A family of ubiquitin-like proteins binds the ATPase domain of Hsp70-like Stch

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Received 17 December 1999
Edited by Horst Feldmann

Abstract We have isolated two human ubiquitin-like (UbL) proteins that bind to a short peptide within the ATPase domain of the Hsp70-like Stch protein. Chap1 is a duplicated homologue of the yeast Dsk2 gene that is required for transit through the G2/M phase of the cell cycle and expression of the human full-length cDNA restored viability and suppressed the G2/M arrest phenotype of dsk2 rad23a Saccharomyces cerevisiae mutants. Chap2 is a homologue for Xenopus scythe which is an essential component of reaper-induced apoptosis in egg extracts. While the N-terminal UbL domains were not essential for Stch binding, Chap1/Dsk2 contains a Sti1-like repeat sequence that is required for binding to Stch and is also conserved in the Hsp70 binding proteins, Hip and p60/Sti1/Hop. These findings extend the association between Hsp70 members and genes encoding UbL proteins, Hip and p60/Sti1/Hop. These findings extend the broader role for Hsp70-like ATPase family in regulating cell cycle and cell death events.

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Key words: Cell cycle; Hsp70; Stch; Ubiquitin-like gene

1. Introduction

The Hsp70-like gene family encodes a group of related protein chaperones that are required for the viability of all living organisms. The structure of all Hsp70 proteins is similar and consists of a highly conserved 45 kDa N-terminal ATPase domain and a 25 kDa C-terminal domain that functions to reversibly capture nascent or denatured cellular polypeptides to initiate a wide range of protein processing events [1–3]. The complexity of Hsp70 activity has become increasingly apparent with the recognition that they interact with other co-chaperones, including Hsp40, Hsp90, and p60/Sti1, to form a functional unit [4]. In addition, non-chaperone ‘Hsp70 interacting proteins’ have been identified which are required for regulating protein folding and/or ATPase activity [5–11].

In contrast to the bi-functional structure of Hsp70 family members, a novel gene product was isolated, designated Stch, which encodes the ‘core ATPase’ domain of Hsp70 but lacks the peptide binding domain [12]. The truncated structure of Stch is conserved in Caenorhabditis elegans, rat, and human tissues where it was observed to resemble a proteolytically cleaved N-terminal ATPase fragment of Hsc70/Hsp70 [13]. To study the role of the Stch product in regulating protein processing, we performed a two-hybrid screen. We isolated multiple, overlapping human cDNA clones that encode distinct, ubiquitin-related proteins that bound efficiently to a conserved 20 amino acid region within the Stch ATPase domain. Analysis of the Chap1/Dsk2 gene showed that it is a homologue of the Saccharomyces cerevisiae DSK2 gene, which, together with RAD23, is important for the proper organization of the yeast mitotic spindle and transit through mitosis [14]. In contrast, Chap2 represents the human Bat3 gene [15] and is a homologue of the Xenopus scythe gene which is essential for reaper-induced apoptosis [16]. The identification of ubiquitin-linked proteins that bind to a conserved peptide motif within the ‘core ATPase’ Stch molecule suggests a broader role for Hsp70 family members in regulating specialized cellular events.

2. Materials and methods

2.1. Isolation of Stch binding proteins

Human Stch cDNA (codons 2–467) was subcloned in-frame into the pGBT9 Gal4p DNA binding domain plasmid (Clontech, Palo Alto, CA, USA). The H7c yeast strain was transformed with the pGBT9-Stch plasmid followed by sequential transformation of a human lung cDNA library fused to the pGad10 Gal4p activation domain plasmid. Plasmids were isolated from yeast transformants on -L/H/W plates and subjected to nucleotide sequencing. Yeast strains were re-transformed with purified plasmids and multiple independent transformants were tested to confirm protein binding by β-galactosidase enzyme activity and by growth on SC-His media in all cases.

2.2. Functional analyses of Chap1/Dsk2

Yeast media and general techniques were as described previously [17]. Wild-type S cerevisiae strain, MY3492, and the dsk2 rad23a mutant strain, MY5156, were transformed with: pMR3429, pGAL vector alone; pMR2757 DSCK2 CEN [14]; pMR4647 (pGAL-human Chap1/Dsk2); pMR2905, pGAL-DSK2; and pMR2906, pGAL-DSK2-1. Serial dilutions of transformants were incubated at the permissive or restrictive temperature and in the presence or absence of galactose and scored for growth [17]. Cultures of yeast transformants were also grown in SC-ura galactose medium until early logarithmic phase at 30°C and were shifted to 37°C for 10 h. Cells were fixed with methanol:aceton (3:1 ratio) on ice for 30 min and stained with DAPI on ice for 30 min. Greater than 100 cells were counted for each culture.

