Association of *FHIT* (fragile histidine triad), a candidate tumour suppressor gene, with the ubiquitin-conjugating enzyme hUBC9

Yufei SHI*[1], Minjing ZOU*, Nadir R. FARID† and Malcolm C. PATERSON*

*Department of Biological and Medical Research (MBC-03), King Faisal Specialist Hospital and Research Center, P.O. Box 3354, Riyadh 11211, Saudi Arabia, and †Department of Medicine, Hemel Hempstead General Hospital, Hillfield Road, Hemel Hempstead, Herts. HP2 4AD, U.K.

FHIT (fragile histidine triad), a candidate tumour suppressor gene, has recently been identified at chromosomal region 3p14.2, and deletions of the gene have been reported in many types of human cancer. However, the biological function of the Fhit protein has not been fully characterized yet. Using the yeast two-hybrid screen to search for proteins that interact with Fhit *in vivo*, we identified a protein that is specifically associated with Fhit. This association was confirmed in both immunoprecipitation and glutathione S-transferase pull-down assays. The sequence of the protein is identical with that of human ubiquitin-conjugating enzyme 9 (hUBC9). The last 21 amino acids at the C-terminus of hUBC9 appear to be unimportant for its biological activity, since an hUBC9 mutant harbouring a deletion of these amino acids could still restore normal growth of yeast containing a temperature-sensitive mutation in the homologue UBC9 gene. Mutational analysis indicated that hUBC9 was associated with the C-terminal portion of Fhit. Neither a single amino acid substitution at codon 96 (His → Asn) nor triple amino acid substitutions (His → Asn) at a histidine triad (codons 94, 96 and 98) affected the association, whereas Fhit triphosphate (diadenosine 5′,5‴-P^1,P^3-triphosphate) hydrolase activity has been reported to be eliminated by either type of mutation, suggesting that the interaction between Fhit and hUBC9 is independent of Fhit enzymic activity. Given that yeast UBC9 is involved in the degradation of S- and M-phase cyclins, Fhit may be involved in cell cycle control through its interaction with hUBC9.

Key words: fragile histidine triad, neoplasm, protein interaction.

INTRODUCTION

FHIT (fragile histidine triad) is a putative tumour suppressor gene located on chromosome 3 at band p14.2 (3p14.2). The gene comprises 10 exons which encode a 1.1 kb transcript and a protein of 147 amino acids [1]. The *FHIT* gene contains the most common fragile site in the human genome, FRA3B, and the t(3;8) chromosomal translocation breakpoint that is associated with familial renal clear cell carcinoma [1]. Alterations in the *FHIT* gene leading to abnormal transcripts containing deletions of one or more coding exons, intragenic homozygous deletions and gross genomic rearrangements have been found frequently in many cancer cell lines, as well as in primary tumours, including those of lung, breast, thyroid, oesophagus, stomach, Merkel cell, and head and neck [1–7].

FHIT is a member of the histidine triad gene family [8]. The cognate protein has 69% identity with the *Schizosaccharomyces pombe* enzyme diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) asymmetrical hydrolase, which cleaves its Ap_4A substrate into ATP and AMP [9]. Ap_4A has been implicated in both DNA replication and cell cycle control [10,11]. The human Fhit protein has been characterized as a diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A) hydrolase [12], and Fhit–Ap_3A complexes have been suggested as the active signalling form of Fhit [13]. However, a number of investigators have raised doubts about the candidacy of *FHIT* as a tumour suppressor gene, based on the following two observations: (i) aberrant transcripts can be amplified by reverse transcription–PCR from normal cells; and (ii) inactivation of the *FHIT* gene by point mutation has rarely been reported [14–16]. On the other hand, it has been shown that replacement of Fhit in cancer cell lines that lack endogenous Fhit suppresses tumorigenicity, and that Fhit enzymic activity is not required for its tumour suppressor function [17,18].

