

Human homologue of *ariadne* promotes the ubiquitylation of translation initiation factor 4E homologous protein, 4EHP

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Abstract Human homologue of *Drosophila ariadne* (HHARI) is a RING-IBR-RING domain protein identified through its ability to bind the human ubiquitin-conjugating enzyme, UbcH7. We now demonstrate that HHARI also interacts with the eukaryotic mRNA cap binding protein, translation initiation factor 4E homologous protein (4EHP), via the N-terminal RING1 finger of HHARI. HHARI, 4EHP and UbcH7 do not form a stable heterotrimeric complex as 4EHP cannot immunoprecipitate UbcH7 even in the presence of HHARI. Overexpression of 4EHP and HHARI in mammalian cells leads to polyubiquitylation of 4EHP. By contrast, HHARI does not promote its own autoubiquitylation. Thus, by promoting the ubiquitin-mediated degradation of 4EHP, HHARI may have a role in both protein degradation and protein translation. © 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Human homologue of *ariadne*; Translation initiation factor 4E homologous protein; Ubiquitin-conjugating enzyme; Ubiquitin-protein ligase; Ubiquitin

1. Introduction

The human homologue of *ariadne* protein (HHARI) was isolated from a yeast 2-hybrid screen in which the ubiquitin-conjugating enzyme (E2), UbcH7, was used as bait [1]. The function of HHARI is unknown. However, its importance in neurodevelopment was inferred from studies on the gene encoding its *Drosophila* orthologue, *ariadne-1*, which interacted with UbcD10, the *Drosophila* orthologue of UbcH7 [2]. Although null mutations in *Drosophila ariadne-1* yielded few adult survivors, they manifested a short life-span, motor behavioural deficits and abnormal connectivity in the central nervous system [2].

HHARI is characterised by the presence of a RING (really interesting gene)-(in between RING) IBR-RING finger signa-

ture that is found in a number of ubiquitin-protein ligases (E3s) including Parkin and Dorfin [1,3–7]. Indeed, the RING finger domain structure characterises the main class of E3s [8–10]. Although many E3s are defined by the presence of this domain structure, not all RING finger proteins are necessarily E3s.

HHARI, Parkin and Dorfin interact with the E2s, UbcH7 and/or UbcH8, through their RING-IBR-RING structures [6,7,11]. Consequently, we proposed that HHARI is either a protein that facilitates protein substrate ubiquitylation as a component of an E3, or it is a substrate for protein ubiquitylation. The aim of this study was to identify novel HHARI-interacting proteins in order to understand the function of HHARI in normal cells and provide clues to the neurological phenotype observed when expression is lost.

2. Materials and methods

2.1. Materials

Mouse monoclonal anti-Myc (clone 9E10), and anti-FLAG M2 antibodies were obtained from Sigma. Mouse monoclonal anti-Myc antibody clone 9B11 was obtained from Cell Signaling. Rabbit polyclonal anti-ubiquitin, and horseradish peroxidase-conjugated secondary antibodies were obtained from Dako. Affinity purified anti-UbcH7 C-terminal peptide rabbit antisera was described previously [1,4]. All reagents for Cytotrap yeast 2-hybrid screening were purchased from Stratagene.

2.2. Cytotrap yeast 2-hybrid screen for HHARI-interacting proteins

The Cytotrap yeast 2-hybrid system was performed according to the manufacturer's instructions (Stratagene) in order to identify HHARI-interacting proteins. This system is based upon the detection of interacting proteins in the cytoplasm of yeast cells [12]. In brief, the open reading frame of HHARI was used as 'bait' as a pSos.HHARI fusion construct. pSos.HHARI (60 µg) was used to screen a human testis cDNA library in pMyr (60 µg) as per the manufacturer's protocol (Stratagene).

Putative interactants were purified prior to transformation into the *Escherichia coli* K12 strain, HB101. Plasmids were purified and the sizes of cDNA inserts were determined by polymerase chain reaction (PCR) using vector-specific oligonucleotide primers. PCR amplicons were sequenced.

2.3. Plasmid constructs

A series of HHARI-Myc deletion constructs in pcDNA3.1(-)/Myc-His (Invitrogen) were generated by PCR using *Pfu* Turbo DNA polymerase (Stratagene) and HHARI-Myc as the template [4]. Similarly, the full coding sequence of 4EHP was amplified from the yeast 2-hybrid 4EHP cDNA clone. *Bam*HI and *Xho*I sites were incorporated into the forward and reverse oligonucleotide PCR primers, respectively. Amplicons were digested with *Bam*HI and *Xho*I, gel purified and cloned into FLAG-pcDNA3 or Myc-pcDNA3 vectors [13].

