Frequenin, a member of a large family of myristoyl-switch calcium-binding proteins, functions as a calcium-ion sensor to modulate synaptic activity and secretion. We show that human frequenin colocalizes with ARF1 GTPase in COS-7 cells and occurs in similar cellular compartments as the phosphatidylinositol-4-OH kinase PI4Kβ, the mammalian homolog of the yeast kinase PIK1. In addition, the crystal structure of unmyristoylated human frequenin has been determined and refined to 1.9 Å resolution. The overall fold of frequenin resembles those of neurocalcin and the photoreceptor, recoverin, of the same family, with two pairs of calcium-binding EF hands and three bound calcium ions. Despite the similarities, however, frequenin displays significant structural differences. A large conformational shift of the C-terminal region creates a wide hydrophobic crevice at the surface of frequenin. This crevice, which is unique to frequenin and distinct from the myristoyl-binding box of recoverin, may accommodate a yet unknown protein ligand.

Frequenin (Frq), or neuronal calcium-sensor 1, is a member of a family of related calcium-myristoyl-switch proteins that have been proposed to function as calcium-ion sensors. Members of this family include recoverin, GCAP, neurocalcin, visinin, and others (1). Recoverin and GCAP have been implicated in multiple membrane trafficking events including the recruitment of the mammalian PIK1 homolog, PI4Kβ, to the Golgi membrane (9). Here we show that human Frq (HuFrq) colocalizes with ARF1 in COS-7 cells and occurs in similar cellular localizations as PI4Kβ. In addition, in a further step toward understanding the cellular function of Frq, we report the crystal structure of unmyristoylated Ca2+-bound human Frq (HuFrq) refined to 1.9 Å resolution. This structure confirms that frequenins belong to the large family of myristoyl-switch Ca2+-binding proteins and reveals the architecture of the Ca2+-binding sites. Most importantly, comparative analysis of the HuFrq structure with those of neurocalcin and recoverin highlights a unique wide crevice and a solvent-exposed carboxyl terminus that could be responsible for ligand recognition and account for the broad substrate specificity among members of the family.

EXPERIMENTAL PROCEDURES

HuFrq Cloning, Expression, and Purification—Human poly(A)RNA was isolated from HER293 cells using the Fast Track II Kit (Invitrogen), and cDNA was synthesized with Superscript II Reverse Transcriptase (Life Technologies, Inc.). The HuFrq-encoding cDNA was amplified in a polymerase chain reaction (PCR) using Pfu Turbo-Polymerase (Life Technologies, Inc.) with the first strand cDNA as template and the primers 5'-ATACATGCGGAAATCCCAAAGC-3' (sense) and 5'-CTATACACGCTCGTTCGAGGG-3' (antisense). Primer sequences were derived from the HuFrq nucleotide sequence.
HuFrq (Ncs1) Crystal Structure

The expression plasmid pET-HuFrq was transformed into Escherichia coli strain BL21(DE3) (Novagen). Transformed cells were grown in Luria-Bertani (LB) medium containing ampicillin (100 μg/ml) at 37 °C. HuFrq expression was induced overnight at an A600 of 0.8 with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and reached average yields of 20 mg/liter. Cells were harvested by centrifugation, resuspended in 20 ml of lysis buffer (50 mM HEPES, pH 7.4; 100 mM KC1; 1 mM DTT; 1 mM diithiothreitol; 1 mM MgCl2) per 1 liter culture, and lysed in a French pressure cell. Proteamine sulfate was added to a final concentration of 0.1% for 10 min and then the lysate was cleared by centrifugation (40,000 × g, 30 min, 4 °C). The supernatant was filtered (0.45 μm), adjusted to 1 mM CaCl2, and applied to a 15-ml phenyl-Sepharose CL-4-B column (Amersham Pharmacia Biotech). The column was washed with buffer A (20 mM Tris/HCl, pH 7.9; 1 mM MgCl2; 1 mM DTT; 10% (v/v) ethylene glycol, flash-cooled at 100 K in the nitrogen gas stream and stored in liquid nitrogen. No single crystal could be selected for form C. Data for forms A and B were collected on beamline ID14-EH2 of ESRF (Grenoble, France). Oscillation images were integrated with DENZO (12) and scaled and merged with SCALA (13). Amplitude factors were generated with TRUNCATE (13). Form A crystals were found to be twinned and the collected data could not be used.

