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A new family of small, palmitoylated, membrane-associated proteins, characterized by the presence of a cysteine-rich hydrophobic motif[1]

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Abstract We recently cloned the *CHIC2* gene (previously *BTL*) by virtue of its involvement in a chromosomal translocation t(4;12)(q11;p13) occurring in acute myeloid leukemias. In this study we show that CHIC2 is a member of a highly conserved family of proteins characterized by the presence of a striking cysteine-rich hydrophobic (CHIC) motif. Our data illustrate that cysteines in this central CHIC motif are palmitoylated and that CHIC2 is associated with vesicular structures and the plasma membrane. The CHIC proteins thus resemble the cysteine string proteins, which function in regulated exocytosis.

Key words: Palmitoylation; Cysteine; Membrane protein; Protein family; Leukemia

1. Introduction

We recently cloned the *BTL* gene (Brx-like translocated in leukemia) by virtue of its involvement in a chromosomal translocation t(4;12)(q11;p13) occurring in cases of acute myeloid leukemia [1]. As a consequence of this translocation an in-frame fusion gene is generated between *BTL* (exon 1-3) and the *ETV6/TEL* gene (exon 2-8) on chromosome 12 [2], which is frequently involved in translocations [3]. Since the function of BTL is unknown, the oncogenic properties of the BTL–ETV6 fusion protein are not yet understood. *BTL* was found to be very similar to murine *Brx*, a brain specific X-linked gene with unknown function, described as a candidate gene for X-linked mental retardation [4,5].

Although BTL/Brx do not contain any known functional domains, the most striking feature of these proteins is the presence of a cysteine-rich hydrophobic stretch of 23 amino acids, that we have named the CHIC motif. Homologous proteins were found in different species as early as *Caenorhabditis elegans*, revealing extreme high conservation of the CHIC motif and of the proteins in their whole. In accordance to the presence of the CHIC motif, the proteins have now been renamed to Chic1 (Brx) and CHIC2 (BTL). The high cysteine content of this motif resembles somehow the cysteine string domain of the cysteine string proteins (CSPs). CSPs are palmitoylated proteins present on vesicular membranes that are involved in Ca^{2+}-regulated exocytosis [6,7]. We have investigated if the CHIC motif has similar characteristics as the cysteine string domain and determined the subcellular localization of CHIC2 by use of myc- and enhanced green fluorescent protein (EGFP)-tagged constructs complemented with cell fractionation experiments.

2. Materials and methods

2.1. Cloning of C. elegans chic

C. elegans total RNA was extracted using TRIzol (Life Technologies). First strand cDNA was generated using a random primer and M-MLV reverse transcriptase (Life Technologies) and used for PCR with the primers Cechic-F (5′-AAAATGTCACATGGCTCACCAG) and Cechic-R (5′-GAAACAGTGGGCGAAAATGC). The 610 bp PCR product was cloned and sequenced.

2.2. Construction of the expression plasmids

The coding region of human *CHIC2*, including the stop codon, was amplified by PCR with the primers CHIC2-F (5′-TAGGATCCAGGATGGCGGATTTC) and CHIC2-R (5′-CCACACGATTCTGTAGAACC) and cloned into pEGFP-C1 (Clontech). Alternatively, the coding region of *CHIC2*, without the stop codon, was amplified by PCR with the primers CHIC2-Fb (5′-CAGGATGGCGGATTTCGACG) and CHIC2-Rb (5′-AATCTGGTCGAAAAATCGGTG) and cloned into pcDNA3.1-myc/his-A (Invitrogen) and pEGFP-N3 (Clontech).

