Minireview

The CREC family, a novel family of multiple EF-hand, low-affinity Ca^{2+}-binding proteins localised to the secretory pathway of mammalian cells

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Abstract The CREC family consists of a number of recently discovered multiple (up to seven) EF-hand proteins that localise to the secretory pathway of mammalian cells. At present, the family includes reticulocalbin, ERC-55/TCBP-49/E6BP, Cab45, calumenin and crocalbin/CBP-50. Similar proteins are found in quite diverse invertebrate organisms such as DCB-45 and SCF in *Drosophila melanogaster*, SCF in *Bombyx mori*, CCB-39 in *Caenorhabditis elegans* and Pfs40/PfERC in *Plasmodium falciparum*. The Ca^{2+} affinity is rather low with dissociation constants around 10^{-4}–10^{-3} M. The proteins may participate in Ca^{2+}-regulated activities. Recent evidence has been obtained that some CREC family members are involved in pathological activities such as malignant cell transformation, mediation of the toxic effects of snake venom toxins and putative participation in amyloid formation.

Key words: Multiple EF-hand; Low-affinity Ca^{2+}-binding; Secretory pathway; Malignant cell transformation; Snake venom toxin mediation

1. Introduction

Ca^{2+} plays fundamental roles in the cell as a secondary messenger, being involved in the regulation of many processes including exocytosis, signal transduction, contraction and gene expression. The cytosolic concentration of free Ca^{2+} is kept at the sub μM level [1], since the Ca^{2+} ion is concentrated in intracellular stores, mainly the endoplasmic reticulum (ER), where the concentration of free Ca^{2+} is probably around the mM level [2]. The proteins involved in Ca^{2+}-regulated processes in the cytosol are thus mainly high-affinity Ca^{2+}-binding proteins, in general with EF-hand motifs such as troponin C, calmodulin, parvalbumin and intestinal Ca^{2+}-binding protein [3]. In the ER lumen, the Ca^{2+}-binding proteins are generally of low affinity with a high capacity such as calsequestrin, BiP, endoplasmin, Erp72, PDI and calnexin [2].

Until the early 1990s, there had been no reports on proteins localised in the secretory pathway containing EF-hand motifs that specifically coordinate the Ca^{2+} ion. The first such protein to be discovered in mammals was mouse reticulocalbin in 1993 [4]. Reticulocalbin localises strictly to the ER, contains as many as six EF-hands and thus represented the first of a growing family of multiple EF-hand proteins present throughout the whole secretory pathway of mammalian cells. At present, besides reticulocalbin, the family now includes ER Ca^{2+}-binding protein of 55 kDa (ERC-55) [5] also called taipoxin-associated Ca^{2+}-binding protein 49 (TCBP-49) [6] or E6-binding protein (E6BP) [7], crocalbin [8] previously termed CBP-50 [9], Cab45 [10,11] and calumenin [12–14]. Yabe et al. [15] have suggested the acronym CREC (Cab45, reticulocalbin, ERC-45, calumenin) for this family. In addition, some invertebrate members have been identified, *Drosophila* Ca^{2+}-binding protein of 45 kDa (DCB-45) and DNA supercoiling factor (SCF) in *Drosophila melanogaster* [16], SCF in *Bombyx mori* [17], a protein that we here term CCB-39 (for *Caenorhabditis* Ca^{2+}-binding protein of 39 kDa) in *Caenorhabditis elegans* (GenBank AAB04578) and *Plasmodium falciparum* 40 kDa sexual stage surface protein (Pfs40) [18] or *P. falciparum* ER-located Ca^{2+}-binding protein (PfERC) [19].

Interestingly, in contrast to what is usually found, the EF-hands, that specifically coordinate the ion in these EF-hand proteins, are found to possess low Ca^{2+} affinity with dissociation constants up to the mM level [13]. The specific subcellular localisation of each of the mammalian CREC proteins in the secretory pathway varies. Reticulocalbin and ERC-55 are strictly localised to the ER, Cab45 is strictly localised to the Golgi complex while calumenin is distributed throughout the whole secretory pathway besides being secreted to the medium of cultured cells. Some of the invertebrate proteins are even found to be localised in the nucleus [16].

Although the functional properties of the members of the CREC family are largely unknown, they may serve important roles in the maintenance of normal cell behaviour as the members of this family are highly conserved between the species. Furthermore, there is a possibility that some may be essential for life since homozygous deletion of a region containing the reticulocalbin gene is lethal [20]. Also, there have been several reports on their important role in pathophysiological processes especially in connection with malignant transformation [7,21] and as mediator of the toxic effects of snake venoms [6,8]. Here we give an overview of the accumulated results during the last few years of this novel growing family.

