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Calcium binding to the first EGF-like module of human factor IX in a recombinant fragment containing residues 1–85

Mutations V46E and Q50E each manifest a negligible increase in calcium affinity

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Abstract The first EGF-like module of human coagulation factor IX contains a single functionally important calcium ion binding site. We have now shown the dissociation constant for this site to be approximately 160 μM in a recombinant protein fragment consisting of residues 1–85 in human fIX. This represents a ≈10-fold increase in affinity as compared with the isolated EGF module (residues 46–85). The Gla module (here with Glu instead of Gla) thus increases the affinity of the EGF module calcium ion binding site. Each of two mutations, V46E and Q50E, made to investigate whether the extra negative charge would increase the affinity of the calcium binding site manifested a negligible increase in affinity.

Key words: EGF-like module; Calcium binding; Dissociation constant; Site-directed mutagenesis; Human factor IX

1. Introduction

Coagulation factor IX (fIX) is a single-chain molecule with 415 amino acid residues. It is the precursor of the serine protease, factor IXa (fIXa), that activates factor X (fX) to fXa in the blood coagulation cascade [1,2]. Factors IX and X are homologues, as are their cofactors, factors VIIIa and Va. Limited proteolysis of fIX in two steps results in the formation of the physiologically active form of fIX, fIXaβ. Factor IX can be activated either by factor XIa, or by factor VIIa in complex with its membrane-bound cofactor, tissue factor. Activation of fX takes place with fIXaβ bound to its cofactor, factor VIIIa, forming a membrane-bound macromolecular complex.

Factor IX is a vitamin K-dependent protein with an N-terminal γ-carboxyglutamic acid (Gla) containing module which is followed by two modules that are homologous to the epidermal growth factor (EGF) precursor. The C-terminal part of fIXa harbors the catalytic serine protease module. The structure of porcine factor IXa has been determined by X-ray crystallography [3]. However, the protein-protein and protein-phospholipid interactions that are required for assembly of the macromolecular complex are still poorly understood. In contrast to the factor VIIa-tissue factor complex, the factor IXa-factor VIIIa complex has not yet been crystallized and studied by X-ray crystallography [4]. It has, however, been amply demonstrated that occurrence of the conformational changes that follow fIX and fX activation is a prerequisite for their high affinity interaction with their respective cofactors. Moreover, three types of calcium binding sites are important for complex assembly and biological activity. The Gla module binds ~10 Ca^{2+} required for the interaction between fIXa and biological membranes, and the first EGF-like module binds one Ca^{2+} which appears to be required to secure an orientation between the Gla and the first EGF-like module that is compatible with biological activity [5,6]. Finally, there is a single Ca^{2+} binding site in the serine protease module. The importance of these Ca^{2+} binding sites has been demonstrated by analysis of naturally occurring mutants causing hemophilia B, and by site-directed mutagenesis studies [7–9].

The Ca^{2+} affinity of the isolated EGF module is low both in fIX (human; $K_d \approx 1.8$ mM) [8] and in fX (bovine; $K_d \approx 1$ mM) [10]. In fX the Ca^{2+} affinity increases approximately 20-fold when the EGF module is linked to the Gla module [11]. This increase does not require the Glu residues to be carboxylated to Gla. The structures of the N-terminal EGF-like modules of fIX and fX have been studied both by NMR spectroscopy (fIX and X) and by X-ray diffraction (fIX) [12–14]. X-ray crystallography determination of the structure of the synthetic N-terminal EGF module from fIX has enabled six ligands for the calcium ion to be identified: Asp-47, Gly-48, Gln-50, Asp-64 (bidentate interaction) and Asp-65 where residues 47, 50 and 64 provide side-chain ligands and residues 48 and 65 provide ligands from their backbone carbonyl groups [13]. This is in complete agreement with results previously obtained with NMR spectroscopy of the calcium-bound EGF module from factor X [14].