2.3. Construction of the mutants

All mutants were constructed by PCR using as template DNA the respective wild-type constructs. For the EGFP-CHIC2-C6S mutant the primer combinations used for the first PCR reactions were: CHIC2-F with CHIC2C6S-R (5′-GCTGCTGGAACTAAGGCTGCCACTAAGTAG) and CHIC2C6S-F (5′-CTTAGTGGCAGCCTTAGTTCCAGCAGCACA) with CHIC2-R. For the CHIC2-C6S-myc construct the primer combinations used were CHIC2-F with CHIC2C6S-R and CHIC2C6S-F with myc-R (5′-AGGCACAGTCGAGGCTG). The products of these first reactions were combined and subjected to a second PCR using the primers CHIC2-F and CHIC2-R or the primers CHIC2-F and myc-R. The final products were subcloned into respectively pEGFP-C1 and pcDNA3.1/zeo (Invitrogen). The deletion mutant of CHIC2 was amplified with the primers CHIC2-F and CHIC2-R19 (5′-ACTGCAGCCATGTTATTCGTTTC) and was subcloned into pEGFP-N3.

2.4. Western blotting

Cell pellets or membrane fractions were resuspended in sample buffer (125 mM Tris pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 40 mM dithiothreitol) by sonication, boiled and separated on 12.5% polyacrylamide gels. Protein transfer and antibody incubation was performed using standard techniques.

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[1] The nucleotide sequence for the *Caenorhabditis elegans chic* cDNA has been deposited in the GenBank database under accession number AF248541.

[2] Jan Cools is an 'Aspirant' of the 'F.W.O.-Vlaanderen'.

Abbreviations: CSP, cysteine string protein; EGFP, enhanced green fluorescent protein; ORF, open reading frame

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2.5. Cell culture and transfection

Cells were grown in DMEM-F12 (Life Technologies), supplemented with 10% fetal calf serum. The K562 cell line, grown in suspension, was transfected by electroporation, while the adherent cell types were transfected using Fugene-6 (Roche). Nocodazole (Sigma) treatment was performed at a concentration of 20 μg/ml as described in [8].

2.6. Immunofluorescence

Cells were fixed 24–36 h after transfection using 4% paraformaldehyde in phosphate-buffered saline (PBS) and blocked with 0.5% blocking reagent (Roche) containing 0.2% Triton X-100 (Merck). Wash steps were performed in PBS containing 0.2% Triton X-100. Slides were mounted in Vectashield mounting medium (Vector Laboratories) containing DAPI (Sigma). The antibodies used are: anti-Golgi 58 kDa protein monoclonal antibody (Sigma), anti-γ-adaptin monoclonal antibody (Sigma), CY3-labeled anti-mouse antibody (Amersham Pharmacia), anti-myc monoclonal antibody (clone 9E10). The samples were examined either by digital imaging microscopy using a cooled charge-coupled device camera (Photometrics) with the Smart Capture software (Vysis) or by use of the laser scanning confocal imaging system MRC1024 (Bio-Rad) attached to an inverted microscope Diaphot 300 (Nikon) with the LaserSharp software (Bio-Rad).

2.7. Cellular fractionation

Membrane fractions were isolated from HEK293T cells, transiently expressing the different constructs. After 48 h the cells were collected in cold PBS. Cells were then resuspended in cold sucrose buffer (250 mM sucrose, 5 mM Tris, 1 mM EGTA, adjusted to pH 7.3) containing protease inhibitors (Complete (Roche)) and subjected to cell lysis by passing 16 times through a 26 G needle. Intact cells and nuclei were removed by centrifugation at $2000\times g$ for 10 min. The supernatant was centrifuged at $100\,000\times g$ for 1 h to pellet the membrane fraction.

2.8. Depalmitoylation and [^{3}H]palmitate labeling

Depalmitoylation was achieved by incubation of the membranes in 1 M hydroxylamine (pH 7) containing protease inhibitors at 15°C for 20 h. As a control, the membranes were treated with 1 M Tris (pH 7) in similar conditions.

