The Divergent Domains of the NEFA and Nucleobindin Proteins are Derived from an EF-Hand Ancestor

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The human protein NEFA (DNA binding, EF-hand, Acidic region) has previously been isolated from a KM3 cell line and immunolocalized on the plasma membrane, in the cytoplasma, and in the culture medium. Sequence analysis of a cDNA clone encoding NEFA identified a hydrophilic domain, two EF-hands, and a leucine zipper at the C-terminus. These characters are shared with nucleobindin (Nuc). In this paper we have further characterized NEFA and probed its evolutionary origins. Circular dichroism (CD) spectra of recombinant NEFA indicated a helical content of 51% and showed that the EF-hands are capable of binding Ca^{2+}. Experiments with recombinant NEFA and synthesized peptides revealed that the leucine zipper cannot form a homodimer. The leucine zipper may allow heterodimer formation of NEFA and an unknown protein. Phylogenetic analyses suggest that this protein is derived from a four-domain EF-hand ancestor with subsequent duplications and fusions. The leucine zipper and putative DNA-binding domains of NEFA have evolved secondarily from existing EF-hand sequences. These analyses provide insights into how complex proteins may originate and trace the precursor of NEFA to the common ancestor of eukaryotes.

Introduction

Calcium functions as a cytosolic messenger for the regulation of diverse cellular processes such as excitation, motility, proliferation, and metabolism (Kawasaki and Kretsinger 1995). The EF-hand superfamily defines a ubiquitous group of proteins that respond to changes in intracellular calcium (Nakayama, Moncrief, and Kretsinger 1992). EF-hand proteins are characterized by one or more conserved domains of length 29 amino acids with a helix-loop-helix secondary structure which are derived from an ancestral one-domain precursor found in the common ancestor of eukaryotes (CTER domain, see Nakayama, Moncrief, and Kretsinger 1992 for details). EF-hand protein families (e.g., calmodulin, troponin C, centrin) have evolved from the CTER ancestor through sometimes complex patterns of gene duplication, translocation, and splicing or by fusions with nonrelated genes; the order of these genetic events cannot always be inferred from sequence analyses (Kawasaki and Kretsinger 1995). When it is possible, the reconstruction of the evolutionary history of EF-hand proteins promises to provide basic insights into how multidomain proteins have evolved.

We have recently reported the nucleotide sequence of a protein that contains a basic region thought to bind DNA, two EF-hands, and a leucine zipper repeat (NEFA, Barnikol-Watanabe et al. 1994). NEFA and its coding region were isolated from the KM3 cell line originating from an acute lymphoblastic leukemia patient and a cDNA library derived from this cell line, respectively. NEFA shares significant sequence identity (61.56%) to the DNA/Ca^{2+}-binding protein, nucleobindin (Nuc, Miura et al. 1992). In this paper we examine the calcium-binding activity of the EF-hands and the dimerization properties of the leucine zipper repeat of NEFA and investigate, using phylogenetic methods, the evolutionary history of this and the Nuc protein. These analyses show that all domains of NEFA/Nuc are derived from a common four-domain CTER, EF-hand ancestor. A complex sequence of duplications/fusions and sequence divergence has resulted in the original Ca^{2+}-activated EF-hand protein gaining, putatively, the abilty to bind DNA and to form a heterodimer.

Materials and Methods

Analysis of NEFA

Extraction of native NEFA from a KM3 cell line was done as in Barnikol-Watanabe et al. (1994), whereas expression of the recombinant protein in *Pichia pastoris* was done according to Hirschfeld et al. (unpublished data). Non-SDS-PAGE was done according to Hames and Rickwood (1981) and the circular dichroism (CD) analysis as described in Geisler et al. (1993). Peptides were sythesized with an automatic peptide synthesizer (Pep Synthesizer 9050, Milligen, division of Millipore GmbH, Eschborn, Germany) employing the Fmoc solid phase strategy. Peptides of the leucine repeat of the NEFA, GCN4, and Fos proteins had cysteine and two glycines placed at the N-terminus (PIN) or at the C-terminus (P1C) as an indicator of helix interaction and ori-

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Abbreviations: NEFA-DNA binding, EF-hand, Acidic region, Nuc—nucleobindin, CD-circular dichroism, CTER-calmodulin, Fos-murine osteogenic sarcoma virus, GCN4—general control nonderepressible, KM3—human acute lymphoblastic leukemia cell line, MRL/1—mouse mesenteric lymph node cell line, PDMS-plasma desorption mass spectrometry.

Key words: calcium binding, EF-hand, leucine zipper, molecular evolution, NEFA, nucleobindin.

