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Proteins encoded by human Down syndrome critical region gene 1-like 2 (*DSCR1L2*) mRNA and by a novel *DSCR1L2* mRNA isoform interact with cardiac troponin I (TNNI3)

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Abstract

Down syndrome critical region gene 1-like 2 (*DSCR1L2*) belongs to the human *DSCR1*-like gene family, which also includes *DSCR1* and *DSCR1L1*. Both DSCR1 and DSCR1L1 proteins interact with calcineurin, a calcium/calmodulin-dependent phosphatase. To date, no interactor has been described for DSCR1L2. The aim of this work was to perform a first functional study of DSCR1L2 using yeast two-hybrid analysis conducted on a human heart cDNA library. Here, we report the interaction between DSCR1L2 and the human cardiac troponin I (TNNI3), the heart-specific inhibitory subunit of the troponin complex, a central component of the contractile apparatus. This interaction was confirmed by both yeast cotransformation and GST (glutathione–sepharose transferase) fusion protein assay. Moreover, a new *DSCR1L2* mRNA isoform, generated by alternative splicing, was identified and cloned in different tissues: it lacks two central exons, encoding the most conserved domains among the DSCR1-like protein family. A quantitative relative reverse transcription-polymerase chain reaction (RT-PCR) assay showed that in heart tissue the normalized expression level ratio for *DSCR1L2* and *DSCR1L2-E2E5* mRNA isoforms is 3.5 : 1, respectively. The yeast cotransformation and GST fusion protein assay demonstrated the interaction between this new DSCR1L2 variant and the human cardiac troponin I and the prominent role of *DSCR1L2* exon 2 in determining binding between both DSCR1L2 isoforms and TNNI3. These data indicate an entirely new role for a *DSCR1*-like family gene, suggesting a possible involvement of DSCR1L2 in cardiac contraction.

Keywords: Two-hybrid system techniques; Human heart; Alternative splicing

Abbreviations: Ade, adenine; BSA, bovine serum albumin; cDNA, DNA complementary to RNA; CDS, coding sequence; *DSCR1*, Down syndrome critical region gene 1; DSCR1, Down syndrome critical region gene 1 encoded protein; *DSCR1L1*, Down syndrome critical region gene 1-like 1; DSCR1L1, Down syndrome critical region gene 1-like 1 encoded protein; *DSCR1L2*, Down syndrome critical region gene 1-like 2; DSCR1L2, Down syndrome critical region gene 1-like 2 encoded protein; *DSCR1L2-E2E5*, DSCR1L2 mRNA spliced form containing exons 2 and 5; DSCR1L2-E2E5, DSCR1L2 spliced form protein; *DSCR1L2-E3E4*, cDNA containing *DSCR1L2* exons 3 and 4; *DSCR1L2-E2*, cDNA containing *DSCR1L2* exon 2; DTT, dithiothreitol; EST, expressed sequence tag; GAL4 AD, GAL4 activation domain; GAL4 DBD, GAL4 DNA binding domain; GST, glutathione–sepharose transferase; His, histidine; HA, hemagglutinin; IPTG, isopropyl β-D-thiogalactoside; kb, kilobase(s) or 1000 bp; kDa, kilodalton(s); *lacZ*, β-galactosidase gene; Leu, leucine; MW, molecular weight; ORF, open reading frame; PA, polyacrylamide; PAGE, PA-gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; QDO, quadruple dropout media lacking leucine, tryptophan, histidine and adenine; RT-PCR, reverse transcription-polymerase chain reaction; SD, synthetic dropout medium; SDS, sodium dodecyl sulfate; T_a, annealing temperature; TAE, tris acetate EDTA (ethylene diamine tetraacetic acid); TNNI3, human cardiac troponin I; Trp, tryptophan; 5′-UTR, 5′ untranslated region.

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1. Introduction

Human Down syndrome critical region gene 1-like 2 (*DSCR1L2*; RefSeq mRNA entry no. NM_013441) belongs to the *DSCR1*-like gene family (Strippoli et al., 2000a), which includes three human genes: *DSCR1* (Fuentes et al., 1995) (also named *MCIP1* — Myocite Interacting Protein 1 or adapt78 or calcipressin 1) located on chromosome 21 (21q22.12), *DSCR1L1* (Miyazaki et al., 1996) (also named *MCIP2* or *ZAKI-4*) on chromosome 6 (6p21.1-p12.3) and *DSCR1L2* (Strippoli et al., 2000a) (also named *MCIP3*) on chromosome 1 (1p36.11). The *DSCR1*-like gene family was also characterized in mouse (Strippoli et al., 2000b) and *DSCR1* homologues are conserved from yeast to humans, sharing an ISPPXSPP box in their encoded proteins (Strippoli et al., 2000a).

DSCR1L2 gene codes for a 241 amino acids protein with an estimated MW of 27.5 kDa. Although *DSCR1L2* gene was originally described to be composed of four exons (Strippoli et al., 2000a), further availability of cDNA sequences from a large-scale cDNA sequencing project allows identification of five exons, without modification of the coding sequence that is now included between exon 2 and exon 5 (GenBank DNA entry no. AL034582; Strausberg et al., 2002). An alternative splicing form (GenBank accession no. AF176117), which presents an in-frame loss of 30 bases with consequent absence of 10 amino acids in the middle of the predicted protein, has been also described (Strippoli et al., 2000a).

