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Research article

THE CYTOPLASMIC DOMAIN OF CHONDROLECTIN INTERACTS WITH THE β-SUBUNIT OF RAB GERANYLGERANYL TRANSFERASE

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Abstract: Mouse chondrolectin (*chodl*) was isolated out of the tail tip of four-day old 129/SvJ mice as a by-product of a PCR-based subtractive cDNA library screening. The gene is predominantly expressed in adult skeletal muscle, heart, testes and lungs and in embryonic stadia. *Chodl* is the mouse homologue of human chondrolectin *(CHODL*), a gene that encodes for a type Ia transmembrane protein and that is expressed in human testis, prostate, heart and skeletal muscle tissue. *CHODL*-splice variants ($CHODL_f$, $CHODL_{f\Delta E}$, $CHODL_{\Delta E}$) are detected in human leukocytes. The proteins of the chondrolectin family belong to the family of C-type lectins. As the members of this protein family are important for a wide array of biological processes, the function of chodl was investigated by searching for its protein interaction partners. The β-subunit of Rab geranylgeranyl transferase (Rabggtb) was isolated 8 times after a complete Sos recruitment system (SRS) screen with the cytoplasmic domain of chodl. The interaction was confirmed with *in vitro* transcription/translation and co-immunoprecipitation (co-IP) experiments.

Key words: C-type lectin, Chondrolectin, SRS

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Abbreviations used: chodl – chondrolectin; co-IP – co-immunoprecipitation; CRD – carbohydrate recognition domain; dpc – days post coitum; ECM – extracellular matrix; Rabggtb – Rab geranylgeranyl transferase β; SRS – Sos recruitment system

INTRODUCTION

Mouse chondrolectin (*chodl*) was isolated out of the tail tips of 4-day old 129/SvJ mice as a by-product of a PCR-based subtractive cDNA library screening. The gene is located at chromosome 16C3 and consists of 6 exons and 5 introns. Chodl is predominantly expressed in adult skeletal muscle, heart, testes and lungs, and can already be observed at the embryonic stage of 8 days post coitum (dpc). *In situ* hybridization at 15 dpc reveals that chodl is predominantly expressed in skeletal muscle [1].

Chodl is the mouse homologue of human chondrolectin (*CHODL*), which encodes for a type Ia transmembrane protein and is located at chromosome 21q21. CHODL is predominantly expressed in human testis, prostate, heart and skeletal muscle tissue [2]. *CHODL*-splice variants are detected in human leukocytes. They are expressed from a distal promoter and arise by alternative splicing in which an additional exon 3 is inserted ($CHODL_f$) or exon E is removed ($CHODL_{\Delta E}$) or a combination of the two occurs ($CHODL_{f\Delta E}$). The insertion of exon 3 does not alter the protein sequence, but the removal of exon E results in soluble variants of *CHODL* with a frame shift in the C-terminal region. The soluble variants have a functional endoplasmic reticulum retention signal (QDEL) at their C-terminus [3, 4].

The proteins of the chondrolectin family belong to the family of C-type lectins as they have a carbohydrate recognition domain (CRD) in their N-terminal domain [2, 4]. C-type lectins are Ca^{2+}-dependent lectins which express a common structural motif (CRD) that is responsible for their sugar-binding activity [5, 6]. The C-type lectin-containing protein family consists of transmembrane and soluble proteins classified into 17 groups. They are involved in diverse processes including cell recognition and communication, cell-cell adhesion, and extracellular matrix-cell interactions [6, 7]. For example, the proteoglycans play an important role in the structure and stability of the ECM and the interaction between the ECM and the cell [8]. Lectins are also important in embryonic development and immune responses. They function as recognition molecules in important processes in the immune system: the selectines, for example, function in leukocyte-leukocyte and leukocyte-endothele interactions [5]. Some lectins have a role inside the cell: the mannose receptor is involved in the binding and endocytosis of exogenous pathogens and endogenous glycoproteins [6]. Calnexin and calreticulin play important roles as molecular chaperones in the endoplasmic reticulum [9].