[^{3}H]Palmitate labeling was performed 24 h after transfection of HEK293T cells grown in a 6-well plate. Prior to labeling, cells were washed with PBS and starved for 1 h in serum free medium. Cells were then incubated in serum free medium containing 0.5 mCi/well [9,10-^{3}H]palmitic acid (NEN) for 1 h. After labeling, the cells were directly lysed in 1 ml DIPA buffer (50 mM Tris, 150 mM NaCl, 1% Na-deoxycholate, 0.1% SDS, 1% Triton X-100) and subjected to immune precipitation using the anti-myc monoclonal antibody in combination with protein G agarose (Amersham Pharmacia). The immune-precipitated proteins were separated on a 12.5% polyacrylamide gel and the dried gel was exposed for 20 h.

2.9. Database searches and sequence alignment

Database searches were performed using the BLAST program available at http://www3.ncbi.nlm.nihgov/BLAST. For sequence alignment, Vector NTI Suite (Informax) was used. The phylogenetic tree was calculated using the Megalign program (DNASTAR).

3. Results and discussion

3.1. Identification of the CHIC family

Based on the amino acid sequence of CHIC2 (BTL) [1] and the reported sequence of Chic1 (Brx) [4], database searches were performed to investigate the existence of homologous proteins in different species (results shown in Table 1). Members of this family were found in the worm, the fly and in vertebrates. Since the genomes of *C. elegans* and *Drosophila melanogaster* are now completely sequenced [9,10], it is clear that they contain only a single *chic* gene, whereas in fish, mouse and human at least two different members exist. Based on subtle amino acid sequence differences (Fig. 1), these can be subdivided in two groups, named Chic1 and Chic2, as is also indicated by the phylogenetic tree (Fig. 2A). The absence of these proteins in yeast indicates that these proteins do not play a role in basic eukaryotic cellular functions.

The CHIC protein from *C. elegans* was previously predicted as two separate proteins W06E11.5 and W06E11.6, clearly distinct from all other CHIC proteins. To solve this discrepancy, the potential *C. elegans chic* cDNA was amplified from total RNA. The PCR product encoded the expected protein (shown in Fig. 1), combining the predicted proteins W06E11.6 and W06E11.5 to one single protein (this cDNA sequence was deposited to GenBank with accession number AF248541).

Alignment of all identified members of the CHIC family indicates an extreme good conservation through evolution. Except from the differences at the N-terminus, the all over similarity between the deduced protein sequences is very high (Fig. 1). The N-terminus of mouse and human Chic1/CHIC1 is characterized by the presence of poly-S and poly-E stretches, which are lacking in *Danio rerio* Chic1 and the other known CHIC proteins, indicative for the late occurrence of these repeats during evolution. Fig. 2B schematically illustrates the similarity between the exon–intron structures of

Table 1
Identification of CHIC family members using database searches

Species	Gene	cDNA clone	EST or Unigene cluster	Genomic clone
Homo sapiens (*Hs*)	*CHIC1*	Y11897	Hs.66831	AL355528
	CHIC2	AF159423	Hs.119488	AC069068
Mus musculus (*Ms*)	*Chic1*	Y11896	Mm.42223	X99946
	Chic2	–	Mm.27267	–
Rattus norvegicus (*Rn*)	*Chic2*	–	Rn.41048	–
Bos taurus (*Bt*)	*Chic2*	–	AW344465	–
Xenopus laevis (*Xl*)	*Chic2*	–	AW872240	–
D. rerio (*Dr*)	*Chic1*	–	AW077256	–
	Chic2	–	AW153802, AW343214	–
D. melanogaster (*Dm*)[a]	*chic*	CG5938	AI512125, AI062125	AE003760
C. elegans (*Ce*)[a]	*chic*	W06E11.6 W06E11.5 AF248541[b]	–	U20862
Saccharomyces cerevisiae[a]	–	–	–	–

All numbers refer to the corresponding accession numbers of the clones, except for Unigene clusters (underlined) and predicted proteins (double underlined). Only some representative EST clones are given. The identity of the genes as being *Chic1* or *Chic2* was determined by comparing their corresponding protein sequences using the similarity plot and phylogenetic tree shown in Figs. 1 and 2.
[a]Complete genome sequence available.
[b]This report.