In this study, we wanted to investigate whether the Gla module has the same effect on the Ca^{2+} affinity of the EGF module in human fIX as in bovine fX. A feature specific to factors VII, IX and X and protein C is the presence of a Gln residue N-terminal to the first Cys in the EGF module (position 50 in human fIX), where most other EGF modules have a Glu residue. Moreover, the fourth EGF module in protein S, which binds Ca^{2+} with very high affinity, has a Glu residue instead of the Val found at position 46 in fIX. To investigate whether we could significantly increase the Ca^{2+} affinity of the

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Abbreviations: EGF, epidermal growth factor; hfIX, human factor IX; fX, factor X; Gla, γ-carboxyglutamic acid; Gla*, uncarboxylated glutamic acid; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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EGF module, as a preparatory step for subsequent studies of the effect of such a mutation on the biological activity of fIX, particularly its effect on membrane and cofactor interactions, we expressed the Gla*-EGF fragment (the asterisk denoting Glu instead of Gla) from fIX with a $Gln^{50}Glu$ or $Val^{46}Glu$ mutation and measured the calcium affinity of the site in the EGF module.

2. Materials and methods

2.1. Materials

AmpliTac polymerase was from Perkin Elmer. Restriction enzymes were from Boehringer Mannheim. *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA and lipofectin were from Invitrogen. Phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin, neutral red, leupeptin and luminol were from Sigma. Diisopropyl fluorophosphate (DFP) and citraconic anhydride were from Fluka. TC-100 and SF 900 cell medium were from Gibco. Gelatin-Sepharose and Sepharose S-100 were from Pharmacia Biotech. Molecular weight standards and Chelex 100 resin were from Bio-Rad. Goat anti-mouse antibodies labeled with horseradish peroxidase were from Dako. The monoclonal antibody AW binds the N-terminal EGF module of hfIX, is conformation specific and has no affinity for the reduced EGF module (unpublished results). The antibody was a kind gift from Dr. A. Wallmark, Department of Medicine, University Hospital, Malmö, Sweden.

2.2. Construction of vectors for expression of gelatin binding fIX fragments

Amplification of hfIX cDNA sequences was accomplished with standard PCR techniques. The following primer was used to create the sense for all the fragments, starting at N-18, and to create a larger primer for the mutations: 5′-GGCCATCCCCGTGGACAGTTTTTCTTGATCATGAAAAC. For all fragments, the C85 antisense is 5′-GGCCACGGGGATGGATCTAATTCACAGTTCTTTCCTTC. The mutation V46E was made in two steps with the following antisense primers: first 5′-ACACTGATCTCCATCA**T**CATACTGCTTCCAAAA, and then 5′-TCACACTGATCTCCATC**CT**CATACTGCTTCCAAAA. For mutation Q50E, the antisense primer was 5′-GATTGGACTCACACT**C**ATCTCCATCAACAT. The bold letters represent mutated nucleotides. Underlined sequences represent nucleotides added to create a *Bst*XI site. The amplified DNA was ligated into the *Bst*XI-cleaved expression vector GE-1/pGEM4, transformed into the *Escherichia coli* strain DH5α and subcloned into the transfer vector pAcYM1 as previously described [15].

The baculovirus transfer vectors were cotransfected with AcMNPV DNA into *Spodoptera frugiperda* (Sf21) cells with lipofectin. Recombinant viruses were plaque purified. For protein expression, Sf9 cells were used as previously described [16]. After infection with the recombinant viruses, leupeptin was added and the cells were incubated for 3 days at +27°C in serum-free SF900 II medium. PMSF and DFP were added to a final concentration of 2 mM. The proteins were expressed as fusion proteins with fibronectin, and after centrifugation at 10 000 rpm for 30 min the supernatant was loaded onto a fibronectin binding gelatin-Sepharose 4B column, equilibrated in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS). After washing with 1.0 M NaCl in 20 mM Tris-HCl, pH 7.4, elution was carried out with 3.0 M guanidine-HCl, 20 mM Tris-HCl, pH 7.4. The fusion proteins were dialyzed against TBS and stored at −70°C. To prevent cleavage at lysine residues, the proteins were citraconylated as described previously, and then digested with 0.05% (by weight) trypsin in 5 mM $CaCl_2$ for 15 min in 37°C [15]. The reaction was terminated by addition of DFP to a final concentration of 5 mM. The lysine blocking groups were removed by lowering the pH to 3.0 with glacial acetic acid, and subsequent incubation overnight at +8°C. pH was then adjusted to 7.4 with NaOH before a second chromatography on the gelatin-Sepharose column to remove free fibronectin and uncleaved fusion protein. The digested protein was concentrated in a Filtron Omegacell (cut off 5 kDa) and gel filtered on a Sepharose-100 column loaded with 50 mM NH_4HCO_3. Before entering the Sepharose column, the buffer was made Ca^{2+}-free by passage through a Chelex 100 column.