The function of the chondrolectin proteins is unknown. Since many biological processes are mediated by lectines, it is important to elucidate the function(s) of chondrolectin and its variants. The identification of one or more binding partners would be a first step towards defining the putative function(s) of chondrolectin. In this study, we used the Sos recruitment system (SRS) to search for any intracellular binding partners for chondrolectin. The SRS system is an *in vivo* assay where the interacting proteins occur in the cytoplasm of yeast cells, which

is an advantage when looking for cytoplasmic ligands [10]. The β-subunit of Rab geranylgeranyl transferase (Rabggtb) was isolated 8 times after a complete SRS screening with the cytoplasmic domain of chodl as the bait protein. The interaction was confirmed by *in vitro* transcription/translation and co-IP of c-myc-labelled Rabggtb and HA-labelled Chodl.

MATERIALS AND METHODS

Antibodies

The mouse anti-Sos1-antibody was purchased from BD Transduction laboratories. The HRP-labelled goat anti-mouse secondary antibody was obtained from Roche. For the co-Ips, we used a monoclonal mouse anti-c-myc antibody and a rabbit polyclonal anti-HA antibody (Clontech).

Plasmids

The CytoTrap bait construct (pUA900) was made by amplifying the sequence encoding the chodl cytoplasmic domain from the *chodl* cDNA construct (pUA855) with the primers 5'-gtcgacggggaggaggaggaggaggaacctgctgtttccagatgttg and 5'-gcggccgctatacctccatgccactttcc in a PCR program of: 94ºC for 5 min, 38 cycles of (94ºC for 30 s, 65ºC for 30 s, 72ºC for 1 min), and 72ºC for 10 min. The PCR products were fractionated on a 1% agarose gel, purified by gel extraction with the GenElute Gel extraction kit (Sigma), and subcloned into the TOPO2.1-vector (Invitrogen). Inserts were excised out of the subcloning vector with *Sal*I/*Not*I, and ligated into the same restriction enzyme-digested pSos vector (Stratagene). We used the same methodology for the *in vitro* transcription/translation constructs. The inserts of the pMyr-cDNA plasmids of Rabggtb (NM_011231) and titin (BC025840) were amplified with the primers 5'-gctatcccagtgtgctgggccgccaccatggaggagcagaagctgatctcagaggaggacctgcgccggtc tagagaattc and 5'-gctatcgcggccgccggccctctagagcttatta, which respectively contain a *Bst*XI restriction site, and a c-myc-sequence and a *Not*I restriction site. Arm1 (BC008974), RIKEN cDNA 260017A12 (NM_028242), Gapdh (AC116997), Acta1 (NM_009606), Cfl2 (NM_007688) and the cDNA sequence BC020447 were amplified with the primers 5'-gctatcaagcttgccgccaccatggagg agcagaagctgatctcagaggaggacctgcgccggtctagagaattc and 5'-gctatcctcgagcggccctct agagcttatta, which respectively contain a *Hind*III restriction site, and a c-myc sequence and *Xho*I restriction site. The fragments were amplified using a PCR program of: 94ºC for 5 min, 38 cycles of (94ºC for 30 s, 52ºC for 30 s, 72ºC for 2 min), 72ºC for 10 min, subcloned and cloned into pcDNA3 (Invitrogen). The complete *chodl* sequence was already provided with an HA-tag coding sequence at its 5' end (pUA853; [1]).

SDS-PAGE, Western blotting and immunodetection

The expression of the Sos-bait protein was analysed via SDS-PAGE and immunodetection. Cdc25-2 yeast cells were transformed with pUA900 and subsequently grown in Glc(-L)-medium for 2-3 days. The yeast cells were lysed

in 1.85 M NaOH/7.5% 2-mercaptoethanol, and proteins were precipitated with 55% TCA. The protein pellet was resolved in SU-buffer and subsequently analysed by SDS-PAGE, Western blotting and immunodetection with the anti-Sos1-antibody and the HRP-labelled goat anti-mouse secondary antibody. After ECL-detection, the signal was analysed by autoradiography.

