Chp-1 and melusin, two CHORD containing proteins in vertebrates

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Abstract Melusin is a muscle specific protein required for heart hypertrophy in response to mechanical overload. Here we describe a protein 63% homologous to melusin, named chp-1, expressed in all tissues tested, including muscles, and highly conserved from invertebrates to human. Both proteins are characterized in their N-terminal half by a tandemly repeated zinc binding 60 amino acid domain with a motif of uniquely spaced cysteine and histidine residues. These motives are highly conserved from plants to mammals and have been recently named CHORD (for cysteine and histidine rich domain) domains. At the C-terminal end melusin contains a calcium binding stretch of 30 acidic amino acid residues which is absent in chp-1. While invertebrate genome contains only one gene coding for a chp-1 homolog, two genes coding for CHORD containing proteins (chp-1 and melusin) are present in vertebrates. Sequence analysis suggests that the muscle specific CHORD containing protein melusin originated by a gene duplication event during early chordate evolution.

Key words: chp-1; Melusin; Cysteine and histidine rich domain containing protein; Conserved protein domain

1. Introduction

Melusin is a muscle specific protein involved in cardiomyocyte hypertrophy response, localized at actin membrane junctions [1]. In mouse, gene inactivation indicated that melusin is not required for skeletal muscle and heart development during embryogenesis and does not affect basal cardiac and skeletal muscle function. However, melusin null mice subjected to chronic pressure overload develop dilated cardiomyopathy and failure, indicating a crucial role of melusin in transducing signaling in response to mechanical stretch in cardiomyocytes [2].

Melusin is a multidomain protein with unique structural features. The C-terminal half of the molecule contains an integrin binding site followed by a region highly enriched in aspartic and glutamic acid closely resembling to a C domain, known to bind calcium at high capacity and low affinity [3,4]. The N-terminal half of melusin consists of a tandemly repeated cysteine and histidine rich region, spaced by an intervening sequence of approximately 90 amino acid residues. The cysteine and histidine residues in these repeats are characteristically spaced with a unique pattern [1]. Proteins consisting of similar domains, called CHORDs (for cysteine and histidine rich domain), have been identified in barley and *Arabidopsis* and shown to be essential components of the innate immune resistance upon pathogen attack in plants [5]. Sequences coding for CHORD domains have been found in dbEST collections from different species indicating the existence of proteins highly conserved during phylogenesis named CHORD containing proteins.

Here we report the full length sequence, gene structure and partial characterization of a new mouse CHORD containing protein highly homologous to melusin, named chp-1. Than, while plant, protozoa and invertebrate genomes contain only one gene coding for a CHORD containing protein, two different genes coding for this class of proteins are present in vertebrates.

2. Materials and methods

2.1. Plasmid construction

Two IMAGE cDNA clones coding for chp-1 were obtained as bacterial stock cultures from IMAGE consortium. The two bacterial stocks were cultured and plasmids were purified and sequenced using ABI Prism big dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). Both clones carried the entire chp-1 coding sequence. 1200 bp containing the complete chp-1 coding sequence were cloned in pMALc2 vector (New England Biolabs) and pGEX 4T2 vector (Pharmacia Biotech) in frame with maltose binding protein (MBP) and with glutathione S-transferase (GST) coding sequences. Melusin was fused to MBP by cloning the cDNA into the pMALc2 vector. To obtain truncated versions of melusin fused to MBP we amplified by polymerase chain reaction (PCR) partial mouse cDNA sequences encoding amino acids 1–320 for MBP-Δ-melusin (5′-CGGAATTCATGTCTCTGCTCTGCTAT-3′ and 5′-CCCTCGAGTTATAGTAAAACCCCTGCCCT-3′) and amino acids 1–149 for MBP-melCHORD1 (5′-CGGAATTCATGTCTCTGCTCTGCTAT-3′ and 5′-CGGGATCCTTAGCTGGAACCAGTCCAGAT-3′).