We reported previously that both benign thyroid adenomas and malignant papillary and anaplastic carcinomas frequently harbour abnormalities in the *FHIT* gene [5]. The present study was undertaken to identify Fhit-interacting proteins, in the hope of elucidating its biological role in tumorigenesis. To this end, we have used the yeast two-hybrid system to search for proteins that interact with Fhit *in vivo*. A protein was identified by screening a human placenta cDNA library using the full-length human *FHIT* cDNA as a 'bait'. A protein that was found to associate with the C-terminal portion of Fhit proved to be identical with the recently cloned human ubiquitin-conjugating enzyme 9 (hUBC9).

MATERIALS AND METHODS

Yeast two-hybrid screen

Full-length human *FHIT* cDNA was obtained by reverse transcription–PCR, as described previously [5], and subcloned into *Eco*RI and *Xho*I sites of the plasmid pHybLex/Zeo (Invitrogen, Carlsbad, CA, U.S.A.) to create a LexA–Fhit fusion protein as a 'bait' (pHyblex/Fhit). To screen for Fhit-interacting proteins, *Saccharomyces cerevisiae* L40 cells were transformed by the lithium acetate method with pHyblex/Fhit, and subsequently with the B42 activation domain/human placenta cDNA library constructed in pYESTrp according to the manufacturer's pro-

Abbreviations used: Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; ATF2, activating transcription factor 2; CMV, cytomegalovirus; *FHIT*/Fhit, fragile histidine triad; GST, glutathione S-transferase; NF-κB, nuclear factor-κB; IκBα, NF-κB inhibitory protein; SUMO-1, small ubiquitin-like molecule-1; (h)UBC9, (human) ubiquitin-conjugating enzyme 9; WT1, Wilms' tumour suppressor gene product.

[1] To whom correspondence should be addressed (e-mail yufei@kfshrc.edu.sa).

© 2000 Biochemical Society

cedure (Invitrogen). Briefly, 8×10^8 transformants were selected by their growth on plates lacking histidine. A total of 267 His^+ colonies were subsequently analysed for β-galactosidase activity. Some 20 cDNA library plasmids derived from colonies with strong LacZ activity (His^+ and $LacZ^+$) were sequenced with the aid of a cyclic sequencing kit from Applied Biosystems (DNA sequencing kit # 402079) following the protocol provided by the manufacturer. Sequence analysis was undertaken in an Applied Biosystems 373A automated sequencing apparatus using 373A version 1.2.1 data collection and analysis software (Applied Biosystems). The LexA–Fhit fusion protein of pHyblex/Fhit did not spontaneously transactivate the transcription of either reporter gene (*HIS3* or LacZ) used in this system, and the expression of the appropriate bait protein was confirmed by Western blot using anti-LexA antibody (Invitrogen).

To map the Fhit interaction domain(s), four Fhit mutants were constructed by PCR and oligonucleotide-based site-directed mutagenesis [19], whereupon all four were subcloned in-frame with the LexA DNA-binding domain of pHybLex/Zeo: pHyblex/Fhit-NT (N-terminal part of Fhit: amino acids 1–81), pHyblex/Fhit-CT (C-terminal part of Fhit: amino acids 82–147), pHyblex/Fhit-H96N (His → Asn substitution at codon 96), and pHyblex/Fhit-H94,96,98N (His → Asn substitution at codons 94, 96 and 98). hUBC9 cDNAs were subcloned in-frame with the B42 activation domain of the plasmid pYESTrp (Invitrogen). Yeast strain L40 was co-transformed with pYESTrp-hUBC9 and each one of the bait constructs indicated above. Protein interaction was assessed by the β-galactosidase filter assay and semi-quantified using the β-galactosidase liquid assay kit according to the manufacturer's procedure (Invitrogen).

