Calcium Affinity, Cooperativity, and Domain Interactions of Extracellular EF-hands Present in BM-40*

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The structure and function of cytosolic Ca\(^{2+}\)-binding proteins containing EF-hands are well understood. Recently, the presence of EF-hands in an extracellular protein was for the first time proven by the structure determination of the EC domain of BM-40 (SPARC (for secreted protein acidic and rich in cysteine)/osteonec-tin) (Hohenester, E., Maurer, P., Hohenadl, C., Timpl, R., Jansonius, J. N., and Engel, J. (1996) Nat. Struct. Biol. 3, 67–73). The structure revealed a pair of EF-hands with two bound Ca\(^{2+}\) ions. Two unusual features were noted that distinguish the extracellular EF-hands of BM-40 from their cytosolic counterparts. An insertion of one amino acid into the loop of the first EF-hand causes a variant Ca\(^{2+}\)-coordinating residues, and a disulfide bond connects the helices of the second EF-hand. Here we show that the extracellular EF-hands in the BM-40 EC domain bind Ca\(^{2+}\) cooperatively and with high affinity. The EC domain is thus in the Ca\(^{2+}\)-saturated form in the extracellular matrix, and the EF-hands play a structural rather than a regulatory role. Deletion mutants demonstrate a strong interaction between the EC domain and the neighboring FS domain, which contributes about 10 kJ/mol to the free energy of binding and influences cooperativity. This interaction is mainly between the FS domain and the variant EF-hand 1. Certain mutations of Ca\(^{2+}\)-coordinating residues changed affinity and cooperativity, but others inhibited folding and secretion of the EC domain in a mammalian cell line. This points to a function of EF-hands in extracellular proteins during biosynthesis and processing in the endoplasmic reticulum or Golgi apparatus.

The EF-hand is a highly conserved Ca\(^{2+}\)-binding motif found in a large number of intracellular proteins. EF-hands, as present in calmodulin, troponin C, and calcineurin, are responsible for signal transduction by the second messenger Ca\(^{2+}\), leading to the activation or inactivation of numerous and diverse target proteins and thus influencing a wide range of cellular processes (1–4). The term EF-hand was introduced by Kretsinger and Nockolds (5) in describing the structure of parvalbumin. Two helices (E and F in parvalbumin) flank a loop of 12 amino acids in which the Ca\(^{2+}\) ion is 7-fold coordinated in a pentagonal-bipyramidal arrangement. The Ca\(^{2+}\) ion is coordinated by side chain oxygen atoms at the X, Y, and Z vertices, by a backbone carbonyl at −Y, by a water molecule at −X, and by a carboxylate (mostly glutamate) at −Z, which makes a crucial bidentate interaction with the metal ion (see Fig. 1). This canonical EF-hand structure was subsequently found in a number of proteins of the Ca\(^{2+}\) second messenger system. Usually, EF-hands occur in pairs, although in some cases one or even both EF-hands in a pair may no longer be functional because of mutations of Ca\(^{2+}\)-coordinating residues (for reviews see Refs. 6–8).

Several variations of the canonical Ca\(^{2+}\)-binding mode are known. In the S100 subfamily of EF-hand proteins, two amino acids are inserted in the N-terminal EF-hand loop, while the C-terminal EF-hand is of the canonical type. The insertions lead to a rearrangement in the loop, such that the Ca\(^{2+}\) ion is coordinated by backbone carbonyl oxygens at positions X, Y, Z and −Y, with the bidentate ligand at −Z providing the only side chain oxygen atoms (9). Despite the unusual coordination of this site, the affinities of the two EF-hands for Ca\(^{2+}\), e.g. in calbindin D9k, are nearly identical (10). A further variation of the canonical EF-hand was found in myosin essential light chain. The loop in the first EF-hand of the EF-hand pair also contains two inserted amino acids but at different positions compared with S100 proteins. In essential light chain the insertions result in a more pronounced rearrangement of Ca\(^{2+}\) ligands, with several residues coordinating Ca\(^{2+}\) with both their backbone and side chain oxygens (11, 12). Finally, in the Ca\(^{2+}\)-binding domain of calpain, the first of the five EF-hands binds Ca\(^{2+}\) noncanonically. This EF-hand lacks the typical side chains at positions X and Y and uses a carbonyl oxygen and a second water molecule instead (13, 14).