2.2. Northern blot

Poly A+ RNA isolated from mouse tissues and immobilized onto a nitrocellulose filter after electrophoretic separation, was obtained from Clontech (multiple tissue Northern blot). The filter was probed at 65°C with chp-1 cDNA labeled with ^{32}P using a random prime labeling system (rediprime II, Amersham Pharmacia) and was washed twice with 2× standard saline citrate (SSC) 1% sodium dodecyl sulfate (SDS) and twice with 0.4% SSC 1% SDS at 65°C and exposed to X-ray film (Kodak).

2.3. Antibody preparation

MBP-chp-1 fusion protein was produced by expressing the entire sequence of the mouse chp-1 cloned in pMALc2 vector (New England Biolabs) in *Escherichia coli* BL21 bacterial strain. MBP-chp-1 was purified on an amylose column according to the manufacturer's instructions (New England Biolabs). Rabbits were immunized by re-

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peated intramuscular injections of the purified fusion protein (500 μg) emulsified in complete Freund's adjuvant. The specificity of the antiserum was demonstrated in Western blots on protein extracts from *E. coli* expressing GST-chp-1 fusion protein, GST-melusin and GST alone (Pharmacia Biotech) as negative control. In order to affinity purify antibodies from rabbit serum MBP and MBP-chp-1 fusion proteins were purified and coupled to Sepharose. Rabbit serum was loaded on the MBP column to eliminate antibodies to MBP and then reloaded on the MBP-chp-1 column. Antibodies adsorbed on the MBP-chp-1 Sepharose column were eluted with pH 3 glycin–HCl buffer.

2.4. Affinity purification of MBP fusion proteins

E. coli BL21 bacterial cells transformed with plasmids coding for MBP fusion proteins were grown in Luria–Bertani (LB) medium containing 1 μM $ZnCl_2$, at 30°C up to an OD_{600} value of 0.6. Isopropyl thiogalactose (IPTG) (0.1 mM) was then added to the culture and the incubation was continued for 4 h. MBP-chp-1, MBP-melusin, MBP-Δ-melusin (without the acidic domain at the extreme C-terminal of the protein), MBP-chp-1 CHORD1 (containing only the first CHORD domain and the intervening sequence of melusin) were purified according to the manufacturer's instructions (New England Biolabs). We measured the zinc binding to the fusion proteins by a colorimetric assay based on complex formation with 4-(2-pyridylazo) resorcinol (PAR) [6].

0.4 nmol of each recombinant protein were separated on polyacrylamide gel in the presence of SDS and subsequently blotted to polyvinylidene difluoride (PVDF) membrane. Ca^{2+} overlay assay was performed as described [7].

2.5. Cell culture and Western blot

C2C12 mouse skeletal muscle cell line was maintained in Dulbecco's modified Eagle's medium with 20% fetal calf serum. Cells were induced to differentiate into myotubes by switching to culture medium with 2% horse serum.

Western blots on cell and tissue extracts were performed as follows. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in 120 mM NaCl, 50 mM Tris–HCl pH 8, 1% Triton X-100, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 trypsin-inhibiting unit (TIU)/ml aprotinin for 10 min at 4°C. Extracts were centrifuged at 14 000 rpm for 10 min to remove insoluble material. Tissues were frozen and triturated in liquid nitrogen and extracted in lysis buffer containing 120 mM NaCl, 50 mM Tris–HCl pH 8, 1% Triton X-100, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 TIU/ml aprotinin. Tissue extracts were centrifuged at 14 000 rpm for 10 min to remove insoluble material. Western blots were performed as described [1].