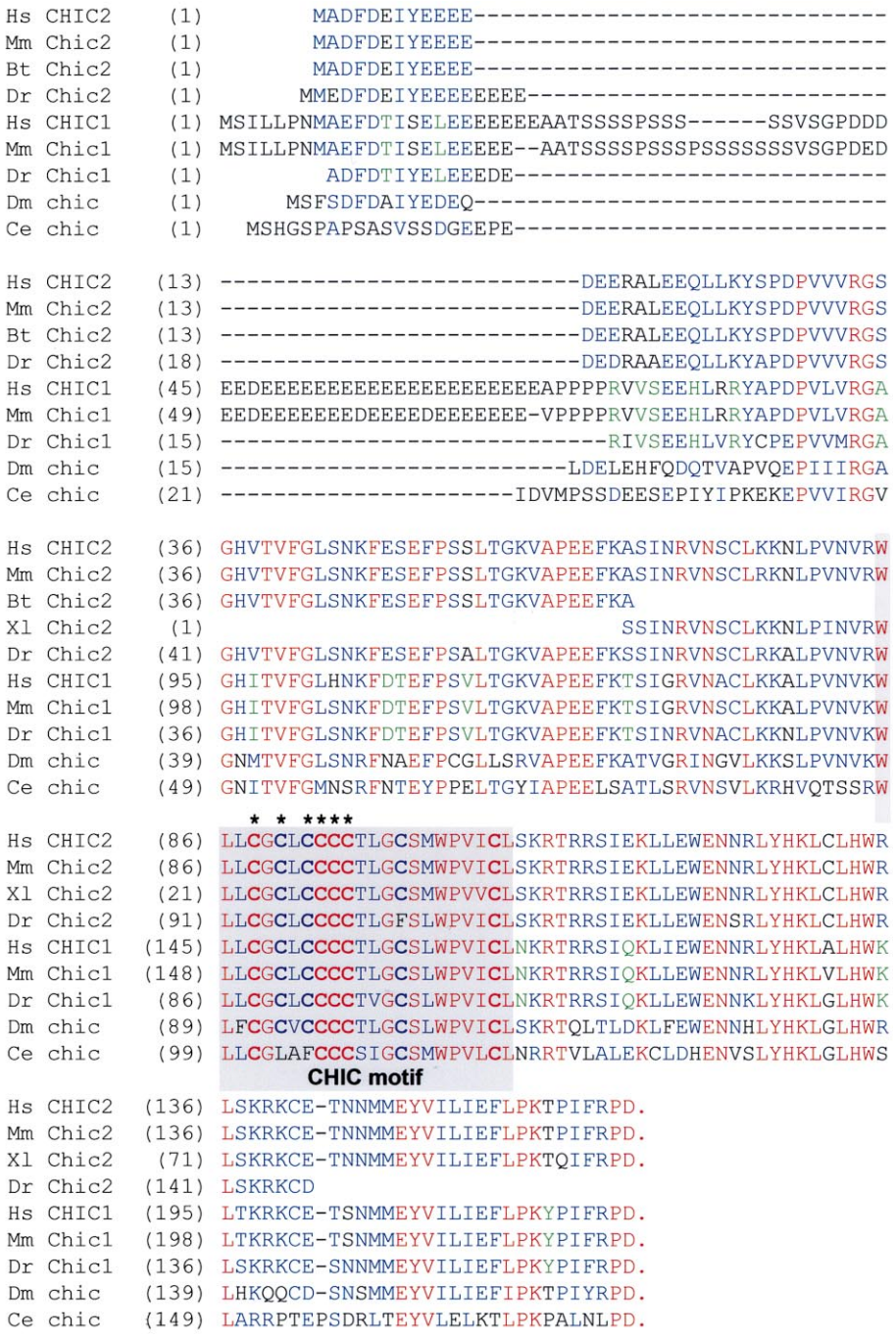

Fig. 1. Alignment of all known CHIC family members. *Rn* Chic2 is not shown, but is 100% identical to *Mm* Chic2. Similar residues are shown in blue, residues that are 100% conserved are in red, the residues specific for the chic1 subfamily are shown in green; the CHIC motif is the shaded area with cysteines shown in bold; cysteines mutated in the CHIC2-C6S construct are marked with an asterisk. Protein sequences are deduced from cDNA clones, ESTs or genomic clones, listed in Table 1. The 5′ ends of the ORFs of *Dr Chic1* and *Xl Chic2* are not known at this moment, as well as the 3′ ends of the ORFs of *Bt Chic2* and *Dr Chic2*. We have used the genomic sequence to determine the ORF of *Mm Chic1*.

worm and fly *chic* and human *CHIC1* and *CHIC2*. Two out of five exon–intron boundaries as well as the phases at these boundaries are conserved from worm to human, confirming these genes to be derived from one ancestral gene.

No significant similarities were found between the CHIC proteins and any other protein or protein domain. However, a striking feature of the CHIC proteins is the presence of a highly conserved CHIC stretch of 23 amino acids, flanked by charged residues, that we have named the CHIC motif. This motif contains eight cysteines of which five cysteines are 100% and three others 90% conserved. This cluster of cysteines resembles the cysteine