The CytoTrap™ two-hybrid system

The CytoTrap™ screening (Stratagene) was performed with a murine skeletal muscle cDNA library which was cloned into the pMyr plasmid (Stratagene) as prey with pUA900 as the bait, according to the manufacturer's instructions. The *Saccharomyces cerevisiae* strain cdc25-2 was used for serial transformations with pUA900 and the murine skeletal muscle cDNA library. Positive clones were selected by the ability of cdc25-2 cells to grow on galactose at 37ºC. To eliminate p21-Ras and cdc25-Sos false positives, a multiplex PCR reaction was performed on the cdc25-2 co-transformed yeast cells [11]. The following primers were used: p21-forward, 5'aagagtgccctgaccatc; p21-reverse, 5'tcaggacagcacacattt, cdc25-forward, 5'tgctgtgctggagatcac and cdc25-reverse, 5'gcacacgctggagaacaa, in a PCR program of: 94ºC for 5 min, 35 cycles of (95ºC for 15 sec, 53ºC for 30 sec, 72ºC for 1 min), and 72ºC for 10 min. The PCR reactions were analysed on a 1% agarose gel, and yeast cells that were positive were eliminated. The pMyr-cDNA plasmids from colonies that showed galactose-dependent growth and did not grow on glucose at the restrictive temperature (37ºC) were isolated according to the Cytotrap™ protocol. Interactions between the putatively positive Myr-proteins and hSos-Chodl were confirmed by co-transformations of cdc25-2 cells with pSos-Chodl or pSos and the isolated pMyr-cDNA plasmid. Only those clones with interaction of the Myr-protein with hSos-Chodl (but not with hSos alone) were defined as positive. The pMyr-cDNA plasmids of these putative positive clones were sequenced and the derived cDNA sequence was used to search the GenBank database with the BLAST program (NCBI).

***In vitro* transcription/translation**

The c-myc-labelled proteins of Rabggtb, titin, Arm1, RIKEN cDNA 260017A12, Gapdh, Acta1, Cfl2, cDNA sequence BC020447 and HA-Chodl were generated by *in vitro* transcription-coupled translation using the TNT® T7 Quick-Coupled Transcription/Translation System (Promega). In brief, 40 ul of the TNT® quick mastermix was incubated along with 20 µCi ^{35}S-methionine and 1 µg of the pcDNA3-construct at 30ºC for 90 min. To verify the expression of the proteins, the radioactive labelled translated proteins were separated on a 10% SDS-PAGE, the gel was dried at 80ºC for 90 min, and then exposed to a X-Omat AR film (Kodak).

***In vitro* co-immunoprecipitation**

The *in vitro* co-immunoprecipitations were performed using the Matchmaker™ Co-IP kit (Clontech). *In vitro* translated HA-Chodl and c-myc-protein were

respectively combined with c-myc monoclonal antibody or HA-tag polyclonal antibody. Subsequently, protein A beads were added. The beads were washed according to the manufacturer's protocol, and resuspended in SDS-PAGE loading buffer. The reactions were boiled for 5 min, centrifuged briefly and separated on a 10% SDS-PAGE gel. The gel was exposed to an X-Omat AR film (Kodak) after drying at 80ºC for 90 min.