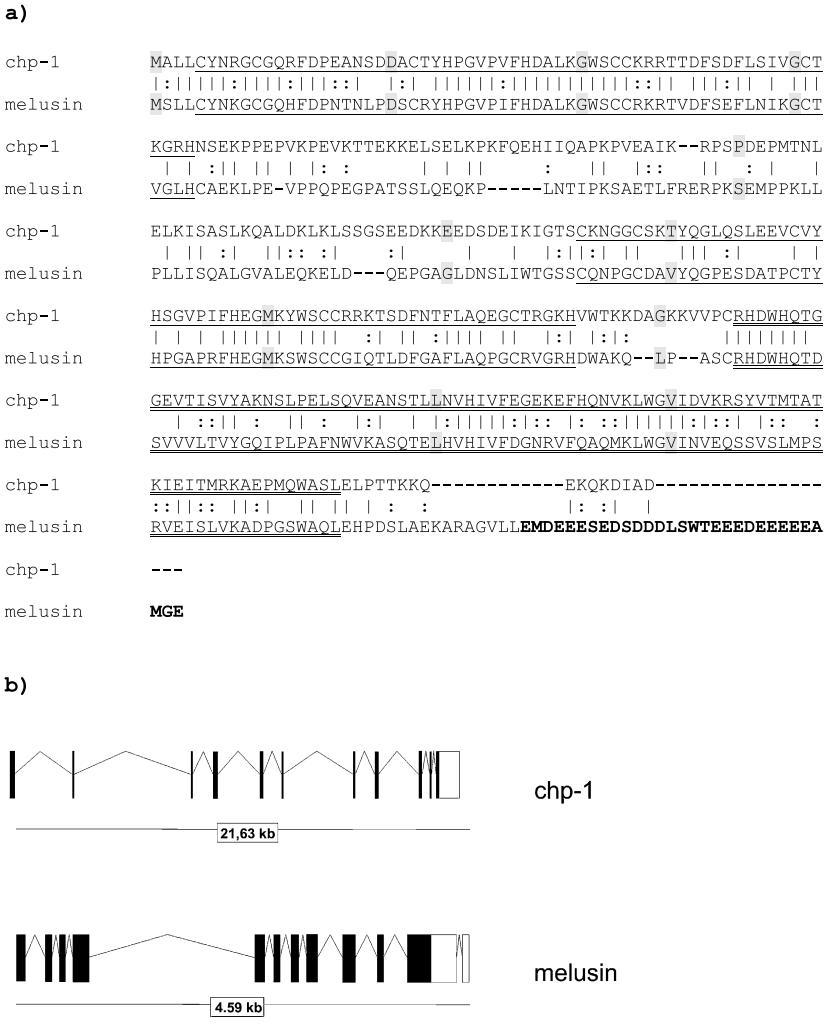

Fig. 1. Amino acid sequences and gene organization of mouse chp-1 and melusin. a: The amino acid sequences were deduced from the full length mouse cDNAs (mouse melusin accession number AF140691; mouse chp-1 accession number NM_025844). Identical residues are indicated by vertical bars and conservative substitutions by double dots. The two cysteine rich domains are underlined and the CS domains (Sgt1-like) are double underlined. The acidic amino acid stretch at the extreme C-terminal is in bold. The coding sequences of both genes are organized in 11 coding exons. The first amino acid residue coded by each exon is shadowed. b: Intron extensions are very different between the two genes, in fact melusin coding exons span a chromosome region of about 4.5 kb, while chp-1 coding exons are dispersed over a 21.6 kb stretch. This information has been obtained using Ensembl mouse database [12].

3. Results

3.1. Identification of a melusin homolog in mouse

To identify proteins homologous to melusin in human and mouse, we used 5′ melusin cDNA sequences to perform BLAST searches in dbest database. We found partial cDNA sequences coding for a protein highly homologous to melusin in mouse and human databases. The conceptual translation of the sequence of a murine melusin homolog cDNA clone revealed the presence of an open reading frame of 331 amino acids coding for a protein with theoretical molecular weight of 37 247.43 Da (Fig. 1a). The overall amino acid sequence showed 47% identity (63% homology considering conservative substitutions) to melusin. This protein, like melusin, contained two CHORDs, that are separated by an intervening region of 92 amino acids in the N-terminal half of the molecule [5]. The C-terminal half consists of 85 amino acids sharing significant homology with a portion of Sgt1, an essential component of the ubiquitin ligation machinery and of the yeast kinetochore assembly pathway [8]. In melusin this C-terminal region is followed by an aspartic and glutamic acid rich sequence, which is absent in the new protein (Fig. 1a). The homology

