

Interaction of a novel cysteine and histidine-rich cytoplasmic protein with galectin-3 in a carbohydrate-independent manner

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Abstract We have used the yeast two-hybrid system to search for cytoplasmic proteins that might assist in the intracellular trafficking of the soluble β -galactoside-binding protein, galectin-3. We utilised as bait murine full-length galectin-3 to screen a murine 3T3 cDNA library. Several interacting clones were found to encode a partial open reading frame and a full-length clone was obtained by rapid amplification of cDNA ends methodology. In various assays *in vitro* the novel protein was shown to bind galectin-3 in a carbohydrate-independent manner. The novel protein contains an unusually high content of cysteine and histidine residues and shows significant sequence homologies with several metal ion-binding motifs present in known proteins. Confocal immunofluorescence microscopy of permeabilised 3T3 cells shows a prominent perinuclear, as well as cytoplasmic, localisation of the novel protein.

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Key words: Galectin-3; Two-hybrid screen; Novel protein

1. Introduction

Galectins are intracellular, carbohydrate-binding proteins that are present in many organisms and are believed to have a variety of functions intra- and extracellularly [1–3]. Galectin-3, although lacking any conventional signal sequence, can be secreted by novel, as yet incompletely understood, pathways circumventing the endoplasmic reticulum–Golgi complex involved in classical secretory pathways [4–7]. Galectin-3 has also been found within the cell nucleus [8,9]. In murine 3T3 fibroblasts, the lectin appears to re-locate from the cytoplasm predominantly to the nucleoplasm in proliferating cells [10–13]. The pathway(s) by which galectin-3 enters the nucleus are unknown. The galectin-3 sequence contains no reasonable classical nuclear localisation signal [14].

By analogy to the case of β -catenin, that shows a similar shuttling from the cytoplasm either to plasma membrane domains or into the nucleus [15] regulated by complex formation with the LEF-1 transcription factor [16] and other factors [17], we considered the possibility that intracellularly galectin-3 may combine with cytoplasmic proteins that function to direct the lectin into the nucleus or into secretory pathways. Therefore, we have undertaken a yeast two-hybrid screening in an attempt to identify cytoplasmic binding partners of galectin-3.

2. Materials and methods

2.1. Yeast two-hybrid screening

Bait plasmid was generated by inserting the complete open reading frame (ORF) of mouse galectin-3 [18], kindly provided by J.L. Wang, University of Michigan, East Lansing, MI, USA, into the yeast vector pGBD-C3 [19], using *EcoRI*–*BglII* sites, to produce in-frame fusions with the GAL4 DNA-binding domain. Plasmids were transformed into *Saccharomyces cerevisiae* strain CG1945 (Clontech) and selectants were checked for expression of fusion proteins by immunoblotting yeast lysates with anti-galectin-3 [20] or with anti-GAL4 DNA-binding domain antibodies (Clontech). A cDNA library of mouse embryonic NIH/3T3 fibroblasts cloned in the pACTII vector carrying a haemagglutinin (HA) tag (Clontech) was transformed into *S. cerevisiae* strain Y187 and the yeast transformants carrying the bait were used to screen this library using an improved mating strategy [21]. Approximately 10^7 clones were screened and the His⁺ clones were further tested for LacZ expression by β -galactosidase assay using 5-bromo-4-chloro-3-indolyl- β -D-galactoside as a substrate. Interacting cDNA clones were retrieved by transformation of competent *Escherichia coli* DH5 α cells with DNA extracted from yeast clones and identified by sequencing with GAL4 activation domain primers. Positive clones were checked for interaction by reintroduction into strain Y187 and mating with yeast cells containing the bait. The specificity of interaction was checked by mating library clones with CG1945 containing the empty pGBD-C3 vector as well as various combinations of constructs in pGBD-C3, such as that encoding the mouse desmocollin-1a cytoplasmic domain.