Both *DSCR1* and *DSCR1L1* are highly expressed in adult heart and skeletal muscle in both humans and mice (Fuentes et al., 1995; Miyazaki et al., 1996), whilst *DSCR1L2* transcripts are expressed in many tissues such as heart, skeletal muscle, liver, kidney, peripheral blood leukocytes (Strippoli et al., 2000a), as well as in foetal and neoplastic tissues (as shown by analysis of expressed sequenced tag (EST) database).

An increasing number of papers has been published on the DSCR1 and DSCR1L1 protein functions (reviewed in Rothermel et al., 2003). Both DSCR1 and DSCR1L1 proteins interact physically and functionally with calcineurin, a calcium/calmodulin-dependent phosphatase (Rothermel et al., 2000; Fuentes et al., 2000). DSCR1 and DSCR1L1 proteins bind directly to calcineurin and they are potent inhibitors of calcineurin in vitro (Fuentes et al., 2000; Gorlach et al., 2000; Kingsbury and Cunningham, 2000). Moreover, different studies focus on possible roles of DSCR1 in cardiac hypertrophy and failure (Rothermel et al., 2003) with dysregulation of calcium and activation of calcineurin.

To date no functional studies have been performed on DSCR1L2, although a common role of DSCR1, DSCR1L1 and DSCR1L2 in binding calcineurin has been hypothesized (Rothermel et al., 2003) only on the implicit basis of the high sequence homology between *DSCR1L2* and the other two *DSCR1*-like family members.

The aim of this work was to perform a first functional study of DSCR1L2 protein based on a yeast two-hybrid analysis performed on a human heart cDNA library, to evaluate the possible protein–protein interactions between DSCR1L2 and cardiac human proteins. The human heart library was chosen because of both the high expression of *DSCR1L2* in this tissue and the important DSCR1 and DSCR1L1 functional roles recently identified in the adult human heart.

In this study, we detail the interaction between DSCR1L2 and the human cardiac troponin I (TNNI3), confirmed by both yeast cotransformation and GST fusion protein assay. TNNI3 is the heart-specific inhibitory subunit of the troponin complex, a central component of the contractile apparatus (Vallins et al., 1990).

Furthermore, the product of a new *DSCR1L2* mRNA isoform (*DSCR1L2-E2E5*) generated by alternative splicing was identified and demonstrated to interact with human cardiac troponin I too. Finally, interaction was mapped to a domain encoded by *DSCR1L2* exon 2, suggesting that DSCR1L2 is able to interact with TNNI3 via the region least conserved among the other DSCR1L family members.

These findings suggest a new role in cardiac contraction for a *DSCR1*-like family gene.

2. Materials and methods

2.1. Polymerase chain reaction (PCR)

Standard PCR conditions for all amplifications described in this work were: 50 μL final volume, primers 0.3 μM each, dNTPs 0.2 mM each, $MgCl_2$ 2 mM, Taq polymerase (TaKaRa, Shigan, Japan) used with its 1× companion buffer; initial denaturation: 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at the indicated annealing temperature (T_a), 30 s at 72 °C; final extension 7 min at 72 °C. Deviations from these data conditions are given below when appropriate.

2.2. Plasmid construction

Specific primers (Table 1, #1 and #2) were used to clone *DSCR1L2* cDNA from 1.5 μL of commercial human heart cDNA (Clontech, Palo Alto, CA) using standard conditions

Table 1
Primer list

No	Sequence 5′→3′
#1	GGAAAAGGCCCACTTTGGGAATTCATGCTGA (F)[a]
#2	CCGCCTCGCACCACAACCAGGATCCTCACAG (R)[b]
#3	CCGCCTCGCACCACAACCAGAATTCTCACAG (R)
#4	CATGAAGCAGTGTTTGAGGAATTCGAGCAG (F)
#5	CGACTCTGTTCCCGCGTGAATTCCATATT (R)
#6	GCAGAAGTGAGAATTCGAAGCACTCTTC (F)
#7	GAAGAGTGCTTCGAATTCTCACTTCTGC (R)
#8	TAATACGACTCACTATAGGGC (F)
#9	GGGCTGGCAAGCCACGTTTGGTG (F)
#10	CCGACAACCTTGATTGGAGACTTGAC (F)
#11	GTATGGCTTACCCATACGATGTTCCAG (R)
#12	GCCAGTCACATCTGTGTAGCAC (F)
#13	TCCCGCGTGAAGTTCATATTTCTCTCCTGG (R)
#14	TCCCGCGTGAAGTTCATATTTCTCTCCTTC (R)
#15	GCGGGCATTCCTGAAGCTGACAGCA (F)
#16	TACATCAAACATGGAGACAGCACTC (R)

[a] F, forward primer.
[b] R, reverse primer.

(Section 2.1), except that KlenTaq polymerase (AB Peptides, St. Louis, MO) 0.8 U was used with its 1× PCR companion buffer including $MgCl_2$ and cycles were 25 with annealing time of 2 min at 68 °C. RT-PCR product was evaluated to contain both the full length *DSCR1L2* cDNA and the *DSCR1L2* spliced form containing exons 2 and 5 (*DSCR1L2-E2E5*) (deposited in GenBank accession no. AY906854). Full length *DSCR1L2* and *DSCR1L2-E2E5* cDNAs were first cloned in pT-Adv vector by AdvanTAge Kit (Clontech). *DSCR1L2* and *DSCR1L2-E2E5* cDNAs were amplified from pT-Adv plasmid by using specific forward and reverse primers, each containing an *Eco*RI site (Table 1, #1 and #3), starting from 10 μL of a bacterial colony resuspended in 50 μL of water under standard PCR conditions (Section 2.1), with T_a of 68 °C. The PCR products were then cloned into the *Eco*RI site of pGBKT7 (Clontech) to yield pGBKT7/*DSCR1L2* and pGBKT7/*DSCR1L2-E2E5* plasmids, suitable for the yeast two-hybrid test (the former) and yeast cotransformation (both plasmids).

