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Undercarboxylation of recombinant prothrombin revealed by analysis of γ-carboxyglutamic acid using capillary electrophoresis and laser-induced fluorescence

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Abstract The γ-carboxyglutamic acid (Gla) content of several variants of human prothrombin has been measured by using capillary electrophoresis and laser-induced fluorescence (CE-LIF). Both plasma-derived prothrombin and recombinant prothrombin contain ten residues of Gla per molecule of protein. In contrast, a variant of human prothrombin (containing the second kringle domain of bovine prothrombin) was separated into two populations that differed in their Gla content. Direct measurement of the Gla content showed an association with the presence or absence of the calcium-dependent conformational change that is required for prothombinase function. Thus, the CE-LIF assay is useful in determining the carboxylation status of recombinant proteins.

Key words: Gamma carboxy glutamic acid; Prothrombin; Capillary electrophoresis; Laser-induced fluorescence; Recombinant protein; Baby hamster kidney cell

1. Introduction

Gamma-carboxyglutamic (Gla) acid is a naturally occurring amino acid that is formed by the post-translational modification of glutamic acid during protein synthesis. Gla residues are found predominantly in blood proteins (coagulation factors II, VII, IX, X, XI, XII, proteins C, S, and Z) [1] and in bone and calcified tissue (bone Gla protein or osteocalcin and bone matrix protein) [2]. For plasma proteins, the conversion of Glu to Gla is a post-translational step that involves a vitamin K-dependent pathway in the rough endoplasmic reticulum of hepatocytes (see [3] for a recent review). The presence of Gla residues imparts unique calcium-binding properties to a protein due to the extra carboxyl group in this residue. In prothrombin, ten Gla residues in the amino-terminal region (termed the Gla domain) bind calcium leading to a calcium-dependent conformational change of the protein [1]. This conformational change is absolutely required for interaction with phospholipid membrane surfaces during prothrombin activation in the prothrombinase complex.

Several previous studies have reported the qualitative and quantitative determination of Gla in proteins. Typically, the assays involve the hydrolysis of the protein to free amino acids followed by derivatization, separation and quantification. A variety of reagents and methodologies have been developed including chemical modification [4], ion exchange chromatography and analysis on an amino acid analyzer [5–7], detection of an *N*-isobutyloxycarbonyl trimethyl ester derivative by gas-liquid chromatography [8], specific tritium labeling or tritium exchange of Gla in protein or free Gla followed by HPLC or mass spectrometry [9–11], and derivatization with phenylisothiocyanate followed by reverse-phase HPLC [12]. Recently, capillary electrophoresis was used to compare quantitatively the differences in Gla content in small peptides [13]. Currently, the favored method [14,15] involves hydrolysis of the protein with base followed by a pre-column derivatization with *o*-phthalaldehyde and anion exchange HPLC. This method yields the most consistent result for the quantification of Gla by calculating the relative ratio of the peak areas of Glu to Gla in a protein and using Asp as an internal standard.

Recently, we developed a new method for the analysis of Gla in proteins by labeling a base hydrolysate with fluorescein isothiocyanate (FITC) followed by separation and detection by capillary electrophoresis and laser-induced fluorescence (CE-LIF) [16]. In this paper, we have used the assay to determine the Gla content in several recombinant prothrombin variants expressed in baby hamster kidney cells. The Gla contents have then been correlated with the physical and biological properties of these prothrombin variants.

2. Materials and methods

2.1. Materials

Electrophoresis reagent grade Tris, FITC isomer I (98%), and the amino acid standards L-aspartic acid, L-glutamic acid, and L-γ-carboxyglutamic acid were purchased from Sigma-Aldrich Canada Ltd., Mississauga, Ont., Canada. HPLC grade acetone and perchloric acid (69–72%), and capillary tubes (open ends, 1.5-1.8-100 mm, KIMAX-51) were from Fisher Scientific Canada, Nepean, Ont., Canada. Human plasma prothrombin was purchased from Haematologic Technologies Inc., Essex Junction, VT, USA. A sample of recombinant ferric iron-binding protein from *Neisseria gonnhoreae* was prepared in this laboratory using a published procedure [17]. A polyclonal anti-human prothrombin antibody was purchased from Affinity Biologicals, Hamilton, Ont., Canada. All other chemicals were reagent grade or better, and were obtained from either Fisher Scientific Canada or Sigma-Aldrich Canada Ltd.