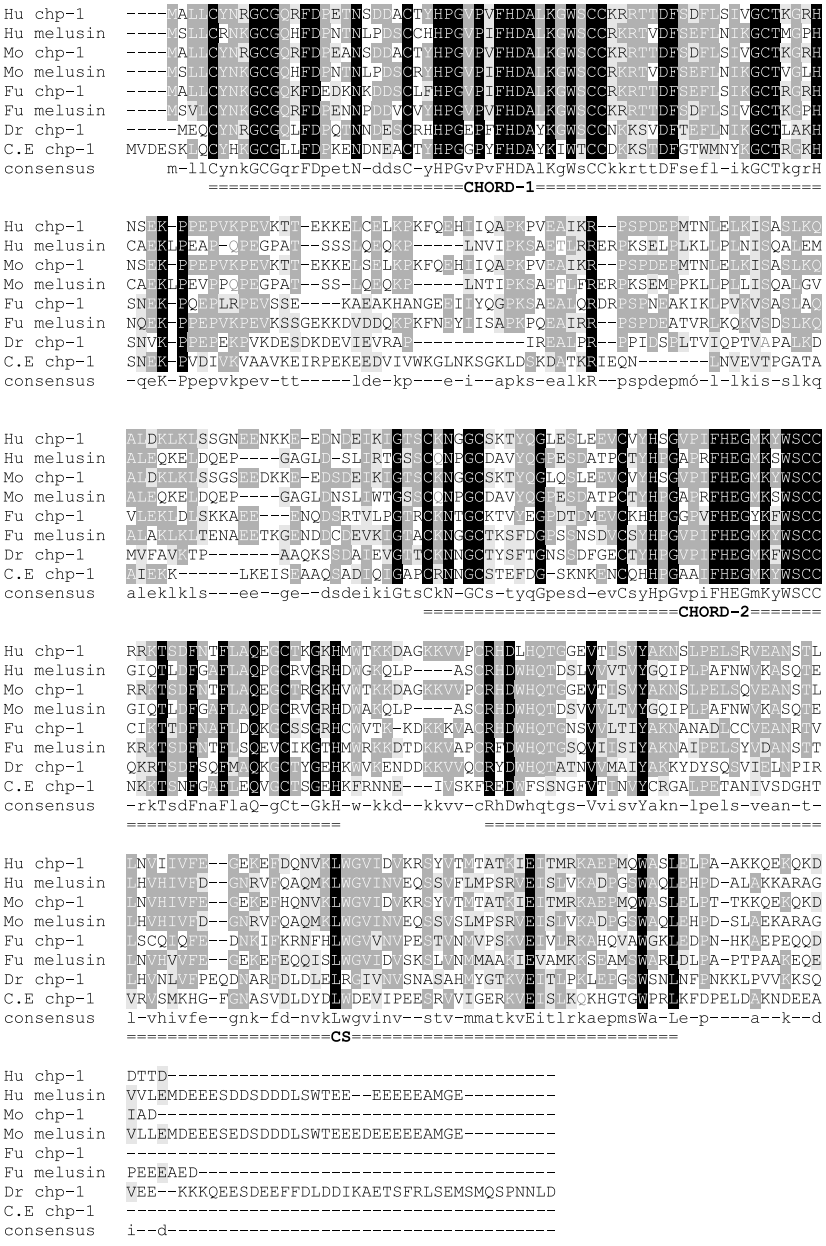

Fig. 2. Sequence alignment among vertebrate and invertebrate CHORD containing protein sequences. Multiple alignment of human (Hu), mouse (Mo), fugu (Fu), *Drosophila* (Dr) and *C. elegans* (C.E) CHORD containing proteins. Black boxes denote invariant residues among all proteins, dark gray boxes indicate invariant residues among a subset of proteins and light gray boxes indicate conservative amino acid substitutions (Boxshade version 3.3.1, by Kay Hofmann and Michael D. Baron). In the consensus shown, upper and lower case letters refer to all identical and similar residues, respectively. CHORD-1, CHORD-2 and CS domains (Sgt1-like) are double underlined. Gene accession numbers: human chp-1 (AF192466), human melusin (AF140690), mouse chp-1 (NM_025844), mouse melusin (AF140691), fugu chp-1 (lcl|SINFRUP00000058559 from fugu genome predicted proteins), fugu melusin (lcl|SINFRUP00000074060 from fugu genome predicted proteins), *Drosophila* chp-1 (AF192465), *C. elegans* chp-1 (AF192264).

between the two proteins is particularly high in the CHORD domains (72% identity and 87% homology in the first domain and 57% identity and 65% homology in the second one) (Fig. 1a) and in the Sgt1 like C-terminal domain (47% identity and 74% homology) (Fig. 1a).