A cDNA containing *DSCR1L2* exons 3 and 4 (*DSCR1L2-E3E4*) and a cDNA containing *DSCR1L2* exon 2 (*DSCR1L2-E2)* were amplified from 1 μL of a human *DSCR1L2* RT-PCR product (obtained as described above with primers #1 and #3) using specific forward and reverse primers, each containing *Eco*RI site (Table 1, #4 and #5; #6 and #7, respectively). PCR was performed under standard PCR conditions (Section 2.1), except that cycles were 40, at T_a of 63 °C for primers #4 and #5 and 58 °C for primers #6 and #7. The PCR products were then cloned into *Eco*RI site of pGBKT7 (Clontech) to yield pGBKT7/*DSCR1L2-E3E4* and pGBKT7/*DSCR1L2-E2* plasmids, suitable for yeast cotransformation. *DSCR1L2*, *DSCR1L2-E2E5*, *DSCR1L2-E3E4*, *DSCR1L2-E2* cDNAs were cloned into *Eco*RI site of pGEX-4T-1 vector (Amersham Biosciences, Uppsala, Sweden) to yield pGEX-4T-1/*DSCR1L2*, pGEX-4T-1/*DSCR1L2-E2E5*, pGEX-4T-1/*DSCR1L2-E3E4* and pGEX-4T-1/*DSCR1L2-E2* plasmids, suitable for GST fusion protein assay.

To check the sequences of the plasmid inserts, PCR products were obtained from 10 μL of a bacterial colony resuspended in 50 μL of water under standard PCR conditions (Section 2.1), with T_a of 55 °C using a vector-specific forward primer (Table 1, #8 for pGBKT7 and #9 for pGEX-4T-1) and a reverse insert-specific primer corresponding to the reverse primer used for the respective PCR amplification. These products were then sequenced as described in Section 2.4, with the same primers used in the respective PCR.

2.3. Yeast two-hybrid assay

Yeast two-hybrid Matchmaker System 2 (Clontech) was used to screen for potential DSCR1L2-interacting proteins. The human heart cDNA library (Clontech, code #PT3183-1, containing $>1\times10^6$ independent clones) was cloned into pACT2 plasmid, encoding a GAL4 activation domain (GAL4 AD) and a hemagglutinin (HA) amino-terminal tag, and it was pretransformed by the manufacturer into the yeast strain Y187. cDNA library was then screened by mating Y187 with PJ692A yeast containing the *DSCR1L2* bait construct (pGBKT7/*DSCR1L2*). pGBKT7/*DSCR1L2* encodes a fusion protein that contains amino acids 1–147 of the GAL4 DNA binding domain (GAL4 DBD), a Myc tag, and the whole human DSCR1L2 amino acid sequence. PJ692A contains two conditional reporter genes, *HIS3* and *ADE2*, which are under the control of GAL4-responsive upstream activation sites. Y187 contains a conditional reporter gene, *lacZ* (β-galactosidase gene). PJ692A and Y187 were mated and were initially selected on triple-dropout media lacking vector-selective markers Leucine (Leu), Tryptophan (Trp) and Histidine (His). Positive clones were then re-streaked on the most stringent selection media, i.e., quadruple-dropout (QDO) media lacking Leu, Trp, His and Adenine (Ade). The plates were incubated at 30° C for 15 days. A membrane filter assay for β-galactosidase activity (Ausubel et al., 1999) was performed to assess the expression of reporter gene *lacZ* of QDO positive clones. β-galactosidase positive colonies were then grown in selective media, and plasmids were recovered from putative yeast positive colonies by YEASTMAKER Yeast Plasmid Isolation Kit (Clontech). Each putative interactor from positive clones was PCR-amplified using pACT2-specific primers (Table 1, #10 and #11) flanking the insert, to confirm uniqueness of the insert and hence of the clone. PCR was performed starting from 10 μL of a bacterial colony resuspended in 50 μL of water under standard PCR conditions (Section 2.1), with T_a of 55 °C. PCR products were sequenced (Section 2.4) with the same primers used in the respective PCR reaction.

2.4. DNA sequencing and sequence analysis

All sequences were determined using the Big Dye Terminator Cycle Sequencing-ready Reaction kit and automated DNA sequence analyzer ABI-PRISM 310 (Applied Biosystems, Foster City, CA). Searches of nucleotide databases were performed using BLAST programs – accessed via the NCBI homepage (http://www.ncbi.nlm.nih.gov/) – to identify the putative interactors and to analyze the EST database. Sequence alignments were made using ClustalW 1.8 software (http://www.ebi.ac.uk/clustalw).