string domain of the CSPs, but is clearly distinct from it since there are no similarities between non-cysteine residues of the cysteine string domain and the CHIC motif and CSPs contain more cysteines [7]. To further document on this, we have investigated whether the CHIC motif is indeed a variant of the cysteine string domain.

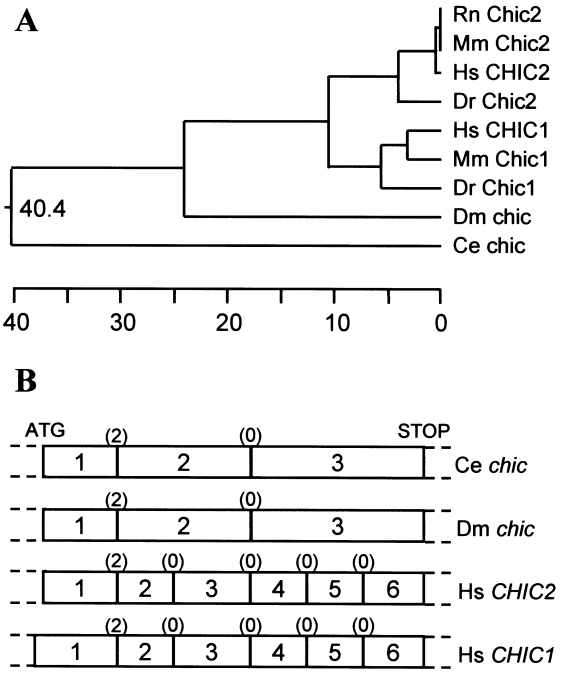

Fig. 2. (A) Phylogenetic tree of the CHIC family. Only members for which almost the complete ORF is known were included. The numbers at the bottom indicate the number of substitution events. (B) Schematic comparison of the exon–intron boundaries of *C. elegans chic*, *D. melanogaster chic*, human *CHIC1* and human *CHIC2*. The exons are shown as numbered boxes and the phases of the exons are indicated above each exon boundary.

3.2. CHIC2 localizes at the plasma membrane and at vesicular structures

To determine the subcellular localization of CHIC2, we fused its open reading frame (ORF) N-terminal or C-terminal to the EGFP or to the myc epitope and transfected these constructs into four different cell lines: HEK293T (human embryonic kidney), NIH3T3 (murine fibroblasts), CV-1 (African green monkey) and K562 (human erythroleukemia). No major differences were observed in localization between the protein products of these three different constructs, indicating that the tags do not interfere with the correct folding and homing of CHIC2.