2. General structure and subcellular localisation

Structurally, the members of this family are very similar

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Abbreviations: ER, endoplasmic reticulum

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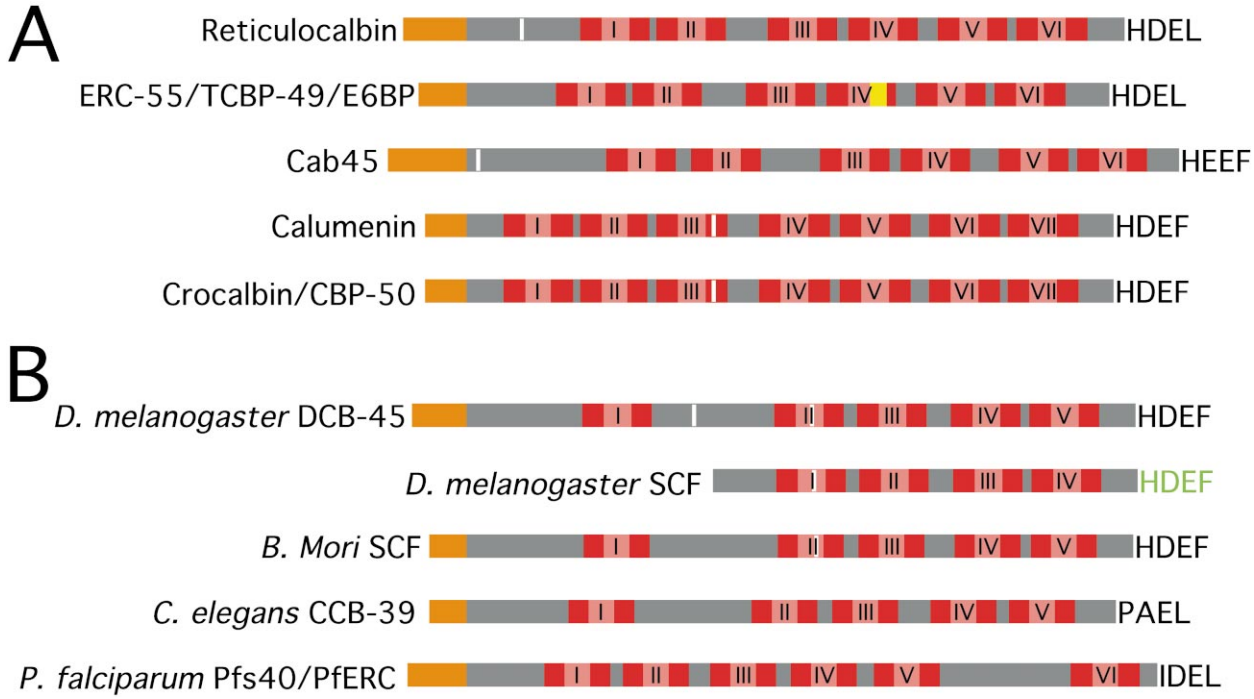

Fig. 1. Structure of the mammalian (A) and invertebrate (B) CREC family members. Each protein except for *D. melanogaster* SCF contains a signal sequence (orange). The length of the signal sequence and the putative cleavage site were predicted by a method based on neural networks trained on eukaryotic signal sequences using a publicly available WWW server, http://www.cbs.dtu.dk/services/SignalP/ [23]. The EF-hands are shown with red and the Ca^{2+}-binding loops with pink. Glycosylation sites are shown with white. The yellow peptide in EF-hand No. IV of ERC-55 mediates the association with the E6 oncoprotein. In *D. melanogaster*, two proteins exist, one is a 45 kDa ER variant (DCB-45) synthesised with a signal sequence, the other is a 30 kDa nuclear variant (SCF) that represents a truncated version of the DCB-45 protein lacking the N-terminal signal peptide and the first N-terminal EF-hand. The C-terminal HDEF sequence (green) of *D. melanogaster* SCF mediates the interaction with topoisomerase II

(Fig. 1). The proteins are synthesised as pro-proteins with leader peptides containing the typical characteristics of a signal sequence with few charged amino acids at the N-terminal followed by a stretch of hydrophobic amino acids that directs the synthesis into the lumen of the ER [22,23]. The mammalian pro-proteins (Fig. 1A) are acidic with pIs between 4.1 and 4.7 consisting of 315–362 amino acids giving molecular masses between 37 and 42 kDa. The signal sequences are between 19 and 36 amino acids giving molecular masses of the mature proteins between 35 and 40 kDa. The ERC-55 rat homologue purified from brain membranes is cleaved at various positions after the signal sequence resulting in molecular mass heterogeneity [6]. The mature proteins are generally hydrophilic and none of them contains hydrophobic transmembrane segments, suggesting that the entire proteins are transferred into the lumen of the ER after synthesis. Four of the mammalian proteins reticulocalbin, Cab45, calumenin and crocalbin contain N-glycosylation sites and mouse reticulocalbin [4], Cab45 [10] as well as calumenin [12,14] have been shown to be glycosylated. Although there have been no reports on the three dimensional structure of any of the proteins yet the main feature is the presence of six, or perhaps seven in case of calumenin and crocalbin, specific Ca^{2+}-binding domains of the EF-hand type [3]. By nuclear magnetic resonance spectroscopy, it has been demonstrated [24] that a synthetic peptide representing EF-hand No. 4 of ERC-55 folds into a classical EF-hand motif consisting of two perpendicular α-helices connected by a 12 residue Ca^{2+}-binding loop as described in detail below.

