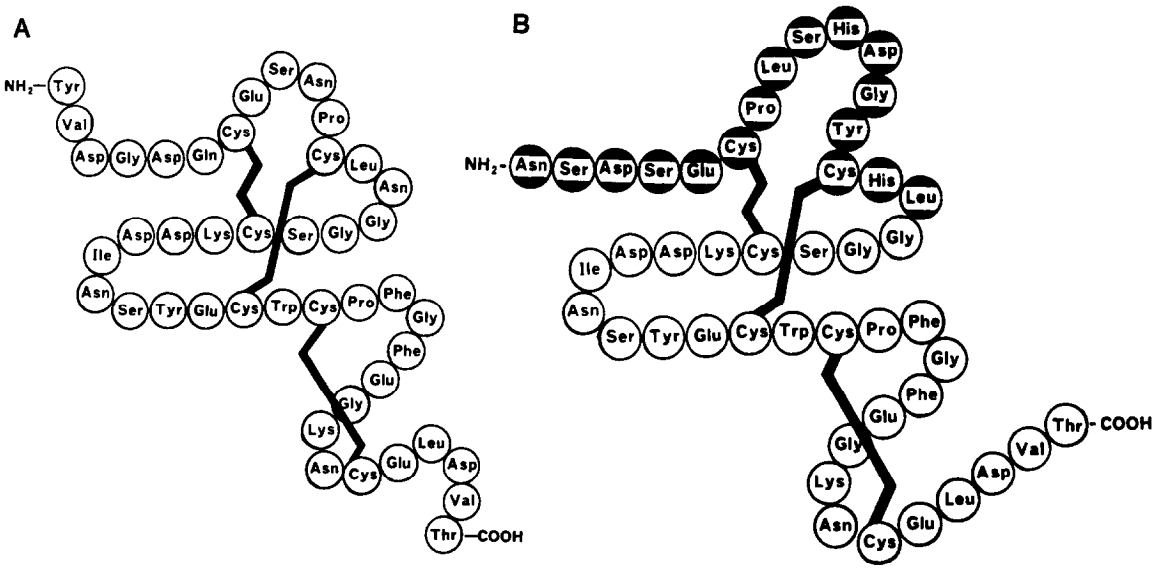

Figure 1. Structure of (A) the first GFD (residue 45-87) of human Factor IX and (B) a chimeric EGF(1-16) GFD-FIX(59-87).

evidence of the dimer corresponding to the $(2M + 3H)^{3+}$ was found. To determine the disulfide pairing of the synthetic product, the purified peptide was subjected to proteolytic digestion by thrice recrystallized thermolysin. The digested fragments were separated by HPLC and analyzed by amino acid analysis (Table 1). The results showed that the first disulfide loop was paired by Cys-51 and Cys-62, and third disulfide loop paired by Cys-73 and Cys-82. Such disulfide linkages were similar to those in EGF, with the structure indicated in Fig.1.

The putative biological activities of EGF consisting of binding to EGF receptor and stimulation of mitogenicity were tested for synthetic GFD-FIX. The results showed that GFD-FIX was inactive at concentrations lower than 1 mM in its ability to compete with [^{125}I]-EGF in binding to EGF receptor in A-431 cells and to stimulate [^{3}H]-thymidine uptake in normal rat kidney fibroblast (6).

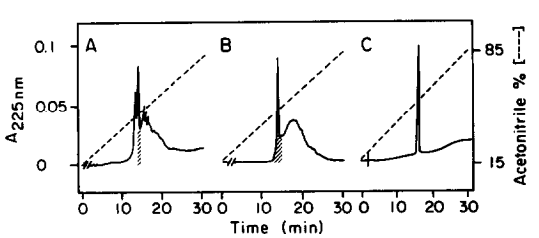

Figure 2. C_{18} reverse phase HPLC analysis of GFD-FIX. Two sequential HPLC (panel A and B) were used for the purification of GFD-IX to obtain the purified product (panel C). Note: the shaded area in panel A was used for the second purification.

Table 1. Amino acid composition of peptide fragments obtained from thermolysin digestion

Factor IX Amino acid composition			Peptide fragment corresponding to
Amino acid	Theory	Found	
Cys	2	2	
Asp	2	1.75	Asp-Gly-Asp-Gln-Cys-Glu
Ser	1	0.66	Cys
Glu	2	1.29	Ser
Gly	2	1.66	Gly
Cys	2	2.0	
Asp	1	1.36	Cys-Pro
Glu	2	2.03	Glu-Cys-Asn-Lys-Gly-Glu
Pro	1	1.23	
Gly	1	1.35	
Lys	1	1.06	

In initial attempts to probe calcium binding, Yb^{3+} was used as an NMR shift reagent. Although ^{1}H-NMR spectra recorded at low Yb^{3+} concentrations exhibited changes typical of Yb^{3+} binding, concentrations higher than 4 mM induced protein precipitation. For this reason, the less sensitive approach of using calcium ion directly was employed (Fig. 3).