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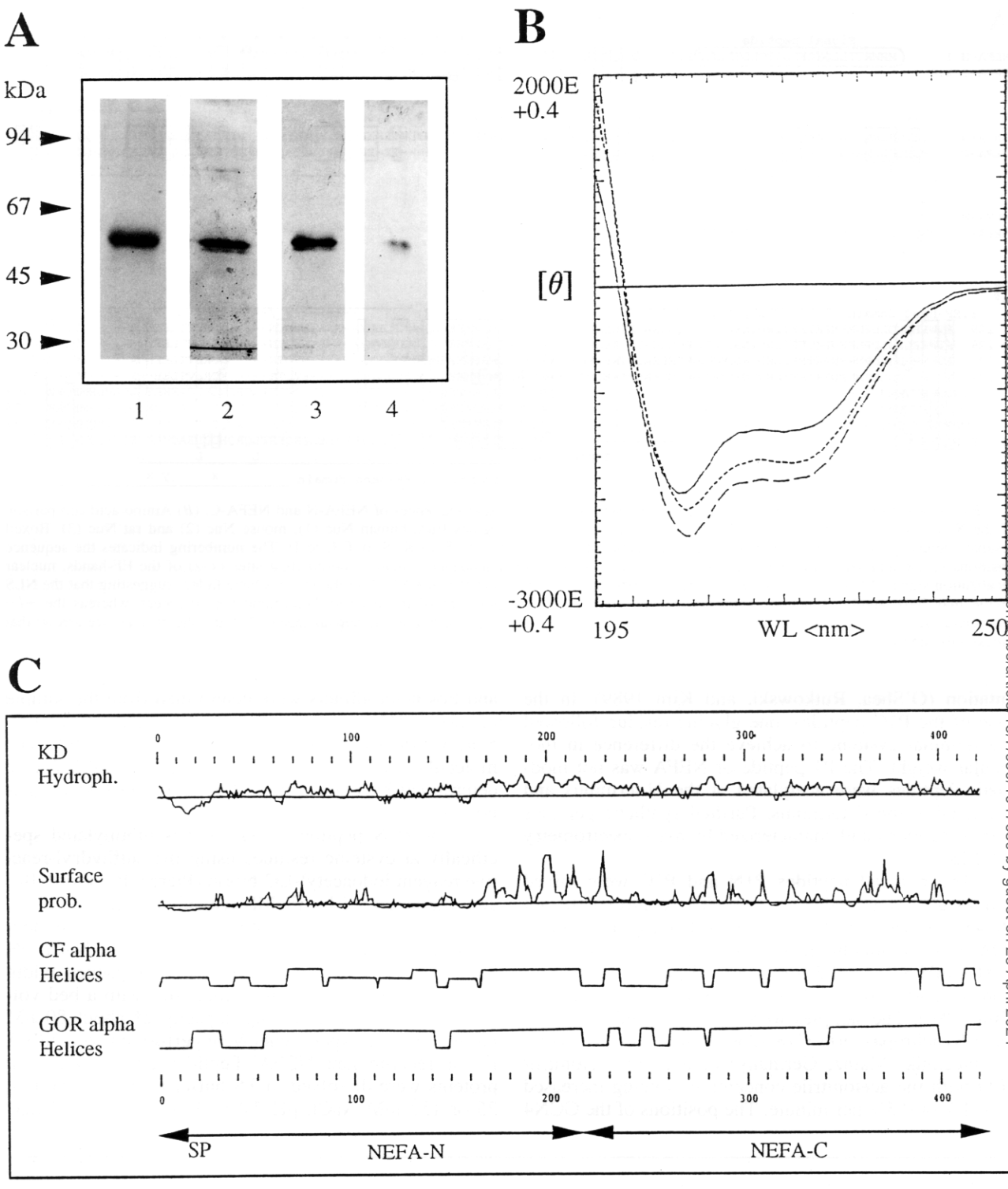

FIG. 1.—Biochemical analyses of the NEFA protein. (*A*) Lane 1: photo of SDS-PAGE gel of purified cytoplasmic NEFA, used as a control; lanes 2 and 3: photo of SDS-PAGE gel and corresponding immunoblot of purified recombinant NEFA (unpublished data); lane 4: photo of immunoblot of recombinant NEFA after non-SDS-PAGE using a nondissociating buffer system; this result suggests that NEFA cannot self-interact. (*B*) Circular dichroism spectroscopy of the recombinant NEFA protein measured in the presence of 1 mM EDTA (solid line), 1 mM $CaCl_2$ (dashed line) and 5 mM $CaCl_2$ (dotted line). The molar ellipticity was calculated from the mean residue molar mass of 46,345 Da calculated with Lysin-34 as the N-terminal residue. The reduction of helicity at high Ca^{2+} concentrations probably reflects the ability of the helical non-EF-hand regions of NEFA to weakly bind Ca^{2+}. (*C*) Secondary structure predictions and hydropathy analysis of NEFA using the GCG software package. Note the similar pattern of helical regions within NEFA-N and NEFA-C. The acronym SP denotes the position of the signal peptide.