Reticulocalbin and ERC-55 terminate with the four amino acids HDEL that resemble the well-known KDEL retrieval signal for the ER [25]. HDEL also functions as retrieval signal for the ER in case of reticulocalbin and ERC-55 explaining the localisation of both proteins to the ER of the secretory pathway with no detection of secretion [4,5]. The retrieval function of the HDEL signal has been verified by the observation that reticulocalbin [4] and ERC-55 [5] with deletion of the HDEL signal may be seen in the medium. However, in case of ERC-55, the secretion proceeds slowly suggesting that a HDEL independent ER retention mechanism acts [5]. Since ultrastructural analyses have shown that ERC-55 [5] and calumenin [14] are localised in close association with the ER membrane, it has been suggested that the proteins may interact with yet unidentified membrane proteins. This is in line with the fact that TCBP-49 and crocalbin may be purified from membrane-enriched samples [6,9].

Cab45 terminates with a HEEF motif which does not function as retrieval signal for the ER since the protein localises strictly to the Golgi complex although the molecular mechanism for its retention here is unknown at present [10].

The localisation of calumenin is less strictly defined. Calumenin contains an HDEF sequence at the C-terminal. While mouse calumenin has been shown to be localised to the ER suggesting a retrieval function of the HDEF sequence [12], recent results with the human homologue [14] have shown a heterogeneous type of localisation in cultured fibroblasts with some cells possessing calumenin strictly in the ER while other cells also contain it in the Golgi complex, indicating that the

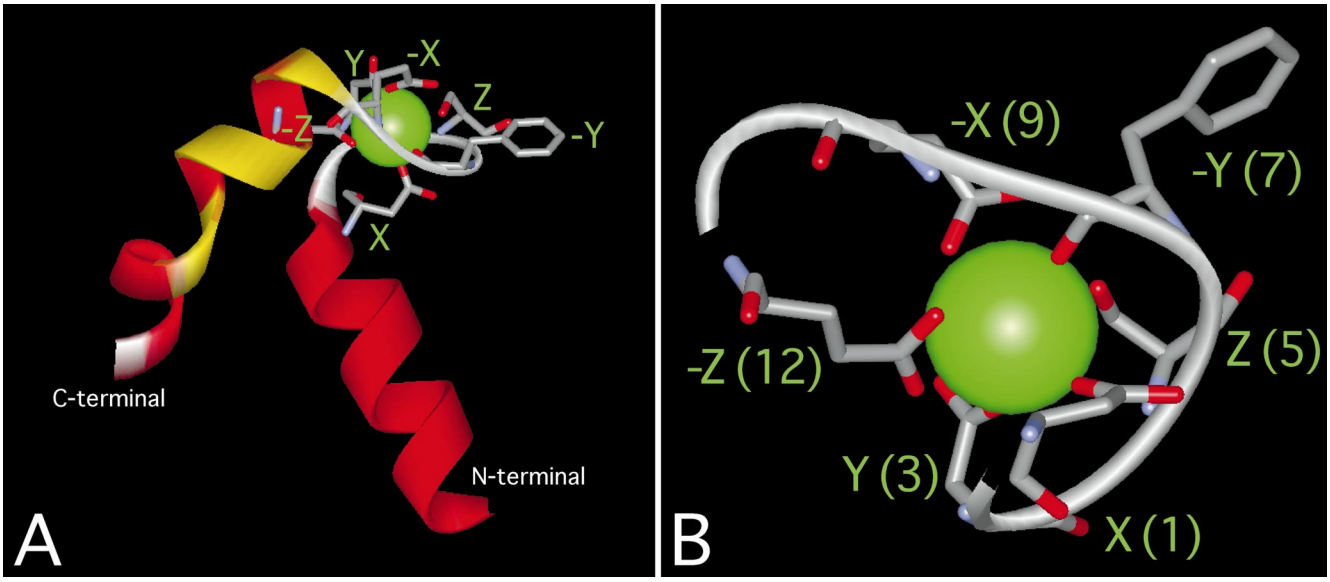

Fig. 2. Three dimensional structure of the main motif of the CREC family members represented by the classical EF-hand from parvalbumin. It consists of (A) two perpendicular α-helices (red) with a Ca^{2+}-binding loop of 12 amino acids (grey). The amino acids in the loop that provide oxygen ligands to the Ca^{2+} ion (green) are indicated with X (No. 1), Y (No. 3), Z (No. 5), −Y (No. 7), −X (No. 9) and −Z (No. 12), which are Asp, Asp, Ser, Phe, Glu and Glu in the case of parvalbumin [28]. A synthetic peptide representing the fourth EF-hand of ERC-55 forms this classical structure. The position of the four amino acids in the C-terminal α-helix, that are most critical for the interaction of the fourth EF-hand of ERC-55 with the papillomavirus E6 oncoprotein, are indicated with yellow in the α-helical backbone. (B) Close view of the six amino acids in the loop that provide oxygen ligands to the Ca^{2+} ion in a pentagonal bipyramidal fashion. The structures were constructed with the WebLab® ViewerLite program.

HDEF signal does not function as an efficient retrieval signal. This has further been verified with calumenin mutants where the HDEF signal is exchanged with HDEL or KDEL. Such mutants are more efficiently retrieved to the ER [14]. Human calumenin is the only protein in the family that has been reported to be secreted, suggesting that it possesses extracellular as well as intracellular functions [14]. This is in line with the finding that an extracellular protein, serum amyloid P component (SAP), can be affinity-purified on a column with immobilised calumenin [26]. No localisation has been reported for crocalbin that also terminates with an HDEF signal.