A number of small changes are observed in the ^{1}H-NMR spectrum if Ca^{2+} is added. One of the most shifted resonances is a resolved peak near 5.4 ppm which shifts approximately 35 Hz at high Ca^{2+} concentration (86 mM). At lower Ca^{2+} concentrations the

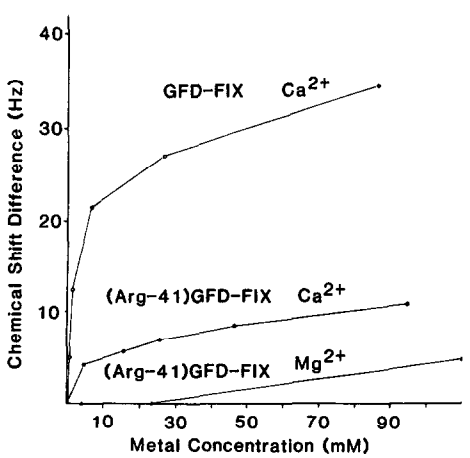

Figure 3. NMR titration curves of Ca^{2+} and Mg^{2+} to GFD-FIX and [Arg-41] GFD-FIX.

resonance is found at intermediate positions, indicating that the Ca^{2+} is exchanging rapidly on the NMR time scale. By observing its chemical shift as a function of Ca^{2+} concentration, an estimate of 2-5 mM was obtained for a putative calcium dissociation constant.

Two chimeric derivatives were also examined in a similar manner. One of these, human factor IX(45-80,82-87 FIX numbering system)/ human EGF(41 EGF numbering system), differs from GFD-FIX by a single amino acid replacement of Arg for Asn at position 81. The NMR spectrum of this derivative is similar to that of GFD-FIX, and a similar though smaller change was noted for the resonance near 5.4 ppm on addition of calcium. A change in chemical shift of only 15 Hz was observed at high calcium concentration, and a calcium dissociation constant near 20 mM was estimated from the NMR data. Prior treatment with EDTA followed by dialysis had no significant effect on the titration. As a control, a similar titration was performed using $MgCl_2$. No shift was observed at 4 or 23 mM Mg^{2+}. At 110 mM Mg^{2+} a small shift of 5 Hz was observed, comparable to the shift observed with 10 mM Ca^{2+}. The observed difference between Ca^{2+} and Mg^{2+} binding (Fig. 3) suggest that binding is being detected.

The third derivative examined was human EGF(1-16)/human factor IX(59-87). The NMR spectrum suggests that this peptide has less well defined structure than the others, as evidenced by smaller chemical shift dispersion and the absence of slowly exchangeable resonances. There were no detectable changes in the spectrum upon addition of 126 mM calcium, nor did the peptide precipitate in the presence of 50 mM Yb^{3+}.

DISCUSSION

Our results show that the growth factor domain (GFD) of Factor IX contains disulfide linkages similar to those found in EGF and probably the characteristic three-dimensional motif formed by three disulfides of EGF. However, this GFD-FIX is devoided of the putative EGF-like activities. The GFD-FIX, like those repeating EGF-like domains found in large proteins, lack six conserved residues found in EGF or other active EGF members such as transforming growth factor-type α. These include Tyr-13, Arg-41, Asp-46 and Leu-47 (EGF numbering system) and which may be important determinants in forming the binding surface to the EGF receptor. Thus, significant differences both in the size of the disulfide loops and the lack of highly conserved residues may contribute to the putative activities attributable to EGF.

Although calcium binding has been associated with the γ-carboxyglutamic acid (Gla) domain of factor IX, high affinity Gla-independent calcium binding has also been observed, with a dissociation constant of 0.08 mM (7). Studies of Gla-independent calcium binding in human factor IX (1) have suggested the involvement in calcium binding of two Asp residues in the N-terminal tail (residues 47 and 49) and a β-hydroxyaspartic acid residue in position 64 (8).

The results of the calcium and magnesium binding experiments described here are consistent with calcium binding by GFD-FIX, although with a higher dissociation constant

than found for the native protein. The higher dissociation constant, indicating weaker calcium affinity, may reflect the absence of the β-hydroxyaspartate in position 64. For the human factor IX(45-80, 82-87)/human EGF(41) derivative, the replacement of Asn with Arg in the third disulfide loop weakens this apparent calcium binding. The third chimeric derivative, human EGF(1-16)/GFD factor IX (59-87), which shows no calcium-binding capacity, lacks one of the two proposed calcium binding aspartic acid resides (2).

In conclusion, our results show that GFD-FIX and its chimeric analogs are chemically synthesized and refolded correctly; the different amino acid seqences at the first disulfide loop and N-terminal may contribute to the the conformation required for Ca^{2+} binding; and these EGF-like proteins may have similarity in overall structural motif but differences in biological functions compared to EGF.

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