2.3. Amino acid composition and sequence analysis

Amino acid compositions were determined after 24 h hydrolysis as previously described [15]. Absorbance coefficients ($A^{1\%}_{1cm}$ at 280 nm) were determined by quantitative amino acid analysis of a solution with known absorbance, and were found to be 20.2 for the non-mutated recombinant fragment, 21.4 for the V46E fragment and 23.6 for the Q50E fragment. The molecular weight used was 12 552 Da. N-terminal sequences were determined on an Applied Biosystems 475 A Pulsed Liquid Phase Sequencer.

2.4. SDS-PAGE and immunoblotting analysis

SDS-PAGE was performed according to Laemmli [17]. For immunoblotting, the proteins were transferred to an Immobilon transfer membrane and detected by chemiluminescence as described [18], using the monoclonal antibody AW that recognizes the first EGF module of human fIX.

2.5. Solid phase immunoradiometric assay

To determine whether the recombinant protein fragments were folded to their native conformations, a solid phase immunoradiometric assay was used. Microtiter plates were coated overnight at +4°C with the monoclonal antibody AW. The antibody, 50 μl (10 μg/ml), was in sodium carbonate buffer, pH 9.6. The plates were washed five times with washing buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4), incubated at room temperature for 15 min with blocking buffer (washing buffer with 1% BSA) and then washed again. Increasing amounts of the recombinant proteins (after proteolytic removal of the fibronectin part and purification, see above) or purified hfIX were mixed with [^{125}I]hfIX in blocking buffer and incubated at room temperature for 3 h. The plates were washed and the bound radioactivity measured.

2.6. Intrinsic protein fluorescence measurements

Intrinsic protein fluorescence was measured at 25°C in an SLM 4800S spectrofluorometer (SLM Instruments, Rochester, NY) at a protein concentration of 2 μM in 50 mM Tris, 0.1 M NaCl, pH 7.4. The excitation wavelength was 280 nm. Emission spectra were recorded between 300 and 400 nm with excitation and emission bandwidths of 2 and 8 nm, respectively. Titrations with Ca^{2+} were monitored at an emission wavelength of 350 nm (corresponding to the maximum fluorescence emission intensity of the fragments). The emission intensity was measured 2 min after each addition of 1–5 μl 0.11 M $CaCl_2$ by averaging 30 signal readings of 0.25 s each. Dissociation constants and maximal fluorescence increases at calcium saturation were determined by fitting the data to the equilibrium binding equation by non-linear regression [19]. For each fragment, the maximal fluorescence increase and the K_d presented are means of 3–4 titrations with the standard deviation noted in parentheses.

3. Results

A cDNA fragment consisting of the coding region for the Gla* and N-terminal EGF-like modules was introduced into a baculovirus-derived vector. The protein was expressed in Sf9 cells as a fusion protein with a deletion mutant of fibronectin [20]. The fibronectin part of the recombinant protein allowed rapid purification on gelatin-Sepharose. As cleavage with thrombin, used in the original procedure, does not work adjacent to the fIX EGF module, the gelatin binding part of fibronectin was removed by limited tryptic cleavage at Arg-259 of fibronectin (removing residues 260–572) after reversible protection of Lys residues by citraconylation. This precaution was necessary in order to avoid cleavage at Lys-43 in the fIX fragment. A fibronectin-derived tail of 26 amino acids, which can be used to incorporate dansyl cadaverine and render the protein fluorescent, remained attached to the C-terminus of the EGF module (Fig. 1). The purified fragments were analyzed by SDS-PAGE (Fig. 2). Prior to tryptic cleavage the fusion proteins yielded two bands as the fibronectin domain was probably partially cleaved, either intracellularly or in the