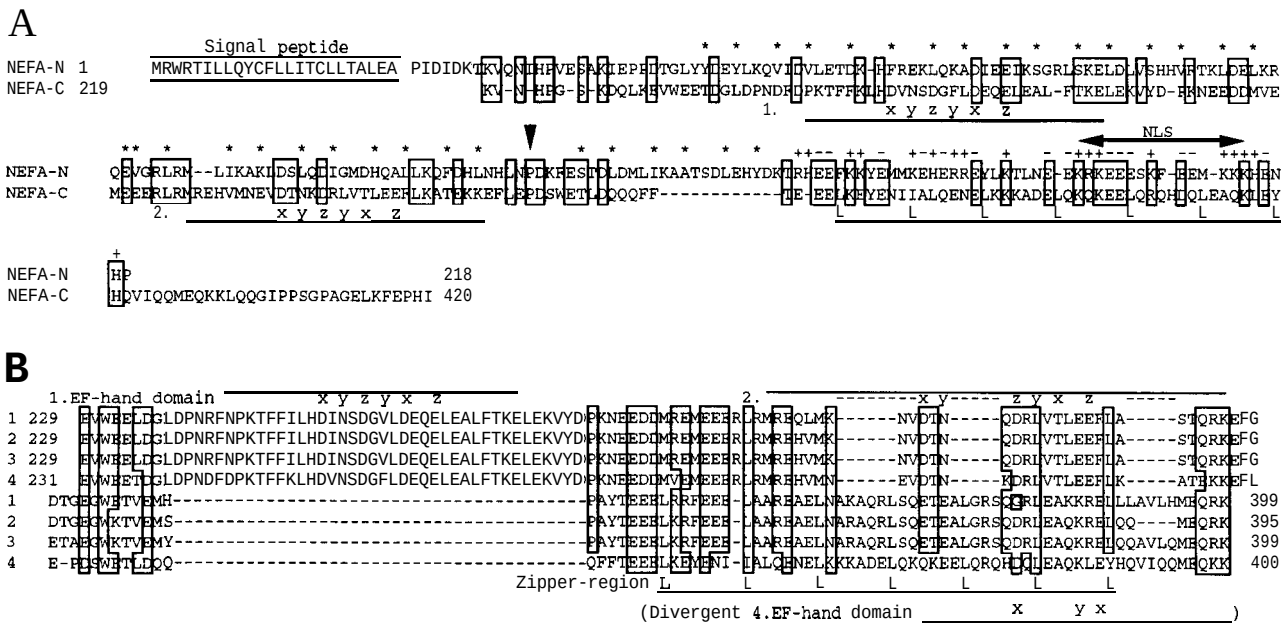

FIG. 2.-Sequence analysis of NEFA. ***(A)*** Comparison of the amino acid sequences of NEFA-N and NEFA-C. (*B*) Amino acid comparison of the N- and C-terminal halves of NEFA-C (4) with the homologous regions from human Nuc (I), mouse Nuc (2) and rat Nuc (3). Boxed residues denote identical amino acids or conserved substitutions (i.e., D to E, R to K, S to T, L to I). The numbering indicates the sequence positions of the aligned amino acids in NEFA/Nuc. The putative signal sequence, calcium coordinating sites (x-z) of the EF-hands, nuclear localization signal (NLS) and leucine zipper (L) are also shown. Attempts to localize NEFA in the nucleus have failed, suggesting that the NLS region may have another function. Asterisks (*) indicate the **a** and **d** positions of the putative N-terminal heptad repeat, whereas the +/- characters identify the hydrophilic region of NEFA-N. Note the change of phase within the heptad repeat (**) and the proline (see arrow) that breaks this repeat.

entation (O'Shea, Rutkowski, and Kim 1989). In the case of the P1C peptides, one glycine residue followed the terminal cysteine to achieve the difference in molecular weight. The P1 peptide of NEFA was not modified. Peptides were acetylated at the N-terminus and amidated at the C-terminus. Purified synthetic peptides were sequenced and characterized by mass spectrometry (PDMS).

The reduced peptides P1N and P1C were mixed and incubated in either 2 mM HCl (control data not shown) or redox buffer (1 mM oxidized glutathione, 1 mM reduced glutathione, 0.2 M KCl, 0.1 M Tris, 1 mM EDTA, pH 8.7) for 1.5 hours at total peptide concentrations of 340, 170, and 34 μM (O'Shea, Rutkowski, and Kim 1989). The samples were then analyzed by HPLC on a Synchropak 300 Cl8 (250 X 4.6 mm, MZ-Analyzentechnik, Mainz, Germany) column in acetonitril/H_2O with the acetonitrile concentration being increased by 0.1% or 0.5% per minute. The positions of the GCN4 and Fos heterodimers were determined from the sample of mixed P1N and P1C peptides that were oxidized by 5 mM oxidized DTT/10 mM Tris in 5N GuHCl. The molecular masses of all species detected by HPLC were determined by PDMS, non-SDS, and SDS-PAGE (data not shown).

The P1N peptide of NEFA was biotinylated specifically at cysteine residues using the sulfhydryl-reactive reagent iodoacetyl-LC-biotin (Pierce, Rockford, Ill.) and characterized by PDMS (calculated M_r was 4,613, determined 4613,4) and amino acid analysis. The peptides, dissolved in 10 mM Tris and 150 mM NaCl, pH 7.4 (TS buffer), were passed through a prepacked avidin agarose column (Pierce, Rockford, Ill.) with a bed volume of 1 ml and a binding capacity of about 80 nM biotin. Binding experiments were performed with NEFA P1 or recombinant NEFA. Peptides and recombinant proteins were dissolved in TS buffer (5 or 10 mM Tris, 75 or 150 mM NaCl, pH 7.4 with addition of 0.5 mM