Human wild-type and mutant hUBC9 each complement loss of the yeast UBC9 gene

The yeast strain Y0233 (YWO103,ubc9▲::TRP1,ubc9-1::LEU2,bar1::HIS3) carrying a temperature-sensitive ubc9-1 allele [20] was generously donated by Dr Thorsten Hoppe (University of Heidelberg, Germany). The wild-type and truncated hUBC9 genes were subcloned into the plasmid pYESTrp under the control of the GAL1 promoter (Invitrogen). Cells were transformed with pYESTrp alone, pYESTrp-Jun expressing the transcription factor Jun (control), pYESTrp-hUBC9, or pYESTrp-$hUBC9^{mutant}$ lacking the final 21 amino acids at the C-terminus of hUBC9. Transformants were streaked on to galactose-containing plates and incubated for 4 days at either a permissive (28 °C) or a restrictive (37 °C) temperature.

Generation of a polyclonal antibody to Fhit

Full-length human *FHIT* cDNA was subcloned into *Eco*RI and *Xho*I sites of the plasmid pGEX-5X-1 in-frame with the glutathione S-transferase (GST) gene to create a GST–Fhit fusion protein. The production of the GST–Fhit protein was induced in *Escherichia coli* and was purified using glutathione–Sepharose 4B beads, as recommended by the manufacturer (Pharmacia, Vienna, Austria). For immunization and three subsequent boosts, two rabbits were injected with beads carrying at least 100 μg of purified GST–Fhit fusion protein to obtain a polyclonal serum.

GST pull-down assay

Full-length hUBC9 cDNA was subcloned into *Eco*RI and *Xho*I sites of the plasmid pGEX-5X-1 in-frame with GST and expressed in *E. coli* (strain BL21) as a GST–hUBC9 fusion protein. GST–hUBC9 or GST alone was bound to glutathione–Sepharose 4B, and each complex was washed three times with PBS. [^{35}S]Methionine-labelled Fhit was generated using the TNT T7 Quick Coupled *in vitro* transcription/translation system (Promega, Madison, WI, U.S.A.) following the subcloning of the full-length *FHIT* cDNA into a TA vector (Invitrogen). [^{35}S]Methionine-labelled Fhit (5 μl) was incubated with the beads (20 μl) in 0.5 ml of 50 mM NaCl/BSA (1 mg/ml) at 4 °C for 1 h. The mixture was then washed four times with 0.1 % Nonidet P-40 in PBS, and heated in standard SDS sample buffer. The eluted proteins were resolved on an SDS/15 %-polyacrylamide gel, stained with Coomassie Blue, and exposed to Kodak X-ray film at room temperature.

Co-immunoprecipitation of Fhit and hUBC9

Full-length *FHIT* and hUBC9 cDNAs were each subcloned into the pcDNA 3.1 expression plasmid (Invitrogen) under the control of the cytomegalovirus (CMV) promoter (pCMV-FHIT and pCMV-hUBC9 respectively). CHO cells were transfected with pCMV-FHIT, pCMV-hUBC9 or pCMV-FHIT plus pCMV-hUBC9. At 48 h after transfection, the CHO cells were washed with PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 % Nonidet P40, 0.1 % SDS and 0.5 % sodium deoxycholate) supplemented with a cocktail of four protease inhibitors: 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 50 μg/ml PMSF. The lysates were precleared with normal rabbit IgG. Equal amounts of protein (1–2 mg in 0.5 ml) were incubated with 10 μl of rabbit anti-Fhit antibody for 3 h at 4 °C with constant rotation. Immune complexes were precipitated by incubation with 20 μl of Protein A/G–agarose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 1 h. The agarose beads were pelleted by centrifugation at 3000 ***g*** for 5 min), washed four times with RIPA buffer, and heated in SDS sample buffer. The eluted proteins were resolved on an SDS/15 %-polyacrylamide gel, transferred on to a nitrocellulose membrane (Bio-Rad, Melville, NY, U.S.A.) and analysed using an anti-hUBC9 monoclonal antibody (Transduction Labs, Lexington, KY, U.S.A.). Immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (Amersham, Arlington Heights, IL, U.S.A.) according to the manufacturer's protocol.