2.5. Yeast cotransformation

Four different yeast cotransformations were performed: one to confirm the putative DSCR1L2 interactor obtained from the yeast two-hybrid assay; one to verify the interaction between the putative DSCR1L2 interactor and the spliced form DSCR1L2-E2E5; two to verify the interaction between the putative DSCR1L2 interactor and the *DSCR1L2-E3E4* or the *DSCR1L2-E2* encoded products. Purified plasmid with the hypothetical interactor (pACT2/*TNNI3*) and pGBKT7/*DSCR1L2* or pGBKT7/*DSCR1L2-E2E5* or pGBKT7/*DSCR1L2-E3E4* or pGBKT7/*DSCR1L2-E2* plasmids were cotransformed into the PJ692A yeast strain (0.1 μg for each plasmid). Four cotransformations were performed in each assay as negative controls, to exclude the presence of non-specific interactions: pACT2/*TNNI3* (0.1 μg) and the empty pGBKT7 (0.1 μg) expressing only the GAL4 DBD (with no *DSCR1L2*); pACT2/*TNNI3* (0.1 μg) and no pGBKT7; pGBKT7/*DSCR1L2* (or pGBKT7/*DSCR1L2-E2E5* or pGBKT7/*DSCR1L2-E3E4* or pGBKT7/*DSCR1L2-E2*) (0.1 μg) and no

pACT2; pGBKT7/*DSCR1L2* (or pGBKT7/*DSCR1L2-E2E5* or pGBKT7/*DSCR1L2-E3E4* or pGBKT7/*DSCR1L2-E2*) (0.1 μg) and the empty pGADT7 (0.1 μg, now provided by Clontech in place of pACT2) (Table 2). Cotransformed products were streaked on QDO media, incubated at 30 °C and observed after 5 days. Three different dilutions (1 : 1, 1 : 10, 1 : 100) of cotransformation products were streaked on SD/-Trp/-Leu (synthetic dropout medium lacking Trp and Leu) to evaluate the cotransformation efficiency.

2.6. GST fusion protein assay

2.6.1. Purification of GST-DSCR1L2 fusion proteins

GST fusion protein assay was performed as described by Spisni et al. (2001).

GST-DSCR1L2, GST-DSCR1L2-E2E5, GST-DSCR1L2-E3E4 and GST-DSCR1L2-E2 fusion proteins and GST non-fusion protein were expressed in *Escherichia coli*, following 2 h of induction with 0.4 mM IPTG (isopropyl β-D-thiogalactoside). Fusion proteins were purified from cell lysates using glutathione–agarose beads (Sigma, St. Louis, MO). The beads were washed three times in 1× PBS (phosphate buffered saline), pH 7.4, containing 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiothreitol), and a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The yield of the proteins was determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and blue Coomassie staining of the gel, using BSA (bovine serum albumin) as a standard.

2.6.2. Interaction of GST-DSCR1L2, GST-DSCR1L2-E2E5, GST-DSCR1L2-E3E4 and GST-DSCR1L2-E2 fusion proteins with human cardiac troponin I (TNNI3)

About 100 pmol of GST or GST-DSCR1L2 fusion proteins (DSCR1L2, DSCR1L2-E2E5, DSCR1L2-E3E4, and DSCR1L2-E2) prebound to glutathione–agarose beads (Sigma) were incubated overnight (shaken at 4 °C) with an equimolar amount of TNNI3 (Abcam, Cambridge, UK). Following the overnight binding reaction, the beads were washed six times in 1 mL of wash buffer containing 1 mM PMSF and protease inhibitors as above. Bound proteins were eluted with 50 μL of glutathione elution buffer (Amersham Biosciences).

SDS-PAGE buffer was added to the eluted proteins and after boiling they were separated on 12% SDS-polyacrilamide gel, transferred to nitrocellulose paper, and immunoblotted with a monoclonal anti-TNNI3 antibody (Abcam) diluted 1 : 150 or with a polyclonal antibody anti-GST (kindly provided by Stefania Trazzi, Dept. of Experimental Biology, Bologna). Detection was performed using the ECL reagents developed by Amersham Biosciences. The amount of TNNI3 bound to GST-DSCR1L2 and GST-DSCR1L2-E2E5 was detected under white light in unsaturated pixel modality with the Gel Doc 2000 Imaging System and analyzed with Quantity One software (Bio-Rad, Hercules, CA), using determined amounts of commercial TNNI3 (Abcam) as a quantitative reference.

Table 2
Experimental design of yeast cotransformation

Plasmid 1[a]	Plasmid 2[a]	Colonies on QDO[b]
pGBKT7/*DSCR1L2*	pACT2/*TNNI3*	Yes
Empty pGBKT7	pACT2/*TNNI3*	No
No plasmid	pACT2/*TNNI3*	No
pGBKT7/*DSCR1L2*	Empty pGADT7	No
pGBKT7/*DSCR1L2*	No plasmid	No
pGBKT7/*DSCR1L2-E2E5*	pACT2/*TNNI3*	Yes
pGBKT7/*DSCR1L2-E2E5*	Empty pGADT7	No
pGBKT7/*DSCR1L2-E2E5*	No plasmid	No
pGBKT7/*DSCR1L2-E3E4*	pACT2/*TNNI3*	No
pGBKT7/*DSCR1L2-E3E4*	Empty pGADT7	No
pGBKT7/*DSCR1L2-E3E4*	No plasmid	No
pGBKT7/*DSCR1L2-E2*	pACT2/*TNNI3*	Yes
pGBKT7/*DSCR1L2-E2*	Empty pGADT7	No
pGBKT7/*DSCR1L2-E2*	No plasmid	No

[a] Plasmid 1 and Plasmid 2 indicate the plasmids cotransformed in PJ692A yeast strain.

[b] Colonies on QDO indicate the grow (YES) or the not-grow (NO) of colonies on the selective media after cotransformation.