2.2. 5'-Rapid amplification of cDNA ends (RACE)

The 5'-RACE System Version 2 (Life Technologies) was used to carry out 5'-RACE. 1 μ g of mouse kidney mRNA (Clontech) was converted to first strand cDNA using gene-specific reverse primer 5'-TGATCATATAGTTGTGTGAAGCTGCTGCCA-3' corresponding to 67 bp downstream of the putative stop codon in the two-hybrid positive clones, and poly(A)-tailed using recombinant terminal deoxynucleotidyl transferase. 5 μ l of the tailed cDNA was amplified using a second gene-specific primer 5'-GGACAGCACTACCGGACTG-CAGCC-3' located 28 bp downstream of the putative stop codon, and the abridged anchor primer 5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTTTTTTTT-3'. PCR was carried out using AmpliTaq Gold polymerase (Perkin-Elmer). 5 μ l of a 1–100 dilution of the PCR products was re-amplified using gene-specific primer 5'-GATATCGATTACTTCTGTATCTGGAACAG-3' and anchor primer 5'-GGCCACGCGTCGACTAGTAC-3'. PCR products were gel-purified and digested with *SalI* and *Clal* restriction enzymes and cloned into pBluescript II SK+ vector (Stratagene). Single clones were grown overnight and plasmid DNA was prepared using a Hybaid kit.

2.3. DNA sequencing and analysis

DNA sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems), and the reactions were analysed using an ABI Prism DNA Sequencer (Perkin-Elmer). Sequence analysis of two-hybrid-positive clones was performed using an on-line BLAST search and the Laser Gene Software for Macintosh (DNASTAR, Madison, WI, USA). The EMBL/GenBank accession number for the full-length cDNA is AJ251516.

2.4. Production of antibodies

An *EcoRI*–*XhoI* digest of the positive clone 4.4 was ligated to

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pGEX-6P-2 (Pharmacia) to make an in-frame fusion with glutathione-S-transferase (GST). After bacterial expression, the GST fusion protein was purified on a glutathione-Sepharose column (Pharmacia) and used to raise polyclonal antibodies in rabbits. Absorption of the anti-serum was carried out using GST protein coated onto glutathione-Sepharose beads.

2.5. Immunoprecipitation

Yeast cells carrying the bait and the positive clones were grown in LWH medium (2 ml). Cells were pelleted and lysed in ice cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40, pH 7.4) containing protease inhibitors (Boehringer). The lysate was cleared of cell debris by centrifugation and immunoprecipitated with anti-galectin-3 polyclonal antibody raised [20] in rabbits. Immune complexes were collected onto protein A-Sepharose at 4°C overnight and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose, the blot was probed with anti-HA mouse monoclonal antibody 12CA5 (Babco, Richmond, CA, USA). The filters were then stripped and reprobed with the Mac-2 rat monoclonal antibody (Boehringer Mannheim) to reveal the presence of galectin-3.

3T3 cells were grown to confluency in F 15 medium (Gibco) containing 10% FCS and the cells were washed once with phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 1.0% NP-40, 0.1% SDS). Lysates were cleared of cell debris and immunoprecipitated with anti-galectin-3 or anti-GST fusion protein antibodies. Cell lysates were pre-cleared with anti-GST antibodies (Pharmacia) prior to immunoprecipitation with anti-GST fusion protein antibodies. Immune complexes were separated by SDS-PAGE and blotted with Mac-2 antibody.