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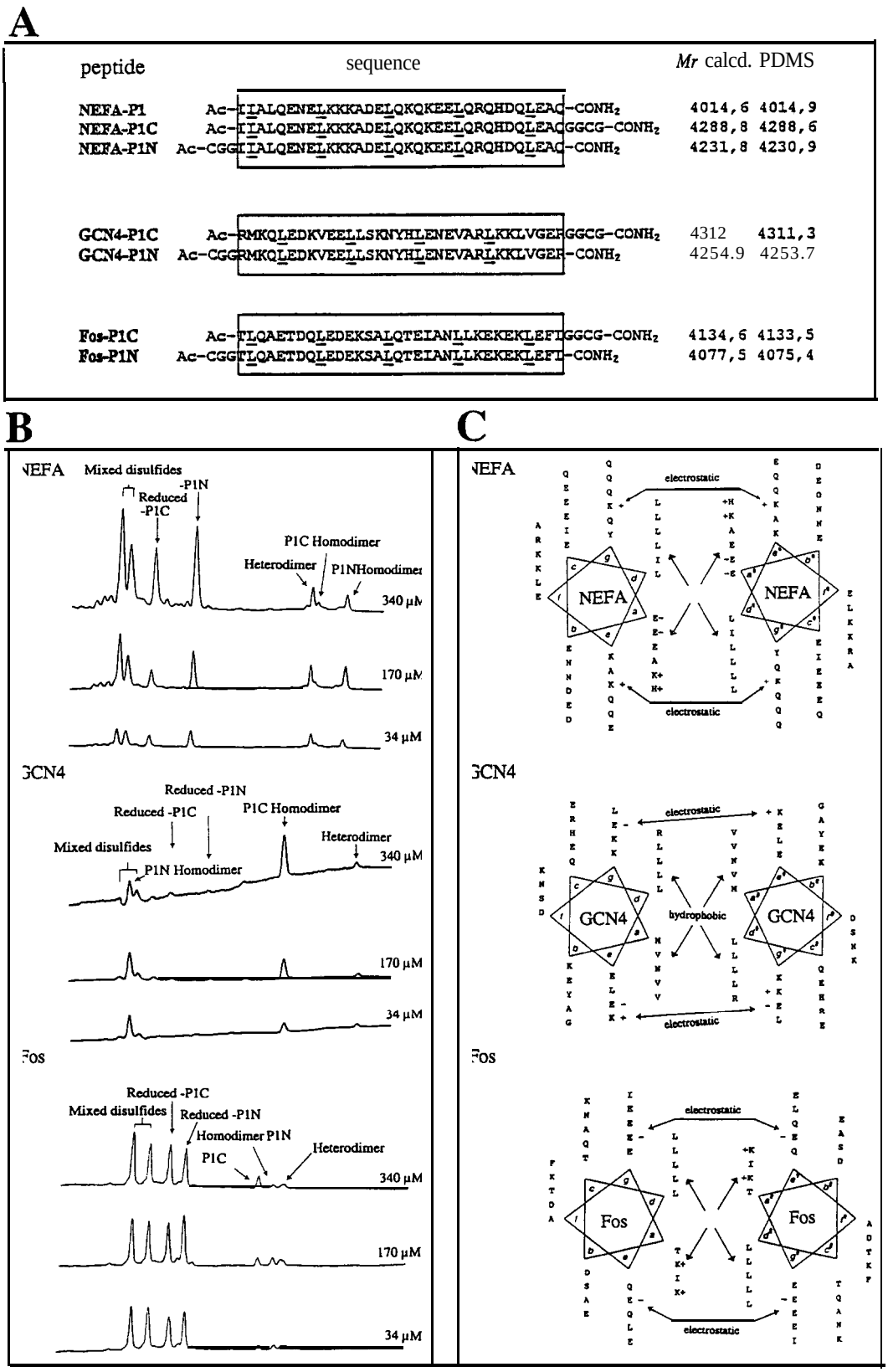
FIG. 3.-Structural analysis of the leucine zipper repeat peptides. ***(A)*** Amino acid sequences and molecular masses calculated (M_r calculated) or determined by PDMS of the synthesized leucine zipper peptides. The leucines (isoleucines) that define the proposed leucine zipper structure are underlined. All peptides were acetylated at the N-terminus and amidated at the C-terminus. The Gly-Gly-Cys (Gly) modifications were necessary to stabilize the dimerization products by the formation of disulfide bonds and to differentiate between parallel and antiparallel dimerization by PDMS. (*B*) High-performance liquid chromatography (HPLC) of synthesized NEFA, GCN4, or Fos (PIN and P1C) leucine zipper peptides after incubation in redox buffer. P1N and P1C peptides with free cysteines were incubated for 1.5 h in the redox buffer described by O'Shea, Rutkowski, and Kim (1989) at decreasing concentrations (340-34 pm). Nearly complete dimerization in parallel orientation based on specific helical interactions was observed with GCN4 peptides. Antiparallel (hetero) dimerization was negligible. In contrast, the monomers of Fos and NEFA peptides represent the main species at all concentrations. (*C*) Helical wheel representation of parallel coiled coils of NEFA, GCN4, and Fos leucine zipper depicted according O'Shea, Rutkowski, and Kim (1989) and Schuermann et al. (1991). The view is from the N-terminus toward the C-terminus. Ionic and hydrophobic interactions (positions **a, d, e,** and g) determined by X-ray crystallography



for GCN4 (O'Shea et al. 1991) or biochemically and genetically for GCN4, Fos, and **Jun** (Schuermann et al. 1991; O'Shea, Rutkowski, and Kim 1992) are transferred to the parallel model of the NEFA domain. The NEFA, GCN4, and Fos sequences correspond to residues 344-385, 250–281, and 164–196, respectively (fig. 2*A*). Electrostatic attraction or repulsion in the corresponding positions are marked by arrows. Note the strong electrostatic repulsion in the position d for the parallel NEFA and Fos LZ-dimer as well as no electrostatic attraction in positions e and g' and vica versa there, shown to stabilize interhelical interactions of GCN4 dimer or Fos/Jun heterodimer. These could explain the pure dimerization properties without the orientation preference of NEFA LZ-peptides, in contrast to that of GCN4.