2.7. Cloning of DSCR1L2 mRNA isoforms

Commercial RNA (Clontech) from a normal adult whole heart was used to clone *DSCR1L2* and *DSCR1L2-E2E5* cDNAs. *DSCR1L2-E2E5* cDNA was also cloned from adult whole skeletal muscle (pooled from two subjects) and brain commercial RNAs (Clontech). Specific primers were designed using Amplify software (Engels, 1993), following standard criteria (Sharrocks, 1994). The data source for the primer design were the GenBank RNA sequences no. NM_013441 (*DSCR1L2*) and no. AY906854 (*DSCR1L2-E2E5*). To clone the two *DSCR1L2* isoforms, three primers were designed: a forward primer common to both gene isoforms on exon 2 (Table 1, #12) and two reverse primers specific for *DSCR1L2* and *DSCR1L2-E2E5* isoforms (Table 1, #13 and #14, respectively). These reverse primers were needed due to the presence of two mismatches at the last three bases of the 3′ end (one being the 3′-residue) with respect to the sequence of the other isoform. The specific reverse primers were designed encompassing exon 4–exon 5 (#13) and exon 2–exon 5 (#14) junctions. Reverse transcription conditions were: 2 μg total RNA, Moloney murine leukemia virus reverse-transcriptase (Promega, Medison, WI; used with companion buffer) 400 U, oligo dT-15 2.5 μM, random hexamers 2 μM, dNTPs 500 μM each. Reaction was performed in a final volume of 50 μM for 60 min at 37 °C. PCR conditions were as described (Section 2.1) except that final volume was 25 μL, the primer concentrations were 0.15 μM each, cycles were 45 with a T_a of 65 °C.

2.8. Quantitative relative RT-PCR

RT-PCR for the relative quantification of *DSCR1L2* isoforms was performed following the advice of Freeman et al. (1999). *B2M* housekeeping RNA was used for RNA quantity normalization and it was amplified from heart total RNA using primers #15 and #16 (Table 1). In preliminary PCR experiments, we evaluated PCR products after 25, 30, 35, and 40 cycles (data not shown), in order to find the conditions allowing quantification of *B2M* (25 cycles), *DSCR1L2* and *DSCR1L2-E2E5* (40 cycles both) RT-PCR products, at the maximum distance from the reaction

Fig. 1. Representative *TNNI3* sequences obtained from two hybrid assay positive clones aligned by ClustalW software. Sequence downstream the first base following *Eco*RI site corresponds in each case to *TNNI3* 5′-UTR followed by the start codon (ATG, in bold) of the reported reference *TNNI3* CDS (not shown). The different length of the sequences indicates that all of them derived from independent clones.

plateau. 10 μL aliquots of every PCR product were separated on 1.7% agarose TAE gel. Marker M5 (Fermentas, Hanover, MA) was used as a quantitative reference. After separation, the gel was stained in TAE buffer containing ethidium bromide (0.5 μg/mL) and detected under ultraviolet light in "unsaturated pixel" mode with the Gel Doc 2000 Imaging System. The digital image was analyzed by Quantity One software (Bio-Rad), selecting the "Volume Rect Tool" function to acquire intensity pixel data for each band and subtracting gel image background. Intensity values of the PCR product bands were calculated in comparison with a regression line with correlation coefficient 0.995 generated from measurements of four Marker M5 bands with different concentration values. The mean for each replicate data point (expressed as PCR product mass in ng) and the *DSCR1L2*/*B2M* or *DSCR1L2-E2E5*/*B2M* product mass ratios (to normalize the *DSCR1L2* isoforms expression level) were determined.

2.9. Accession number

The nucleotide sequence of the *DSCR1L2-E2E5* cDNA has been deposited in GenBank under accession no. AY906854.

3. Results

3.1. Yeast two-hybrid assay

The mating efficiency and number of clones screened corresponded to the values indicated in the manufacturer's instructions.

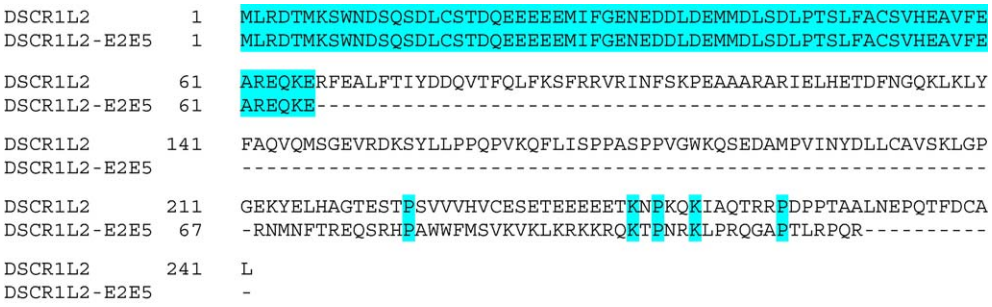

Fig. 2. DSCR1L2 and DSCR1L2-E2E5 predicted protein sequences aligned by ClustalW software. Due to out-of-frame joining between exons 2 and 5, the carboxy terminus sequence encoded by *DSCR1L2-E2E5* cDNA is not similar to that of DSCR1L2 product sequence. Exon 2 encoded domain is the same in both the alternative DSCR1L2 isoforms.

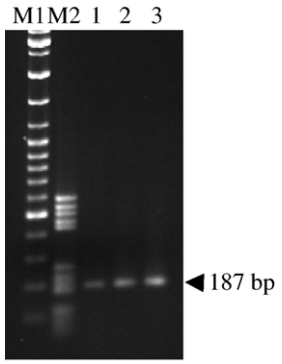

Fig. 3. *DSCR1L2-E2E5* mRNA isoform cloning in human heart, skeletal muscle and brain. Exemplificative agarose gel loaded with RT-PCR products, lanes 1–3: *DSCR1L2-E2E5* (band of the expected size: 187 bp) amplicon in heart, skeletal muscle and brain, respectively. M1 and M2 are size marker GeneRuler (500 ng) and size marker MBI 5-pBR322 DNA/BsuRIGeneRuler (250 ng).