RESULTS

Identification of a Fhit-binding protein in the yeast two-hybrid system

We used the two-hybrid system to isolate any proteins that may interact with Fhit. To this end, full-length *FHIT* cDNA was subcloned in-frame with the LexA DNA-binding domain in the yeast expression vector pHybLex/Zeo as a LexA–Fhit fusion protein (bait) vector. pHybLex-Fhit was transformed into the L40 strain of yeast containing *LacZ* and *HIS3* reporter constructs integrated into the genome. Expression of LexA–Fhit fusion protein was confirmed by Western blot analysis using anti-LexA antibody. Non-specific activation of the *LacZ* and *HIS3* reporter genes by pHybLex-Fhit was ruled out by the absence of activation in cells transformed with pHybLex-Fhit alone or co-transformed with the pYESTrp vector. Thus pHybLex-Fhit was used to screen a human placenta cDNA library constructed in-frame with the B42 activation domain of the yeast expression vector pYESTrp.

A total of 8×10^8 transformants were screened, and 267 His^+ colonies were obtained after 4–8 days at 30 °C. Of these colonies,

© 2000 Biochemical Society

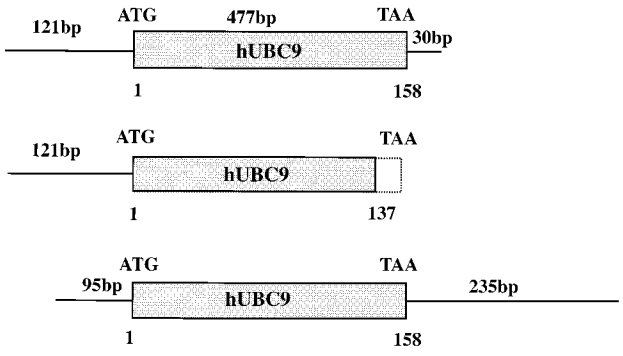


Figure 1 Schematic representation of different hUBC9 clones detected by the yeast two-hybrid screen

Three different hUBC9 clones were identified, two of which contained the full-length hUBC9 coding sequence. The remaining one was truncated, missing 21 amino acids at the C-terminus. Shaded and open boxes indicate translated and truncated regions respectively. The flanking lines represent untranslated regions.

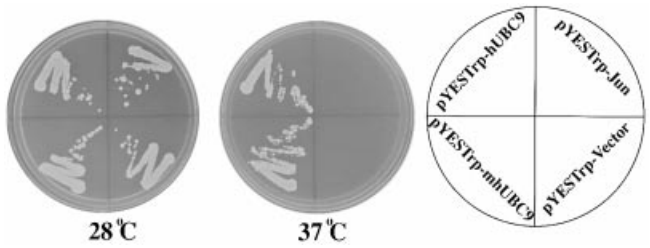


Figure 2 Human wild-type and mutant UBC9 complement the loss of the homologous yeast UBC9 gene

Yeast stain Y0233 (YWO103) carrying the temperature-sensitive ubc9-1 allele was transformed with either a control vector (pYESTrp or pYESTrp-Jun) or a construct expressing either a full-length (pYESTrp-hUBC9) or truncated (pYESTrp-mhUBC9) hUBC9 gene. Cells were streaked on to galactose-containing plates and incubated for 4 days at either the permissive (28 °C) or the restrictive (37 °C) temperature.

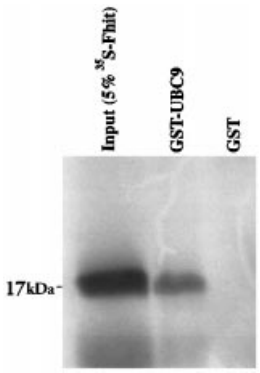


Figure 3 *In vitro* interaction of Fhit and hUBC9 in a GST pull-down assay

The full-length hUBC9 cDNA was subcloned in-frame with GST and expressed in *E. coli* as a GST–hUBC9 fusion protein. GST–hUBC9 or GST alone was bound to glutathione–Sepharose beads and incubated with an equal amount of [^{35}S]methionine-labelled Fhit prepared by *in vitro* transcription and translation. Specifically bound protein was separated on an SDS/15%-polyacrylamide gel. Lane 1 contained 5% (2 μl) of *in vitro* translated Fhit protein.