3.2. Genomic organization of chp-1 and melusin genes

Melusin gene was previously localized in human on chromosome Xq12.1-13 and on the synthetic region X band D in mouse [1]. BLAST searching of the mouse and human genome sequences showed that chp-1 gene is localized on chromosome 9 in mouse and on chromosome 11q14.3 in human.

Chp-1 and melusin genes share a conserved exon–intron organization. The coding sequences of these genes are organized in 11 coding exons in both mouse and human genomes (Fig. 1a and b). On the contrary, intron extensions between the two genes are very different, in fact melusin coding exons span a chromosome region of about 4.5 kb in mouse and 3.6 kb in human genome, while chp-1 coding exons are dispersed over a 21.6 kb stretch both in mouse and in human genome (Fig. 1b). The first four exons (1–4) encode for the first CHORD domain as well as for the intervening region, while the second CHORD is entirely encoded by three exons (from exon 6 to exon 8). The Sgt1 like domain is specified by the three terminal coding exons (exons 9–11) (Fig. 1a).

We searched the database to identify the presence of chp-1 and melusin hortologs in other species and we found the presence of homologs of both genes in fugu (*Fugu rubripes*) genome and two partial dbest cDNAs in zebrafish, coding for melusin and chp-1 homologs. In contrast, in *Drosophila melanogaster* and *Caenorhabditis elegans*, only one homolog was present (Fig. 2). The invertebrate proteins do not contain the acidic stretch in the extreme C-terminal portion, typically present in melusin, suggesting that the *Drosophila* and *C. elegans* proteins represent the hortolog of the mammalian chp-1. These conclusions are further supported by analyzing the relative degree of homology. In fact, *Drosophila* and *C. elegans* unique CHORD containing proteins showed a higher degree of homology with mouse and human chp-1 than with melusin (Table 1).

3.3. Chp-1 and melusin are metal binding proteins

As already described, plant CHORD domains are able to bind Zn^{2+} [5]. To test whether chp-1 and melusin have retained this ability, we measured the zinc binding to a number of recombinant MBP fusion proteins by a colorimetric assay based on complex formation with PAR. Chp-1, melusin and melusin lacking the extreme C-terminal acidic stretch (Δ-melusin) were found to bind very similar mol equivalents of Zn^{2+} (1.6, 1.8, 1.65 means) (Table 2). A melusin construct containing only CHORD1 bound 0.7 mol equivalents of zinc (Table 2). These data showed that both CHORD domains of mammalian proteins have retained the ability to coordinate zinc ions.

Since the acidic amino acid stretch located at the extreme C-terminal portion of melusin closely resembles calreticulin and calsequestrin C domain, known to bind calcium at high capacity and low affinity [3,4], we performed a Ca^{2+} overlay

Table 1
Comparative sequence analysis among vertebrate and invertebrate CHORD containing proteins

	Chp-1		Melusin	
	Human	Mouse	Human	Mouse
Drosophila chp-1	42	42	35	38
C. elegans chp-1	33	32	30	31

Comparative sequence analysis among vertebrate (mouse and human) CHORD containing proteins (chp-1 and melusin) and invertebrate (*Drosophila* and *C. elegans*) unique chp-1. The numbers refer to Clustal W sequence alignment score [13,14]. *Drosophila* and *C. elegans* CHORD containing protein showed a higher degree of homology with vertebrate chp-1, than with melusin.