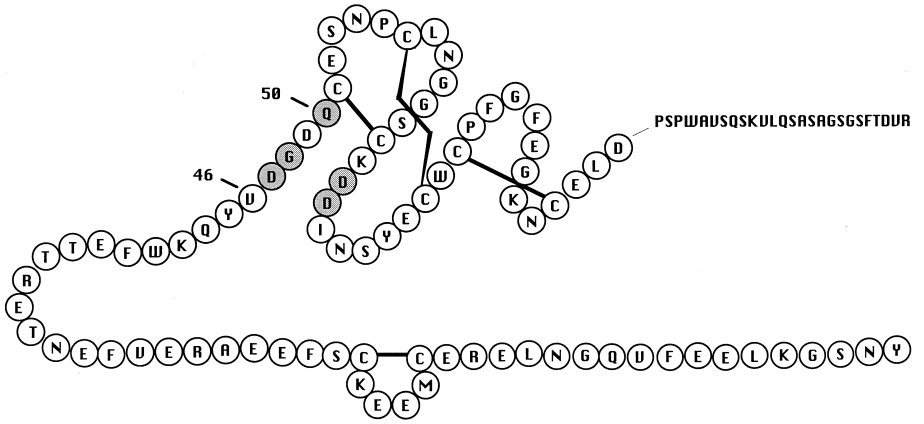

Fig. 1. Schematic model of the recombinant fragment 1–85 of hfIX, including the N-terminal EGF module with the Ca^{2+} ligands in gray. The mutated residues are denoted by numbers 46 and 50. At the C-terminal end the sequence of the fibronectin part linked to the recombinant fragment is shown.

medium (Fig. 2). The EGF module of fIX contains two unique potential glycosylation sites [21,22]. Aberrant glycosylation of these sites may account for the rather broad bands seen on the gel. The amino acid compositions of the purified fragments were in agreement with the composition deduced from the known sequence of the fragments (not shown). N-terminal sequence analysis of the cleaved and isolated fragments showed the fragments to be more than 90% homogeneous (Table 1). There was no evidence of intrinsic cleavage in the fragments. The fragments were made in insect cells, which do not have the vitamin K-dependent carboxylase that converts certain Glu residues to Gla [23]. However, decarboxylation of a bovine fIX fragment that contains the Gla and the N-terminal EGF module does not affect the Ca^{2+} binding to the N-terminal EGF module [24]. The β-hydroxyaspartic acid content was 0.08–0.15 mol/mol of protein, as compared with approximately 0.3 found in plasma-derived fIX [25].

Western blot experiments, using a conformation-dependent antibody recognizing an epitope in the first EGF module of hfIX, indicated the non-reduced recombinant protein to be

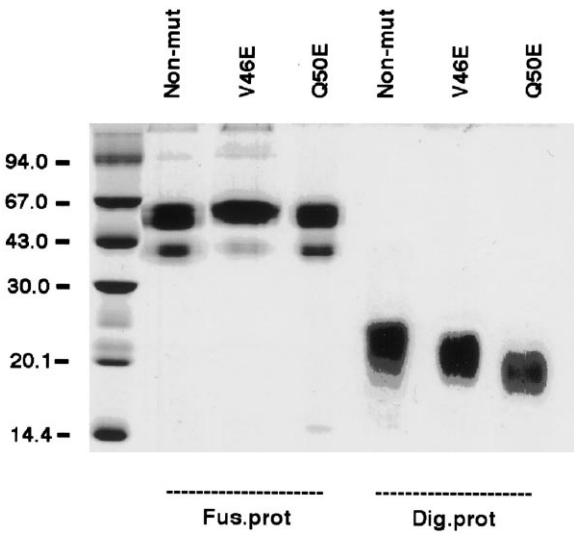

Fig. 2. SDS-PAGE of recombinant fusion proteins and isolated fragments. The sample load of each protein was 20 μg, and the gel was stained with Coomassie brilliant blue. Fus. prot. = fusion protein; Dig. prot. = digested protein; Non-mut = non-mutated.

folded to a native conformation (not shown). The antibody did not bind the reduced EGF module. As the cleaved recombinant proteins bound very poorly to the Immobilon membrane, the fold of the Gla*EGF modules was probed in a solid phase immunoradiometric assay (Fig. 3). The mutant recombinant protein fragments and plasma-derived hfIX competed equally well with [^{125}I]hfIX for binding to the monoclonal antibody, suggesting that the EGF module was folded to a native conformation.

The calcium binding properties of the recombinant fIX fragments were characterized by fluorescence spectroscopy (Fig. 4). In a fragment from bovine fIX including the Gla module and the N- and C-terminal EGF modules, there is a 10% increase in fluorescence emission intensity at low calcium concentrations, due to high affinity Gla-independent binding to the N-terminal EGF module [24]. This is followed by a quenching of approximately 50% at higher calcium concentrations, caused by binding of calcium to sites with lower affinity in the Gla module. Somewhat smaller, although highly reproducible, fluorescence enhancements than that noted for the bovine fragment were also observed at micromolar calcium concentrations with the recombinant human fragments. However, the quenching at higher concentrations was absent,

Table 1
N-terminal amino acid sequence analysis of purified fragment hfIX 1–85

Non-mutated		46E		50E	
Y	133	Y	87	Y	50
N	110	N	80	N	41
S	60	S	32	S	22
G	98	G	66	G	34
K	64	K	14	K	30
L	60	L	71	L	33
E	53				
E	61				
F	98				
V	65				
Q	42				
G	37				
N	24				
L	41				
E	18				
R	9				

The numbers indicate the amount (in pmol) recovered in each cycle.