2.6. In vitro transcription/translation

PCR was carried out on pBluescript SK+ vector carrying the clone obtained by 5'-RACE using primers: 5'-ATAGAATTCATGGTTTCAAACCCAGGACT-3' and 5'-AGAGGATCCTTACTTCTGTATCTGGAACAGG-3'. The amplified ORF was cloned into expression vector pSG5 [22] using *EcoRI* and *BamHI* restriction sites. Proteins were synthesised in medium containing [³⁵S]methionine (Amersham-Pharmacia Biotech) using the above plasmid DNA carrying the amplified ORF, or pSG5 carrying a full-length mouse galectin-3 insert, with the Promega TNT Quick Coupled Transcription/Translation System. Empty pSG5 vector was used as a control. Translated proteins were either analysed directly or the reaction mixtures were diluted with lysis buffer, mixed 1:1 by volume and incubated at 4°C on a rocking platform for 1 h. After addition of Mac-2 antibody, immune complexes bound to protein A-Sepharose were subjected to SDS-PAGE and autoradiography.

2.7. Bead binding assay

E. coli BL 21DE3 cells were transformed with pGEX-6P-1 vector carrying in-frame fusions of the full-length ORF of the interacting protein and cells were induced for 4 h with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were washed with PBS, solubilised in lysis buffer and lysates were incubated with glutathione-Sepharose beads for 2 h followed by extensive washing with lysis buffer. 100 µl of beads was incubated for 2 h with 100 µl (40 µg) of recombinant full-length hamster galectin-3 or a C-terminal galectin-3 carbohydrate recognition domain (CRD) fragment lacking the first 94 residues [20]. As a control, beads were incubated with GST-transformed BL 21DE3 lysates. The beads were washed with lysis buffer and subjected to SDS-PAGE and Western blotting with antibodies recognising the galectin-3 CRD [20].

2.8. Northern analysis

1 µg of mRNA prepared from 3T3 fibroblasts was electrophoresed through 1.1% agarose/6.6% formaldehyde gels, transferred to Hybond N⁺ membranes (Amersham) and hybridised at 68°C with a 1065 bp *EcoRI*-*XhoI* fragment from clone 4.4 that was ³²P-labelled using Ready-To-Go DNA labelling beads (Pharmacia). The RNA hybridisation buffer contained 6×SSC, 2×Denhardt's reagent, 0.1% SDS and 20 µg sonicated salmon sperm DNA. The blots were hybridised overnight and washed once with 1×SSC, 0.1% SDS for 20 min at room temperature and three times with 0.2×SSC, 0.1% SDS for 20 min each at 68°C, followed by autoradiography. Tissue-specific expression of the newly identified gene was tested by probing mouse

multiple tissue Northern blots (Clontech) with a PCR-derived fragment from the full-length clone encoding a 453 bp region showing homology to a human brain mRNA, according to the manufacturer's instructions.

2.9. Immunofluorescence microscopy

Sub-confluent 3T3 cells grown on glass coverslips were fixed in 3% paraformaldehyde in PBS for 30 min at room temperature, permeabilised with 0.1% NP-40 in PBS for 5 min and non-specific binding sites were blocked with 2% BSA in PBS for 15 min. For double staining, the cells were first incubated with rat monoclonal Mac-2 antibody, washed and incubated with goat anti-rat Ig-FITC. After washing with PBS, the cells were incubated with rabbit polyclonal antibodies against the GST-clone 4.4 fusion protein, washed and incubated with goat anti-rabbit Ig-Texas red. As controls, cells were stained only with second antibodies or with goat anti-GST antibody (Pharmacia) followed by sheep anti-goat Ig-FITC.

3. Results and discussion

By screening a mouse 3T3 embryonic fibroblast library, using the yeast two-hybrid system, we identified 34 out of 51 positive clones encoding fragments apparently belonging to a single class of cDNA. One clone, 4.4, had an interacting ORF consisting of 209 amino acids and a putative 3'-untranslated region of 415 bp, containing a potential polyadenylation site ahead of the poly(A) tail. To identify the 5'-sequence of the interacting clone, a 5'-RACE strategy was performed that yielded a further 544 bp at the 5'-end with a putative start codon in a Kozak consensus sequence [23] and an in-frame stop codon upstream of the start codon. The predicted amino acid sequence of this cDNA (Fig. 1) indicated an exceptionally high content of cysteine (17 residues) and histidine (11 residues) in a total of 311 residues. 14 cysteine and seven histidine residues reside within an N-terminal domain (residues 30–140) of the protein. Hence this protein is referred to provisionally as cysteine/histidine-rich protein (Chrp).