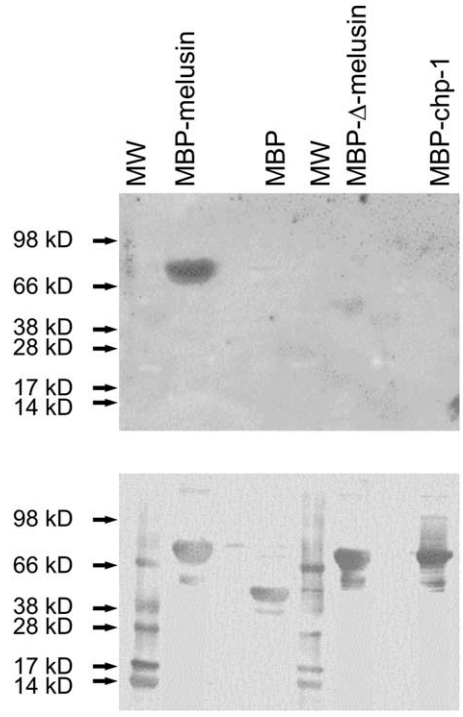

Fig. 3. Calcium binding assay. Same amounts of purified MBP-melusin, MBP-Δ-melusin, MBP-chp-1 and MBP alone were subjected to SDS–PAGE, blotted on nitrocellulose membrane and incubated with $^{45}Ca^{2+}$. Upper panel: the image of the exposed film; lower panel: the same blot was stained with Ponceau red. While MBP-melusin was able to bind calcium, MBP-Δ-melusin, lacking the acidic terminal domain, lost completely this ability. Moreover MBP-chp-1, that does not possess the acidic domain, did not bind calcium. MW: molecular weight.

Table 2
Zinc binding to chp-1 and melusin fusion proteins

Recombinant proteins	Zn++ mole equivalents	Schematic structure of recombinant proteins
MBP	0.1/ 0.2	
MBP-chp-1	1.6/ 1.8/ 2.1/ 1.5/ 2	
MBP-melusin	1.5/ 1.7	
MBP-Δ-melusin	1.5/ 1.8	
MBP-melCHORD1	0.6/ 0.7/ 0.8/ 0.7	

Purified fusion proteins were tested for their Zn^{2+} binding by a colorimetric assay based on complex formation with PAR. ⬭: CHORD; ⬭: intervenient sequence; ⬭: Sgt1 like domain; ▭: acidic amino acid stretch. The numbers indicated are zinc mol equivalents obtained from independent experiments.