RESULTS

Isolation of chodl binding partners

Mouse chondrolectin (chodl) consists of a large N-terminal extracellular domain which contains the CRD, a transmembrane region and a small cytoplasmic C-terminal domain [2]. We used the coding region of the carboxyterminal domain of chodl to perform a CytoTrap screening with a murine skeletal muscle cDNA library. The CytoTrap system is studied in a temperature-sensitive yeast strain, cdc25-2, wherein the yeast cdc25, a Ras guanine nucleotide exchange factor (GEF), is inactive at the restrictive temperature (37ºC). The system is based on the observation that the N-terminal fragment of the Ras-GEF, hSos, suppresses temperature-sensitive growth caused by the *cdc25-2* mutation in *S. cerevisiae* if hSos is targeted to the plasma membrane in the vicinity of Ras.

A hSos-tagged cytoplasmic domain of chodl (pUA900) was used as the bait. The expression of the bait was confirmed by SDS-PAGE and Western blot detection with the anti-Sos antibody (Fig. 1). A protein with an approximate molecular weight of 130 kDa was detected corresponding to the theoretical molecular weight of this fusion protein.

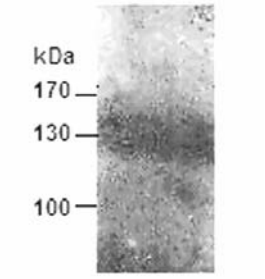

Fig. 1. Expression of the hSos-chodl fusion proteins in cdc25-2 yeast cells. Cdc25-2 yeast cells were transformed with the pSos-chodl construct and analysed via SDS-PAGE and Western detection with a primary mouse anti-Sos1 antibody.

The yeast cells expressing hSos-chodl were subsequently transformed with the skeletal muscle cDNA library (Stratagene). Approximately 2.3×10^6 transformants were screened. Among the positive clones revealing galactose-dependent growth at 37ºC, only 10% of the yeast colonies were kept after the elimination of temperature revertants. Subsequently, a multiplex PCR reaction was performed

on the remaining yeast cells to eliminate p21-ras and cdc25-sos. These proteins are frequently occurring false positives of the SRS [11]. 30% of the remaining yeast cells were eliminated after this step. The pMyr-cDNA plasmids were isolated from the putative positive yeast cells. To confirm the interaction between hSos-chodl and the Myr-protein, we performed a co-transformation with pSos-chodl and the purified pMyr-cDNA. A co-transformation between the pSos-plasmid and every pMyr-cDNA was performed to exclude interactions between hSos and the Myr-protein. The Myr-proteins that showed an interaction with hSos-chodl but not with hSos were retained and these pMyr-cDNA constructs were sequenced subsequently. We verified whether the open reading frame (ORF) of the potential ligand was cloned into the same reading frame as the myristylation signal sequence. Almost 50% of the putative positive binding partners could be eliminated. Because the cytoplasmic domain of chodl was used in the screening, only binding partners that localize at the plasma membrane or in the cytosol were withdrawn. This screen resulted in the isolation of 7 clones: RAB geranylgeranyl transferase, β-subunit (Rabggtb), ras-like CAAX1 (Rit1), adhesion regulating molecule-1 (Arm-1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), titin, alpha 1 actin (Acta1) and cofilin 2 (Cfl2) (Tab. 1).

Tab. 1. Results of the Cytotrap™ screening.

Insert of pMyr plasmid	Accesion number	Amount
RAB geranylgeranyl transferase, b subunit (Rabggtb)	NM_011231	8
Ras-like without CAAX1	BC012694	3
Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	XM_132897	3
Adhesion regulating molecule 1 (Arm1)	BC008974	1
cDNA sequence BC020447	BC020447	1
Titin	BC025840	1
Actin, alpha 1, skeletal muscle (Acta1)	NM_009606	1