2.3. GST-Stch pull-down analysis with endogenous Bat3/Scythe

Glutathione-S-transferase (GST) and GST-fusion proteins were purified in Escherichia coli on glutathione-Sepharose beads as previously described [18], blocked with 10 μg/ml bovine serum albumin for 30 min, and washed three times in egg lysis buffer (ELB: 250 mM

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PII: S0014-5793(00)01135-2
sucrose, 2.5 mM MgCl₂, 1 mM dithiothreitol, 50 mM KCl, 10 mM HEPES pH 7.4). A crude Xenopus egg extract was prepared in ELB as previously described [16] and added to the GST-fusion proteins at 10× the bead volume. Following a 60 min incubation at 4°C, the GST-fusion proteins were pelleted and washed three times in ELB. The washed pellets were resolved by SDS-PAGE and subjected to protein immunoblotting using 1:500 dilution of an K-Scythe antisera raised against the carboxy-terminal 16 amino acids of the X. scythe protein.

2.4. Protein sequence analysis

The non-redundant database of protein sequences at the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD, USA) was tested using the gapped BLASTP program and the Position-Specific Iterating (PSI) BLAST program [19]. Conserved signaling and interaction domains in protein sequences were identified using the SMART searching engine [20]. Multiple sequence alignments were constructed using the Clustal W program [21]. The accession numbers for the amino acid sequences used: S. cerevisiae DSK2 (P48510), S. cerevisiae SSA4 (P22202), human Stch (U04735), and human BiP (P11021).

3. Results and discussion

We obtained several HF7c His+ transformants after screening a human lung two-hybrid cDNA library using a pGBT9-Stch bait plasmid. Three overlapping clones were isolated for

Fig. 2. The human Chap1/Dsk2 gene suppressed the dsk2Δ rad23Δ phenotype. Serial dilutions of wild-type or temperature-sensitive dsk2Δ rad23Δ mutant yeast colonies were grown at the permissive or restrictive temperatures as indicated. The yeast colonies contained either wild-type yeast DSK2 (yDSK2), dominant mutant yeast DSK2 (yDSK2-1), the human Chap1/Dsk2 (hDsk2), or the empty vector. All of the DSK2 genes were expressed as galactose-inducible proteins.
the Chap1 gene, one of which represented a full-length cDNA spanning 3360 nucleotides and encoding an open reading frame of 624 residues (Fig. 1). A Blastp search [22] revealed that this gene represented the human homologue for the yeast DSK2 gene [14]. Inspection of the amino acid alignment between the human and yeast homologues shows that the human gene encodes a 21 residue leader peptide, resembling a mitochondrial import sequence, followed by a conserved 70 amino acid, ubiquitin-like (UbL) domain at the amino-terminal end. Chap1/Dsk2, however, is approximately twice the size of the yeast homologue, apparently resulting from a duplication of the yeast sequence distal to the UbL domain. In addition, both the yeast and human genes contain a ubiquitin-associated (UbA) domain at their C-termini [23].

Neither the N-terminal UbL domain nor the C-terminal UbA domain of Chap1/Dsk2 were required for binding to Stch and the smallest cDNA clone isolated in our screen spanned 200 residues of one monomer DSK2 unit suggesting that the duplication of the yeast sequence was not essential for Stch binding. Interestingly, this minimal binding domain contained two Sti1-like repeat sequences with strong sequence similarity to the conserved C-terminal domain of the chaperone binding proteins p60/Sti1 and Hip [24] (Fig. 1) (Chap1 residues: 190–237, 360 to 412, and 433–460 align with the S. cerevisiae Sti1 residues: 149–191 and 540 to 579) that in Sti1 is implicated in Hsp70 and Hsp90 binding [24,25]. We observed that an in-frame deletion of Chap1/Dsk2 removing amino acid residues 418 to 431 that lie between the last two Sti1 repeat sequences within the minimal binding region had no effect on Stch binding using the yeast two-hybrid binding assay. In contrast, a carboxy-terminal deletion that disrupted only the final Sti1 repeat sequence showed complete loss of Stch binding (data not shown) suggesting that this region is required for binding to the ATPase domain of Stch. In addition, the minimal binding domain of Chap1/Dsk2 spans a 55 residue glycine/proline collagen-like repeat region which resembles, but is distinct, from the GGMP repeat observed in the Hip product [24].