2.2. Instrumentation

All oligodeoxyribonucleotides in this study were synthesized on an Applied Biosystems Model 391 DNA Synthesizer, using chemicals supplied by Perkin Elmer (Applied Biosystems Division), Foster City, CA, USA. Capillary electrophoresis was performed on a Beckman P/ACE 5000 automated CE-LIF system, manufactured by Beckman Instruments Inc., Palo Alto, CA, USA. Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 75 μm, an outer diameter of 360 μm, and a length of 57 cm were used. The detection system utilized an argon laser (4 mW) and a fluorescence detector that were also manufactured by Beckman Instruments Inc. Calcium-binding and phospholipid-binding proper-

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ties of proteins were measured by using a Perkin Elmer Luminescence Spectrometer Model LS 50 obtained from the Perkin Elmer Corporation, Norwalk, CT, USA.

2.3. Plasmid construction

The expression of recombinant human prothrombin (rhFII) using the pNUT-baby hamster kidney (BHK) cell system has been described previously [18]. A mutant form of human prothrombin cDNA [19] was prepared in which the coding region for the second cringle domain (Cys-170–Cys-248) was replaced with the equivalent region of bovine prothrombin cDNA [20] by using PCR-directed mutagenesis. Oligo 1 (5′-ATCGAATTCTCTTCTGTGTCCCGGACCGCGGCCGG-3′) and Oligo 2 (5′-ATCGAATTCTCTTCTCACAGTAGTTCAGGTCACAATA-3′) were used to amplify the kringle 2 region of bovine prothrombin cDNA; the PCR introduced *Ear*I sites at the ends of the amplified fragment. Oligo 3 (5′-ATCGAATTCTCTTCACACACTGCTCCAATGGAGG-3′) and Oligo 4 (5′-ATCGAATTCTCTTCTGTGAGGAGGCCGTGGAGGA-3′) were then used to amplify the human prothrombin cDNA regions flanking the kringle 2 domain; the PCR procedure also introduced *Ear*I sites that correspond to those sites introduced by the bovine PCR amplification. The human and bovine prothrombin PCR products were then cleaved with *Ear*1, *Sst*I and *Xba*I, and ligated into the *Sst*I and *Xba*I sites of Bluescript to reconstruct the human prothrombin cDNA now containing the bovine kringle 2 sequence. The nucleotide sequences of all junctions were then determined to ensure that no PCR errors had been introduced during the PCR steps, and to ensure the correct orientation of fragments after the ligation step. The mutated cDNA was then ligated into the pNUT expression vector.

2.4. Protein expression and purification

The mutated prothrombin expression vector was introduced into BHK cells by using the calcium phosphate co-precipitation method followed by selection with methotrexate [18]. Individual colonies were picked, and cloned by plating to limiting dilution. The cloned cell lines were expanded and analyzed for recombinant protein expression by Western blotting using an anti-human prothrombin antibody. The highest expressing cell line was used for further analysis. For large scale production of recombinant protein, cells were grown in roller bottles using DMEM-F12 medium (GIBCO-BRL, Life Technologies, Burlington, Ont., Canada) containing ITS-K supplement (bovine pancreatic insulin at a final concentration of 10 mg/l, human apo-transferrin at 5.5 mg/l, sodium selenite at 6.7 μg/l (all from Sigma-Aldrich), ethanolamine (ICN Biochemicals, OH, USA) at 2 mg/l, AlbuMAX (Life Technologies) at 0.5 g/l, and vitamin K1 (Abbott Laboratories, Montreal, Que., Canada) at 10 μg/l). The medium containing recombinant protein was harvested every 48 h, and stored at 4°C in 10 mM benzamidine until further purification. The purification of the recombinant protein was carried out essentially as described previously for recombinant human prothrombin [18], and consisted of precipitation with barium citrate, anion exchange chromatography by Fast Performance Liquid Chromatography (FPLC) using a column of high Q resin (Pharmacia Biotech, Baie D'Urfé, Que., Canada), and pseudo-affinity FPLC using a calcium gradient on a column of Mono-Q HR 5/5 (Pharmacia Biotech); the last step was used to resolve the different Gla-containing protein species [21].