BM-40 (SPARC (for secreted protein acidic and rich in cysteine)/osteonec-tin) is the only example of an extracellular EF-hand protein that is secreted by virtue of a classic signal peptide and for which a structure determination has proved the presence of EF-hands (15, 16). BM-40 is an abundant glycoprotein and has been suggested to participate in the modulation of cell-matrix interactions, bone mineralization, wound repair, and angiogenesis (17). It is highly expressed in some malignant tumors and has been reported to play a crucial role in the tumorigenicity of human melanomas (18). Developmental anomalies are induced by overexpressing or suppressing BM-40 in nematodes (19, 20). Furthermore, microinjection of BM-40 RNA, protein, peptides, or antibodies against BM-40 interfere with Xenopus embryonic development (21, 22). In contrast, deletion of the BM-40 gene in mice resulted in a relatively mild phenotype, namely late onset cataract formation in the eye, whereas development proceeded normally (23, 24). However, further defects, such as severe osteopenia, are under investigation (25). It is suspected that homologous pro-

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Construction of BM-40 Mutants—The point mutant EC-EF1-E234K was produced by the method of Deng and Nickoloff (37) using the transformer site-directed mutagenesis kit (CLONTech) and the cDNA encoding the EC domain of human BM-40 (34). The mutation primer 5'-CCCCACGACCATCTCAGGC-3' caused a change of the GAG codon for Glu234 to the AGG codon of lysine. An additional silent T to G mutation resulted in a novel NalI site which was used to identify mutated clones. Mutagenesis was performed as described by the manufacturer (CLONTech, CA). Production and purification of full-length BM-40, deletion mutants FS-EC and EC, and mutants BM-40-EF2-D257K and BM-40-EF2-E268K was done as described previously (24). Site-directed mutagenesis in EF2 were obtained by KpnI/XhoI restriction cleavages of BM-40-EF2-D257K and BM-40-EF2-E268K (33) and were cloned into the pDNA of the EC domain resulting in EC-EF2-D257K and EC-EF2-E268K. The double mutant EC-EF1.2-E234K.E268K was produced by subcloning the 136-base pair NcoI/XhoI fragment of EC-EF2-E268K into EC-EF1-E234K fragment. NheI/XhoI fragments of EC-EF1-E234K, EC-EF2-D257K, EC-EF2-E268K, and EC-EF1.2-E234K.E268K were excised and ligated with the NheI/XhoI restricted episolial expression vector pCPE-Pu (38). This vector contains the signal peptide of BM-40 and enables the secretion of recombinant protein into the cell culture medium. Correct mutagenesis and cloning was verified by cycle sequencing using the Alphapex DNA sequencer (Amersham Pharmacia Biotech).

Human embryonic kidney cells (EBNA-293) were transfected with 20 µg of pCPE-Pu vectors using LipofectAMINE (Life Technologies, Inc.) or electroporation. Cells were selected with 0.5 µg/ml puromycin in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium with 10% fetal calf serum for 14 days as described (38). For purification of proteins from the cell culture media, cells were maintained under serum-free conditions and culture medium was collected every 3 days.

Reversing Transcription-Polymerase Chain Reaction of Transfected Cellular mRNA—Transfected and nontransfected EBNA-293 cells were lysed in trypsinized from confluent plates, and 10^9 cells were used for isolation of mRNA performed according to the manufacturer’s protocol (Amersham Pharmacia Biotech). mRNA concentrations were determined using UV spectrophotometry. RT-PCR was performed in a one-tube reaction by addition of 1 unit of reverse transcriptase and 1 unit of Tso polymerase in RT-PCR buffer (0.1 mM Tris/HCl, pH 8.4, 0.5 mM KCl, 25 mM MgCl2, 1 mM dithiothreitol, 0.2% gelatin, 6.25% W1, 2.5 mM of each dNTP). After addition of 100 ng of mRNA, 50 pmol of sense primer 5’-GTCCCACTTCCAGCATGGGG and 50 pmol of antisense primer 5’-GATCCGTAGGT-TAGATCTACAAGATGC reverse transcription was allowed to proceed for 5 min at 72°C. Five cycles of polymerase chain reaction were then performed at an annealing temperature of 50°C followed by 30 cycles at an annealing temperature of 65°C. Denaturation and elongation temperatures were 95 and 72°C, respectively. Half of the reaction product was subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. In controls reverse transcriptase was omitted.

Immunoblot of Transfected EBNA-293 Cells—Transfected EBNA-293 cells of confluent plates (diameter, 10 cm) were washed with phosphate-buffered saline and lysed with 1 ml of 0.5% Triton X-100. Aliquots of serum-free culture medium (1 ml) were precipitated with trichloroacetic acid. The pellets were resuspended in reducing sample buffer. Resuspended pellets and cell lysates were subjected to electrophoresis in 15% SDS-PAGE gels according to Laemmli (39). Proteins were transferred onto nitrocellulose, and blocking was done with 5% milk powder solution. A rabbit polyclonal antiserum against human BM-40 (40) was used as a primary antibody, and detection was performed with a goat antibody against rabbit IgG coupled to peroxidase (DAKO, Denmark) and the enhanced chemoluminescence kit (Amersham Pharmacia Biotech).

Purification of EC-EF1-E234K and EC-EF2-D257K—Serum-free cell culture media (about 2.5 liters) were dialyzed against 0.15 M NaCl, containing 2 M urea. EC domains eluted in a broad peak between 0.15 and 0.4 M NaCl. Fractions containing the recombinant proteins were pooled and applied to a Sephadex G-75 column equilibrated in 50 mM Tris/HCl, pH 8.6, 150 mM NaCl. Final concentration

The abbreviations used are: RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
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was achieved on a Resource Q column followed by extensive dialysis against the appropriate buffer.