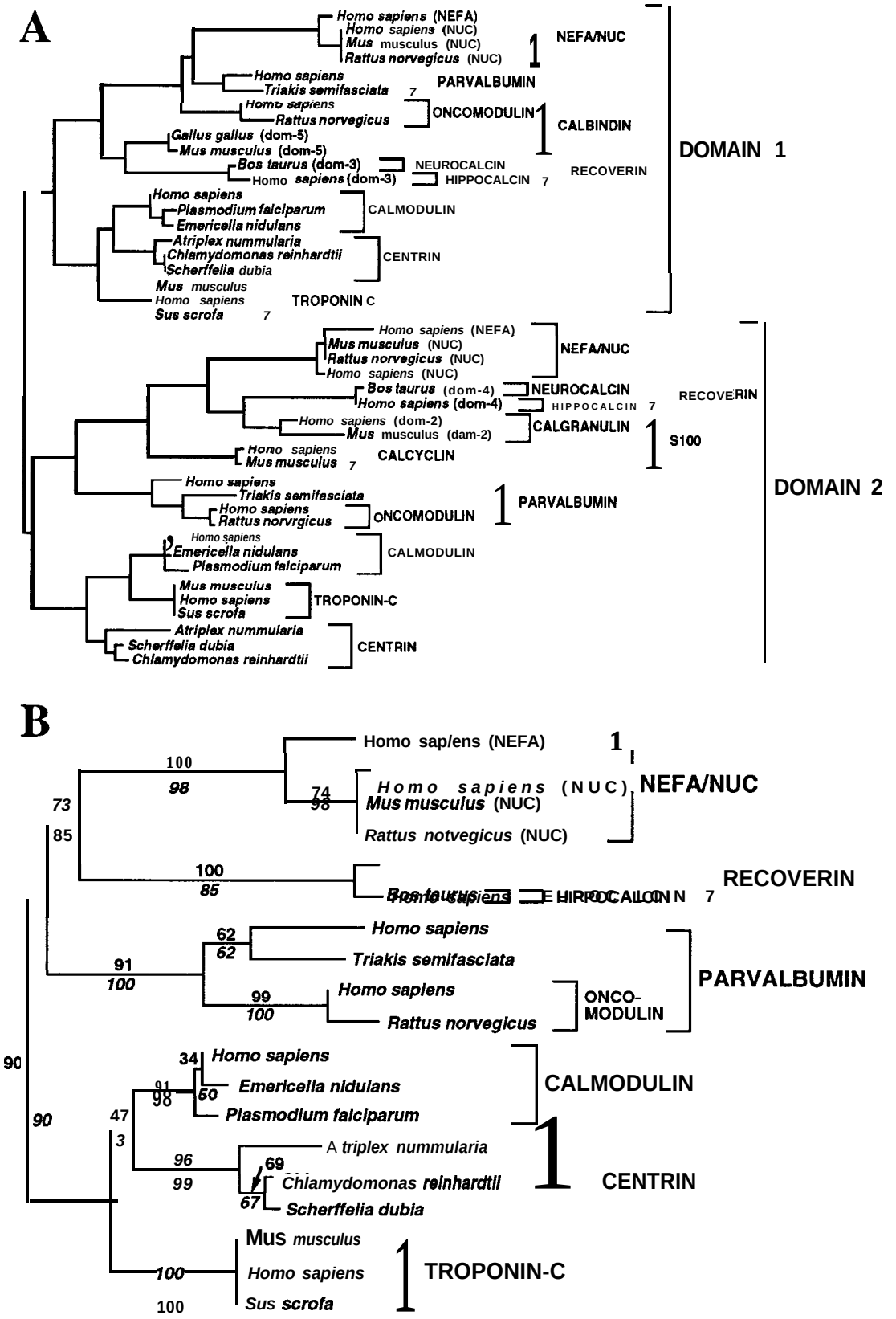


FIG. 4.-Phylogenetic analyses of NEFA/Nuc. *(A)* Majority rule consensus phylogram of four equally parsimonious trees of individual, congruent EF-hand domains from CTER proteins (Nakayama, Moncrief, and Kretsinger 1992) inferred with a weighted maximum-parsimony method (PAUP V3.1.1, Swofford 1993). This tree has a consistency index of 0.72. (B) Majority rule consensus phylogram of a weighted bootstrap (1,000 replications) maximum-parsimony analysis (PAUP V3.1.1, Swofford 1993) of domains 1 and 2 together (and other congruent domains) of CTER proteins and NEFA/Nuc. This tree has a consistency index of 0.88. The bootstrap values inferred from this analysis are shown above the internal nodes. The bootstrap values (1,000 replications) shown in italic script below the internal nodes were inferred from a

EDTA or $CaCl_2$) and passed through the columns. Columns were washed with 5 ml of the TS buffer and bound material was eluted with 2 ml of 0.1 M glycin, pH 2.2; with 0.1 M diethylamine, pH 11.5; 0.5 M NaCl; or with 5 mM of EDTA or $CaCl_2$. Peptides and proteins were then analyzed by HPLC.

Phylogenetic Analyses

The amino acid sequences of the two conserved EF-hand domains from NEFA were used to query the GenBank/EBI database using the BLITZ program (Sturrock and Collins 1993). Individual EF-hand sequences identified with this search were aligned and these regions (29 amino acids) were submitted to a weighted (rescaled consistency index over an interval of 1–1,000) maximum-parsimony phylogenetic analysis (PAUP V3.1.1, Swofford 1993). Random additions (10 cycles) of the data were analyzed with a heuristic method with a branch-swapping algorithm (tree bisection-reconnection, TBR).