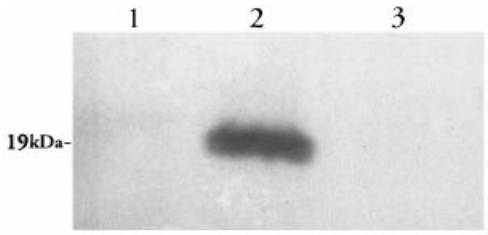


Figure 4 Co-immunoprecipitation of Fhit and hUBC9

Full-length *FHIT* and hUBC9 cDNAs were subcloned into pcDNA 3.1 expression plasmid (denoted pCMV-FHIT and pCMV-hUBC9 respectively). CHO cells were transfected with pCMV-FHIT alone, pCMV-hUBC9 alone, or pCMV-FHIT and pCMV-hUBC9 together. Cell extracts were prepared from CHO cells 48 h after transfection, and extracts expressing Fhit alone (lane 1), Fhit and hUBC9 (lane 2), and hUBC9 alone (lane 3) were immunoprecipitated with anti-Fhit antibody, separated on an SDS/15%-polyacrylamide gel, blotted and probed with anti-hUBC9 monoclonal antibody.

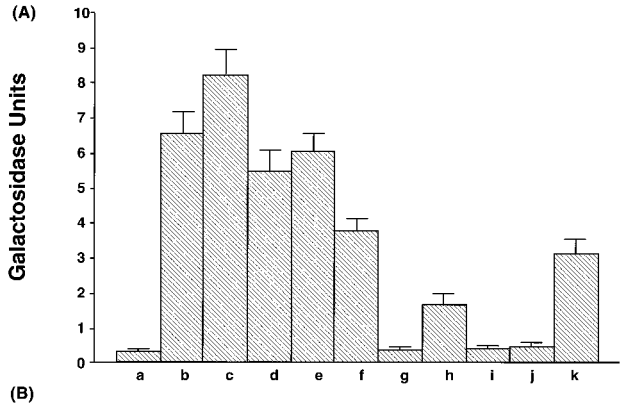
20 showed strong β-galactosidase activity, as judged by the filter assay [blue colour that developed in the presence of X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactoside) in 30–60 min], and these were characterized further by DNA sequencing and re-transformation into L40 cells to verify the specific interaction. Among the 20 clones, 16 formed three groups of cDNAs which encoded a single gene and were fused in the same reading frame at two different positions in the 5′-untranslated region (Figure 1). Sequence analysis revealed the full-length and truncated cDNAs to be identical with that of the previously cloned enzyme hUBC9 [21]. The remaining four clones were unrelated to hUBC9 and have sequence similarity to human 18 S rRNA. Both the full-length and the truncated (missing 21 amino acids at the C-terminus) hUBC9 interacted strongly with the LexA–Fhit bait, but failed to interact with the LexA DNA-binding domain alone. This result suggests that the interaction between Fhit and hUBC9 may be specific, and that the C-terminal 21 amino acids of hUBC9 are not required for the interaction. To establish that mutant hUBC9 is also functional, full-length and mutant hUBC9 cDNAs were each expressed in a yeast strain (Y0233) carrying a temperature-sensitive (ts) yUBC9 mutation [19]. Cells harbouring the yUBC9ts mutation grow normally at 28 °C, but fail to grow at the non-permissive temperature (37 °C). As shown in Figure 2, both wild-type and mutant hUBC9 fully restored normal growth to the yeast at the otherwise non-permissive temperature for the mutant strain.

Specific association of Fhit and hUBC9

To address further the specificity of the interaction, we performed both *in vitro* and *in vivo* binding assays in a context other than yeast. Full-length hUBC9 was expressed in bacteria as a GST fusion protein, coupled to glutathione–Sepharose beads and incubated with [^{35}S]methionine-labelled Fhit produced by *in vitro* transcription and translation. Specifically bound protein was separated on an SDS/15%-polyacrylamide gel. As shown in Figure 3, Fhit interacted with GST–hUBC9, but not with GST alone, in an *in vitro* GST pull-down assay.