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Abbreviations: E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; 4EHP, translation initiation factor 4E homologous protein; RING, really interesting gene; IBR, in between RING; HHARI, human homologue of *ariadne*; PBS, phosphate-buffered saline; eIF, translation initiation factor

2.4. Mammalian cell transfection, immunoprecipitation (IP) and Western blot analysis

Procedures for transfection of human embryonic kidney (HEK) 293t cells using LipofectAMINE 2000 (Invitrogen), IP and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), protein transfer and immunodetection were performed as described in [4,13]. Working dilutions of primary antibodies were diluted in 1% (w/v) non-fat milk in phosphate-buffered saline (PBS) as follows: 1:1000, mouse anti-FLAG antibodies; 1:100 and 1:1000, mouse 9E10 and 9B11 anti-Myc antibodies, respectively; 1:5000, affinity purified rabbit anti-UbcH7 peptide antibodies; 1:1000, rabbit anti-ubiquitin antibodies. HRP-conjugated secondary antibodies were used at 1:3000 in 0.1% (w/v) non-fat milk in PBS.

3. Results

3.1. Isolation of HHARI-interacting proteins using the Cytotrap yeast 2-hybrid system

cdc25H yeast cells were co-transformed with pSos.HHARI and pMyr human testis cDNA library plasmids in order to identify HHARI-interacting clones. Ninety yeast colonies were initially identified, however only four of these proved to be true interactants. The identities of the four clones

were determined by DNA sequence analysis to be UbcH7, 4EHP (twice – accession number AF047695) and an EST of unknown function (accession number BM471831) (Clone 4). 4EHP is 30% identical (and 60% similar) to the human translation initiation factor, eIF4E, at the amino acid level [14].

Co-transformation of plasmids containing pMyr.4EHP, pMyr.UbcH7 and pMyr.Clone 4 with different control plasmids confirmed the specificity of interaction with pSos.HHARI. Interestingly, strong interactions were observed between pSos.HHARI and pMyr.4EHP or pMyr.UbcH7, by contrast, pSos.HHARI appeared to interact only weakly with Clone 4.

3.2. HHARI interacts with 4EHP in mammalian cells

Co-IP of HHARI-Myc and FLAG-4EHP from transfected HEK293t cells was performed in order to confirm the interaction between HHARI and 4EHP (Fig. 1A). HHARI-Myc and FLAG-4EHP were expressed at approximately the same levels in both singly and co-transfected cells (Fig. 1A, compare lanes 6, 7 and 8). Equal loading of protein lysates was