In yeast two-hybrid assay, using *DSCR1L2* cDNA as bait, 57 QDO and β-galactosidase activity positive clones were successfully sequenced. 22 out of 57 clones encoded the cDNA for human cardiac troponin I (TNNI3). Other sequenced clones were already described as two-hybrid assay aspecific clones by the manufacturer, or they were present in only one copy, or they contained cDNA insert out-of-frame with the activation domain coding sequence of the plasmid (data not shown). All *TNNI3* sequenced clones showed the whole coding sequence (GenBank accession no. NM_000363) or part of it; all of them were in-frame with the activation domain coding sequence of the plasmid. Differences in the length of the cDNA inserts were due to the different length of the 5′-UTR upstream the start codon and indicated that all putative interactors derived from independent clones (Fig. 1).

3.2. DSCR1L2-E2E5 isoform cDNA cloning

Primers #1 and #2 were used to clone *DSCR1L2* cDNA from human heart RNA by RT-PCR. PCR product was cloned in pT-Adv and the sequence of several transformed clones was determined. Sequence analysis revealed the presence of clones containing a *DSCR1L2* alternatively spliced form containing exons 2 and 5 (*DSCR1L2-E2E5*) and lacking exons 3 and 4 (deposited in GenBank under accession no. AY906854). An EST database search failed to retrieve existing EST sequences corresponding to the *DSCR1L2-E2E5* mRNA isoform. DSCR1L2 and DSCR1L2-E2E5 predicted protein sequences were aligned using ClustalW software (Fig. 2). Due to out-of-frame joining between exons 2 and 5, the carboxy terminus sequence encoded by *DSCR1L2-E2E5* cDNA is not similar to that of the DSCR1L2 product sequence. *DSCR1L2-E2E5* mRNA isoform has been cloned from human heart, skeletal muscle and brain RNA (Fig. 3).

3.3. Yeast cotransformation

Four different yeast cotransformations were performed with their appropriate negative and positive controls (Table 2). All cotransformation efficiencies corresponded to the values indicated by the library manufacturer. Yeast cotransformation with plasmids containing the hypothetical interactor (pACT2/*TNNI3*) and pGBKT7/*DSCR1L2* showed a specific interaction between DSCR1L2 and TNNI3 gene products, confirming the yeast two-hybrid assay results. Yeast cotransformation with plasmid containing the *DSCR1L2* spliced form (pGBKT7/*DSCR1L2*-

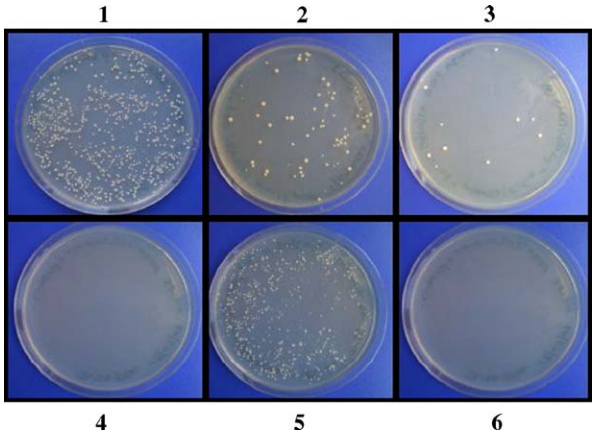

Fig. 4. Exemplificative pictures of yeast cotransformation plate results (see Table 2). 1. Cotransformation in SD/-Leu-Trp selective media to evaluate the cotransformation efficiency; 2–6. cotransformations in PJ692A yeast evaluated on QDO selective media: 2, pGBKT7/*DSCR1L2* and pACT2/*TNNI3* plasmids, (positive); 3, pGBKT7/*DSCR1L2-E2E5* and pACT2/*TNNI3* plasmids, (positive); 4, pGBKT7/*DSCR1L2-E3E4* and pACT2/*TNNI3* plasmids, (negative); 5, pGBKT7/*DSCR1L2-E2* and pACT2/*TNNI3* plasmids, (positive); 6, pGBKT7 and pACT2/*TNNI3* plasmids, (negative).

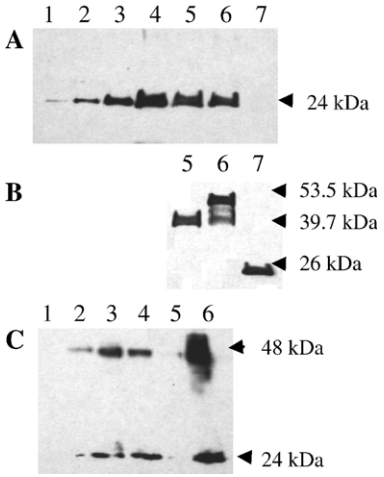

Fig. 5. Interaction of GST fusion proteins with TNNI3. Panel A: Western blotting experiment performed with anti-TNNI3 monoclonal antibody revealing expected band size of 24 kDa. Lanes 1–4: commercial TNNI3 75, 150, 300 and 600 ng respectively; lanes 5–7: TNNI3 incubated with GST-DSCR1L2-E2E5, GST-DSCR1L2 and GST alone (as negative control), respectively. Panel B: Western blotting experiment performed with anti-GST polyclonal antibody on the same nitrocellulose paper described in Panel A. Lane 5: GST-DSCR1L2-E2E5 fusion protein (39.7 kDa); lane 6: GST-DSCR1L2 fusion protein (53.5 kDa); lane 7: GST alone (26 kDa). Panel C: Western blotting experiment performed with anti-TNNI3 monoclonal antibody. Lanes 1–5: TNNI3 incubated with GST-DSCR1L2-E3E4, GST-DSCR1L2-E2, GST-DSCR1L2-E2E5, GST-DSCR1L2 and GST alone, respectively; lane 6: commercial TNNI3 300 ng. TNNI3 appears both in the monomer (24 kDa) and in the dimer (48 kDa) forms as declared by the manufacturer. Images are representative of four experiments performed under the same conditions.