In order to demonstrate the specific interaction *in vivo*, Fhit and hUBC9 were co-expressed in mammalian (CHO) cells, and

© 2000 Biochemical Society



(B)

Lex A DBD Fusion Protein	Fhit	Fhit	Fhit	Fhit-H94 96,96N	Fhit H96N	Fhit-CT	Fhit NT	Fhit-CT	DBD alone	Fhit-NT	Fos
B42 Ad Fusion Protein	AD alone	UBC9	UBC9 mutant	UBC9	UBC9	UBC9	UBC9	AD alone	UBC9	AD alone	Jun
β-gal Activites	-	++++	++++	++++	++++	++++	-	+++	-	-	++++

Figure 5 Interaction of Fhit mutants with hUBC9 in a yeast two-hybrid assay

Yeast strain L40 containing the LacZ reporter gene was co-transformed with the indicated Fhit mutants fused into the LexA DNA-binding domain (DBD) of the plasmid pHybLex/Zeo (baits) or vector alone, and full-length or truncated hUBC9 fused into the B42 activation domain (Ad) of the plasmid pYESTrp. Protein interaction was assessed by both β-galactosidase liquid assays (**A**) and β-galactosidase filter assays (**B**). The Fhit mutants were: Fhit-NT, Fhit in which the C-terminal half (amino acids 82–147) was deleted; Fhit-CT, Fhit in which the N-terminal 81 amino acids were deleted; Fhit-H96N, His → Asn mutation at codon 96; Fhit-H94,96,98N, His → Asn mutations at codons 94, 96 and 98. (**A**) Individual colonies containing the indicated protein pairs were taken from each plate and grown overnight in the liquid dropout medium. Equal amounts of cells were collected and β-galactosidase activity was measured in duplicate in the liquid assay. Data represent means ± S.E.M. (A_{420}/A_{600}) of three independent experiments. (**B**) At least six independent transformants were scored for the intensity of the galactose-inducible blue colour that developed in the presence of X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactoside): ++++ (strong interaction), dark blue developed in 30 min; +++, dark blue observed in 2 h; ++, dark blue observed in 6 h; +, dark blue seen in 16 h; —, white colonies after 16 h (no interaction).

extracts were immunoprecipitated with anti-Fhit polyclonal antibody. The immunoprecipitates were separated on an SDS/15%-polyacrylamide gel and analysed by Western blot with an anti-hUBC9 monoclonal antibody. hUBC9 was detected (co-immunoprecipitated) only in lysates from cells expressing both Fhit and hUBC9, and was not found in cells expressing either hUBC9 or Fhit alone (Figure 4). The fact that anti-Fhit antibody can precipitate hUBC9 only when Fhit is present indicates the presence of FHIT–hUBC9 immunocomplexes. No detection was observed in cells expressing both Fhit and hUBC9 when rabbit pre-immune serum was substituted for the anti-Fhit antibody (results not shown). Taken together, the results support the conclusion that Fhit and hUBC9 interact specifically both *in vitro* and *in vivo*.

Mutational analysis of the interaction between Fhit and hUBC9

To define the regions of Fhit that interact with hUBC9, we made two deletion constructs: pHyblex/Fhit-NT, in which the C-terminal half (amino acids 82–147) of Fhit was deleted, and pHyblex/Fhit-CT (N-terminal 81 amino acids deleted). Fhit-NT and Fhit-CT were detected as LexA–Fhit-NT and LexA–Fhit-CT fusion proteins respectively by Western blot analysis, using anti-LexA antibody (results not shown). As shown in Figure 5, Fhit-NT did not interact with hUBC9, whereas Fhit-CT interacted strongly with hUBC9, suggesting that the C-terminal half of Fhit may be involved in the interaction between Fhit and hUBC9. No interaction was observed when Fhit was co-expressed with the B42 activation domain alone or when hUBC9 was co-expressed with the LexA DNA-binding domain alone. Interestingly, Fhit-CT could modestly activate the B42 activation domain in the absence of hUBC9, whereas Fhit itself could not (Figure 5). Therefore the N-terminal half of Fhit may play a role in inhibiting the C-terminal half self-activating the B42 activation domain. Self-activation of the activation domain happens frequently with transcription factors in yeast two-hybrid assays. It is thus tempting to speculate that Fhit may have the capacity to act as a transcription factor.