Structure Determination and Refinement—Initial phases for form B crystals were obtained by molecular replacement using the structure of neurocalcin (14) (PDB code 1BJF) as a search model with the AMoRe package (15), giving a correlation coefficient of 36% and a R-factor value of 48% in the 15 to 4 Å resolution range. Rigid-body refinement, performed on each molecule with CNS (16) using data between 20 and 3 Å, gave an R-factor of 49%. For 2% of the reflections against which the model was not refined, R-free was 48%. The model was refined to 1.9 Å resolution using CNS, including bulk solvent and anisotropic B-factor corrections; the resulting 2Fo−Fc and Fo−Fc electron density maps were used to correct the model with the graphics program TURBO-FRODO (17). Solvent molecules automatically added using CNS were carefully examined on the graphics display. The final model comprises residues Asn2−Val190 and Asn2−Gly188, respectively, for the two molecules in the asymmetric unit. High temperature factors and weak electron density are associated with residues 1–7, 49–60, and 133–138. The average r.m.s.d. between the two HuFrq molecules is 0.6 Å for 182 C atoms.

RESULTS AND DISCUSSION

Chromosomal Localization—A search in the HTGS data bank with the HuFrq cDNA sequence revealed that the HuFrq gene is located on chromosome 9. A comparison of the publicly available chromosome 9 DNA sequence with the frequenin cDNA sequence showed that the exon-intron organization between Drosophila (4) and HuFrq genes has been conserved. Both open reading frames are interrupted by the same exon-intron borders and each are composed of same 8 exons (Fig. 1A). About 50 kilobases upstream of the first Freq

| TABLE I Data collection and refinement |
|----------------------------------------|
| Crystal form | B |
| Resolution (Å) | 20–1.9 |
| No. of observations | 251,683 |
| No. unique | 35,881 |
| Rmerge (%) | 6.0 (55) |
| LipL (%) | 8.9 (2.0) |
| Redundancy | 3.3 |
| Completeness (%) | 98.9 (98.9) |
| Rmerge (%) | 0.216 |
| bond angles (°) | 1.7 |
| dihedral angles (°) | 21.3 |
| improper angles (°) | 1.2 |
| Mean B factors (Å²) | 26/30 |
| main/side chain | 34/22 |
| solvent/Ca²⁺ | 1.6 |
| Rmerge (%) | 2.3 |
| R-factor = | | |
exon are located several STS markers, e.g. DGS1924, A001W37, STSG22304, placing the Frq gene at 9q34.11. To our knowledge, a human disease has not been associated yet with this locus.

Molecular Identification and Immunocytochemical Localization—Previously, mammalian homologs of Drosophila Frq have been cloned (22, 23). We have used this information to clone HuFrq from human first strand cDNA (Fig. 1A). The predicted HuFrq sequence contains 190 amino acids (Fig. 1B) with a theoretical monoisotopic mass of 21,865.91 and exhibits four EF hand motifs that represent potential Ca\textsuperscript{2+}-binding domains. In HuFrq, as in other members of the family, the first of the four EF hand motifs is not likely to be a functional Ca\textsuperscript{2+}-binding site as it lacks two Ca\textsuperscript{2+}-coordinating amino acids. The HuFrq N terminus contains the consensus sequence MGXXX(S/T)K for myristoylation (24); hence it could be myristylated like the rat homolog (5). The HuFrq sequence is 100% homologous to those of rat (5) and mouse (23) and it differs by a single amino acid from that of Xenopus (25). Remarkably, the yeast (6) and HuFrq protein sequences also show a high degree of conservation: 143 of 190 amino acids (75%) are either identical or correspond to conservative replacements (Fig. 1B).

In yeast, Frq has been shown to stimulate the activity of the PtdIns-4-OH kinase PIK1 (6), an enzyme that is essential for normal secretion, Golgi and vacuole membrane dynamics, and endocytosis (7). Xenopus Frq rescues a yeast Frq deletion mutant, indicating that Frq from higher eukaryotes is able to fulfill similar functions like yeast Frq (25). Consistent with the functional role of Frq in yeast are the phenotypes that have been described for Drosophila mutants (4) and for mammalian cells overexpressing Frq (5). In both cases, it appears that evoked secretion is stimulated by Frq (26).