EGFP-CHIC2, CHIC2-EGFP and CHIC2-myc were localized at the plasma membrane, which was most easily seen in the round-shaped K562 and HEK293T cells (Fig. 3B,F), but was also observed in the other cell lines (Fig. 3G,H,I,M). The proteins were also present at a Golgi-like vesicular compartment and at scattered vesicles. To confirm that the intracellular vesicular structure was a part of the Golgi complex, we performed two experiments: (a) localization of the Golgi 58 kDa protein, an epitope located on the microtubuli-binding peripheral Golgi membranes [8] and (b) nocodazole treatment, which depolarizes the microtubuli thereby disintegrating the Golgi apparatus [8]. In K562 cells CHIC2 was always present in the same region as the 58 kDa protein, on one side of the nucleus, indicating that the observed vesicles were found at the site where the Golgi complex resides (Fig. 3C). Disturbance of this vesicular structure was observed after nocodazole treatment (Fig. 3D), confirming that this was a part of the Golgi complex. Partial co-localization of EGFP-CHIC2 with γ-adaptin [11] was demonstrated in CV-1 cells (Fig. 3J,K,L). Co-localization was observed at the Golgi apparatus/*trans* Golgi network surrounding the nucleus (yellow in Fig. 3L). Scattered vesicles, those not co-localizing with the γ-adaptin marker, were observed as well. Although our overexpression results illustrate that CHIC2 is associated with these different membrane structures, it should be considered that endogenous CHIC2 could be more restricted to one of these sites.

3.3. The CHIC motif is palmitoylated and is involved in membrane association

To confirm the membrane localization observed by immunostaining, membrane fractions of HEK293T cells, transiently expressing the CHIC2-myc construct, were collected. Analysis of complete cells by Western blotting revealed the presence of the expected protein of 23 kDa (the CHIC2-myc construct has an ORF of 194 amino acids) together with a 27 kDa protein, which could only be generated by post-translational modifications (Fig. 4). Analysis of membrane pellets showed that this fraction only contained the 27 kDa form, suggesting that the post-translational modification was required for membrane association (Fig. 4). In accordance to the presence of eight clustered cysteine residues in the CHIC motif, we investigated if these cysteines were palmitoylated, as was previously described for the CSPs [12,13]. Depalmitoylation of the proteins present in the membrane fraction resulted in a shift of CHIC2-myc from 27 to 23 kDa, indicating that the 27 kDa form of the CHIC2-myc construct was indeed a palmitoylated form (Fig. 4). This was also confirmed by metabolic labeling with [^{3}H]palmitic acid, yielding a 27 kDa radioactive band after immune precipitation with the anti-myc antibody (Fig. 4).

We further demonstrated that the palmitoylation of the CHIC motif was indispensable for membrane association, as was also reported for the CSPs [13]. The mutation of six cysteines to serines (mutated cysteines are indicated in Fig. 1) abolished the membrane association of CHIC2 so that its subcellular distribution became cytosolic (Fig. 3N). The mutant CHIC2-C6S-myc construct showed a similar migration on Western blot as the unpalmitoylated CHIC2-myc construct and was not detected by autoradiography after [^{3}H]palmitate labeling (Fig. 5), even after exposure for 5 days, illustrating that at least some of the mutated cysteine residues are indispensable for palmitoylation of CHIC2.

3.4. The C-terminal residues of CHIC2 are indispensable for its subcellular localization

The C-terminal region of the CHIC proteins is well conserved and all proteins contain the stop codon at exactly the same position. To document the role of this region, we constructed a deletion mutant of CHIC2 lacking the last 19 amino acids and fused it to EGFP. This mutant showed a combined nuclear/cytosolic localization, mostly associated with a reticular pattern that might reflect an association with the endoplasmic reticulum (Fig. 3O), indicating that the C-terminal part of CHIC2 is indispensable for its proper subcellular localization. This region might be important for the tertiary structure of the protein or alternatively might contain indispensable motifs involved in protein sorting and/or association with the palmitoyl-*S*-transferase system. It will be of interest to address the potential presence of such motifs by specific point mutations.