A weighted maximum-parsimony analysis was done with the domain 1 + domain 2 data (58 amino acids) using a heuristic search method with a branch-swapping algorithm (TBR) to infer the evolutionary relationships of the EF-hand proteins. A bootstrap analysis (1,000 replications) was used to infer a majority rule consensus phylogram that was rooted with the calmodulin/centrin/troponin C sequences as outgroup. The EF-hand sequences were also analyzed with a bootstrapped (1,000 replications) distance method (PROTDIST, Dayhoff distance matrix, PHYLIP V3.5, Felsenstein 1993). Accession numbers of the EF-hand sequences used in this study are as follows: NEFA-Homo ***sapiens,*** X76732; ***Nut-H. sapiens,*** *Mus* ***musculus, Rattus*** *norvegicus,* M96824, M96823, Z36277, respectively; Neurocalcin-Bos ***taurus,*** P29554; Hippocalcin-H. ***sapiens,*** P4 12 11; Parvalbumin-H. ***sapiens, Triakis*** *semifasciata,* P20472, P30563, respectively; Oncomodulin—***H. sapiens, R. norvegicus,*** P32930, ***PO263 1,*** respectively; Calmodulin—*H.* ***sapiens, Emericella nidulans,*** *Plasmodium* ***falciparum, P02594,*** P19533, P24044, respectively; Centrin—*Atriplex* ***nummularia,*** *Scherffelia* ***dubia, Chlamydomonas reinhardtii, P4 12 10, P05434,*** Q06827, respectively; Troponin C—*M.* ***musculus, H. sapiens, Sus scrofa,*** P20801, ***P02585, PO2587,*** respectively; Calbindin—*Gallus gallus, M.* ***musculus, P04354, Pl2658,*** respectively; Calgranulin-H. ***sapiens, M. musculus,*** P06702, ***P3 1725,*** respectively; Calcyclin-H. ***sapiens,*** *M.* ***musculus,*** P06703, P14069, respectively.

Results and Discussion

Analysis of NEFA

The CD spectroscopy of NEFA, using the recombinant protein expressed in ***P.*** *pastoris* ***(fig.*** 1*A*), showed the CD spectrum to be dominated by α-helices. The ellipticity of − 19,000" $cm^2\,dmol^{-1}$ at 208 nm predicted an a-helical content of about 51% using the formula of Greenfield and Fasman (1969). This value increased to 65% on addition of 1 mM $CaCl_2$ (fig. 1*B*); NEFA is, therefore, capable of interacting with Ca^{2+}, leading to an alteration of its secondary structure.

Inspection of the amino acid sequence of the predicted (Chou and Fasman 1978; Gamier, Osguthorpe, and Robson 1978) N-terminal helices (NEFA-N [fig. 1*C*], residues l-218) revealed that the first region (44-169, interrupted by Proline-147, shown with an arrow in fig. 2A) comprised a putative heptad repeat in which 75% of residues in the **a** and **d** positions of the heptad repeat cycle (shown with asterisks [*] in fig. 2A) as well as 28% of the remaining residues are apolar (Cohen and Parry 1986). These hydrophobic residues may directly, or through protein-protein contacts, mediate the NEFA/cell membrane interaction (Barnikol-Watanabe et al. 1994). This result is in agreement with our preliminary electron microscopic analyses which show that recombinant NEFA is not capable of filament formation (unpublished data). The second region had a high proportion of hydrophilic residues (over 70%) as revealed by hydropathy (Kyte and Doolittle 1982) and surface probability analyses (fig. 1*C*) and contained a bipartite nuclear localization signal (Dingwall and Laskey 1991; fig. 2A). This region is likely to bind DNA by analogy with the closely related Nuc protein (Miura et al. 1992; Barnikol-Watanabe et al. 1994). The C-terminal helical region (NEFA-C [fig. 1*C*], residues 219-420) encoded two conserved EF-hands (245-325) followed by a leucine zipper (344-392); the latter domain contained a repeat with the apolar residues in the **d** but not in the **a** position.

To study the role of the proposed leucine zipper of NEFA, two peptides (P1N, P1C) corresponding to the 33 amino acids of this repeat and those of the leucine zippers of GCN4 and Fos proteins (used as controls) were synthesized with N- and C-terminal modification (fig. 3A and ***Materials and Methods).*** The CD intensity at 208 nm indicated that folded NEFA peptides are >70% helical, whereas those from GCN4 and Fos are >90% and >80% helical, respectively (data not shown). Biochemical analyses of the leucine zipper repeat peptides (see fig. 3A) of GCN4 revealed formation of concentration-independent parallel oriented dimers. The monomeric GCN4 peptides were not observed, indicating complete dimerization. A small amount of GCN4 heterodimers (antiparallel form) was detected, which presumably is due to an unspecific nonhelical interaction (fig. 3B). In the case of the Fos leucine zipper peptides, the monomers were the dominant fractions at all concentrations of peptides studied. In addition, the small amount of dimers (mainly parallel oriented) was not concentration-dependent and could be observed if the peptide concentration was below 34 μM (fig. 3B). These results were also reported by O'Shea, Rutkowski, and

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distance analysis of the EF-hand sequences using the program PROTDIST (Dayhoff distance matrix, PHYLIP V3.5, Felsenstein 1993). The arrow is used to show a bootstrap value that did not fit on the branch length.