2.5. Protein hydrolysis and Gla analysis

The protein sample (1 μg of a 1 mg/ml solution in 20 mM Tris-HCl buffer, pH 7.5 containing 150 mM NaCl) was added to 30 μl of 2.5 M KOH for alkaline hydrolysis, or 30 μl of 6 N HCl for acid hydrolysis. The mixture was transferred into a 1.5×100 mm capillary tube and heat-sealed at both ends such that the air space was minimal. The tubes were then submerged completely in the wells of a heating block filled with industrial oil, and the hydrolysis was carried out at 110°C for 16 h. After hydrolysis, the sample was transferred to an Eppendorf tube and silicate byproducts were removed by centrifugation at $13\,000\times g$ for 5 min.

Prior to derivatization, the alkaline hydrolysate was neutralized by the addition of 7% perchloric acid. After neutralization, the mixture was left on ice for 15 min. and the insoluble potassium perchlorate was removed by centrifugation at $13\,000\times g$ for 10 min. at room temperature. The supernatant (16 μl) was transferred to a new Eppendorf tube containing 12 μl 0.2 M sodium bicarbonate buffer, pH 9.0, and 2 μl of 50 mM fluorescein isothiocyanate (FITC) in acetone:pyridine (80:20, v:v) was added. The derivatization reaction was carried out in the dark at room temperature for 24 h. The mixture was diluted 400-fold with distilled water prior to CE.

The fluorescein thiocarbamyl (FTC) derivatives of the amino acids were separated by using an automated CE system. Before each separation, the capillary was rinsed with 0.1 M NaOH for 2 min followed by a 4 min rinse with separation buffer (100 mM Tris-HCl, pH 8.25). The sample was introduced by a 3 s low pressure injection (0.5 psi) and the separation was carried out for 20 min at 30 kV and 25°C. An argon ion laser was used for detection; excitation was at 488 nm and the emission was monitored at 520 ± 20 nm with a fluorescence detector. Data were collected and processed using System Gold software (Beckman Instruments Inc.).

2.6. Analysis of Ca^{2+}- and phospholipid-binding properties

The Ca^{2+}-binding properties of recombinant proteins were inferred by the decrease in intrinsic tryptophan fluorescence [22,23]. The protein sample (2 ml, 10 μg/ml in 20 mM HEPES buffer, pH 7.4 containing 150 mM NaCl) was stirred in a thermostated (25°C) quartz cuvette in the Luminescence Spectrometer. Excitation was at 280 nm and intrinsic fluorescence was monitored continuously at 300 to 400 nm, with excitation and emission band widths of 5 and 15 nm respectively, and with a 290 nm cut-off filter for the emission beam. An aliquot (10 μl) of 1 M $CaCl_2$ was added, and the decrease in fluorescence was monitored over the emission bands. The ratio of the magnitude of the decrement (ΔI_{340}) over the initial fluorescence (I_{o340}) of the recombinant proteins was extrapolated and compared to that obtained for plasma prothrombin.

The phospholipid-binding properties of the proteins were monitored by right angle light scattering [24,25]. Phospholipid vesicles (phosphatidyl choline-phosphatidyl serine, 75:25 mol:mol) were prepared (10 μg/ml in 20 mM Tris-HCl buffer, pH 7.4 containing 150 mM NaCl [26]), and an aliquot (2 ml) was stirred in a quartz cuvette at 25°C in the Luminescence Spectrometer. The right angle scattering intensity was monitored continuously over 10 min with an excitation wavelength of 400 nm and an emission wavelength of 410 nm, both with a 2.5 nm slit width. An aliquot of protein was added to give a final concentration of 20 μg/ml. After stirring for a further 5 min., an aliquot (10 μl) of 1 M $CaCl_2$ was added, inducing the calcium-dependent protein-phospholipid interaction. Data were collected at intervals of 1 s, and the increase in light scattering was monitored for 5 min or until a stable reading was obtained.