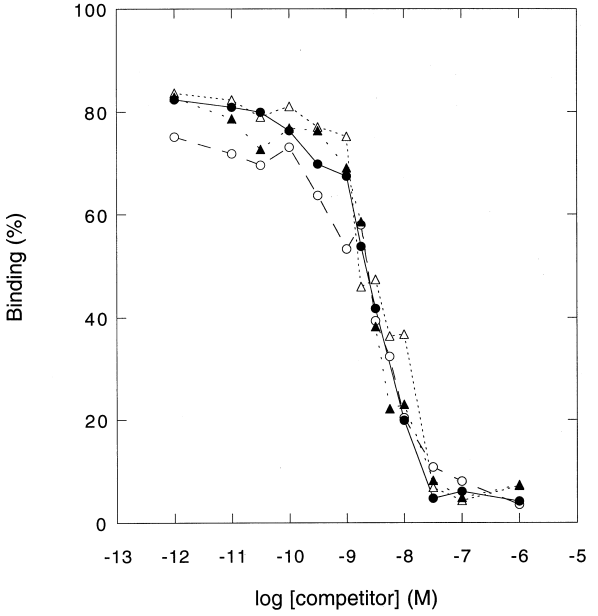

Fig. 3. Solid phase immunoradiometric assay of recombinant proteins and native hfIX. The competitors were (○) hfIX, (△) non-mutated, (●) V46E and (▲) Q50E.

as the recombinant fragments lack Gla residues. Quantitative evaluation of titrations monitored by the fluorescence increase gave a dissociation constant of 160 (±40) μM for the wild type fragment, consisting of residues 1–85 of human fIX. The two mutant fragments, V46E and Q50E, showed a negligible increase in Ca^{2+} affinity, the dissociation constants being 90±40 μM and 90±60 μM, respectively. The experimental error is quite large, due to the small change in fluorescence, but the increase in fluorescence is clearly reproducible. The maximum fractional increase in fluorescence intensity at calcium saturation was larger for the Q50E fragment (0.027±0.01) than for the wild type (0.016±0.009) and V46E fragments (0.016±0.006), though the reason for this is not known.

4. Discussion

The first EGF module of human fIX linked to the uncarboxylated Gla module has now been expressed in insect cells. The conformation of the EGF module was native as judged by a solid phase immunoradiometric assay using a conformation-specific antibody. However, this experiment does not prove that the uncarboxylated Gla module is folded to a native conformation. The three-dimensional structure of an uncarboxylated Gla module has not yet been determined, but is assumed to be similar to the structure of the carboxylated Gla module from factor X in the absence of calcium, as determined by 2D NMR spectroscopy [6]. The recombinant proteins were secreted from the insect cells, and in this respect behave like the uncarboxylated forms of Gla modules secreted from human and bovine hepatocytes, suggesting that the proteins had attained a native conformation [26].

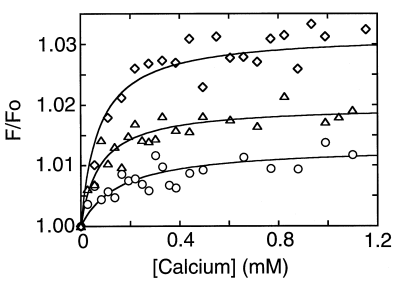

Fig. 4. Ca^{2+} titration, monitored by intrinsic protein fluorescence, of the recombinant digested fragments. Examples of titrations for the (○) non-mutated, (△) V46E and (◇) Q50E fragments. F and F_0 are the emission intensities in the presence and absence of Ca^{2+}, respectively.