Northern blotting of mRNA derived from 3T3 cells revealed two transcripts of approximately 4.3 kb and 1.7 kb (Fig. 2A). cDNA library screening indicated that the smaller transcript is derived by alternative polyadenylation (results not shown). Northern blotting of mouse tissues (Fig. 2A) revealed variable expression of Chrp mRNA with highest expression in liver, heart and kidney. Interestingly, although Chrp (Fig. 2A) and galectin-3 (Fig. 2B) mRNAs are co-expressed at similar levels in liver and kidney, they show divergent expression in other tissues.

The specificity of Chrp-galectin-3 interactions was demonstrated by co-precipitation of these proteins from lysates of yeast cells (Fig. 3a) as well as from 3T3 cells (Fig. 3b) expressing both proteins. Since, in the former case, the cells carry the partial clone 4.4 encoding only a small part of the cysteine/histidine-rich region, it appears likely that this region is not involved in interactions with galectin-3. However, more work is required to map more exactly the galectin-3 interacting site

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MVS KPRTEWSTVL SHLVLAGVSLHA AVSSVQCTNGHLMCAGCFIHL
ADARLKEEQATC PNCRC EISKSLCCRNLA VEKAVSELPSECGFCLRQFP
LLERHQKEECQDRVTQCKYKRIGCPWHPGFHELTVHEAACAHPTKTGN
ELMEILDMDQSHRKEMQLYNSIFSLLSFEKIGYTEVQFRPYRTDDFITRLY
YETPRFTVLNQTWVLKARVNDSE RPNLSCKRTL SFQLLLKSKVTAPLE
CSFLLKGPYDDVRISPVYHFVFTNESNETDYVPLPIIDSVECNKLLAAK
NINLRFLPFQIQK

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Fig. 1. Deduced amino acid sequence of Chrp. The cysteine/histidine-rich region is underlined.

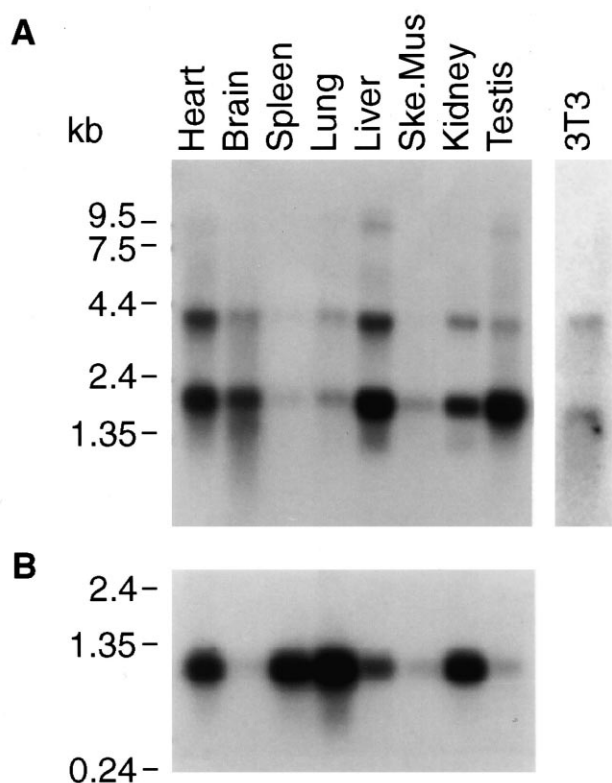


Fig. 2. Northern blot analysis. 1 μ g of poly(A)⁺ RNA obtained from 3T3 cells was hybridised with clone 4.4 insert. 2 μ g of poly(A)⁺ RNA from each indicated mouse tissue was probed with a 453 bp Chrp cDNA fragment (upper panel). This blot was stripped and reprobed with galectin-3 cDNA (lower panel). A parallel blot was hybridised with a β -actin probe to ensure normalisation of the amount of RNA loaded in each lane (data not shown). The sizes of RNA standards are indicated.