2.7. Coagulation assays

Coagulation assays were performed with recombinant prothrombin and variants using human-prothrombin deficient plasma under conditions recommended by the manufacturer (Sigma Diagnostic, St. Louis, MO, USA).

3. Results

3.1. Gla analysis of human prothrombin

Previously, we have shown that CE-LIF analysis of Gla is rapid, reproducible and sensitive with a limit of detection for FTC-Gla of 5.0×10^{-11} M [16]. Initially, the assay was used to detect Gla in commercial preparations of plasma prothrombin, plasma factor X and osteocalcin that had been hydrolyzed with base, as well as to detect Gla directly in plasma and urine [16]. To assess the carboxylation status of prothrombin expressed using the pNUT-BHK system [18], samples of human plasma prothrombin (phFII) and recombinant human prothrombin (rhFII) were hydrolyzed, derivatized with FITC and the FTC-labeled acidic amino acids were analyzed by CE-LIF. As a control, the non-Gla-containing ferric iron-binding protein from *N. gonnhoreae* was also analyzed. Fig. 1 shows the results of CE-LIF analyses of both acid and base hydrolysates of rhFII. The expected FTC-Gla peak was observed in the alkaline hydrolysate of rhFII (panel C); however, no FTC-Gla peak was observed in the acid-hydrolyzed samples of rhFII (panel B) nor in the base-hydrolyzed sample

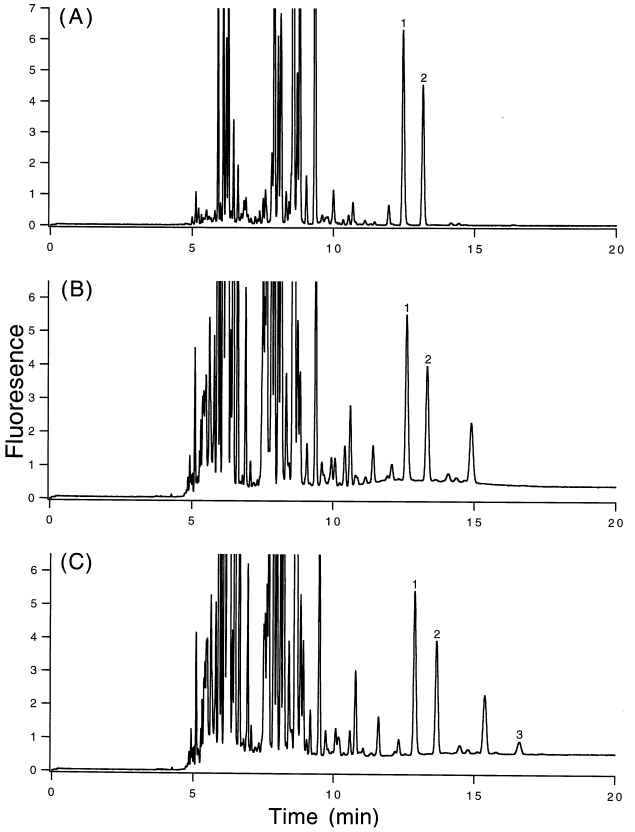

Fig. 1. Electropherograms from hydrolysates of recombinant human prothrombin analyzed by CE-LIF. Panel A: Alkaline hydrolysis of ferric iron-binding protein. Panel B: Acid hydrolysis of rhFII. Panel C: Base hydrolysis of rhFII. The peaks corresponding to the FTC-labeled acidic amino acids were determined from their migration times; where necessary, the identity of the FTC-Gla peak was confirmed by adding an aliquot of standard FTC-Gla to the sample, and repeating the CE-LIF analysis. The peaks corresponding to FTC-Glu (1), FTC-Asp (2) and FTC-Gla (3) are labeled.