The invertebrate CREC proteins (Fig. 1B) are also synthesised with a signal peptide and thereby localised in the ER, except for *Drosophila* SCF that represents a truncated version of DCB-45, devoid of the signal sequence. SCF is localised in the nucleus where it participates in the DNA supercoiling reaction [16,27].

3. The CREC EF-hand motif and its affinity to Ca^{2+}

The predominant motif found in the CREC family is the presence of multiple helix-loop-helix motifs of the EF-hand type (Fig. 2A). This motif was first identified from the crystal structure of parvalbumin [28]. The motif consists of a 12 amino acid loop that directly coordinates the Ca^{2+} ion flanked by two perpendicular α-helices of 10–12 amino acids (Fig. 2A). The amino acids in the loop that provide oxygen ligands to the Ca^{2+} ion are positioned in place No. 1 (X), No. 3 (Y), No. 5 (Z), No. 7 (−Y), No. 9 (−X) and No. 12 (−Z), see Fig. 2B. In parvalbumin, all amino acids in these positions provide oxygen ligands to the Ca^{2+} ion and the amino acid in position −Z provides both of its oxygens so that seven oxygen ligands from the amino acid side chains of the loop coordinate the Ca^{2+} ion in a pentagonal bipyramidal fashion [3]. In other EF-hand proteins, one or more of the oxygen atoms is usually provided by water molecules instead of an amino acid side chain [3]. The consensus sequence of the EF-hand as described by Szebenyi and Moffat [29] is given in Fig. 3 with the alignment of the EF-hand domains of each of the CREC proteins. Human calmodulin and carp parvalbumin are included as examples of classical EF-hand proteins. As can be seen, the CREC proteins contain between two and five EF-hand domains that comply perfectly with the consensus sequence in the binding loop.

The sequence comparison suggests that the CREC proteins bind Ca^{2+} and this has been verified for all mammalian CREC proteins [4] and some of the invertebrate proteins [17,19] with the Ca^{2+} overlay technique [30]. It has been observed that ERC-55 may bind a Ca^{2+} amount of 1.5 relative to calmodulin [5]. However, the overlay technique does not provide any direct information about the strength of the interaction nor the capacity of the proteins for binding the ion. To date, only one study has been published where the affinity of the EF-hands is determined with a CREC protein in solution [13] using a rate dialysis technique [31]. In this study, it was not possible to demonstrate the presence of any high-affinity Ca^{2+}-binding site in human calumenin. In fact, the study showed that all seven EF-hands in calumenin bind the Ca^{2+} ion with a similar rather low affinity with a dissociation constant at 0.6×10^{-3} M at 37°C [13]. Furthermore, our studies have shown that human calumenin is not an exception in this respect since also reticulocalbin and ERC-55 demonstrate the absence of a high-affinity binding site when measured with the rate dialysis technique (unpublished). In general, the EF-hand is considered to possess high affinity towards Ca^{2+}. As seen from the alignment in Fig. 3, there is no obvious struc-