The activity of PI4K\textbeta, the likely mammalian homolog of yeast PIK1 (26), is recruited by the small GTPase ARF1 to the Golgi and contributes to the regulation of Golgi membrane dynamics and Golgi-dependent vesicle formation (9). Accordingly, in immunocytochemical experiments we have compared the immunostaining patterns obtained with anti-PI4K\textbeta, anti-ARF1, and anti-HuFrq antibodies, respectively; overlapping
immunostaining reactions were observed (Fig. 2). For comparison, we also included in our investigations experiments with anti-γ-adaptin antibodies, a typical trans-Golgi network (TGN) marker. Paraformaldehyde-fixed COS-7 cells were first incubated with primary antibodies, e.g. polyclonal anti-HuFrq rabbit antibodies, monoclonal anti-γ-adaptin mouse antibodies, polyclonal anti-PI4Kβ rabbit antibodies, and polyclonal anti-ARF1 goat antibodies, respectively. Then, we used secondary Cy2-, Cy3-, or Cy5-labeled antibodies for immunocytofluorescent staining and localization of γ-adaptin, HuFrq and PI4Kβ, and ARF1, respectively, using confocal microscopy (Fig. 2). The anti-HuFrq antibodies revealed a pattern with a crescent of staining on one side of the nucleus and some punctuate staining within the cytoplasm (Fig. 2A). Staining could be eliminated by preincubation of the primary antibody with the immunizing HuFrq protein (not shown), indicating that the observed immunofluorescence is generated by HuFrq-specific antibodies. A similar staining pattern was obtained with γ-adaptin (Fig. 2B), which in double-labeling experiments co-localized with the HuFrq-immunostaining pattern (Fig. 2C). Previously, γ-adaptin has been shown to be localized in the TGN and the late endosomes (27). The double-immunostaining patterns also indicate a co-localization for γ-adaptin and PI4Kβ (Fig. 2, D–F) in agreement with a recent report (28). Finally, we immunostained the COS-7 cells with anti-HuFrq and anti-ARF1 antibodies (Fig. 2, G–I). Again, we observed a crescent of staining on one side of the nucleus (presumably the TNG) and some punctuate immunostain extending to the plasma membrane, which colocalized with the HuFrq immunostain (Fig. 2I). The results indicate similar subcellular distributions for HuFrq, ARF1, and PI4Kβ. The colocalization is consistent with the proposal that frequenin proteins modulate PtdIns-4-OH kinase activity both in yeast and in mammalian cells and thus may have similar regulatory functions in secretion and Golgi membrane dynamics.

**Calcium-binding Properties**—We mutated EF hands EF2, EF3, and EF4 of HuFrq together or in pairwise combinations utilizing in vitro mutagenesis. In all four cases, we mutated the amino acid residues at the X position as previously described for Drosophila Frq (Fig. 1B) (4). Accordingly, we generated four HuFrq mutants: E81V/T117A/T165A (Frq2,3,4), E81V/T117A (Frq2,3), E81V/T165A (Frq2,4), and T117A/T165A (Frq3,4). Bacterial lysates containing approximately equal amounts of each HuFrq mutant were blotted onto nitrocellulose. The blot was incubated with 45Ca2⁺ to investigate the Ca2⁺-binding capacity of the HuFrq mutants in comparison to wild-type HuFrq (Fig. 3). The results showed that wild-type HuFrq yielded the highest 45Ca2⁺ signal. We noted for the recombinant HuFrq mutants with pairwise mutations attenuated 45Ca2⁺-signals of comparably reduced intensity. The pairwise HuFrq mutants each contained a single intact EF hand (EF2 in Frq2,3, EF3 in Frq2,4, and EF4 in Frq3,4), yet they bound Ca²⁺ with high affinity; this suggests that EF hands EF2, EF3, and EF4 not only are functional in HuFrq but also are independent from each other. Previously, it was shown that single mutations in yeast Frq1 EF hands did not display a temperature-sensitive phenotype like the quadruple mutant in the freq1-1⁰ allele, consistent with our observations. By contrast, the triple mutant Frq2,3,4 did not bind 45Ca²⁺ to a significant extent; hence in HuFrq, EF hand 1 does not constitute a high affinity Ca²⁺-binding site in HuFrq, as predicted earlier from sequence analysis.