of ferric iron-binding protein (panel A). In addition, the FTC-Glu to FTC-Asp ratio of the acid-hydrolyzed sample of rhFII was slightly higher than that of the base-hydrolyzed sample. These results are consistent with the decarboxylation of Gla to Glu during the acid hydrolysis step. In some electropherograms, an unidentified peak appeared that eluted between the FTC-Asp and FTC-Gla peaks; however, this peak did not interfere with the quantification of the three acidic amino acids of interest. The prothrombin analysis shown in Fig. 1 represents the hydrolysis of 1 μg of protein followed by a 400-fold dilution of the FTC-labeled hydrolysate prior to CE-LIF. Thus, nanogram quantities of protein are required for the Gla analysis making this CE-LIF method the most sensitive assay for Gla analysis reported to date. A sample of rhFII was then analyzed for Gla content, as shown in Fig. 2 (panel B). As summarized in Table 1, both the phFII and rhFII samples had Gla/Glu ratios corresponding to the ten residues of Gla per molecule of protein [27,28]. The high Gla content is consistent with the purification of rhFII by pseudo-affinity chromatography using calcium gradients; this method was developed to enrich for fully carboxylated species of recombinant human protein C [21].

3.2. Gla analysis of mutated forms of recombinant human prothrombin

Samples of two mutant forms of human prothrombin were then investigated. A chimeric protein was prepared in which the second kringle of human prothrombin was replaced with the equivalent region of bovine prothrombin; the mutant form was designated rhBK2. After expression in BHK cells, rhBK2 was purified in the same way as rhFII. During the pseudo-affinity chromatography step, rhBK2 eluted as two populations (designated rhBK2A and rhBK2B) eluting at 10 mM $CaCl_2$ and 17.5 mM $CaCl_2$, respectively. These results suggest that the two sub-populations of rhBK2 have different affinities for Ca^{2+} that reflect the presence or absence of the Ca^{2+}-dependent conformational change. The Gla content of the two sub-populations were then determined (Fig. 2, panels C and D). As shown in Table 1, rhBK2A and rhBK2B contain 8.6 ± 0.1 and 6.2 ± 0.7 residues of Gla, respectively.

3.3. Calcium- and phospholipid-binding properties

To correlate the Gla content with the Ca^{2+}-induced conformational change, the calcium-binding and phospholipid-binding properties of rhBK2A and rhBK2B were assessed by using intrinsic tryptophan fluorescence and right angle light scattering, respectively. The results were compared with those obtained with phFII and rhFII. The results of tryptophan fluorescence quenching on calcium binding are shown in Fig. 3A. The rhFII and rhBK2A showed very similar tryptophan fluo-