The first EGF module in human fIX contains a single calcium binding site with a dissociation constant of 1.8 mM at physiological ionic strength and 200–300 μM at low salt [8,27,28]. In bovine factor IX it has been shown that a fragment consisting of the decarboxylated Gla and the two EGF modules contains a calcium binding site in the N-terminal EGF-module with a dissociation constant of around 60 μM at physiological ionic strength [24]. The corresponding EGF module of bovine fX binds calcium with a dissociation constant of ≈2.2 mM at physiological salt. However, when linked to the Gla module the calcium affinity increases to 120 μM at physiological salt, i.e. approximately 20-fold [11]. The site is thus essentially saturated at physiological free calcium ion concentrations in extracellular fluids such as blood plasma. The calcium affinity of the corresponding sites in factor VII and protein C, which have the same domain structure as factors IX and X, appears to be similar to those of the EGF modules in these proteins [28]. We have now shown that the dissociation constant for calcium in the first EGF module of hfIX, when attached to the Gla* module, is around 160 μM at physiological ionic strength, an approximately 10-fold increase relative to the free EGF module. Human fIX thus appears to behave as bovine fX in this respect.

The physiological significance of this calcium binding site is illustrated by the fact that numerous mutations that affect the calcium ligands in the EGF module of factor IX result in the biosynthesis of a protein with low biological activity (1–10%), and causing a hemorrhagic disorder (hemophilia B) [7]. It has been shown that, in the absence of calcium, both the Gla module and the EGF module in fX are folded to characteristic structures [5]. However, the two modules are very mobile relative to each other. This mobility is lost when a calcium ion is bound to the site in the EGF module; the calcium ion locks the hinge between the two modules. The low biological activity of fIX mutants which affect the calcium ligands in the EGF module is thus presumably due to an increased mobility between the Gla and EGF modules that is incompatible with biological activity. Recently it was proposed that the calcium binding site in the N-terminal EGF module of factor VII is important to orient the Gla relative to the N-terminal EGF module in such a manner that interaction with tissue factor is facilitated [29]. It thus appears as if the role of calcium binding to EGF modules is to induce an intermodule orientation that is compatible with biological activity.

The characteristic sequence, immediately N-terminal of the first Cys residue, in calcium binding EGF modules that occur in tandem in, for instance, protein S, fibrillin, the Notch protein and the TGF-β binding protein, is Asp-Ile/Val-Asp-Glu whereas factors VII, IX, and X and protein C all have the sequence Asp-Gly-Asp-Gln in the corresponding position [18]. The isolated EGF modules from the coagulation factors bind calcium with approximately the same affinity as the isolated

EGF modules from protein S and fibrillin. However, when two or more EGF modules are linked together, the calcium affinity is increased [30,31]. In protein S, in which the N-terminal sequence of the last EGF module has the sequence Glu-Asp-Ile-Asp-Glu, the calcium affinity of this site is very high, i.e. nanomolar at physiological ionic strength. Handford and colleagues found the Q50E mutation in a synthetic fIX EGF module to increase the calcium affinity 3.6 times [8]. Based on the rationale that there might be a more drastic increase in the calcium affinity of the EGF module site if it was linked to the Gla module, we expressed two Gla*-EGF modules with mutations V46E and Q50E, that might increase the calcium affinity of the site. Both mutations appeared to have at most a slightly increased calcium affinity (K_d around 90 μM for both), as compared with the wild type fragment. Q50 is a Ca^{2+} ligand, but it is interesting to note that V46 when mutated to E also seems to imply an increase in Ca^{2+} affinity, even though it is not considered to be a direct ligand for Ca^{2+} binding [13]. However, similar effects of changing the charge around a calcium binding site have been observed before [32].

In conclusion, we have found that the first EGF module of hfIX binds Ca^{2+} with a K_d of around 160 μM when linked to the Gla* domain, an approximately 10-fold increase in affinity as compared with the free EGF module. We have also demonstrated the two mutations V46E and Q50E each to result in a negligible increase in calcium affinity.