Circular Dichroism—Proteins were dialyzed against 5 mM Tris/HCl, pH 7.4, or 50 mM Tris/HCl, pH 7.4, 150 mM NaCl (TBS). Protein concentrations were calculated from the absorption at 280 nm as described (34). For the EC domain and EC domain mutants, an extinction coefficient of 1.39 ml mg⁻¹ cm⁻¹ was used (41). CD spectra in the far UV region were recorded in a thermostated quartz cell of 1-mm optical path length in a Jasco 715 CD spectropolarimeter. Spectra were measured with the protein dissolved in the appropriate dialysis buffer, after adding 2 mM CaCl₂, and after subsequent addition of 3 mM EDTA.

Molar ellipticities [Θ] expressed in degrees cm² dmol⁻¹ were calculated on the basis of a mean residue molecular mass of 110 Da. Ten spectra were accumulated to improve the signal/noise ratio. Spectra of buffers were subtracted. The percentage of change in circular dichroism at 222 nm was calculated as ∆[Θ] = 100 × ([Θ]solvent - [Θ]protein)/[Θ]solvent with [Θ] protein representing the signal at Ca²⁺ saturation and [Θ]solvent representing the CD signal in the presence of excess EDTA.

Fluorimetry and Ca²⁺ Titrations—Fluorescence was measured with a Perkin-Elmer LS50B spectrophotometer in 10-mm pathlength rectangular cells at 25 °C. Intrinsic fluorescence was excited at 280 nm for all recombinant proteins. Emission maxima were between 333 and 337 nm. Spectra were recorded in TBS in the presence of 2 mM CaCl₂ and after subsequent addition of 4 mM EDTA. The percentage of change in fluorescence intensity at 337 nm was calculated as ∆F = 100 × (F sat - F 0)/F 0, with F sat representing the signal at Ca²⁺ saturation and F 0 representing the fluorescence signal in the presence of excess EDTA.

Ca²⁺ titrations were performed with the EGTA/CaEGTA buffering system as described by Tsien and Pozzan (42). 100 mM EGTA and 100 mM CaEGTA stock solutions were purchased from Molecular Probes. An equilibrium dissociation constant K 1 = 35 mM for the CaEGTA complex was used for the titrations in TBS. Proteins (about 1 μM) were dissolved in buffer containing 10 mM EGTA, and fluorescence intensity was measured. Defined aliquots of the solution were removed and substituted by the same volume of protein dissolved at the same concentration in 10 mM CaEGTA, thus increasing the free Ca²⁺ concentration while keeping the protein concentration constant. This was repeated until finally the fluorescence intensity was measured for protein in 10 mM CaEGTA. Alternatively, proteins were dissolved in buffer containing 10 mM EGTA, and small aliquots of 100 mM CaEGTA stock solutions were added. In this instance, fluorescence intensity was corrected for dilution. Recombinant BM-40 mutants with low affinity for Ca²⁺ (K 1 = 20 μM) were directly titrated with CaCl₂ in the absence of EGTA. The degree of saturation Y was calculated from the signal S as Y = (S - S 0)/(S ca - S 0), where S ca and S 0 represent the signals at Ca²⁺ saturation and zero Ca²⁺, respectively.

Data Evaluation—Two potential mechanisms for binding of Ca²⁺ to BM-40 and its mutants were considered. The single-site model describes the measured changes in fluorescence as a function of the binding of a single Ca²⁺ ion to the protein as shown in the following equation:

\[ K_1 \] P + Ca ⇌ PCa \hspace{1cm} (Eq. 1)

The degree of saturation Y is then described by Y = [Ca]/(K 1 + [Ca]), where [Ca] represents the free Ca²⁺ concentration and K 1 represents the equilibrium dissociation constant. For a protein containing two binding sites, which may be either independent (no cooperativity present) or interacting (cooperativity present), four microscopic equilibrium dissociation constants are needed to fully describe the situation. However, the spectroscopic methods used do not allow for discrimination of Ca²⁺ binding to the individual sites, and thus the dissociation constants cannot be resolved (44). We therefore used a macroscopic two-site model with two Ca²⁺-binding sites, in which binding of one Ca²⁺ is described by the macroscopic dissociation constant K 11, followed by binding of a second Ca²⁺ with K 12.

\[ K_{D1} \] P + 2Ca ⇌ PCa + Ca ⇌ PCa 2 \hspace{1cm} (Eq. 2)

This model also allows detection of cooperativity between the two sites. If positive cooperativity is present, K 12 will be less than 4 × K 11, because the second Ca²⁺ ion binds with higher affinity when the first site is occupied than when it is empty. If there is no positive cooperativity, K 12 will be equal or greater than 4 × K 11. The degree of saturation can be described by Y = ([PCa] + [PCa 2])/[Ptot] with [PCa] and [PCa 2] representing the concentrations of the corresponding Ca²⁺-bound protein species and [Ptot] representing the total protein concentration. Assuming that contributions to the fluorescence signal are identical for PCa and PCa 2, this equation can be transformed into the following equation:

\[ Y = (K_{D11} \cdot [Ca] + [Ca]²)/(K_{D11} \cdot [Ca] + [Ca]² + K_{D12} \cdot K_{D11}) \] \hspace{1cm} (Eq. 3)

The program COSY (43) or Grafit (Erithacus Software) were used to fit both models to the experimental data with nonlinear least square fit procedures.