In vitro transcription/translation and co-immunoprecipitation

The 7 putative positive interaction partners for chodl were tested via *in vitro* transcription/translation and co-immunoprecipitation (co-IP). The inserts of the prey plasmids were amplified and provided at their 5' side with an ATG-codon in a Kozak consensus sequence followed by the c-myc-tag sequence by PCR, and cloned into the pcDNA3 vector (Invitrogen). The complete *chodl* sequence was already provided with an HA-tag coding sequence at its 5' end, and will be referred to as *HA-chodl* (pUA853; [1]). The TnT® Quick Coupled Transcription/Translation System (Promega) and [^{35}S]-methionin was used to produce the c-Myc-potential ligands and HA-chodl. The pGADT7-T and pGBKT7-53 vectors (Clontech) were used as a positive control. The [^{35}S]-methionin labelled HA-chodl and c-Myc-potential ligands were subsequently applied for co-immunoprecipitation using the Matchmaker™ Co-IP kit

(Clontech). The reaction mixtures were analysed via SDS-PAGE and detection on a photographic film. The *in vitro* translation step was performed successfully for all putative ligands. Only a positive interaction between chodl and Rabggtb could be confirmed by *in vitro* co-immunoprecipitation analysis of the *in vitro* translated fusion proteins HA-chodl and c-myc-Rabggtb. The theoretical molecular weight of c-Myc-Rabggtb is 38 kDa, while the theoretical molecular weight of HA-chodl is 31 kDa, which corresponds with the signals on the autoradiograph (Fig. 2A).

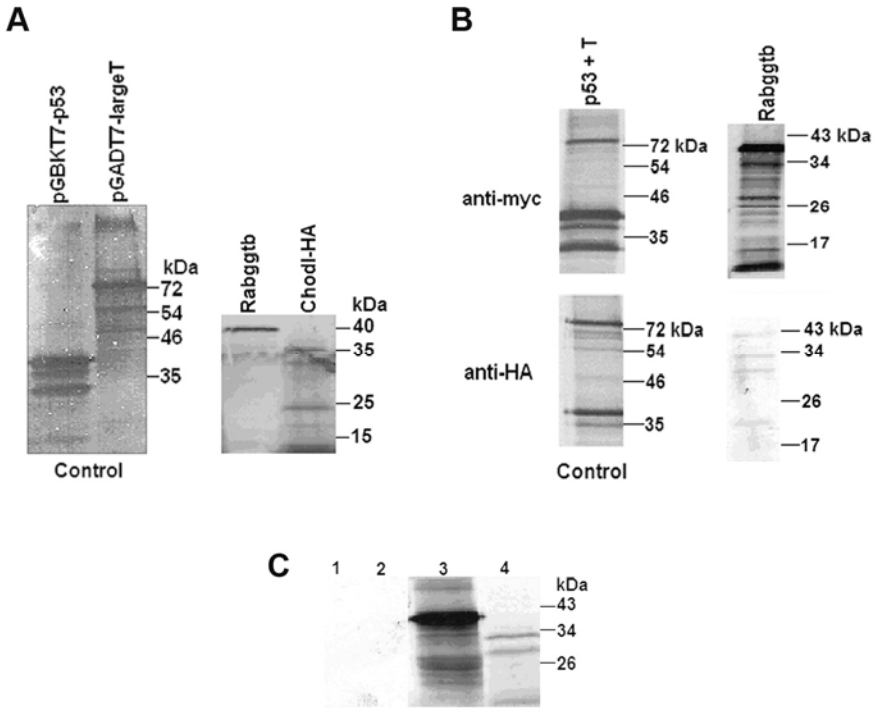

Fig. 2. *In vitro* transcription/translation and co-immunoprecipitation of chodl and Rabggtb. A – The TnT® Quick Coupled Transcription/Translation System and [^{35}S]-methionin were used to produce c-Myc-Rabggtb, HA-chodl and the control proteins HA-p53 and c-myc-T. HA-p53 and c-myc-T have approximate molecular weights of 35 kDa and 70 kDa, respectively. B – The [^{35}S]-methionine labelled HA-chodl, c-Myc-Rabggtb, HA-p53 and c-myc-T were subsequently applied for co-immunoprecipitation using the Matchmaker™ co-IP kit. The reactions were run on a 10% SDS-PAGE gel, the gel was dried, and the proteins were visualised on a photographic film. C – After *in vitro* transcription/translation, different combinations of the *in vitro* translated proteins were incubated with protein A beads and an anti-HA antibody or an anti-c-myc antibody. The reaction mixtures were run on a 10% SDS-PAGE gel, the gel was dried, and the reactions were visualized on a photographic film. Lane 1: Rabggtb + anti-HA; lane 2: HA-chodl + anti-c-myc; lane 3: HA-chodl + anti-HA; lane4: Rabggtb + anti-c-myc.