To examine the functional activity of Chap1/Dsk2, a temperature-sensitive dsk2Δ rad23Δ S. cerevisiae strain (MY5156) was tested for cell growth following transformation with the full-length yeast DSK2 gene (yDsk2), a dominant yeast DSK2 mutation in the ubiquitin domain (yDSK2-1), the human Chap1/Dsk2 cDNA (hDsk2), or the vector alone (Fig. 2). All of the proteins were expressed under the control of the GAL1 galactose-inducible promoter. While single mutants for either dsk2Δ or rad23Δ do not exhibit cell growth defects at 37°C, double mutants at the restrictive temperature are arrested at G2/M with defects in duplication of the mitotic spindle pole body [14]. We observed that high levels of yDSK2 and yDSK2-1 expression were toxic in wild-type S. cerevisiae cells [14]. However, as reported previously, when expressed under its own promoter, yDSK2 could reverse the block at G2/M observed in dsk2Δ rad23Δ cells (Fig. 3 and data not shown) [14]. Following galactose induction, the human Chap1/Dsk2 gene efficiently suppressed the growth arrest of the dsk2Δ rad23Δ cells, and, in contrast to yeast DSK2, high level expression of the human homologue was not toxic to wild-type cells. In addition, we analyzed the cell cycle distribution of the dsk2Δ rad23Δ transformants following 10 h incubation at the restrictive temperature. We observed a reduction in the frequency of aberrant large-budded cells at G2/M in cells transformed with the human Chap1/Dsk2 cDNA clone, confirming the ability of the human homologue to complement the dsk2Δ rad23Δ defect (Fig. 3). These results indicate that the sequence conservation between the yeast and human proteins extends to functional conservation.
The role for these interactions with UbL-linked proteins, however, is still unknown. The best-characterized UbL-linked protein is Rad23p, which controls UV sensitivity [26,27], regulates Rad4p activity, and directly interacts with the 26S proteasome through its amino-terminal ubiquitin domain [28]. Since Rad23p binds to the Rad4p DNA repair protein through its carboxy-terminal region [29], this protein represents a direct link between DNA repair and the ubiquitin/ proteasome pathways [28]. While we have shown that Rad23 and Chap1/Dsk2 share conserved UbL and UbA domains [23,30] and are functionally redundant for the ability to complement cell growth in dsk2Δ and rad23Δ S. cerevisiae cells, there is no evidence to date that either Chap1/Dsk2, Scythe, or Bag1/Rap46 are associated with the proteasome.

In the case of Bag1/Rap46, recent studies have shown that the binding of Bag1/Rap46 with Hsp/Hsc70 serves to both inhibit chaperone activity and to mediate multimeric protein complexes that include the anti-apoptosis molecule, Bel-2, the nuclear oncogene, c-jun, as well as several hormone and growth factor receptors [5,9,11]. These observations suggested that Bag1/Rap46 may serve to regulate the cellular stress response and provide a link with cell signalling and the cell death machinery. Although we have not yet defined the subcellular localization and the specific functional role for Chap1/Dsk2 and Chap2/Scythe, we have now identified two additional ubiquitin-linked proteins that retain the capacity to bind to the ATPase domain of an Hsp70-like molecule and which can modulate, respectively, transit through the G2/M phase of the cell cycle or the apoptotic machinery. Of interest, a recently identified Hsp70 interacting protein, CHIP [7] also encodes a discrete ubiquitin box domain (U-box) identified in a novel ubiquitin conjugation factor involved in multiquitin assembly and cell survival under stress conditions [31]. These findings propose a broader role for the Hsp70-like family in regulating cell cycle and cell death events.

Acknowledgements: This work was supported in part by a grant from the National Institutes of Health (to M.D.R.), an NJCCF fellowship (to I.I.), and an NIH grant: RO1 GM56518 (to S.K.). S.K. is a Scholar of the Leukemia Society of America. K.T. is a predoctoral fellow of the USARMC breast Cancer program. We thank Ahad Rehmattulla for technical assistance.

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