Cooperativity in a two-site system can be characterized by ∆ΔG, which gives the difference in free binding energies between the binding to a given site when the other site is occupied and when the other site is empty. For calculation of this free energy of interaction, ∆ΔG, knowledge of the microscopic equilibrium dissociation constants is required. However, these are not directly accessible from Ca²⁺ titrations. A lower limit of the free energy of interaction ∆ΔG can be calculated from the microscopic Kd values with ∆ΔG = −RT ln (Kd1/Kd2) (44). ∆ΔG is identical to ∆ΔG if the microscopic affinity of both sites is identical. If microscopic affinities are not identical, ∆ΔG is always smaller than ∆ΔG.

RESULTS

BM-40 Binds Ca²⁺ with High Affinity and Cooperativity—The affinity of EF-hands for Ca²⁺ varies for the different cytosolic members of the EF-hand family with dissociation constants (K 1) ranging from nM to μM values. Our first aim was to determine the affinities of the EF-hands of BM-40. We were unable to obtain BM-40 in a sufficiently Ca²⁺-free form either by dialysis against EDTA or Chelex-100 or by chromatography on an EDTA-agarose column (data not shown). We therefore used an EGTA/CaEGTA system (42) to follow Ca²⁺ binding. Briefly, this method relies on the use of two stock solutions of 100 mM EGTA and 100 mM CaEGTA, respectively. The latter is exactly titrated using a pH-metric method. Using different ratios of EGTA and CaEGTA, the free Ca²⁺ concentrations in a solution can be adjusted with high precision to [Ca²⁺]free = K d × [CaEGTA/EGTA]. Knowledge of the concentrations of residual Ca²⁺ from buffer solutions and bound to proteins (usually between 1–20 μM) is not necessary because this is buffered by the excess EGTA (normally 10 mM) in the system. For a residual Ca²⁺ concentration of 20 μM the error in the calculated free Ca²⁺ concentration is less than 2%.

The reversible change in intrinsic fluorescence of full-length BM-40 (36) was used to monitor Ca²⁺ binding. A simple model of one Ca²⁺ ion binding to BM-40 could not sufficiently describe the measured values, which evidently follow a curve with a steeper slope around the midpoint than predicted from the single-site model. An appropriate description was achieved with the two-site model, which enabled the determination of two macroscopic Kd values of Kd1 = 490 nM and Kd2 = 57 nM, respectively (Fig. 2A). The fact that Kd2 is much smaller than 4 × Kd1 clearly demonstrates that positive cooperativity is present because of interaction between the sites.

Domain Interactions with the FS Domain Influence Ca²⁺ Affinity—The change in fluorescence of the deletion mutant FS-EC was also investigated in the EGTA/CaEGTA system. Cooperative high affinity binding of Ca²⁺ to FS-EC was observed (Fig. 2B). The affinity of FS-EC was very similar to that of full-length BM-40 with macroscopic Kd values of 470 and 26 nM. This indicates that the acid domain I does not significantly influence Ca²⁺ binding to the EC domain. In contrast, removal of both domain I and the FS domain results in the isolated EC domain, strongly decreased Ca²⁺ affinity. Macroscopic Kd values of 11750 and 56 nM were obtained as best fit parameters for the EC domain (Fig. 2B). Ca²⁺ affinity is on average 6-fold lower if one compares (Kd1/Kd2)², which corresponds to a loss in total free binding energy of 9.9 kJ/mol (Table I).
Point Mutations in EF2 Disturb Folding and Secretion of the EC Domain—To investigate the influence of the individual EF-hands on Ca\(^{2+}\) affinity and cooperativity we mutated the conserved glutamic acid at position Z to lysine, a mutation recently shown to strongly decrease affinity of EF-hands (45–48). Because the two disulfides of the EC domain are essential for proper folding, all proteins were expressed in an eukaryotic expression system. When analyzing cell culture supernatants of transfected EBNA-293 cells recombinant EC domains with mutations EC-EF1-E234K and EC-EF2-D257K were readily seen in an immunoblot (Fig. 3). However, EC-EF2-E268K as well as the double mutant EC-EF1,2-E234K,E268K could not be detected in the medium (Fig. 3). The presence of similar amounts of endogenous BM-40 shows that the absence of the two mutants was not caused by cell death or a general inhibition of the secretory pathway. To prove successful transfection and selection of the EBNA-293 cells, we isolated mRNA from transfected cells and performed RT-PCR with primers specific for the recombinant EC domain. For all transfected cell lines the 540-base pair cDNA fragment could be amplified demonstrating the presence of the respective mRNAs (Fig. 4A). mRNA of endogenous BM-40 was not amplified as the reverse primer partially spanned noncoding region of the vector sequence. Next we prepared cell lysates of the nonsecreting EC-EF2-E268K and EC-EF1,2-E234,268K cells. In an immunoblot a faint band for both proteins could be detected (Fig. 4B). The intensity of the bands was comparable with that of the endogenous BM-40 and presumably represents the small amount of protein that is translated and processed in the endoplasmic reticulum and/or Golgi apparatus.