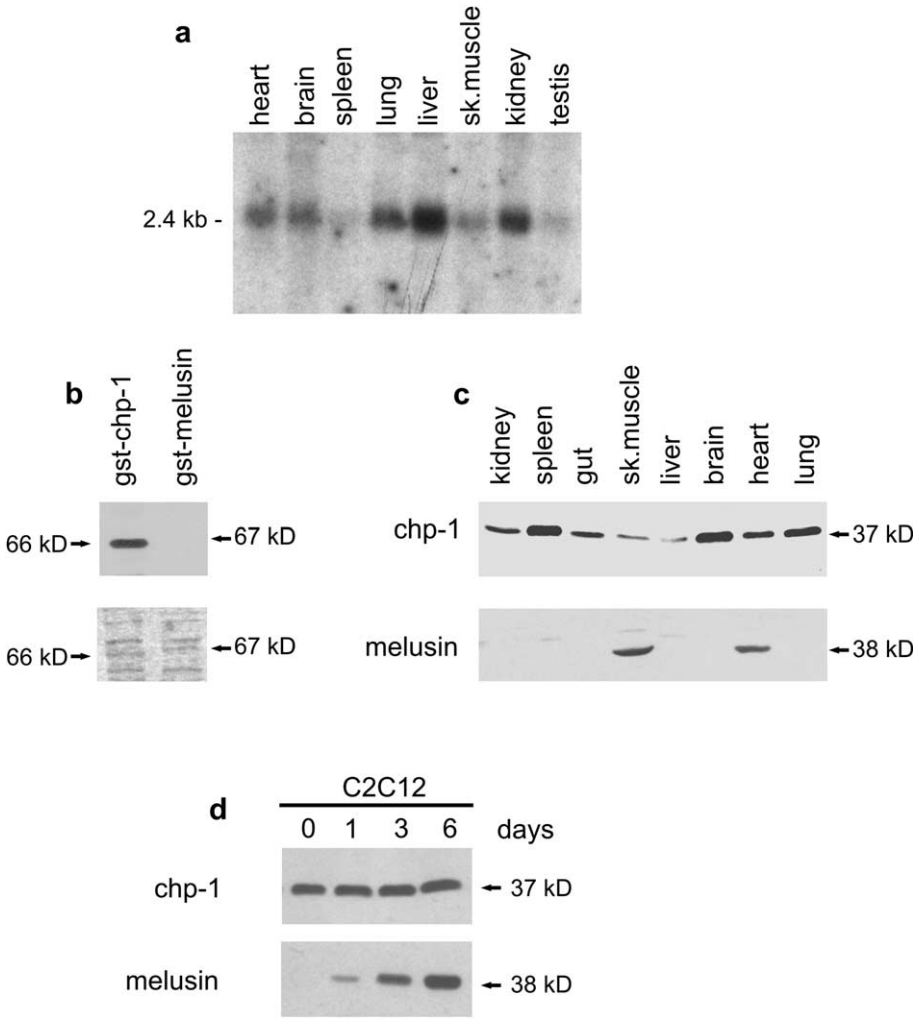

Fig. 4. Analysis of chp-1 and melusin expression. a: Northern blot on poly A+ RNA from mouse tissues using as probe mouse chp-1 cDNA. A single transcript of 2.4 kb was detected in all tissues tested. b: Western blot analysis to test anti-chp-1 antibody specificity. Total protein extracts from *E. coli* expressing GST-chp-1 and GST-melusin were subjected to SDS–PAGE, blotted on nitrocellulose membrane and probed with purified anti-chp-1 polyclonal antibodies. Upper panel: chemiluminescent detection of antibody binding; lower panel: the same blot stained with Ponceau red. Only recombinant chp-1 (66 kDa), but not melusin (67 kDa), was recognized by the purified anti-chp-1 polyclonal antibodies. c: Western blot analysis on total protein extracts from different mouse tissues with the purified polyclonal antibodies to chp-1 (upper panel) and to melusin (lower panel). A chp-1 band (37 kDa) was detected in all tissues tested, while melusin band (38 kDa) was present only in skeletal muscle and heart. d: Western blot of total proliferating (0) and differentiated (1, 3, 6 days) C2C12 protein extracts was probed with chp-1 (upper panel) and melusin antibodies (lower panel). While chp-1 is expressed in proliferating myoblasts and its expression remained steady after differentiation, melusin expression is switched on during C2C12 differentiation.

assay to test the ability of melusin to bind calcium. The same amounts of melusin, Δ-melusin and chp-1 were subjected to SDS–polyacrylamide gel electrophoresis (PAGE), blotted on nitrocellulose membrane and incubated with $^{45}CaCl_2$. While melusin was able to bind calcium, Δ-melusin, in which the terminal acidic domain is deleted, lost this ability. Moreover chp-1, that does not contain the acidic domain, did not bind calcium (Fig. 3).

3.4. *Analysis of chp-1 and melusin expression in mouse tissues and cell lines*

Melusin mRNA and protein are detectable only in skeletal and cardiac muscles [1] (see also Fig. 4c). To investigate chp-1 expression pattern, Poly A+ RNA from mouse tissues was analyzed by Northern blotting. A single transcript of 2.4 kb was detected in all tissues tested (Fig. 4a). Reverse transcription (RT)-PCR analysis of different tissues, with primers covering the entire length of the molecule, did not reveal the existence of alternative spliced forms (data not shown).

To detect the presence of chp-1 protein we produced rabbit polyclonal antibodies against MBP-chp-1 fusion protein. This antiserum specifically reacted with chp-1 and did not cross-react with melusin as shown in Fig. 4b.

Western blotting analysis with affinity purified antibodies confirmed that chp-1 is ubiquitously expressed (Fig. 4c), the higher expression level was detected in spleen, lung and brain. Moreover we detected the presence of chp-1 in cell lines derived from different tissues and animal species: COS (monkey kidney cells), ECV (human epithelial cells), 293 (human embryo kidney cells), FRT (Fischer rat thyroid cells), NIH (mouse embryonal fibroblasts), CHO (Chinese hamster ovary cells), N1E115 (mouse neuroblastoma cells), mouse primary fibroblasts (data not shown).