**Overall Structure**—The crystal structure of HuFrq was solved by the molecular replacement method using neurocalcin
as a search model and was refined to 1.9 Å resolution. The structure consists of residues Asn 5–Val190 with good stereochemistry; clear electron density maps could be observed for all structural elements (Fig. 4A). As predicted, HuFrq shares the typical α-helical fold found in homologous proteins, with overall dimensions of 35 × 60 × 40 Å. HuFrq contains 10 helices labeled A to J (Fig. 4B). Consistent with the recently proposed NMR-derived model of yeast Frq (29), the four EF hands, which all present the typical architectural short β-strand, come in two pairs: an N-terminal pair (EF1, EF2) and a C-terminal pair (EF3, EF4), which are connected by the hinge loop 93–97 and related by an approximate 2-fold axis. The hinge loop conformation positions the four EF hands on one side of the molecule in a tandem linear array. This structural arrangement is similar to the ones seen in other members of the myristoyl-switch Ca²⁺-binding proteins, e.g. neurocalcin, recoverin, and GCAP (1). It is, however, different from the dumbbell arrangement found in e.g. calmodulin and tropinin C (30). The HuFrq structure contains three calcium ions bound to EF hands EF2, EF3, and EF4. The Ca²⁺ coordination in HuFrq is virtually identical to recoverin and neurocalcin. The side chains of residues x, y, and z in the 12-residue loop each provide an oxygen atom, as does the main chain carbonyl of residue y. Two additional oxygen atoms come from the conserved glutamic side chain of residue z and a water molecule.

Structural Comparison with Homologous Proteins—For residues 8–175, the HuFrq overall fold is similar to that of neurocalcin (14) with an r.m.s.d. value of 1.3 Å for 164 Ca atoms (Fig. 4C). Extended comparison with the structure of recoverin (31) yields a r.m.s. deviation of 1.7 Å for only 123 Ca atoms. These values indicate that the HuFrq structure is more closely related to that of neurocalcin. This reflects the high sequence identity existing between the two proteins, which is 61% for the whole molecule, compared with only 41% between HuFrq and recoverin. However, whereas neurocalcin dimerizes in solution (14), no dimeric assembly could be observed for HuFrq in concentrated solution or in the crystals (data not shown); this suggests that the distinct oligomeric assembly of these proteins may be related to their biological functions.

Novel Hydrophobic Crevice—The dominant and striking feature of the HuFrq structure is the large positional shift of the C-terminal helix J compared with its position in the recoverin and neurocalcin structures. Indeed, helix J has moved by 45° as a hinge-type rigid-body motion, with the residue pair Asp 176-Pro177 acting as a pivot point, to adopt an original position that is well ordered in our structure (Fig. 5, A and C). As a result,
the helix is tightly packed against loop aE-aF where it establishes numerous polar and hydrophobic interactions, consistent with an average mobility similar to that of other structural elements in the molecule. As a consequence of helix J motion, a large hydrophobic crevice, with dimensions $30 \times 15 \times 15 \text{ Å}$, is unmasked on the HuFrq face that is opposite to the four EF hands (Fig. 5A). The crevice is made of 46 residues provided by 8 helices, of which helices D, E, and F contribute to the floor of the crevice, helices C, G on one side and helix J on the other side contribute to the walls, and helices H and B contribute to the top and bottom, respectively. Hence, a quarter of the HuFrq molecule contributes to the structure of the crevice.

The major structural significance of the helix J repositioning for ligand binding is obvious from the complete exposure of the hydrophobic surface region. This new conformation dramatically alters the solvent-accessible molecular surface of the molecule when compared with those of neurocalcin or recoverin. In HuFrq, a large patch of hydrophobic residues line the crevice and still are fully accessible to the solvent (Fig. 5A), whereas they are sequestered into the neurocalcin and recoverin protein cores (Fig. 5, B and C). Actually, fragments of the polyethylene glycol used for crystallization are found well ordered within the crevice, where they could mimic an interacting ligand/membrane partner and shield the crevice from the solvent. Presence of the detergent octyl-glucoside was a requisite to solubilize and reduce aggregation of yeast Frq (29). In HuFrq, the unique disordered surface loop, loop 133–137, along with the C-terminal part of helix C that also presents high mobility, are located at the periphery of the crevice and may also play a role upon ligand/membrane recognition.