Control immunoprecipitation reactions were performed with the two fusion proteins c-myc-Rabggtb and HA-chodl (Fig. 2C). These immunoprecipitation reactions show that Rabggtb does not react on its own with the anti-HA antibody and that Rabggtb is precipitated with the anti-HA antibody through its interaction with chodl (Fig. 2B).

DISCUSSION

Mouse chondrolectin is a protein that belongs to the C-type lectin family. Chodl is a transmembrane protein with a large extracellular domain which contains the CRD and a small cytoplasmic region [1]. Since C-type lectins have a wide variety of functions, it is important to find the biological function of chondrolectin. In this study, we searched for the binding partner(s) of the cytoplasmic domain of chodl in an attempt to assess its putative biological role. We used the Cytotrap^{TM} system, which is based on the Sos recruitment system (SRS), to search for such binding partners. Since we are searching for cytoplasmic ligands, we took advantage of the SRS as a screening system to detect interactions between bait and prey occurring in the cytoplasm of yeast cells [10]. One of the disadvantages of SRS is the occurrence of temperature revertants, which could be eliminated by plating the colonies on different selective plates at different temperatures, and the occurrence of Sos and Ras false positives, which could be eliminated easily by a multiplex PCR reaction [11]. We could only retain 10% of the possible candidates after the elimination of revertants, and 30% after the multiplex PCR reaction. The reading frame between the myristylation signal and the cDNA-sequence of the possible interaction partner was verified by sequence analysis. More than 50% (thirteen out of twenty-four) of the inserts of putative partners turned out to be cloned out of frame in the pMyr-vector. According to Clontech, yeast shifts the frames while expressing the proteins. The correct protein may be expressed from an out-of-frame cassette, as described in Beil *et al.* [12]. We decided to omit all the out-of-frame inserts in this study because we could not find any reasonable scientific explanation for these repaired frame shifts in yeasts.

After co-IP analyses of 7 possible interaction partners for the cytoplasmic region of chodl, we were able to confirm the interaction of chodl with the Rab geranylgeranyl transferase β subunit (Rabggtb). This protein is a part of the Rab geranylgeranyl transferase enzyme, which contains a component A and a component B, consisting of α and β subunits. Component A is involved in escorting Rab proteins during and after the prenyl transfer reaction, and is also known as Rab escort protein. Component B is the catalytic component. The β subunit of component B, Rabggtb, is ubiquitously expressed in adult tissues and was already detected in the embryo at 7.5 dpc. Rab geranylgeranyl transferase isoprenylates Rab GTPases, and this is essential for the function and membrane association of those proteins, which function in the biosynthetic and endocytic pathway of membrane vesicle docking and fusion [13, 14]. The

isoprenylation occurs at a CC, CXC or CCXX (X = any amino acid) motif in the carboxyterminus of the Rab GTPases [15]. A few Rab GTPases possess a CAAX motif and are modified by a single geranylgeranyl moiety [16]. Chodl does not contain such a C-terminal motif, and therefore it is unlikely that chodl is a substrate for Rab geranylgeranyl transferase. Further experiments are necessary to understand the functional meaning of the chondrolectin-Rabggtb interaction. *In vivo* co-immunoprecipitation experiments should bring more clarity. If chodl does bind Rabggtb *in vivo*, we speculate that it has a structural or stabilizing role for Rabggtb at the cell membrane.

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