### Table I

|      | \(\Delta F337^{a}\) | \(K_{D1}^{b}\) | \(K_{D2}^{b}\) | \(K_{D}^{c}\) | \(\Delta G^{d}\) | \(\Delta G_{\nu-1}^{e}\) |
|------|------------------|----------|----------|---------|-------------|-------------|
| BM-40 | 108               | 490      | 57       | ns       | −77.3       | −8.8        |
| BM-40-EF2-D257K | 55             | 220      | 38.0     | 205400  | −69.5       | −16.7       |
| BM-40-EF2-E268K | 62             | 1200     | 33.8     | 45000   | −97.4       | −10.6       |
| FS-EC  | 90               | 470      | 26       | ns       | −24.8       | −18.4       |
| EC      | 83               | 11750    | 56       | ns       | −21.0       | −18.4       |
| EC-EF1-E234K | 34           | 205000   |          |         |             |             |
| EC-EF2-D257K | 35           | 205000   |          |         |             |             |
| FS-EC-D\(\Delta\) | 39       | 21540    | 52       | ns       | −68.2       | −18.4       |

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\(^{a}\) Percentage of change of intrinsic fluorescence at emission wavelength of 337 nm.

\(^{b}\) Macroscopic equilibrium dissociation constants derived from the model with two binding sites.

\(^{c}\) Equilibrium dissociation constant derived from the model with one binding site.

\(^{d}\) Total free energy of binding \(= RT \ln (K_{D1}K_{D2}) \) or \(= RT \ln (K_{D})\), respectively.

\(^{e}\) Minimal free energy of interaction between two sites \(\Delta G_{\nu-1} = −RT \ln (4K_{D1}K_{D2})\).

### Fig. 2. Ca\(^{2+}\) binding of BM-40 and its fragments.

Ca\(^{2+}\) binding was monitored by intrinsic fluorescence with excitation at 280 nm and emission at 337 nm. The fraction \(Y\) of the total change of fluorescence intensity is plotted against the free Ca\(^{2+}\) concentration adjusted with the EGTA/CaEGTA system. A, for full-length BM-40 two macroscopic constants \(K_{D1} = 490 \text{ nM}\) and \(K_{D2} = 57 \text{ nM}\) were obtained from the best fit of the model with two binding sites to the experimental data (solid line). Fitting a model with only one binding site was insufficient (dashed line). B, comparison of Ca\(^{2+}\) titrations for full-length BM-40 (■), the FS-EC domain pair (□), and the EC domain (○). Best fit curves are shown as solid lines with \(K_{D1} = 470 \text{ nM}\) and \(K_{D2} = 26 \text{ nM}\) for FS-EC domain pair and \(K_{D1} = 11750 \text{ nM}\) and \(K_{D2} = 56 \text{ nM}\) for the EC domain. In addition, the curve calculated with \(K_{D} = 870 \text{ nM}\) for a model with one binding site in the EC domain is shown as a dashed line.

### Table II

| Calcium binding parameter of BM-40 and the fragments |
|-----------------------------------------------|
| \(\Delta F337^{a}\) | \(K_{D1}^{b}\) | \(K_{D2}^{b}\) | \(K_{D}^{c}\) | \(\Delta G^{d}\) | \(\Delta G_{\nu-1}^{e}\) |
| % | ns | ns | ns | kJ/mol | kJ/mol |
| BM-40 | 108 | 490 | 57 | −77.3 | −8.8 |
| BM-40-EF2-D257K | 55 | 220 | 38.0 | 205400 | −69.5 | −16.7 |
| BM-40-EF2-E268K | 62 | 1200 | 33.8 | 45000 | −97.4 | −10.6 |
| FS-EC | 90 | 470 | 26 | ns | −24.8 | −18.4 |
| EC | 83 | 11750 | 56 | ns | −21.0 | −18.4 |
| EC-EF1-E234K | 34 | 205000 | ns | ns | −68.2 | −18.4 |
| EC-EF2-D257K | 35 | 205000 | ns | ns | −68.2 | −18.4 |
| FS-EC-D\(\Delta\) | 39 | 21540 | 52 | ns | −68.2 | −18.4 |

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\(^{a}\) Percentage of change of intrinsic fluorescence at emission wavelength of 337 nm.