Is this α-helix conformational shift specific to HuFrq? To our knowledge, such a large positional shift in the C-terminal portion is novel and unique to HuFrq compared with other members of the family and exemplifies how a structurally related fold can exhibit a distinct binding site. Within the crystal asymmetric unit, a sodium ion is bound in the N-cap of helix J and connects residue Ala$^{175}$ in the α1-αJ loop that precedes helix J of one molecule to residues Thr$^{17}$, Thr$^{20}$, and Phe$^{22}$ of the second molecule via the carbonyl atoms; however, there is no evidence for an active role of this ion in the α-helical shift. Instead, sequence differences in the C-terminal ends of proteins of the family may explain the conformational features specific to the HuFrq structure. Indeed, seven residues are conserved in the C-terminal region of HuFrq compared with other members: Ala$^{182}$, Asp$^{187}$, Gly$^{188}$, and Leu$^{189}$, which are exposed at the periphery of the crevice and may have a role for ligand recognition, and Ser$^{184}$, Tyr$^{186}$, and Asp$^{187}$, which are located on the opposite face where they tightly anchor helix J to the protein core (Fig. 4B). A recent report on a yeast Frq model, designed from a combination of partial NMR data and homology modeling based on the recoverin structure, might suggest a similar location of helix J in the yeast homolog (29). Hence, the fact that Drosophila Frq possesses a glycine doublet in place of the conserved residue pair Tyr$^{186}$/Asp$^{187}$ appears most surprising because these substitutions would be expected to destabilize helix J. Finally, a Kv channel-interacting protein has recently been identified as a novel member of the calcium-binding protein family (32), and its C-terminal sequence presents high similarity with the C-terminal region of HuFrq. This suggests that a hydrophobic crevice similar to that found in HuFrq may exist and be involved in the binding of the cytoplasmic N termini of Kv4 α-subunits.

**The Calcium Myristoyl Switch—HuFrq**

HuFrq may share the molecular mechanisms of calcium-myristoyl switch proteins as seen from the solution structures of recoverin, where the myristoyl group is sequestered in a deep hydrophobic box in the Ca$^{2+}$-free state (33) and ejected into the solvent to interact with a lipid bilayer membrane in the Ca$^{2+}$-bound state (34). HuFrq possesses two discrete conformations at the hinge point Lys$^{7}$/Leu$^{8}$ in the N-terminal region, which therefore is a flexible arm. Residues Gly$^{42}$ and Gly$^{96}$ are the two hinge points that distinguish between the Ca$^{2+}$-free and Ca$^{2+}$-bound conformations in recoverin, and these two residues are conserved in HuFrq. Of the five helices that participate in the formation of the myristoyl box in recoverin, four (helices B, C, E, and F), line the large crevice in HuFrq. In addition, most of the hydrophobic residues that are recruited to accommodate the myristoyl group in recoverin are conserved in HuFrq and are solvent-exposed at the crevice surface. In HuFrq, residue Gly$^{96}$, which...
is one of the two hinge points in recoverin, establishes van der Waals contacts with helix J, suggesting that a conformational shift at this position might modify the position of this helix. Would HuFrq use the same structural switch as seen in recoverin, the shape and/or size of its hydrophobic crevice would be dramatically altered, a modification that could reflect a regulatory mechanism. Further biochemical experiments must await production of myristoylated HuFrq to test this hypothesis, although recent studies suggest that the presence of a N-myristoyl group in yeast Frq does not affect its overall structure whether Ca$^{2+}$ is present or not (29).

Rat Frq, as opposed to recoverin, neurocalcin, and hippocalcin, showed a Ca$^{2+}$-independent interaction with membranes (35), suggesting that the presence of the hydrophobic crevice, so far a feature unique to the Frq, may account for this difference in calcium dependence. As well, yeast Frq binds to and activates PIK1 in a Ca$^{2+}$-independent manner (6), a feature also consistent with our proposal of the hydrophobic crevice as a functionally important binding site. In contrast, the Ca$^{2+}$-enhanced interaction of yeast Frq with membranes (29) would suggest that in Ca$^{2+}$-free yeast Frq the shape of the hydrophobic crevice might be altered.

In summary, we have cloned HuFrq from human cDNA and analyzed its cellular localization in COS cells. HuFrq was overexpressed in E. coli, purified to homogeneity, and crystallized in the Ca$^{2+}$-bound state. This Ca$^{2+}$-bound HuFrq structure is a critical step toward identification of a physiological protein partner using biological experiments. Further crystallographic investigations will help identify the interactions involved in complex formation and conformation.

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