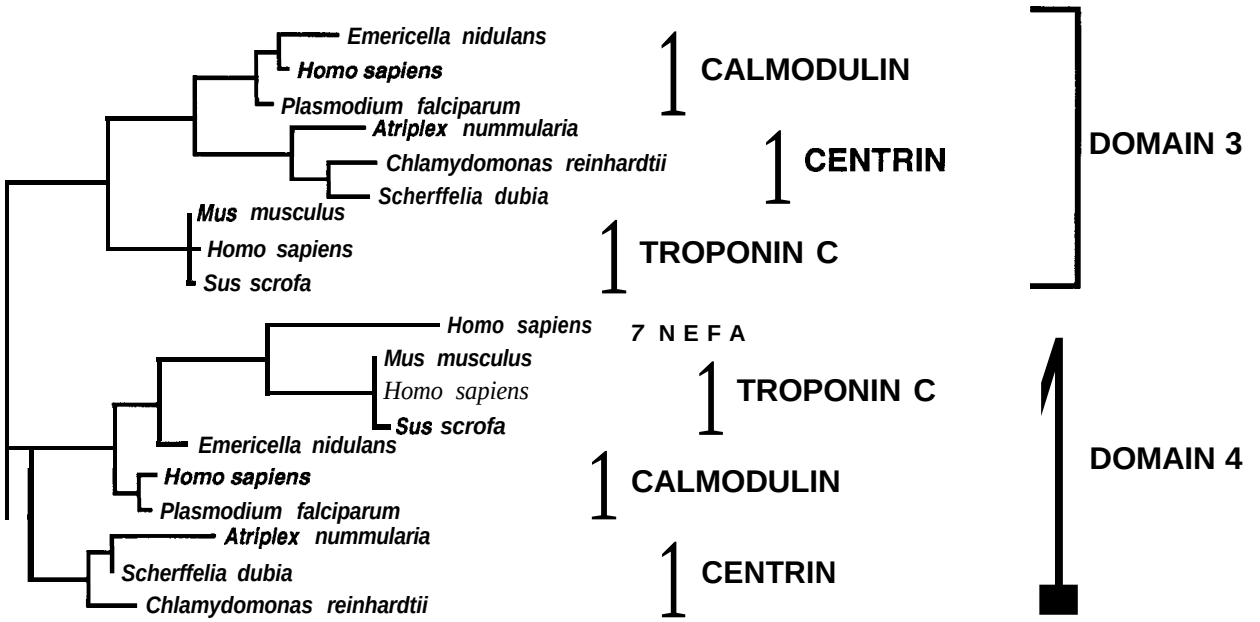

FIG. 5.—Phylogenetic analysis of the fourth EF-hand domain of NEFA-C. This tree is the majority rule consensus phylogram of three equally parsimonious trees of individual, congruent (Nakayama, Moncrief, and Kretsinger 1992) third and fourth EF-hand domains from CTER proteins and the fourth EF-hand domain of NEFA-C inferred with a weighted maximum-parsimony method (PAUP V3.1.1, Swofford 1993). This tree has a consistency index of 0.87.

Kim (1989) and Schuermann et al. (1991). In the case of the NEFA zipper peptides, the monomer was the dominant fraction but, in contrast to the Fos peptides, slightly stronger dimerization with no orientation preference was detected (fig. 3B).

In the experiment with the biotinylated P1N peptides of NEFA, there was no indication of an affinity of the studied material to the immobilized peptide matrix (data not shown). In addition, only one monomeric band of recombinant protein was observed in non-SDS-PAGE using a nondissociating buffer system (fig. 1*A*). In light of these results, and considering the unfavorable charge characteristics of the NEFA zipper (fig. 3*C*), we conclude that the NEFA leucine zipper cannot drive the self-assembly of this protein or promote stable homodimer formation. We suggest that the leucine zipper of NEFA may form a heterodimer, as does the Fos protein, and that NEFA is not a typical leucine zipper (i.e., as in GCN4).

Evolutionary Analyses of NEFA

The maximum-parsimony analysis of the first and second domain sequences resulted in four equally parsimonious phylograms. The strict consensus of these trees (fig. 4A) demonstrates congruence (Nakayama, Moncrief, and Kretsinger 1992) of the NEFA/Nuc EF-hand domains with the first two domains of members of the CTER family (i.e., calmodulin, troponin C, and essential and regulatory light chains of myosin) used in our study (fig. 4A). Congruence of EF-hand domains supports a direct common ancestry of all these proteins from a four-domain precursor (i.e., the common ancestor of the CTER family) without subsequent domain shuffling (Nakayama, Moncrief, and Kretsinger 1992; see also Doolittle 1995, for a review about protein domain shuffling). The NEFA/Nuc EF-hand sequences were positioned as relatively late divergences within the CTER-derived family of EF-hand proteins. The tree shown in figure 4A is a rooted phylogeny since the domains act as mutual outgroups of each other.

Phylogenetic analyses of the two NEFA/Nuc domains together with other homologous EF-hand domains positioned NEFA/Nuc as an independent lineage within the EF-hand superfamily with bootstrap analyses, suggesting (73% and 85% in the maximum-parsimony and distance analyses, respectively) a most recent common ancestry with the C-terminal region (domains 3 and 4) of recoverin-related proteins (fig. 4B). The sister group relationship of NEFA/Nuc and recoverins is interpreted as a provisional result, since the analyses of the individual EF-hand domains do not support this topology. Taken together, these results led us to hypothesize that the third and fourth EF-hand domains of the four-domain precursor of the CTER subfamilies (with the exception of the calbindin and recoverin-related proteins, shown to be only partly congruent with this precursor; Nakayama, Moncrief, and Kretsinger 1992) may comprise the remaining C-terminal sequence of NEFA-C. A BLITZ search done with the C-terminal sequence of NEFA against the GenBank/EBI database identified a region with significant similarity (31% identity, 68% similarity) to the fourth EF-hand domain of calmodulin from *Emericella nidulans.* Sequence conservation patterns, in an alignment of the leucine zipper and remaining C-terminal sequence of NEFA-C with the predomain, l-2 interdomain (linker) region and second EF-hand in the N-terminal region of NEFA-C, supported an evolutionary relationship between these divergent sequences (fig. 2B). We suggest that the ancestral third