\(^{b}\) Macroscopic equilibrium dissociation constants derived from the model with two binding sites.

\(^{c}\) Equilibrium dissociation constant derived from the model with one binding site.

\(^{d}\) Total free energy of binding \(= RT \ln (K_{D1}K_{D2})\) or \(= RT \ln (K_{D})\), respectively.

\(^{e}\) Minimal free energy of interaction between two sites \(\Delta G_{\nu-1} = −RT \ln (4K_{D1}K_{D2})\).

### Point Mutations in EF-hands Abolish Cooperativity of Ca\(^{2+}\) Binding—Both EC-EF1-E234K and EC-EF2-D257K were produced and could be purified to homogeneity from cell culture media (Fig. 5), even though expression levels were decreased compared with the nonmutated EC domain. The proteins eluted in broad peaks from ion exchange columns, and as a consequence, yields were very low (<25 µg/liter of medium). Nonetheless, circular dichroism spectra demonstrated that the mutated EC domains in the Ca\(^{2+}\)-free form had similar folds to that of the nonmutated EC domain (Fig. 6). Upon addition of Ca\(^{2+}\), the EC domain showed a 39% decrease in molar ellipticity at 222 nm, whereas only a 28% decrease was observed for EC-EF1-E234K. The conformational change was even more compromised in mutant EC-EF2-D257K, where the signal changed by only 10%.

In fluorescence studies, EC, EC-EF1-E234K, and EC-EF2-D257K showed emission maxima at about 340 nm when excited at their excitation maxima at 280 nm (data not shown). Titration of EC-EF1-E234K and EC-EF2-D257K revealed no change in fluorescence at submicromolar Ca\(^{2+}\) concentrations. However, changes in intrinsic fluorescence intensities could be observed when Ca\(^{2+}\) concentrations were raised significantly (Fig. 7). In contrast to results obtained with the EC domain, analysis of Ca\(^{2+}\) titrations of EC-EF1-E234K and EC-EF2-D257K revealed no sign of cooperativity. A model assuming a single binding site with \(K_{D} = 45 \mu M\) for EC-EF1-E234K and \(K_{D} = 205 \mu M\) for EC-EF2-D257K, respectively, could adequately describe the data (Fig. 7).

The mutations EF2-D257K and EF2-E268K have been introduced previously into full-length BM-40 (BM-40-EF2-D257K...
and BM-40-EF2-E268K) (33). To analyze cooperativity in the context of the full-length protein, we reinvestigated Ca²⁺ binding to these mutants. The fluorescence change upon Ca²⁺ addition could be described with a model assuming a single binding site, and a $K_D = 0.22 \mu M$ for BM-40-EF2-E268K was evaluated (Fig. 8), confirming the earlier results (33). For BM-40-EF2-E268K, the Ca²⁺ affinity was clearly decreased and not cooperative ($K_D = 1.2 \mu M$). In the mutant FS-EC-ΔC, the helix C is lacking in the EC domain. This mutation leads to enhanced collagen binding to the EC domain (35). The structure of FS-EC-ΔC was recently solved and showed two Ca²⁺ ions bound to the EF-hands (35). The affinity of FS-EC-ΔC for Ca²⁺ was 10-fold reduced relative to FS-EC when $(K_{D1}K_{D2})^{0.5}$ are compared, but the cooperativity between the sites was enhanced by the deletion (Fig. 8 and Table I).

FIG. 3. Immunoblot of cell culture media. 1 ml of serum-free cell culture medium transfected with EC-EF1-E234K, EC-EF2-E268K, EC-EF2-D257K, EC-EF1,2-E234K,E268K, or from untransfected cells (denoted EBNA 293) were separated by 15% SDS-PAGE under reducing conditions, blotted onto a nitrocellulose membrane, and stained with an anti-BM40 antiserum. Molecular masses of protein standards are given in kDa. The arrowhead marks the endogenous BM-40, and the arrow marks the EC domain.

FIG. 4. RT-PCR of mRNA from untransfected and transfected cells (A) and immunoblot of cell lysates (B). A, mRNA was isolated from untransfected (EBNA 293) cells and cells transfected with EC-EF2-E268K, EC-EF1,2-E234K,E268K, and EC-EF2-D257K. Reverse transcription and PCR were performed with primers specific for the EC domain. Reaction products were separated by agarose gel electrophoresis and stained with ethidium bromide. B, cell lysates of transfected EC-EF2-E268K and EC-EF1,2-E234K,E268K EBNA-293 cells were separated by 15% SDS-PAGE under reducing conditions, blotted onto a nitrocellulose membrane, and stained with an antiserum against BM40. The arrowhead marks the endogenous BM-40, and the arrow marks the EC domain.

FIG. 5. SDS-PAGE of the purified EC domain mutants. Electrophoresis of the purified proteins EC-EF1-E234K and EC-EF2-D257K was performed under nonreducing conditions. The gel was stained with Coomassie Brilliant Blue. Molecular masses of protein standards are given in kDa.