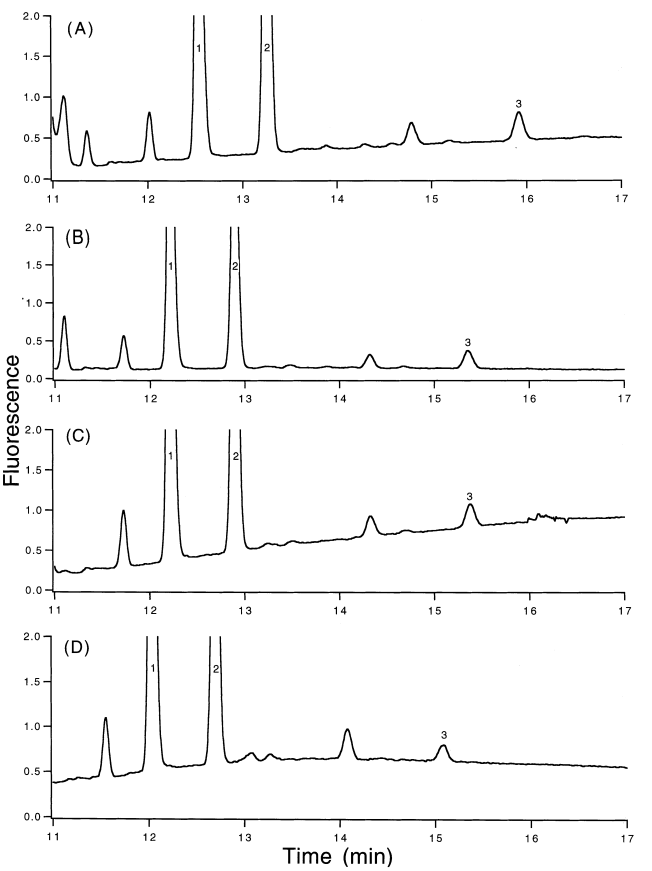

Fig. 2. Electropherograms from alkaline hydrolysates of prothrombin variants analyzed by CE-LIF. Panel A: phFII. Panel B: rhFII. Panel C: rhBK2A. Panel D: rhBK2B. The peaks corresponding to FTC-Glu (1), FTC-Asp (2) and FTC-Gla (3) are labeled.

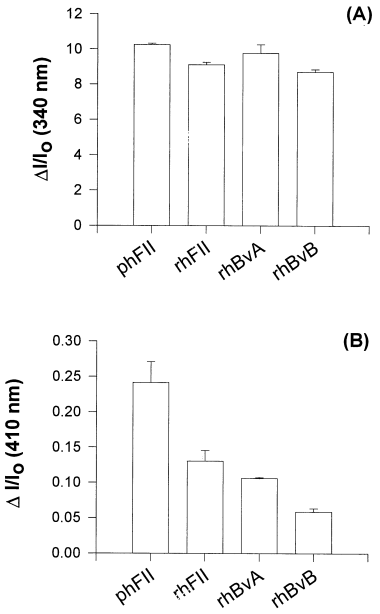

Fig. 3. Calcium-binding and phospholipid-binding properties of prothrombin variants. Panel A: The relative change in intrinsic tryptophan fluorescence after calcium addition. Panel B: The relative change in light scattering on calcium-dependent binding to phospholipid vesicles. Each experiment represents the average of three independent measurements, and the bars represent the standard deviation. phFII, plasma-derived prothrombin; rhFII, recombinant prothrombin; rhBK2A, Gla-rich prothrombin variant with bovine kringle 2; rhBK2B, Gla-poor prothrombin variant with bovine kringle 2.

rescence quenching responses that were similar to those observed for phFII. These results suggest that the proteins undergo a similar conformational change upon calcium binding. Consistent with a lower Gla content, rhBK2B exhibited a slightly smaller response on the addition of calcium (Fig. 3A).

In the right angle light scattering experiments, the addition of calcium to the suspension of protein and phospholipid vesicles promotes the formation of protein-lipid interactions resulting in increased light scattering intensity as the effective radius of the phospholipid vesicles increases. Fig. 3B shows the increment in right angle light scattering of the protein samples upon addition of Ca^{2+}, monitored over a time course of 10 min. On addition of Ca^{2+}, all the recombinant proteins showed diminished binding to phospholipid compared to the phFII (Table 1). The relative response in right angle light scattering of rhFII was 54% compared to phFII. The rhBK2A protein had a lesser response (44%) while the rhBK2B protein (with the least Gla residues) had only a 24% response relative to phFII (Table 1). Thus, the number of Gla residues correlates with the ability of prothrombin to bind to phospholipid vesicles. These results indicate that the phospholipid-binding property of rhBK2B was severely affected when the Gla content was reduced to an average of approximately six residues.

3.4. Clotting activities

To correlate these binding studies with biological activity, clotting assays were performed on phFII and the recombinant molecules (Table 1). As reported previously [18], rhFII has only 62% of the clotting activity of phFII. The mutant rhBK2A has a similar clotting activity to rhFII (Table 1), suggesting that the point mutations introduced with the bovine kringle 2 region do not affect the assembly and activity of the prothrombinase complex under the conditions used. In contrast, the mutant rhBK2B had only 9% of the clotting activity of phFII (Table 1). Thus, the ~6 residues of Gla in rhBK2B are insufficient for the function of prothrombin in the prothrombinase complex.