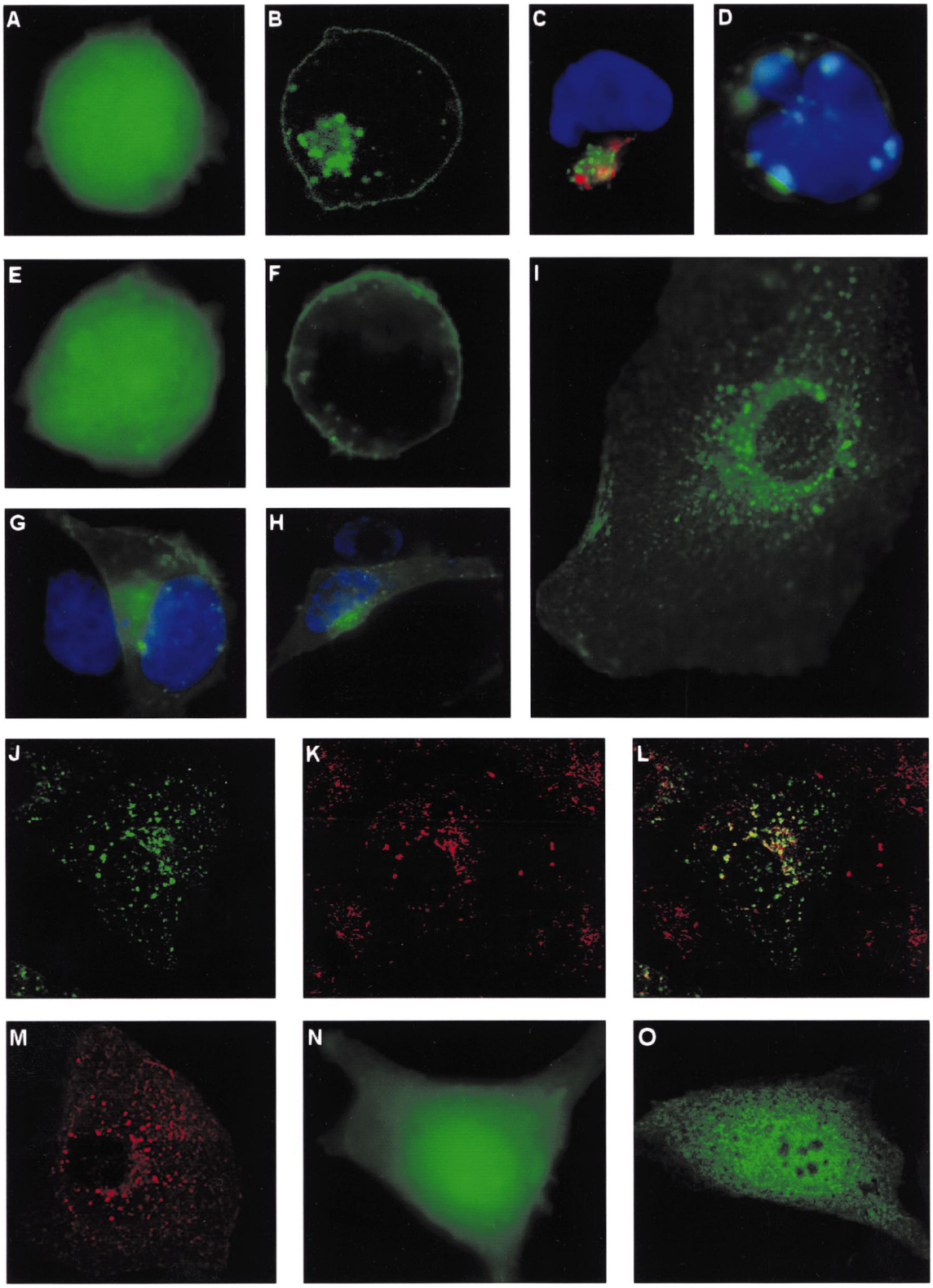

Fig. 3. Subcellular localization of CHIC2 and mutants. In some pictures the nucleus is stained with DAPI (blue). K562 cells: (A) EGFP, (B) EGFP-CHIC2 (confocal image), (C) EGFP-CHIC2+anti-p58 (red), (D) EGFP-CHIC2+nocodazole treatment. 293T cells: (E) EGFP, (F) CHIC2-EGFP, (G) EGFP-CHIC2. NIH3T3 cells: (H) EGFP-CHIC2. CV-1 cells: (I) EGFP-CHIC2, (J,K,L) EGFP-CHIC2+anti-γ-adaptin (red) (confocal images), (M) CHIC2-myc (confocal image). (N) EGFP-CHIC2-C6S. (O) CHIC2-del19-EGFP.

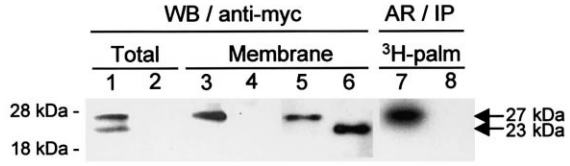

Fig. 4. WB/anti-myc: Western blot analysis of CHIC2-myc detected with the anti-myc monoclonal antibody. Total cell lysates of 293T cells expressing CHIC2-myc (lane 1) or empty control cells (lane 2). Membrane fractions of 293T cells expressing CHIC2-myc (lane 3) or empty control cells (lane 4). Membrane fractions of 293T cells expressing CHIC2-myc, treated with either 1 M Tris as control (lane 5) or 1 M hydroxylamine (lane 6). AR/IP: Autoradiography of immune-precipitated proteins using the anti-myc monoclonal antibody after [^{3}H]palmitic acid labeling of 293T cells expressing CHIC2-myc (lane 7) or empty control cells (lane 8).

In conclusion, our results characterize the CHIC family as small, palmitoylated, membrane-associated proteins, depending on the palmitoylation of cysteines in the CHIC motif for their membrane association. In addition, an important role for the C-terminal part of these proteins in determining their subcellular localization was identified. According to the structural similarities with the CSPs, it