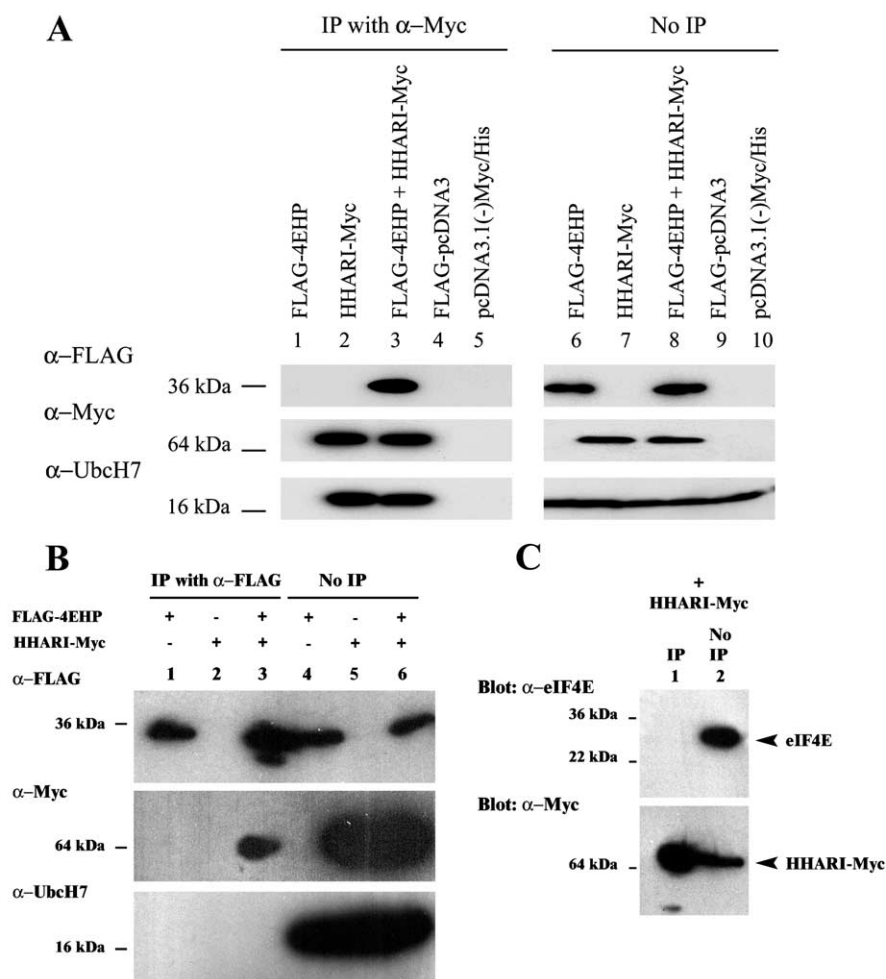


Fig. 1. Interaction of 4EHP, UbcH7 and HHARI in HEK293t cells. A: HEK293t cells were transfected with the plasmid constructs indicated and IPs performed as described in [4]. Immunoprecipitates were then analysed by SDS–PAGE ($T=15\%$; $C=2.5\%$) and Western blotting. Twenty μ l of each total cell lysate was also analysed in parallel (No IP). The membrane was probed sequentially (after stripping) with mouse anti-FLAG (α -FLAG) or anti-Myc antibodies (9B11) (α -Myc), affinity purified rabbit anti-UbcH7 peptide antibodies (α -UbcH7) or anti-eIF4E (α -eIF4E) antibodies. The positions of individual molecular mass markers are indicated. A: HHARI interacts with 4EHP in HEK293t cells. B: Binding of 4EHP to HHARI is independent of binding of UbcH7. C: eIF4E does not interact with HHARI. HEK293t cells were transfected with HHARI-Myc.

confirmed from the approximate equal amounts of UbcH7 (Fig. 1A, bottom right panel).

IP with anti-Myc antibodies from lysates prepared from cells transfected with either HHARI-Myc or FLAG-4EHP alone led to the precipitation of HHARI-Myc (Fig. 1A, middle left panel, lane 2) but not FLAG-4EHP (Fig. 1A, upper left panel, lane 1). Moreover, there was no evidence of other cross-reacting cellular proteins (Fig. 1A, lanes 4 and 5). By contrast, IP of HHARI-Myc with anti-Myc antibodies led specifically to the co-precipitation of FLAG-4EHP in cells expressing both HHARI-Myc and FLAG-4EHP (Fig. 1A, compare lane 3 with lanes 1, 2, 4 and 5). Endogenous UbcH7 was also precipitated (Fig. 1A, lower left hand panel, lane 3). Conversely, IP of FLAG-4EHP with anti-FLAG antibodies (Fig. 1B, upper panel, lanes 1 and 3) did not result in the co-precipitation of endogenous UbcH7 (Fig. 1B, lower panel) although HHARI-Myc was co-precipitated (Fig. 1B, middle panel, lane 3). These data inferred that binding of UbcH7 and 4EHP to HHARI are mutually exclusive.

IP of HHARI-Myc from transfected HEK293t cells did not co-immunoprecipitate endogenous eIF4E (Fig. 1C).

3.3. The RING1 domain of HHARI regulates its interaction with 4EHP

Myc-tagged HHARI deletion constructs (Fig. 2A) were co-transfected into HEK293t cells with FLAG-4EHP. All Myc-tagged HHARI deletion proteins were expressed albeit at slightly variable levels (Fig. 2B, lower panel). Similarly, a consistent level of expression of FLAG-tagged 4EHP was ob-