4. Discussion

A variety of different expression systems have been used to express recombinant vitamin K-dependent clotting factors including prothrombin, factor X, factor IX, factor VII and protein C [29]. Although prothrombin has been expressed at low yields in a fully carboxylated form [30], increasing the yield of recombinant protein is associated with the appearance of partially carboxylated forms [18,30]. It has been proposed that high levels of recombinant protein overwhelm the vitamin K-dependent carboxylation capacity of the tissue culture cell lines [30]. As originally described for plasma-derived prothrombin, there is an abrupt loss of functional activity associated with the loss of as few as three of the ten Gla residues found in the plasma protein [31]. Further studies showed that most if not all of the Gla residues are required for complete prothrombin function [32] with Gla-16, Gla-25 and/or Gla-26 being involved in the formation of a critical high affinity metal-binding site [33]. A second high affinity metal-binding site appears to be comprised of Gla-6, Gla-14, Gla-19 and Gla-20 [33]. Similar studies with recombinant proteins have revealed Gla residues in protein C that are critical for its anti-coagulant activity [34] and calcium- and phospholipid-binding properties [35]. To enrich for fully carboxylated species, purification techniques have been developed such as affinity chromatography using conformation-dependent antibodies [30,36] and pseudo-affinity chromatography with calcium gradients [21].

In this study, the Gla contents of phFII and rhFII have been measured directly. Consistent with previous studies [18], the rhFII produced in the pNUT-BHK cell system has a lower clotting activity than phFII. As the rhFII is fully carboxylated (Table 1), the reason for this reduction in activity is unclear as the only potential difference between phFII and rhFII is heterogeneous post-translational modification

Table 1
Correlation of Gla content and biological activities of various species of human prothrombin

Protein	Gla/Glu ratio	Gla content[1]	$\Delta I/I_o$ Trp (320 nm)	Relative Ca^{2+} binding (%)	$\Delta I/I_o$ (410 nm)	Relative PL binding (%)	Clotting time (s)	Relative clotting time (%)
phFII	0.07377 ± 0.0002	10 ± 0.3	10.25 ± 0.05	100	0.25 ± 0.03	100	20.7 ± 0.6	100
rhFII	0.07371 ± 0.0013	10 ± 0.2	9.10 ± 0.10	89 ± 1	0.13 ± 0.02	53 ± 6	25.3 ± 0.6	62 ± 3
rhBK2A	0.06366 ± 0.0008	8.6 ± 0.1	9.75 ± 0.35	95 ± 3	0.11 ± 0.01	43 ± 1	25.4 ± 0.5	62 ± 3
rhBK2B	0.04584 ± 0.0052	6.2 ± 0.7	8.70 ± 0.10	85 ± 1	0.06 ± 0.01	24 ± 2	61.0 ± 1.0	8.8 ± 0.3

[1]Residues per mol protein.

(such as glycosylation or incomplete propeptide removal) of the recombinant protein. A variant of rhFII (rhBK2) was also expressed in BHK cells at high levels; this variant had the second cringle domain of human prothrombin replaced by its bovine counterpart. During the purification of rhBK2 by pseudo-affinity chromatography, a Gla-rich component was separated from a Gla-poor component. Direct Gla analysis of these sub-populations revealed the presence of 8.6 and 6.2 residues of Gla per molecule of rhBK2A and rhBK2B, respectively. While both species bound Ca^{2+}, only the Gla-rich species bound significantly to phospholipid vesicles, and was active in a clotting assay (Table 1). Thus, the direct measurement of Gla content in the recombinant proteins correlates well with clotting activity.

CE-LIF is the method of choice for the analysis of small amounts of amino acids and peptides [37]. The small sample size and speed of analysis are well suited to the assessment of the carboxylation status of recombinant vitamin K-dependent proteins. Potentially, the CE-LIF-based Gla analysis used in this paper could be used for a protein sample from a partially purified source such as a band from an SDS-polyacrylamide gel. The detection limit for the CE-LIF assay for Gla is well below the detection limit for chromogenic strains. Thus, for some applications, the long sample purification and preparation time could be avoided.

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