			1 3 5 7 9 12 X Y Z-Y-X -Z	
		n__nF__F n n	D_D_DG_n*__E N N F S	n__nn__n F
Reticulocalbin	I	ERLGKIVDRI	DNDGDGFVTTEE	LKTWIKRVQK
	II	DNVAKVWKDY	DRDKDDKISWEE	YKQATYGYYL
	III	PRDERRFKAA	DLNGDLTATREE	FTAFLHPEEF
	IV	IVVLETLEDI	DKNGDGFVDQDE	YIADMFSHEE
	V	SEREQFNEFR	DLNKDGKLDKDE	IRHWILPQDY
	VI	AEARHLVYES	DKNKDEKLTKEE	ILENWNMFVG
ERC-55	I	KRLQAIIKKI	DLDSDGFLTESE	LSSWIQMSFK
	II	QEAKQQFVEY	DKNSDDTVTWDE	YNIQMYDRVI
	III	LKDKKRFEKA	NQDSGPGLSLEE	FIAFEHPEEV
	IV	FVIQEALEEH	DKNGDGFVSLEE	FLGDYRWDPT
	V	VEKDRFVNDY	DKDNDGRLDPQE	LLPWVVPNNQ
	VI	EEALHLIDEM	DLNGDKKLSEEE	ILENPDLFLT
Cab45	I	RKLMVIFSKV	DVNTDRKISAKE	MQRWIMEKTA
	II	EESKTHFRAV	DPDGDGHVSWDE	YKVKFLASKG
	III	NLKDRWYQAD	SPPADLLLTEEE	FLSFLHPEHS
	IV	FMVKEIVRDL	DQDGDKQLSVPE	FISLPVGTVE
	V	DRKKEFEELI	DSNHDGIVTAEE	LESYMDPMNE
	VI	NEAKQMIAVA	DENQNHHLEPEE	VLKYSEFFTG
Calumenin	I	DKVHNDAQSF	DYDHDAFLGAEE	AKTFDQLTPE
	II	ERLGKIVSKI	DGDKDGFVTVDE	LKDWIKFAQK
	III	EDVERQWKGH	DLNEDGLVSWEE	YKNATYGYVL
	IV	VRDERRFKMA	DKDGDLIATKEE	FTAFLHPEEY
	V	IVVQETMEDI	DKNADGLIDLEE	YIGDMYSHDG
	VI	TEREQFVEFR	DKNRDGKMDKEE	TKDWILPSDY
	VII	AEARHLVYES	DQNKDGKLTKEE	IVDKYDLFVG
Crocalbin	I	DKVHNDAQNF	DYDHDAFLGAEE	AKSFGQLTPE
	II	EKLGMIVDKI	DTDKDGFVTEGE	LKSRIKHAQK
	III	DNVENQWQEF	DMNQDGLISWDE	YRNVTYGTYL
	IV	VRDERRFKMA	DQDGDLIATKEE	FTAFLHPEEY
	V	IVLQETMEDI	DQNADGFIDLEE	YIGDMYSHDG
	VI	TEREQFVEFR	DKNRDGKMDKEE	TKDWILPSDY
	VII	AEARHLVYES	DQDKDGKLTKEE	IVDKYDLFVG
D. melanogaster DCB-45 (SCF I)	I	RRLGVIVDRI	DENKDGSVTLAE	LKNWIAYTQR
(SCF II)	II	KRDRYRWSVA	DQDLDDNLTKDE	FTAFLHPEDH
(SCF III)	III	VVLRETITDL	DKDHDGKISVDE	YIGDMYRSTG
(SCF IV)	IV	NEREAFSTHR	DLDKDGYLNEEE	VKQWIAPHDF
	V	AEAKHLLFEA	DADHDDKLTKEE	ILDKYDVFVG
B. mori SCF	I	RRLGEIADKI	DSDQDGFITLVE	LKDWIRYTQK
	II	KRDRRRWTYA	DADQNDALNRTE	FAAFLHPEDH
	III	VVVLETLEDI	DKDQDGKVSLDE	YIGDMYNAGD
	IV	QEREQFTGYR	DTNKDGFMDEHE	VKDWIAPPEF
	V	AEARHLVFEA	DADADEKLTKAE	IIDKYDLFVG
C. elegans CCB-39	I	EKLAKLVPKM	DADSDGFIEENE	LKDHINFMQK
	II	ARDEKRWAVA	DYDSNGALDRTE	YGCFMHPEDC
	III	VVVAETVDDI	DKNKDGSVDLDE	YIGDMYRPDD
	IV	SEREMFKEHR	DKDGDGKLNQEE	MRDWIMPVGF
	V	AEARHLVGIA	DDNKDGKLNLDE	IVAHYDTFVG
P. falciparum Pfs40	I	ERIEKLFHLI	DKNNDKEITEEE	LNTWSSFLKN
	II	KQVQAEMGQI	DSDKDGFISLNE	LNDAFAQNLD
	III	EGLLKRFQIV	DKDKDGKLSINE	VGLLIDPMKD
	IV	LEINEILEHH	DVNKDGKISLDE	FKQTRSDESS
	V	EMALDDFNFF	DANKDGFIDKEE	IIKVYFDPAH
	VI	LPTARSRAFE	DDDMDADNTEDD	KDEADDASQQ
Calmodulin	I	AEFKEAFSLF	DKDGDGTITTKE	LGTVMRSLGQ
	II	AELQDMINEV	DADGNGTIDFPE	FLTMMARKMK
	III	EEIREAFRVF	DKDGNGYISAAE	LRHVMTNLGE
	IV	EEVDEMIREA	DIDGDGQVNYEE	FVQMMTAK
Parvalbumin	I	DDVKKAFAII	DQDKSGFIEEDE	LKLFLQNFKA
	II	GETKTFLKAG	DSDGDGKIGVDE	FTALVKA

Fig. 3. Alignment of each domain to the EF-hand consensus sequence. The consensus sequence is from [29]. X, Y, Z, −Y, −X and −Z refer to the residues that provide oxygen ligands to the Ca^{2+} ion in the Ca^{2+}-binding loop [3]. *n* indicates a non-polar residue, the asterisk a weak preference for a residue with a non-aromatic oxygen-containing side chain (Glu, Gln, Asp, Asn, Ser or Thr) and an underscore that any residue may occur in that position. For comparative purposes, the EF-hands of calmodulin and parvalbumin are included. The mammalian CREC family of proteins (reticulocalbin, ERC-55, Cab45, calumenin and crocalbin) possesses a Ca^{2+} affinity in the mM range whereas calmodulin possesses affinities down to the μM range and parvalbumin down to the nM range [3]. The CREC proteins contain between two and five EF-hand domains that comply perfectly with the consensus sequence. There is no obvious structural explanation for the differences in affinity. *D. melanogaster* has two proteins DCB-45 with five EF-hands and SCF that represents a truncated version of DCB-45 where the first EF-hand from the N-terminal is missing. Thus the numbering of the EF-hands in SCF is different and given in parentheses. ←

tural explanation for the lower affinity of the EF-hands in the CREC family. Most of the deviations to the consensus sequence are at position 6 where Gly is changed to another residue, however, recent mutational studies show that removal of Gly at that position has little effect on the Ca^{2+} affinity [32]. The substitution to a Gly in position 9 of calumenin and crocalbin also seems to be tolerated very well in parvalbumin (Fig. 3) that possesses high affinity. So at most one binding loop in ERC-55 (III), in Cab45 (III) and in Pfs40 (VI) contains substitutions of key coordinating or hydrophobic side chains putatively explaining a low affinity of only a single of the several binding loops present in the proteins. However, it should be kept in mind that also among the classical EF-hand proteins, there are great variations in the affinities with dissociation constants ranging from 10^{-9} M in parvalbumin over 10^{-8}–10^{-6} M in calbindin to 10^{-5} M in the low-affinity sites of calmodulin [3]. Apparently, the CREC family comprise a new set of EF-hand proteins possessing Ca^{2+} affinities with dissociation constants as high as 10^{-4}–10^{-3} M, i.e. up to the mM range.