**DISCUSSION**

Cytosolic EF-hand proteins are designed to fulfill different functions at low Ca²⁺ concentrations in the resting cell (about 0.1 μM Ca²⁺) and when the cell has been activated (about 10 μM Ca²⁺). Their affinity for Ca²⁺ is accordingly tuned to this concentration range, allowing the proteins to switch from Ca²⁺-free to Ca²⁺-bound conformations upon activation. BM-40 is the only example so far of an extracellular protein containing EF-hands of which the three-dimensional structure is known. The role of Ca²⁺-binding to extracellular EF-hands is rather mysterious, because the extracellular free Ca²⁺ concentration is high (about 1.2 mM) and believed to be constant (49). The experiments presented here were designed to shed some light on the possible function(s) of extracellular EF-hands.

We have used a Ca²⁺ buffering system that allows precise adjustment of Ca²⁺ concentrations in the nanomolar range to examine Ca²⁺ binding by BM-40. The two EF-hands in BM-40 were found to bind Ca²⁺ with a free energy of −77.3 kJ/mol. We used two models for analyzing the binding data. The first model assumes a single Ca²⁺ binding site and is described by the microscopic equilibrium dissociation constant $K_D$. The second model assumes two binding sites and is described by two microscopic dissociation constants, $K_{D1}$ and $K_{D2}$. The values obtained for $K_{D1}$ and $K_{D2}$ allow to detect positive cooperativity between the sites. If Ca²⁺ binding to any site is independent from the occupation of the other site (i.e. no cooperativity), $K_{D2}$ will be equal or greater than $4 \times K_{D1}$. If positive cooperativity is present between the sites, $K_{D2}$ is smaller than $4 \times K_{D1}$ because the second Ca²⁺ ion binds with higher affinity when one site is already occupied by a Ca²⁺ ion. This model is an alternative to the use of the Hill equation but allows more detailed insight into the binding process and the energetics of binding (44).

The experimental results for calcium binding to BM-40 could only be fitted satisfactorily with the two-site model. The microscopic dissociation constants, $K_{D1} = 490$ nM and $K_{D2} = 57$ nM, demonstrate that binding of the second Ca²⁺ ion is facilitated by positive cooperativity. A minimum interaction energy of −8.8 kJ/mol can be calculated from these values (Table I). Because a signal specific for EF1 or EF2 cannot be obtained by circular dichroism and fluorescence spectroscopy, it is not pos-
with one binding site. Corresponding dissociation constants were $K_d$ recorded in 5 mM Tris/HCl, pH 7.4. Species were measured in the presence of 2 mM Ca$^{2+}$ (solid line) and after addition of 3 mM EDTA (dashed line).

Far UV circular dichroism mutants is evident from the reduced slope of the binding curves. 56 nM resulted from the best fit of the model with two binding sites to curve for BM-40-EF2-D257K ($\bar{K}_D$ 220 nM. Dissociation constants sites to the experimental data for full-length BM-40 ($\bar{K}_D$ 490 nM, respectively. The loss of cooperativity in the mutants is evident from the reduced slope of the binding curves.

The failure to detect cooperative binding in our previous studies (33, 34) is now seen to have been caused by incomplete removal of Ca$^{2+}$ from BM-40 and buffer solutions. Thus, Ca$^{2+}$ titrations were started with partially saturated proteins, and only the second halves of the saturation curves were recorded. At the time, only one binding site had been predicted for BM-40 (32, 50), and we therefore used a simple single-site model for data evaluation, which happened to describe the experimental data reasonably well. Of course, the data could also be fitted with more complex models including cooperativity, but these models did not produce a significantly better fit. In the present study, high accuracy in the low nanomolar range could be achieved through the use of the EGTA/CaEGTA system, finally allowing the single-site and the two-site model with cooperativity to be clearly distinguished.

BM-40 is half-saturated at a free Ca$^{2+}$ concentration of 170 nM. As already mentioned, free Ca$^{2+}$ concentrations in serum and several other extracellular tissue fluids are much higher (1.2 mM), but local gradients or changes in the extracellular Ca$^{2+}$ concentration down to 0.1 mM have been observed (49, 51, 52). Nevertheless, even at the lowest extracellular Ca$^{2+}$ concentrations reported to date, the EF-hands in BM-40 will be in the fully Ca$^{2+}$-saturated form. This indicates that Ca$^{2+}$ bound to the EF-hands in BM-40 is likely to play a structural rather than a regulatory role. It is known that the structure maintained by Ca$^{2+}$ is essential for the binding of BM-40 to collagens, and the collagen-binding site has indeed been mapped to the EC domain (34, 35).