in the C-terminal domains of Chrp. Further evidence for the interaction between Chrp and galectin-3 was obtained from *in vitro* transcription/translation experiments using rabbit reticulocyte lysates. Galectin-3 and Chrp were independently translated and the synthesised proteins were mixed and immunoprecipitated with lectin-specific antibodies. The immune complexes contained two proteins having molecular weights expected of galectin-3 (approximately 32 kDa) and Chrp (approximately 38 kDa), respectively (Fig. 3c). Finally, recombinant galectin-3 was found to bind to bacterially expressed Chrp (Fig. 3d), as did a galectin-3 CRD fragment lacking the N-terminal domains of the native protein (Fig. 3d). Thus, although interactions between Chrp and galectin-3 are clearly carbohydrate-independent, they do involve the carbohydrate-binding domain of the lectin.

Using confocal immunofluorescence microscopy, double labelling of 3T3 cells showed a distinct difference in the localisation of Chrp and galectin-3. Although both proteins clearly resided at least in part in the cytoplasm, Chrp was strikingly concentrated at the nuclear envelope in a concentric ring (Fig. 4b): this appearance was found in optical cross sections taken at 1 μ intervals throughout the thickness of the cells. Chrp appeared to be excluded from the nucleus (Fig. 4b). By contrast, galectin-3 was found (Fig. 4a) within the nucleus, except for the nucleoli, in agreement with previous findings in 3T3 cells [9].

An initial BLAST search, using the nucleotide sequence of

the partial clone 4.4, showed some homology to a human chromosome 1-specific mRNA transcript (GenBank accession no. KIAA0496) [24]. When the full-length Chrp sequence was compared, a region of 453 bp showed almost complete homology at the nucleotide level to a region in the 6151 bp human mRNA. This encodes 151 amino acids, starting with the ³¹QCTNGHL in the cysteine/histidine-rich N-terminal domain (Fig. 1). A BLAST search using the complete Chrp sequence revealed further homology in this region with cysteine/histidine-rich zinc finger motifs [25] from a variety of proteins. Some representative examples are shown in Fig. 5. Thus, residues 115–140 of the Chrp sequence show (Fig. 5) conserva-