is most likely that both N- and C-terminal parts of the CHIC proteins are cytosolic. CSPs are palmitoylated, vesicular proteins implicated in Ca^{2+}-regulated exocytosis, but are involved in other functions as well [7]. The presence of CHIC2 on vesicular structures and the plasma membrane and its structural similarity to CSPs imply the CHIC proteins as a new family potentially involved in vesicular transport. We believe that our study provides a good starting point for their functional characterization. In addition, it will be of particular interest to investigate the oncogenic properties of the leukemia-associated CHIC2-ETV6 fusion protein and the role the presence of the CHIC motif may play in this.

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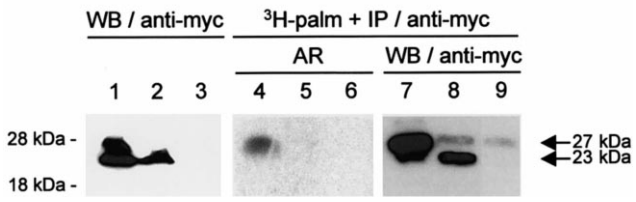

Fig. 5. WB/anti-myc: Western blot analysis of the wild-type CHIC2-myc and the mutant CHIC2-C6S-myc detected with the anti-myc monoclonal antibody. Total cell lysates of 293T cells expressing wild-type CHIC2-myc (lane 1), CHIC2-C6S-myc (lane 2) or empty control cells (lane 3). IP/AR: Autoradiography of immune-precipitated proteins using the anti-myc monoclonal antibody after [^{3}H]palmitic acid labeling of 293T cells expressing wild-type CHIC2-myc (lane 4), CHIC2-C6S-myc (lane 5) or empty control cells (lane 6). IP/WB/anti-myc: Western blot (using the anti-myc monoclonal antibody) of the immune-precipitated proteins to confirm their presence: wild-type CHIC2-myc (lane 7), CHIC2-C6S-myc (lane 8) or empty control cells (lane 9).

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