4. Gene structure and evolution

A phylogenetic analysis of the CREC family shows that the family may have evolved by repeated duplications of a common ancestor [15]. Comparison of the gene organisation of reticulocalbin with that of other EF-hand proteins [33] and a phylogenetic comparison of reticulocalbin and ERC-55 [5] imply that the members of this family diverged very early from the known subfamilies of Ca^{2+}-binding proteins like calmodulin, troponin C or parvalbumin.

The genes encoding three of the members, reticulocalbin, ERC-55 and calumenin, have so far been chromosome-mapped.

The mouse reticulocalbin gene spans 13 kb [33]. Two transcripts of 2 and 2.3 kb are formed by the splicing of six exons [4,33]. In humans, only one transcript of 2.4 kb is found [34]. The human reticulocalbin gene maps to the 11p13 chromosome in the WAGR (Wilms' tumour, aniridia, genitourinary anomalies, mental retardation syndrome) region [20] which is hemizygously deleted in WAGR individuals. Reticulocalbin maps to mouse chromosome 2 in conserved synteny with human 11p13. The mouse region is deleted in the Small eye Harwell (Sey^{H}) mutation and it is suggested that loss of the

Table 1
Percentages of amino acid identity among the mammalian and the invertebrate CREC family members

	Calumenin	Crocalbin/ CBP-50	Reticulocalbin	ERC-55/ TCBP-49/ E6BP	Cab45	SCF[a] (*B. m.*)	DCB-45[a] (*D. m.*)	CCB-39[a] (*C. e.*)	Pfs40/PfERC[a] (*P. f.*)
Calumenin	–	88	61	38	32	54	49	49	26
Crocalbin/CBP-50		–	58	38	30	52	49	47	28
Reticulocalbin			–	40	32	46	45	48	18
ERC-55/TCBP-49/E6BP				–	31	34	35	33	27
Cab45					–	30	31	28	19
SCF[a] (*B. m.*)						–	56	50	22
DCB-45[a] (*D. m.*.)							–	46	28
CCB-39[a] (*C. e.*)								–	26
Pfs40/PfERC[a] (*P. f.*)									–

Sequence comparisons were performed among the human homologues calumenin (accession No. U67280) [13], reticulocalbin (accession No. D42073) [34], ERC-55 (accession No. X78669) [5] and Cab45 [11], except crocalbin (accession No. AJ001929) [8] where only the rat species homologue is available.
[a]These proteins are invertebrate CREC proteins that show a high degree of sequence identity with the mammalian CREC proteins. *B. mori* (*B. m.*) SCF (accession No. A57516), *D. melanogaster* (*D. m.*) calcium-binding protein (DCB-45) (accession No. AB011261), *C. elegans* (*C. e.*) CCB-39 (accession No. AAB04578) and *P. falciparum* (*P. f.*) Pfs40/PfERC (accession No. AAB49899).

reticulocalbin gene could contribute to the early lethality at about 15 days of gestation of *Sey* homozygotes [20].

The human gene encoding ERC-55 spans only about 2.3 kb consisting of two exons and one single intron of 375 bases encoding a transcript of 1.9 kb [5]. In rats, several transcript lengths are seen between 2 and 5 kb [6]. The human gene maps to chromosome 15q22.33–q24.1 [35]. There is no information on the localisation of the gene encoding the mouse homologue.

The calumenin gene has not been sequenced yet. The gene may produce several lengths of transcripts with a major transcript of about 3.4–4 kb [13,15], suggesting that the gene is longer than about 4 kb. The gene maps to human chromosome 7q32 [13]. However, the mouse localisation is also on chromosome 7 which is not in conserved synteny with the human location [12].

A high degree of amino acid conservation is found among the homologues from human, mouse and rat species. In general, the identities between the species of each of the homologues are above 90% except for Cab45 with an identity of 87% between human and mouse. The conservation is not only found in the EF-hand domains but also outside of these regions, suggesting that the proteins serve important roles besides that of binding Ca^{2+}. The relatedness between each family member in terms of amino acid identity is given in Table 1. Interestingly, there is also a high degree of sequence identity

Table 2
CREC interacting proteins and putative functional or pathophysiological aspects