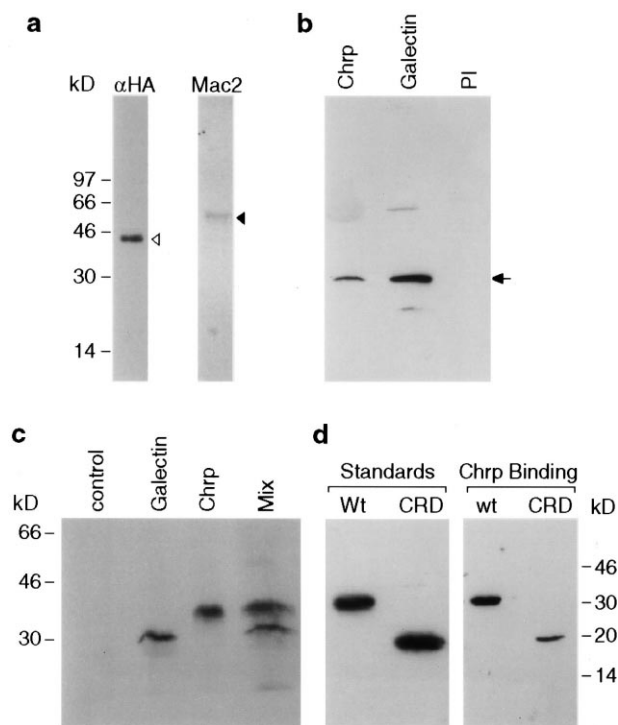


Fig. 3. Interactions of Chrp with galectin-3. a: Yeast cells co-expressing the bait fusion protein galectin-3/GAL4 DNA-binding domain and the HA-tagged clone 4.4 protein were lysed and immunoprecipitated with polyclonal antibody against galectin-3. Immune complexes collected on protein A-Sepharose were subjected to SDS-PAGE and blotted with anti-HA monoclonal antibody. After stripping, the same filter was then blotted with anti-galectin-3 Mac2 monoclonal antibody. The migrations of HA-tagged Chrp (44 kDa, open arrow head) and the galectin-3 fusion protein (60 kDa, closed arrow head) are indicated. b: 3T3 cells were immunoprecipitated with polyclonal antibodies raised against Chrp or galectin-3 as indicated. A control pre-immune serum was also used. Immune complexes were blotted after SDS-PAGE with monoclonal antibody directed against galectin-3. The migration of galectin-3 (approximately 32 kDa, arrow head) is indicated. c: *In vitro* transcription/translation was carried out with control pSG5 vector, vector carrying inserts of either mouse galectin-3 or Chrp. Samples of the [³⁵S]methionine-labelled products were subjected to SDS-PAGE followed by autoradiography. Samples of the *in vitro* produced galectin-3 and Chrp were also mixed, incubated at 4°C for 1 h and immunoprecipitated with polyclonal anti-galectin-3 antibody. Immune complexes were run on SDS-PAGE followed by autoradiography. Note the co-precipitation of a 32 kDa galectin-3 band and a 38 kDa Chrp band. d: Recombinant hamster galectin-3 or a CRD fragment lacking N-terminal residues 1–94 were incubated with GST-Chrp fusion protein adsorbed onto glutathione-Sepharose beads. Beads were washed and bound proteins were subjected to SDS-PAGE followed by Western blotting with anti-galectin-3 CRD antibodies.

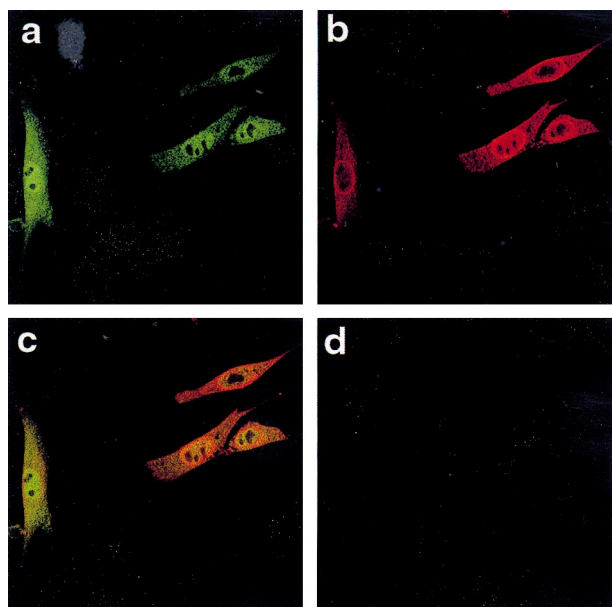


Fig. 4. Subcellular localisation of Chrp in 3T3 cells. Confocal laser immunofluorescence microscopy of unsynchronised permeabilised cells was carried out using a rat monoclonal antibody specific for mouse galectin-3 (a) and a rabbit polyclonal antibody directed against Chrp (b). Bound primary antibodies were located respectively with FITC- or Texas red-conjugated second antibodies directed against rat or rabbit Ig. A merged image (c) and a control staining with a non-immune serum (d) are shown.

tion of all cysteine and histidine residues present in a zinc finger in the *Drosophila* TNF receptor-associated factor 1 [26] and homologous sequences occurring in the *Drosophila* RING finger gene *sina* and its murine and human *siah* homologues [27]. These proteins appear to participate in signal transduction involving tyrosine kinases. Several studies have shown that single zinc finger peptides can fold in the presence of metal ions such as Zn^{2+} or Co^{2+} [28,29]. Therefore, it is an intriguing possibility that the cysteine/histidine-rich region in Chrp may function like the metal-binding domains in other proteins, for example in DNA and RNA-binding [25], in enzyme catalysis [30] as well as in protein–protein interactions and signal transduction [28].