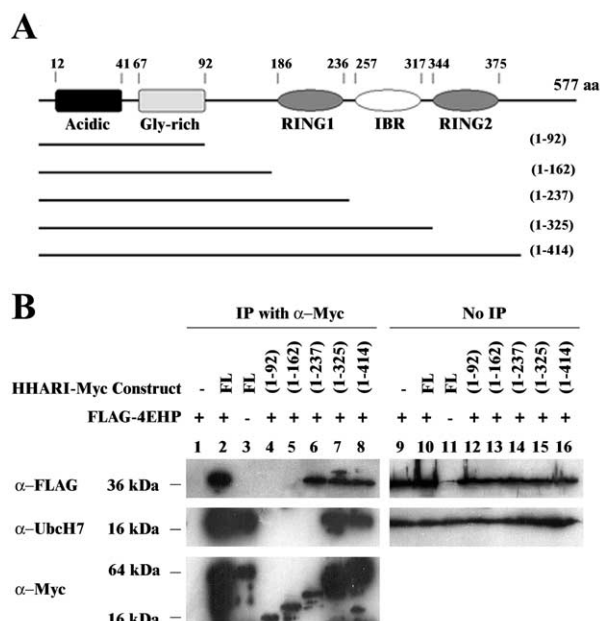


Fig. 2. The RING1 domain of HHARI promotes interaction with 4EHP. A: Schematic of the domain structure of the HHARI-Myc, and deletion constructs generated to establish the region of HHARI that regulates interaction with 4EHP. B: IP of FLAG-4EHP with HHARI-Myc constructs described in A were undertaken as described in Section 2 (IP with α -Myc – lanes 1–8). Twenty μ l of each total cell lysate was also analysed in parallel (No IP – lanes 9–16). The membrane was probed sequentially (after stripping) with mouse anti-FLAG antibodies (α -FLAG), affinity purified rabbit anti-UbcH7 peptide antibodies (α -UbcH7) and mouse anti-Myc antibodies (9B11) (α -Myc). The positions of individual molecular mass markers are indicated.

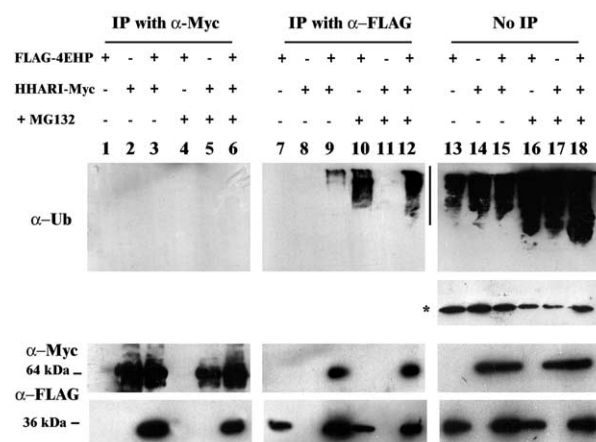


Fig. 3. HHARI promotes polyubiquitylation of 4EHP in HEK293t cells. HEK293t cells were transfected with HHARI-Myc and/or 4EHP constructs and cultured in the presence (+) or absence (–) of 5 μ M MG132 for 16 h. After 48 h, Myc-tagged proteins were immunoprecipitated from 730 μ l transfected cell lysates with either anti-Myc (α -Myc) or anti-FLAG (α -FLAG) antibodies. Immunoprecipitates were then analysed by SDS-PAGE and Western blotting. Twenty μ l of each total cell lysate was also analysed in parallel (No IP). The success of the IP was ascertained by Western blotting with α -Myc (9B11) or α -FLAG antibodies as appropriate (lower panels). The membranes were also probed with anti-ubiquitin antibodies (α -Ub). The positions of individual molecular mass markers are indicated. The vertical black bar at the right hand side of the blots indicates high molecular mass proteins; * – monoubiquitin.

served in those cells transfected with the FLAG-4EHP construct (Fig. 2B, right upper panel). Equal loading of protein was confirmed by the consistent levels of UbcH7 expression observed in the total cell lysates applied to each lane (Fig. 2B, right middle panel).

Full-length HHARI-Myc, HHARI(1-237)-Myc, HHARI(1-325)-Myc and HHARI(1-414)-Myc constructs immunoprecipitated FLAG-4EHP (Fig. 2B, upper left panel). By contrast, HHARI(1-92)-Myc and HHARI(1-162)-Myc did not (Fig. 2B, upper left panel). Although deletion mutant HHARI(1-237)-Myc bound 4EHP, it did not bind UbcH7 (Fig. 2B, compare middle and upper left hand panels, lane 6). These data imply that the RING1 finger domain is a key regulator of the interaction with 4EHP. However, it is not sufficient for binding UbcH7 confirming results of our earlier studies [1,4]. Transfection of deletion mutants lacking the Glycine rich region and acidic domain of HHARI [4] confirmed that these domains did not affect binding to 4EHP (data not shown).