We previously demonstrated that melusin expression is up-

regulated during muscle differentiation using C2C12 myogenic cell line that can be induced to differentiate in vitro to form myotubes. To investigate whether chp-1 expression was also regulated, we performed a Western blot analysis on undifferentiated and differentiated C2C12. As shown in Fig. 4d melusin was absent in undifferentiated myoblasts but during differentiation its expression was turned on and the higher level of its expression was reached in myotubes after 6 days of differentiation. In contrast chp-1 remained steady with no changes in expression (Fig. 4d). These data also demonstrated that melusin and chp-1 are coexpressed in muscle cells.

4. Discussion

We have previously identified melusin, a muscle specific protein involved in transducing mechanical signal in cardiomyocytes [2]. In this paper we report the identification of a gene coding for a protein highly homologous to melusin, called chp-1. Both melusin and chp-1 are characterized by a tandemly repeated domain with a unique cysteine and histidine rich motif in the N-terminal portion of the molecules. Recently, motives containing an identical cysteine and histidine consensus were identified in a protein from barley, *Arabidopsis*, *C. elegans* and in a variety of dbest cDNAs of other animal species and were designated as CHORDs [5]. While the plant CHORD containing proteins consist only of tandemly repeated CHORD domain, metazoan chp-1 and melusin contain a C-terminal extension adjacent to the second CHORD, previously indicated as CS domain [5], sharing significant sequence similarity with yeast protein Sgt1, an essential component of the ubiquitin ligation machinery. Interestingly, barley and *Arabidopsis* CHORD containing protein (Rar1) can form a complex with Sgt1 plant homolog [9]. Thus the presence in metazoans of proteins containing both CHORD domains and Sgt1 homolog C-terminal extension is an interesting example of the Rosetta stone principle, in which two interacting proteins are frequently fused into a single polypeptide during evolution [9,10]. Mutational analysis in plants indicated that the CHORD containing proteins Rar1 and Sgt1 are essential components of the innate immune resistance to pathogen attack [9]. Gene inactivation in mouse revealed that the muscle specific melusin functions as a transducer of mechanical signal in heart, suggesting divergent functional properties of this mammalian CHORD containing protein. The function of chp-1 in mammalian tissues is still unknown and gene inactivation studies will hopefully reveal whether this ubiquitous protein is functionally related to the CHORD containing proteins in plants.

Chp-1 coding gene is located on chromosome 9 in mouse and 11q14.3 in human, while melusin gene was previously mapped on synthetic region X band D in mouse and Xq12.1-13 in human. Both melusin and chp-1 amino acid sequences are coded by 11 exons, and the distribution of the coding sequences in each exon is perfectly conserved, suggesting that the two genes are derived by a duplication event. In contrast intron extensions are very different in chp-1 and melusin, suggesting a phylogenetically far gene duplication. By searching the database we discovered chp-1 and melusin hortologs in *F. rubripes* genome as separate genes, moreover two different partial dbest cDNAs were also present in zebrafish, while *D. melanogaster* and *C. elegans* genomes contained only one homolog. As the CHORD containing proteins in invertebrate do not contain the acidic stretch in the extreme C-terminal portion of the molecule, a domain typically present only in melusin, we suggest that the ancestral gene is the chp-1 homolog that, during early chordate evolution, produced the melusin gene. In addition invertebrate CHORD containing proteins share a higher degree of homology with mouse and human chp-1 than with melusin (Table 1).

Chp-1 and melusin display a different expression pattern in mouse tissues. In fact chp-1 is ubiquitary expressed, while melusin is present only in skeletal and cardiac muscles. Moreover melusin expression is upregulated during C2C12 differentiation demonstrating a typical skeletal muscle transcriptional regulation. In contrast chp-1 expression level does not change during myogenesis. Melusin in muscle fibers is localized at costamers [1], junctional structures connecting Z disk proteins to the plasma membrane and functioning in transducing mechanical stimuli. Recent studies strongly suggest a selective role for the Z disk proteins, such as MLP, ZASP/CYPHER, telethonin, ALP and myotilin, in sensing mechanical stretch [11]. Database searches indicated that among these molecules, telethonin, ALP and myotilin, like melusin, are not present in *C. elegans* and *Drosophila*, but they appear in zebrafish and fugu, indicating that these proteins are functioning as specific Z disk components in vertebrate muscles. Whether these proteins physically interact and are functionally related remains to be established.

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