CREC protein	Interacting protein	Interaction requires divalent cations	Putative functional aspect of the CREC protein	Reference
Reticulocalbin	None detected		Transcript overexpressed in highly invasive breast cancer cell lines	[21]
ERC-55/TCBP-49/E6BP	HPV E6 oncoprotein	No	Mediator of HPV E6 induced transformation of cervical cancer	[7,24]
	VDR	?	Modulation of transcription factor activity of VDR	[37]
	Taipoxin (snake venom toxin)	Yes	Mediator of the toxic effects	[6,42]
	NPR	Yes	Involved in neuronal uptake pathway (including taipoxin)	
	Neuronal pentraxin 1 (NP1)	Yes	Involved in neuronal uptake pathway (including taipoxin)	
	Neuronal pentraxin 2 (NP2)	Yes	Involved in neuronal uptake pathway (including taipoxin)	
Crocalbin/CBP-50	Crotoxin (snake venom toxin)	Yes	Mediator of the toxic effects	[8,9]
Calumenin	SAP	Yes	Participation in the immunological defence system; Participation in amyloid formation	[26]
SCF *D. melanogaster*	Topoisomerase II	No	C-terminal HDEF signal is essential for binding to topoisomerase II; The DNA supercoiling activity of the SCF-topoisomerase II complex is ATP- and Ca^{2+}-dependent	[16,27]
SCF *B. mori*	Topoisomerase II[a]	No[a]	C-terminal HDEF signal is essential for binding to topoisomerase II; The DNA supercoiling activity of the SCF-topoisomerase II complex is ATP- and Ca^{2+}-dependent[a]	[17]

[a]Inferred from the similarity of the *B. mori* SCF protein to the *D. melanogaster* SCF where the observations have been directly shown.

to recently discovered multiple EF-hand Ca^{2+}-binding proteins from invertebrate species including SCF from *B. mori* [17], SCF and DCB-45 from *D. melanogaster* [16], CCB-39 from *C. elegans* (GenBank AAB04578) and Pfs40/PfERC from *P. falciparum* [18,19]. These invertebrate proteins have a very similar structure with the mammalian CREC family members all containing several EF-hands and a high degree of sequence identity (Table 1 and Figs. 1B and 3). *D. melanogaster* has been studied in greater detail. Here, the protein exists in two variants encoded by separate mRNAs. A 1.8 kb transcript encodes a 45 kDa ER variant (DCB-45) containing the signal sequence and five EF-hands and a 1.6 kb transcript encodes a 30 kDa nuclear variant (SCF) that represents a truncated version of the DCB-45 protein lacking the N-terminal signal peptide and the first N-terminal EF-hand, see Fig. 1B [16]. Thus the presence of such similar proteins in these phylogenetically remote organisms suggests that the proteins possess important conserved cellular functions.

5. Putative functional properties and disease association

The CREC proteins are probably necessary for normal behaviour of the cell since homozygous deletion in mice of reticulocalbin is incompatible with life [20]. Since the mammalian Ca^{2+}-binding CREC proteins are localised in the secretory pathway, they could serve a depot or buffer function. However, although not investigated in detail, the concentrations of these proteins are not likely to be very high. The amount of ERC-55 in HeLa cells is below the levels that can be detected by Coomassie- or silver-stained gels, i.e. below that of endoplasmin, BiP, PDI and calreticulin [5]. Also, the rat homologue of ERC-55 is found in a concentration much below 0.01 mg/mg of brain membranes and at lower levels in liver and kidney homogenates with the conclusion that the protein is a low abundance protein [6]. Thus a storage, depot or buffer function of the CREC proteins is not very likely.

Due to their low-affinity binding of Ca^{2+}, it would seem obvious if they serve a role in Ca^{2+}-regulated processes in compartments that may contain a high concentration of free Ca^{2+}, e.g. the secretory pathway of normal cells where the free Ca^{2+} ion concentration probably ranges around the mM level [2] or extracellularly where it also is in the mM range. To date, several CREC interacting proteins have been identified as listed in Table 2. Most of the protein-protein interactions have been shown to be dependent on the presence of divalent cations. Only the interactions between ERC-55 and the HPV E6 oncoprotein and the interaction of SCF with topoisomerase II are known to be independent of the presence of Ca^{2+} [24,27]. It remains to be investigated in detail whether binding of Ca^{2+} to the EF-hands in the CREC family members in general serves a regulatory or a structural role. Regulatory EF-hand proteins expose hydrophobic surfaces upon binding of Ca^{2+} and then interact with their target molecules [36]. The few studies available concerning this indicate that at least some EF-hands of the CREC proteins may serve a regulatory role. Upon binding Ca^{2+}, a 25 amino acid synthetic peptide that corresponds to the fourth EF-hand of ERC-55 aggregates [24]. This is consistent with the interpretation of a conformational change that exposes hydrophobic surfaces. Another observation that favours a regulatory role of the EF-hands comes from functional studies performed by Hirose and coworkers on the *B. mori* and *D. melanogaster* SCF that generates negative supercoils into relaxed DNA in conjunction with topoisomerase II [16,17]. They have shown that the C-terminal HDEF sequence is essential for the binding of SCF to topoisomerase II (Fig. 1B) and that EF-hands No. II and III of SCF are essential for the supercoiling activity. A Glu to Gln substitution at position 12 (−Z) in the binding loop of either EF-hand No. II or III reduces Ca^{2+}-binding as well as supercoiling activity and simultaneous substitution at both sites abolishes the Ca^{2+}-binding as well as the supercoiling activity [27]. The activity of SCF becomes detectable at a Ca^{2+} concentration of 0.01 mM and is maximal at 0.1 mM [17]. Thus EF-hands No. II and III of *D. melanogaster* SCF are regulatory, possibly with a dissociation constant for Ca^{2+} between 10^{-5} and 10^{-4} M. Finally, we have found that the interaction of calumenin with SAP depends on the free Ca^{2+} concentration in the mM range [26]. Further studies, however, are needed before more solid conclusions can be made with respect to the other proteins.