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References

[1] Furie, B. and Furie, B.C. (1988) Cell 53, 505–518.
[2] Davie, E.W., Fujikawa, K. and Kisiel, W. (1991) Biochemistry 30, 10363–10370.
[3] Brandsetter, H., Bauer, M., Huber, R., Lollar, P. and Bode, W. (1995) Proc. Natl. Acad. Sci. USA 92, 9796–9800.
[4] Banner, D.W., D'Arcy, A., Chène, C., Winkler, F.K., Guha, A., Koningsberg, W.H., Nemerson, Y. and Kirchhofer, D. (1996) Nature 380, 41–46.
[5] Sunnerhagen, M., Olah, G.A., Stenflo, J., Forsen, S., Drakenberg, T. and Trewhella, J. (1996) Biochemistry 35, 11547–11559.
[6] Sunnerhagen, M., Forsén, S., Hoffrén, A.-M., Drakenberg, T., Teleman, O. and Stenflo, J. (1995) Nature Struct. Biol. 2, 504–509.
[7] Giannelli, F. et al. (1996) Nucleic Acids Res. 24, 103–118.
[8] Handford, P.A., Mayhew, M., Baron, M., Winship, P.R., Campbell, I.D. and Brownlee, G.G. (1991) Nature 351, 164–167.
[9] Rees, D.J.G., Jones, I.M., Handford, P.A., Walter, S.J., Esnouf, M.P., Smith, K.J. and Brownlee, G.G. (1988) EMBO J. 7, 2053–2061.
[10] Persson, E., Selander, M., Linse, S., Drakenberg, T., Ohlin, A.K. and Stenflo, J. (1989) J. Biol. Chem. 264, 16897–16904.
[11] Valcarce, C., Selander-Sunnerhagen, M., Tämlitz, A.-M., Drakenberg, T., Björk, I. and Stenflo, J. (1993) J. Biol. Chem. 268, 26673–26678.
[12] Baron, M., Norman, D.G., Harvey, T.S., Handford, P.A., Mayhew, M., Tse, A.G.D., Brownlee, G.G. and Campbell, I.D. (1992) Protein Sci. 1, 81–90.
[13] Rao, Z., Handford, P., Mayhew, M., Knott, V., Brownlee, G.G. and Stuart, D. (1995) Cell 82, 131–141.
[14] Selander Sunnerhagen, M., Ullner, M., Persson, E., Teleman, O., Stenflo, J. and Drakenberg, T. (1992) J. Biol. Chem. 267, 19642–19649.
[15] Astermark, J., Sottile, J., Mosher, D.F. and Stenflo, J. (1994) J. Biol. Chem. 269, 3690–3697.
[16] Stenberg, Y., Dahlbäck, B. and Stenflo, J. (1997) Eur. J. Biochem. (in press).
[17] Laemmli, U.K. (1970) Nature 227, 680–684.
[18] Rand, M.D., Lindblom, A., Carlson, J., Villoutreix, B.O. and Stenflo, J. (1997) Protein Sci. 6, 1–13.
[19] Nordenman, B. and Björk, I. (1978) Biochemistry 17, 3339–3344.
[20] Sottile, J. and Mosher, D.F. (1993) Biochemistry 32, 1641–1647.
[21] Hase, S., Kawabata, S.-I., Nishimura, H., Takeya, H., Sueyoshi, T., Miyata, T., Iwanaga, S., Takao, T., Shimonoshi, Y. and Ikenaka, T. (1988) J. Biochem. 104, 867–868.
[22] Nishimura, H., Takao, T., Hase, S., Shimonishi, Y. and Iwanaga, S. (1992) J. Biol. Chem. 267, 17520–17525.
[23] Roth, D.A., Rehemtulla, A., Kaufman, R.J., Walsh, C.T., Furie, B. and Furie, B.C. (1993) Proc. Natl. Acad. Sci. USA 90, 8372–8376.
[24] Astermark, J., Björk, I., Öhlin, A.-K. and Stenflo, J. (1991) J. Biol. Chem. 266, 2430–2437.
[25] Fernlund, P. and Stenflo, J. (1983) J. Biol. Chem. 258, 12509–12512.
[26] Snell, E., Boyer, P., Meister, A. and Richardsson, C. (1997) Annu. Rev. Biochem. 46, 157–168.
[27] Handford, P.A., Baron, M., Mayhew, M., Willis, A., Beesley, T., Brownlee, G.G. and Campbell, I.D. (1990) EMBO J. 9, 475–480.
[28] Stenflo, J. (1991) Blood 78, 1637–1651.
[29] Kelly, C.R., Dickinson, C.D. and Ruf, W. (1997) J. Biol. Chem. 272, 17467–17472.
[30] Stenberg, Y., Linse, S., Drakenberg, T. and Stenflo, J. (1997) J. Biol. Chem. 272, 23255–23260.
[31] Knott, V., Downing, A.K., Cardy, C.M. and Handford, P. (1996) J. Mol. Biol. 255, 22–27.
[32] Linse, S., Brodin, P., Johansson, C., Thulin, E., Grundström, T. and Forsén, S. (1988) Nature 335, 651–652.