3.4. Overexpression of HHARI and 4EHP in HEK293t cells leads to the polyubiquitylation of 4EHP

Observations that HHARI interacts specifically with UbcH7 [1,4], implied that HHARI might possess E3 activity either towards itself or interacting proteins such as 4EHP. Therefore, we analysed immunoprecipitates of HHARI-Myc and FLAG-4EHP from cell lysates of HEK293t cells, cultured in the presence and absence of the proteasome inhibitor MG132, by Western blotting with anti-ubiquitin antibodies (Fig. 3, upper panel). There was no evidence of HHARI autoubiquitylation either in the presence or absence of MG132 (Fig. 3, left upper panel) despite ample evidence of polyubiquitylated products in the total cell lysates (Fig. 3, right upper panel). By contrast, ubiquitylated FLAG-4EHP products were observed when FLAG-4EHP was co-expressed with

HHARI-Myc that were not present in the absence of HHARI-Myc (Fig. 3, upper middle panel, compare lane 9 with lanes 7 and 8). The levels of ubiquitylated FLAG-4EHP increased when transfected cells were cultured in the presence of MG132 both in the presence and absence of HHARI-Myc (Fig. 3, middle panel, lanes 10 and 12).

4. Discussion

We have demonstrated that HHARI interacts specifically with 4EHP and UbcH7 using both Cytotrap yeast 2-hybrid analysis and by co-IP from mammalian cells. The observation that the Cytotrap system detected the interaction between HHARI and UbcH7 confirmed earlier studies [1]. We note that the Cytotrap system avoids problems of transactivation that often occurs when using the classical yeast 2-hybrid system with proteins containing RING finger domain structures [12]. IP of HHARI in the presence of 4EHP resulted in the co-IP of UbcH7. However, 4EHP was not capable of co-immunoprecipitating UbcH7 independently of HHARI, suggesting that a stable heterotrimeric complex does not exist between these three proteins. Although the RING1 domain regulates the interaction between HHARI and 4EHP, we have previously demonstrated that UbcH7 needs an additional 20 amino acid residue sequence C-terminal to RING1 [1,4].

4EHP is homologous to eIF4E [14]. eIF4E is a component of the eIF4F-mRNA cap binding complex that is essential for cap-dependent protein synthesis [15]. eIF4F comprises eIF4A, eIF4E and eIF4G and is responsible for the recruitment of ribosomes to mRNA. [15]. During translation initiation, eIF4F interacts with both the cap of mRNA (through eIF4E) and the ribosome-associated eIF3 (through eIF4G). eIF4F acts as a bridging factor between mRNA and ribosomes. eIF4E is found both in the cytoplasm and the nucleus of cells [16,17]. Whereas cytoplasmic eIF4E functions in cap-dependent translation, the nuclear fraction is involved in selective mRNA transport [18,19]. In this regard, the nuclear import of eIF4E is mediated by binding to 4E-T (eIF4E-Transporter) via the importin $\alpha\beta$ pathway [20]. It is highly unlikely that 4EHP displays a similar function as it appears to be essentially a cytoplasmic protein and although it is capable of binding the 5' mRNA cap structure, it does not bind eIF4G [14]. Furthermore, it is difficult to imagine how 4EHP could significantly interfere with the function of eIF4E as 4EHP is present in cells at only 10% of the level of eIF4E [14] and HHARI does not interact with eIF4E.

We proposed that HHARI may be an E3 due to structural similarities to E3s that include Parkin [6] and Dofin [11]. Overexpression of HHARI and 4EHP in mammalian cells yielded increased levels of polyubiquitylated 4EHP. However, unlike Parkin or Dofin, HHARI was not autoubiquitylated and addition of proteasome inhibitors did not affect the overall levels of HHARI-Myc (data herein and [13]).

HHARI-mediated ubiquitylation of 4EHP may alter the efficiency of its binding to the cap of mRNA. Indeed, we have preliminary evidence to suggest that this may be the case. This may promote translation in a similar way to ubiquitylation of transcription factors aids gene transcription [21].

Alternatively, the function of 4EHP binding to capped mRNA may be to compartmentalise specific mRNA populations inside cells thereby post-transcriptionally regulating gene expression [22]. Loss of such a function may be particularly relevant for mRNA movements down axons and may begin to explain the phenotypic axonal pathfinding defects observed in the *Drosophila ariadne-1* knock-outs [2,22].

Future investigations will focus on elucidating the potentially complex relationship between eIF4E and 4EHP/HHARI, protein mono/polyubiquitylation and their potential roles in the regulation of protein synthesis and/or mRNA transport.

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