Although there so far have been no reports of the presence of any of the mammalian CREC proteins in the cell nucleus, another observation suggests that there also may be nuclear functions associated with some of the mammalian CREC proteins or with some still unidentified nuclear multiple EF-hand proteins possessing similarity to members of the CREC family. By using a yeast two hybrid system, Imai et al. [37] have found that among several nuclear hormone receptors, ERC-55 interacts specifically with the vitamin D receptor (VDR), suggesting that it may act as a cofactor for VDR modulating its function. VDR belongs to a gene superfamily of ligand-inducible transcription factors that play important roles in a number of physiological processes by regulating the expression of a number of genes [38]. Several other receptors like the all-*trans* retinoic acid receptor, the 9-*cis* retinoic acid receptor, the oestrogen receptor and the glucocorticoid receptor do not interact with ERC-55. In vivo, an interaction of VDR with ERC-55 may be explained if a truncated variant of ERC-55 is synthesised without a signal sequence from an alternatively spliced transcript in analogy with the *D. melanogaster* SCF protein thereby avoiding an ER localisation of the CREC protein. However, there is so far no experimental evidence for this and the functional significance of the interaction must await a demonstration of colocalisation of the proteins.

A number of pathophysiological aspects of the proteins have emerged lately as listed in Table 2. There is strong evidence that some of the proteins participate in the pathogenesis of certain diseases, especially cancer that two of the proteins have been associated with. By using a differential display, it was shown that the reticulocalbin transcript is overexpressed in the highly invasive breast cancer cell line MDA-MB-435 but not in the poorly invasive cell line MCF-7, strongly suggesting that reticulocalbin is implicated in tumour cell invasiveness [21]. Also, ERC-55 has recently been shown to be implicated in malignant transformation in connection with papillomavirus infection. The transforming properties of papillomavirus reside in two genes, E6 and E7, which are consistently expressed in HPV-positive cervical cancer cell lines [39]. The mechanism by which papillomavirus promotes cell growth and proliferation has been suggested to be the binding of E6 to p53 with subsequent inactivation of p53 [40]. However, there is growing evidence that E6 has functions that are independent of inactivating p53 in cell transformation. Recently, this has further been substantiated by the observation

that E6 binds to ERC-55 and most importantly that the transforming activity of mutants of E6 is correlated with their ability to bind ERC-55, suggesting an important role for ERC-55 in E6-induced cell transformation [7]. In preliminary immunofluorescence studies, direct evidence has been obtained that E6 colocalises with ERC-55 in the ER, suggesting that the interaction is physiologically relevant [7]. Since ERC-55 might interact with VDR as well as E6, the protein could somehow link the signalling pathway of $1,25(OH)_2D_3$ with the pathway of the tumourigenesis caused by E6. Further studies into this area will be interesting to follow. The ERC-55 site of interaction with E6 has been mapped to the second α-helix of the fourth EF-hand at the sequence FV**S**L**EE**F**LG**D**D**, where especially the four positions shown in bold serve a crucial role [24] (the positions are highlighted with yellow in Figs. 1A and 2A). These amino acids do not participate in the complex formation with the Ca^{2+} ion in line with the observation that the ERC-55 interaction with E6 is independent of whether Ca^{2+} is present or absent [24]. Most of the other members of the CREC family contain rather similar sequences at that position, however, it has not been investigated whether other family members also interact with E6.

Two of the proteins have been shown to interact with snake venoms. ERC-55 interacts with taipoxin, a neurotoxin from the Australian snake Taipan (*Oxyuranus scutellatus*) [6] and crocalbin interacts with crotoxin from the South American rattlesnake (*Crotalus durissus terrificus*) [8,9]. These toxins possess phospholipase A_2 activity and cause flaccid paralysis by blocking the neuromuscular transmission presumably with a presynaptic site of action [41]. It is suggested that ERC-55 interacts with internalised taipoxin and could perhaps mediate its toxic effect [6]. Recently, a neuronal pentraxin receptor, NPR, has been identified in brain tissue [42]. ERC-55 together with NPR and neuronal pentraxins 1 and 2 (NP1 and NP2), which also interact in a Ca^{2+}-dependent way with ERC-55, are suggested to be involved in a pathway responsible for the transport of taipoxin into synapses and may thus represent a novel neuronal uptake pathway involved in the clearance of synaptic debris [42].

Interestingly, NPR, NP1 and NP2 are pentraxins that have high sequence identity to the acute phase pentraxins C-reactive protein and SAP [42]. The latter protein has recently been shown to interact in a Ca^{2+}-dependent way with calumenin [26], the only CREC member known to be secreted [14]. This may suggest a role of calumenin in the immunological defence system and participation in the pathological process of amyloid formation.

6. Concluding remarks

In the last few years, it has become apparent that there exists a family of multiple EF-hand Ca^{2+}-binding proteins that serve important roles for normal cell behaviour possibly in connection with Ca^{2+}-regulated processes in the nucleus, in the secretory pathway and extracellularly. Furthermore, some of the proteins play a central role in pathophysiological processes, especially in cancer, in the mediation of the toxic effects of snake venoms in the nervous system and putatively in amyloid formation. Future studies on this novel family of proteins may thus reveal unknown molecular mechanisms in the pathogenesis of diseases.

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