At present, the functional significance of specific interactions between galectin-3 and Chrp are unknown. We have recently shown that N-terminal residues of galectin-3 are essential for secretion [6,7]. Thus, the observation that Chrp can

bind to a galectin-3 CRD fragment lacking these residues suggests that Chrp is not involved in galectin-3 secretion. By contrast, recent studies indicate that the galectin-3 CRD is sufficient for nuclear expression (J.-C. Gaudin and R.C. Hughes, unpublished results). Galectin-3 binds to ssDNA and RNAs [13,31] and has been implicated in pre-mRNA splicing [32]. Therefore, it is interesting to ask if interactions between Chrp and galectin-3 are involved in any of these events. However, we have not been able to detect the presence of Chrp inside the nucleus, which perhaps argues against any putative role of Chrp/galectin-3 interactions in nuclear import or function. Interestingly, several mammalian inhibitors of apoptosis proteins (IAPs) [33] are known to possess cysteine/histidine-rich motifs termed BIRs [34] and RING-zinc fingers [35–37]. IAPs lacking C-terminal RING fingers and possessing one or more BIRs have been reported [38,39]. Even though the cysteine/histidine-rich motif in Chrp is not in agreement with the consensus BIR- or RING-zinc finger motif found in IAPs, given the proposed role of galectin-3 as an inhibitor of apoptosis [40,41], it is possible that Chrp too is involved in apoptosis regulation, particularly since its cellular localisation is somewhat similar to bcl-2 [42]. However, there is no sequence similarity between Chrp and bcl-2.

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CHRP:	30	VQCTINGHLMCAGCFIHLADARLKEEQATCPNCRCEISKSLCCRNLA	86
SINA (P21461):	85	LQCSSLVLCVSCRSKL-----TCCPTCRGPLAN---IRNLAM-EKVASNVKF	128
STAH-1B (S35754)	53	LQCQSGHLVCSNCRPKL-----TCCPTCRGPLGS---IRNLAM-EKVANSVLF	96
hSTAH2 (gi.2673968):	92	LQCQAGHLVQNCQKQL-----SCCPTCRGALTPS---IRNLAM-EKVASAVLF	136
DTRAF1 (gb.AAD34346.1)	209	-----KSCAKRL-----RRCAHCQREFSADTLPLHAAQCPRAPLACFQ	246
CHRP:	87	ECGFCLRQF----PRSLLEHQQKEECQDRVTQCKYKRIGCPWHGPFHELTVHEA--ACA	140
SINA:	129	PKHSGYGCTASLVYTEKTEHE-ETCECRPYLCPGASCKWQGPLDLVMQHLM---MSH	184
STAH-1B:	97	PKYASGCEITLPHTKAEHE-ELCEFRPYSCPCGASCKWQGSLEAVMPLHM---HQH	152
hSTAH2:	137	PKYATTGCSLTLLHTEKPEHE-DICEYRPYSCPCGASCKWQGSLEAVMSHLM---HAH	192
DTRAF1:	247	RCDAGPI-----PRGELEAHLRDEQCSLAVSCSFKEAGCRFGKPRQMLEAHLESNA	300

Fig. 5. Alignment of the inferred amino acid sequence of Chrp with other protein sequence segments. Conserved cysteine and histidine residues are in red and green, respectively. Other conserved residues are in yellow.

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