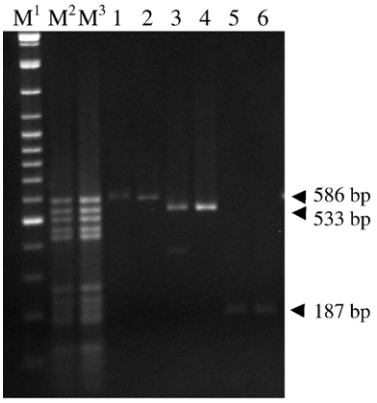

Fig. 6. *DSCR1L2* and *DSCR1L2-E2E5* mRNA relative quantification in heart tissue by RT-PCR. Lanes 1 and 2: *B2M* (band of the expected size 586 bp), lanes 3 and 4: *DSCR1L2* (band of the expected size: 533 bp), lanes 5 and 6: *DSCR1L2-E2E5* (band of the expected size: 187 bp). Lane 3 shows an extra band (360 bp) which corresponds to a not yet described *DSCR1L2* isoform (data not shown). M1: size marker GeneRuler (500 ng); M2 and M3: size marker MBI 5-pBR322 DNA/BsuRIGeneRuler (250 and 500 ng, respectively).

E2E5) and pACT2/*TNNI3* showed a specific interaction between DSCR1L2-E2E5 and TNNI3 gene products too. Yeast cotransformation with plasmid containing the *DSCR1L2-E3E4* cDNA (pGBKT7/*DSCR1L2-E3E4*) and pACT2/*TNNI3* did not show any interaction between DSCR1L2-E3E4 and TNNI3 products. Yeast cotransformation with plasmid containing the *DSCR1L2-E2* cDNA (pGBKT7/*DSCR1L2-E2*) and pACT2/*TNNI3* showed a specific interaction between DSCR1L2-E2 and TNNI3, indicating that *DSCR1L2* exon 2 was sufficient to encode the binding domain of DSCR1L2 or DSCR1L2-E2E5 with TNNI3 (exemplificative results are shown in Fig. 4). This was confirmed by the difference in the predicted amino acid sequence encoded by exon 5 of *DSCR1L2* and *DSCR1L2-E2E5*, respectively (Fig. 2): the exon 2 encoded domain is the only amino acid sequence conserved between DSCR1L2 and DSCR1L2-E2E5.

3.4. GST fusion protein assay

To determine whether DSCR1L2 and DSCR1L2-E2E5 physically interact with TNNI3, four GST fusion proteins (GST-DSCR1L2, GST-DSCR1L2-E2E5, GST-DSCR1L2-E3E4, GST-DSCR1L2-E2) were expressed in *E. coli* and purified as described in Materials and methods. In Fig. 5, the results of a representative experiment are reported, indicating that GST-DSCR1L2 and GST-DSCR1L2-E2E5 bind TNNI3 (Panel A), while GST alone does not appreciably bind TNNI3. Using commercial TNNI3 as quantitative reference, the amount of TNNI3 bound to GST-DSCR1L2 and GST-DSCR1L2-E2E5 was found to be comparable, and the molar ratio between TNNI3 and each of the GST fusion proteins is 1 : 1.5. Fig. 5 Panel B shows the presence of the bands corresponding to GST and GST-fusion proteins in the same blot analyzed using the anti-TNNI3 antibody: the comparability of their amount is consistent within the assay. Results indicate that GST-DSCR1L2-E2 binds TNNI3, while GST alone and GST-DSCR1L2-E3E4 do not appreciably bind TNNI3 (Fig. 5, Panel C). The intensity of the band, showing interaction between TNNI3 and GST-DSCR1L2-E2 (Fig. 5, Panel C, lane 2), appears weaker than the other bands (lanes 3 and 4) because of the possible steric inhibition of GST (26 kDa) on DSCR1L2-E2 (7.5 kDa).

3.5. Quantitative relative RT-PCR

The RT-PCR amplification products for *B2M*, *DSCR1L2* and *DSCR1L2-E2E5* isoform mRNAs were successfully obtained from adult human whole heart RNA. Gel electrophoresis analysis revealed bands of the expected size (Fig. 6). Estimation of the normalized (Section 2.8) expression level for the two *DSCR1L2* mRNA isoforms shows a 3.5 : 1 ratio in heart tissue for *DSCR1L2* and *DSCR1L2-E2E5* isoforms, respectively.

4. Discussion

Five years ago, a new human gene (*DSCR1L2*) and a new human gene family (*DSCR1*-like family) were described (Strippoli et al., 2000a; Kingsbury and Cunningham, 2000).

Some time later the *DSCR1*-like gene family was characterized in mouse (Strippoli et al., 2000b) and orthologous genes have been found in many other species (from yeast to mammals). Several further studies have been performed on the putative functions of *DSCR1*-like family gene products. In particular DSCR1 and DSCR1L1 have been demonstrated to interact with human calcineurin, a calcium/calmodulin-dependent phosphatase which regulates growth and gene expression of striated muscles (Rothermel et al., 2000; Fuentes et al., 2000).