Nearly identical Ca$^{2+}$ binding profiles were observed for the FS-EC domain pair and for full-length BM-40, showing that the acidic N-terminal domain I does not influence the affinity and cooperativity of Ca$^{2+}$ binding to the EC domain. In marked contrast, when both domain I and the FS domain were deleted the free energy of Ca$^{2+}$ binding was increased by 7.8 kJ/mol to −69.5 kJ/mol, demonstrating a strong interaction between the FS and EC domains. The first macroscopic dissociation constant, $K_{D1}$, was increased about 20-fold compared with full-length BM-40, whereas the second constant, $K_{D2}$, was not affected. Interestingly, the cooperativity between the EF-hands, as measured by $\Delta \Delta G$, is higher in the isolated EC domain relative to FS-EC and full-length BM-40 (Table I). Presumably, the strong interaction between the FS and EC domain in FS-EC restricts the conformational changes in the EF-hands that accompany Ca$^{2+}$ binding, whereas in the isolated EC domain binding of the first equivalent of Ca$^{2+}$ leads to a relatively larger rearrangement. The second equivalent then binds to similar structures in both FS-EC and EC, as evidenced by a similar value of $K_{D2}$.

The interface between the FS and EC domain is revealed in the structure of the FS-EC domain pair (16). The relatively small domain interface (550 Å$^2$) is largely formed by $\beta$-strand 5 of the FS domain and $\alpha$-helix E of the EC domain, the second helix of EF1 (Fig. 9). A further contact between the FS domain and EF1 involves His$^{62}$ and His$^{224}$, which participates in the unusual cis-peptide bond in EF1. There are no direct contacts between the FS domain and EF2. A comparison of the EF-hand
structures in FS-EC domain pair and the isolated EC domain does not reveal any significant differences that could explain the large effect of the FS domain on Ca^{2+} binding. This is perhaps not surprising, because both structures were determined under saturating concentrations of Ca^{2+}. The corresponding Ca^{2+}-free X-ray structures of FS-EC, perhaps because of the cis-trans-equilibrium of the His^{22A}. Pro^{225} peptide bond is pushed toward the cis-conformation required for Ca^{2+} binding or because Glu^{284} is positioned appropriately. It is thus tempting to associate \( K_{D1} \) in the interpretation of our binding data with EF1 and \( K_{D2} \) with EF2, although we stress again that the macroscopic dissociation constants are not necessarily linked to microscopic sequential events.

To analyze the contributions of individual EF-hands to Ca^{2+} binding, we replaced the conserved glutamic acid at position \( Z \) in each EF-hand by lysine (Fig. 1). Although the mutant EC-EF1-E234K was readily secreted from the human kidney cells used for recombinant expression, the EC domain with the corresponding mutation in the second EF-hand, EC-EF2-E268K, could not be detected in the cell culture supernatant. However, the corresponding mRNA was present in cellular extracts (Fig. 4). Apparently, mutation of the bidentate Ca^{2+} ligand Glu^{284} in EF2 interfered with proper folding in the endoplasmic reticulum, resulting in protein degradation. These results point to a function of EF-hands in the folding and secretion of extracellular EF-hand proteins. It remains to be elucidated whether chaperones are involved in this process. To determine whether Ca^{2+} binding to EF2 is strictly required for folding and secretion of BM-40, we produced mutant EC-EF2-D257K, in which the aspartic acid at position X was replaced by lysine. This mutant was secreted from the cells. No cooperativity could be detected in the Ca^{2+} titrations, and a model assuming a single binding site with a dissociation constant of \( K_D = 205 \mu M \) fitted the data well. In the mutant EC-EF1-E234K, cooperativity was also lost, and a dissociation constant of \( K_D = 45 \mu M \) was obtained. Most probably, the mutations decreased the Ca^{2+} affinity of the affected site to such an extent that binding could no longer be detected at the Ca^{2+} concentrations used. The remaining Ca^{2+} binding thus represents binding to the nonmutated EF-hand when the other hand in the pair is mutated. The dramatic, several 100-fold reduction in Ca^{2+} affinity of the nonmutated EF-hand is additional evidence for a strong coupling between the two EF-hands, which underlies the cooperativity of Ca^{2+} binding. Further insight into the energetics of Ca^{2+} binding can be gained by comparing the effects of mutations in the EC domain with those in full-length BM-40. Although the EF2-D257K mutation strongly decreased the Ca^{2+} affinity when introduced in the EC domain, the same mutation had only a small effect when introduced in full-length BM-40 (Table I), supporting the interpretation that the FS domain increases Ca^{2+} affinity in the EC domain mainly via interactions with EF1. Interestingly, the interaction of the FS and EC domains allows the mutant BM-40-EF2-E268K to be folded and secreted, whereas the same mutation in the isolated EC domain abolished secretion.

In conclusion, our results show that the EF-hands in the extracellular protein BM-40 bind Ca^{2+} cooperatively and with high affinity, just like their counterparts in cytosolic proteins. However, an additional layer of complexity is introduced by the presence of the FS domain, which physically interacts with the variant EF-hand 1 and, therefore, is intimately involved in Ca^{2+} binding to the EC domain.

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