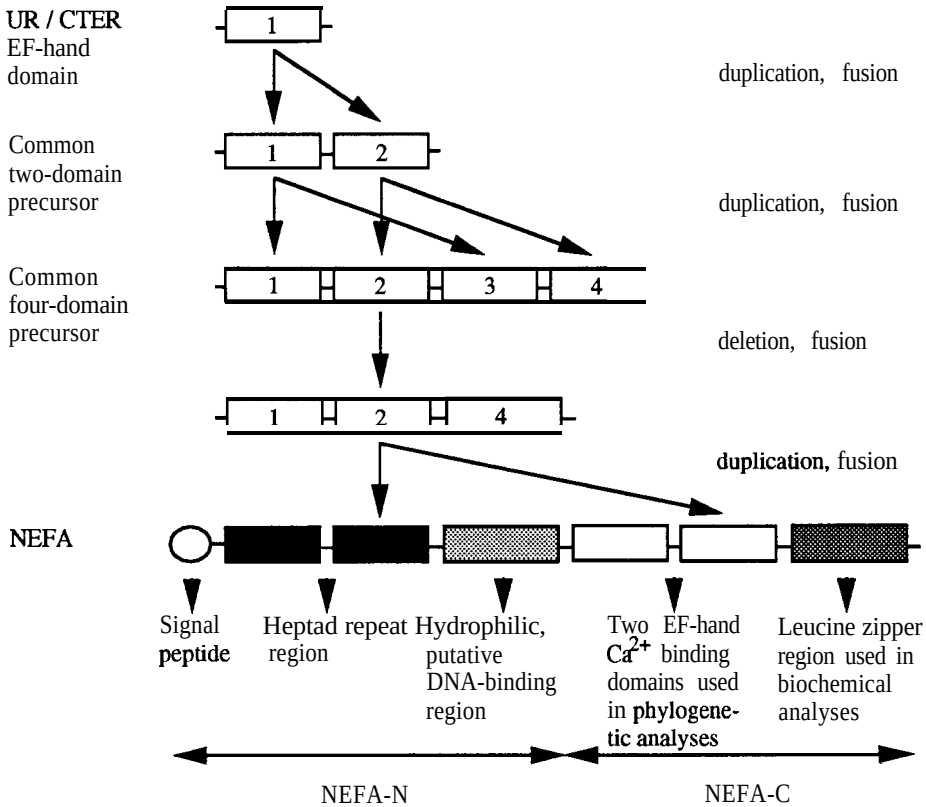

FIG. 6.-Proposed scheme for the evolution of NEFA on the basis of phylogenetic (Nakayama, Moncrief, and Kretsinger 1992) and sequence analyses.

EF-hand domain of NEFA-C has been deleted, whereas the remaining sequence has evolved into a leucine zipper. Furthermore, both dot plot experiments (data not shown) and secondary structure predictions (fig. 1*C*), using the NEFA protein sequence, suggest that NEFA-N was derived from a gene duplication and fusion involving NEFA-C (fig. 2A); these same results were found in analyses of the Nuc protein.

To further study the origin of the remaining (fourth) EF-hand domain of NEFA-C, a phylogenetic analysis was done with this sequence (shown as divergent 4.EF-hand domain in fig. 2B) and those of the third and fourth EF-hand domains of members of the CTER family with the maximum-parsimony method. The results of this analysis (fig. 5) provide phylogenetic evidence for a close evolutionary relationship between the divergent fourth EF-hand domain of NEFA-C and the fourth domain of troponin C and this EF-hand sequence from other members of the CTER family. Analysis of these data with the PROTDIST program gives essentially the same results. The domain 3 and domain 4 EF-hand sequences form independent groupings in the distance analysis but the NEFA fourth domain clusters with the *E. nidulans* calmodulin sequence and not with the troponin C fourth EF-hand domains as in figure 5. Bootstrap analyses were not attempted with these limited sequence data (29 amino acids).

The Origin of NEFA

With these results in mind we have constructed a hypothetical scheme showing the origin of NEFA (fig. 6). The important features of this scheme are the direct common ancestry of NEFA with the UR (Nakayama, Moncrief, and Kretsinger 1992) and then the CTER four-domain ancestor followed by a domain deletion/duplication of this product/fusion, leading to NEFA-N and NEFA-C, putative fusion of a signal peptide sequence at the N-terminus, maintenance of two functional EF-hands at the N-terminal region of NEFA-C, and sequence divergence over the remaining protein, leading to the independent origins of a heptad repeat domain, a putative DNA-binding region and a leucine zipper. This precursor then gave rise via a gene duplication to the NEFA and Nuc proteins. The Nuc coding region subsequently gained a proline-rich C-terminal extension of an approximate length of 30 amino acids (Miura et al. 1992). The NEFA/Nuc coding regions form an unbroken line from the first CTER coding region to the now-complex membrane-associated and/or secretory proteins that are still Ca^{2+}-activated (Miura et al. 1992; Barnikol-Watanabe et al. 1994; Wendel et al. 1995) but can also bind DNA (as shown for Nuc in KML-7 cells derived from a lupus-prone MRL/1 mouse, Miura et al. 1992; Kanai et al. 1995). Solving the function of NEFA/Nuc in the cell poses the next challenge in this study. Recent evidence showing a specific interaction between human Nuc and the G protein, $G_{\alpha i2}$, suggests that these peptides form a heteromer (Mochizuki et al. 1995). Given the close evolutionary relationship between NEFA and Nuc, it is possible that NEFA also interacts with another protein; this hypothesis would explain our finding that

NEFA is not capable of forming a homodimer with its leucine zipper region. These data also suggest the interesting possibility that NEFA/Nuc, through their interaction(s) with G proteins, may play an important role in intracellular Ca^{2+} regulation (Putney and Bird 1993). In any case, the present analyses show that ancestral EF-hands may form templates that give rise to diverse protein functions, and we suggest that other proteins may also trace their origins to a now divergent EF-hand found in the ancestral eukaryote.

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