An amino acid sequence identity between DSCR1L2 and either DSCR1 or DSCR1L1 corresponding to 61% and 68%, respectively, and a central serine–proline motif (ISPPXSPP box) conserved among all the gene family sequences have been described. Probably, on the basis of this homology, a common role of DSCR1, DSCR1L1 and DSCR1L2 in binding calcineurin had been hypothesized in the absence of experimental data.

The purpose of this study was to perform a first functional analysis to search for a DSCR1L2 interactor protein using a yeast two-hybrid assay. 22 clones out of 57 sequenced corresponded to human cardiac troponin I and the interaction between DSCR1L2 and TNNI3 has been confirmed by yeast cotransformation as well as by GST fusion protein assay. Although no calcineurin clone was identified by our two-hybrid test, further investigation will be needed to formally exclude that DSCR1L2 is unable to bind calcineurin, because there is the possibility that calcineurin cDNA was not present or under-represented in the investigated heart library lot.

DSCR1L2 is added to TNNI3K (Zhao et al., 2003), a specific TNNI3 kinase, and Polycistin-2 (Li et al., 2003), the product of the gene for kidney polycystic disease, as an interactor of TNNI3.

Human cardiac troponin I belongs to the troponin family. Troponins are proteins involved in the function of striated muscles, and they exist as a complex with actin and tropomyosin on the thin filament of the contractile apparatus. The

troponin complex consists of three protein subunits: troponin C, troponin T and troponin I, which is the inhibitory subunit preventing contraction in the absence of calcium and troponin C (Cummins and Perry, 1978). Three isoforms of troponin I had been identified: two in fast and slow skeletal muscles, and one form in cardiac muscle (TNNI3) (Cummins and Perry, 1978). Cardiac troponin I is mostly bound to the contractile apparatus in myocardium, but about 8% is found free in the cytoplasm (Bleier et al., 1998). TNNI3 becomes the sole troponin I expressed in myocardial cells during postnatal development; until that time slow skeletal muscle troponin I is the isoform predominantly expressed (Hunkeler et al., 1991). Cardiac troponin I is not expressed in normal skeletal muscle at any time, including development (Bodor et al., 1995). Structure and function of TNNI3 have been broadly studied because of the importance of the cardiac contraction mechanism and the involvement of TNNI3 in heart disease; in addition, the presence of TNNI3 in serum is considered to be a high specific biochemical marker of acute myocardial infarction. Several independent TNNI3 mutations have been uncovered both in patients affected by hypertrophic and restrictive cardiomyopathy (Kimura et al., 1997; Mogensen et al., 2003; Chien, 2003).

To date, research about DSCR1 and DSCR1L1 functions has been focused on the involvement of calcium–calcineurin pathway and some studies have been carried out on cardiac contraction in particular, since the demonstration that DSCR1 is directly induced by mechanical stimuli in cardiac myocytes (Wang et al., 2002). Later, several studies focused on possible roles of DSCR1 in cardiac hypertrophy and failures (reviewed in Rothermel et al., 2003) with dysregulation of calcium and activation of calcineurin. Results from several investigations suggest that an increase in DSCR1 protein levels can have a cardioprotective effect against diverse hypertrophic stimuli without deleterious consequences.

The interaction between DSCR1L2 and TNNI3, a component of the central contractile apparatus in heart muscle involved in congenital cardiac hypertrophy, provides a new functional pathway that enriches the relationship network between *DSCR1*-like gene family products and regulation of cardiac hypertrophy. Due to the presence of *TNNI3* mutations in congenital cardiac hypertrophy, *DSCR1L2* could also be proposed as a candidate gene for human heart diseases. The role of DSCR1L2 in other tissues which express *DSCR1L2* gene (Strippoli et al., 2000a) but not *TNNI3* remains to be determined.

A novel *DSCR1L2* mRNA isoform, showing direct joining of exons 2 and 5 (*DSCR1L2-E2E5*), has been found, in agreement with the recent assessment that human genes have a very high rate of alternative splicing (Kim et al., 2004). Due to out-of-frame joining between exons 2 and 5, the carboxy terminus sequence encoded by *DSCR1L2-E2E5* cDNA is not similar to that of the DSCR1L2 product sequence. A quantitative relative RT-PCR showed in heart tissue that the normalized expression level for the two *DSCR1L2* mRNA isoforms is 3.5 : 1 ratio for *DSCR1L2* and *DSCR1L2-E2E5* isoforms, respectively. The yeast cotransformation and GST fusion protein assay demonstrated the interaction between *DSCR1L2-E2E5* and the human cardiac troponin I; the amount of TNNI3 bound to both DSCR1L2 and DSCR1L2-E2E5 is comparable. It should be underlined that *DSCR1L2* exon 2, which has been found to be sufficient for the binding to TNNI3, encodes the less conserved domain among the human DSCR1-like protein family, suggesting a reason for the presence of specific interactors able to bind products of different members of the same gene family.

From a genomic point of view, the fact that in humans DSCR1L2 and DSCR1L2-E2E5 interact with TNNI3, whilst DSCR1 and DSCR1L1 interact with calcineurin, could indicate a very interesting case of functional divergence, at molecular level, within members of the same gene family, showing high similarity among the respective amino acid product sequences. It should be underlined that the number of DSCR1-like members tends to increase in more complex organisms, and DSCR1L2 has been shown to be the most recent member, appearing only in vertebrates (Strippoli et al., 2002). At the same time it is interesting to observe that, at a broader cell and tissue level, DSCR1, DSCR1L1 and DSCR1L2 products could share an involvement in similar (or in the same) mechanisms related to regulation of cardiac contraction, and remodelling, possible in the common context of the calcium pathway.

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