Since mutation at the histidine triad (H96N) abolishes Fhit triphosphate (Ap_3A) hydrolase activity, but does not interfere with its tumour suppressive function [17], we wondered whether mutation at the histidine triad would disrupt the interaction between Fhit and hUBC9. Thus two constructs were made by site-directed mutagenesis: pHyblex/Fhit-H96N (His → Asn at codon 96) and pHyblex/Fhit-H94,96,98N (His → Asn at codons 94, 96 and 98). As shown in Figure 5, neither a single amino acid substitution at codon 96 nor triple amino acid substitutions at codons 94, 96 and 98 significantly affected the interaction, indicating that the interaction of Fhit and hUBC9 is independent of Fhit enzymic activity.

DISCUSSION

We have used the yeast two-hybrid screen to identify a protein that interacts with Fhit. The protein is identical with the previously cloned hUBC9. The majority of the hUBC9 clones (13 out of 16) identified were full-length, and the ones truncated were those missing 21 amino acids at the C-terminus. Therefore full-length or near full-length hUBC9 may be required to interact with Fhit. The ability of both wild-type and mutant hUBC9 to restore normal growth of yeast that carry a temperature-sensitive mutant UBC9 gene demonstrates evolutionary conservation of function, and that the C-terminal 21 amino acids of UBC9 are not of functional significance. The specific Fhit–hUBC9 interaction has been further confirmed by GST pull-down and co-immunoprecipitation assays. Mutational analysis has shown that the C-terminal half of Fhit may be involved in the interaction between Fhit and hUBC9, and that mutation at the histidine triad does not interfere with that interaction.

UBC9 is a member of a family of ubiquitin-conjugating enzymes (E2) whose members are characterized by a highly conserved domain of 16 kDa. This domain contains a centrally located cysteine residue that is required for ubiquitin–enzyme thiolester formation [22,23]. Ubiquitin/proteasome-dependent proteolysis is involved in the breakdown of abnormal and short- or long-lived proteins in eukaryotes [23]. The hallmark of this pathway is the covalent attachment of ubiquitin to a target protein. Three classes of enzymes participate in ubiquitin conjugate formation. These are ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Studies in the yeast *S. cerevisiae* have identified 13 different ubiquitin-conjugating enzymes that are involved in various cellular processes, such as DNA repair, cell cycle control, transcription and protein translocation, suggesting that E2 enzymes may be involved in establishing the diversity and the specificity of the ubiquitin-dependent proteolysis system [22]. Yeast UBC9 has been shown to be essential for cell viability by

© 2000 Biochemical Society

virtue of its ability to degrade B-type cyclins. Repression of UBC9 synthesis in *S. cerevisiae* prevents cell cycle progression at the G2- or early M-phase [20].

Although its precise role in mammalian cells has not yet been fully characterized, UBC9 has been found to be associated with a large variety of proteins, including the negative regulatory domain of the Wilms' tumour suppressor gene product (WT1) [21], activating transcription factor 2 (ATF2) [24], transcription factor E2A proteins [25], Fas antigen (CD95) [26], Rad51 recombination protein [27], nuclear factor-κB (NF-κB) inhibitory protein (IκBα) [28] and the adenovirus E1A oncoprotein [29]. Apart from degradation of target proteins such as ATF2, IκBα and E2A by ubiquitin conjugation, UBC9 has also been shown to modulate the activity of target proteins through mechanisms other than ubiquitination. For example, UBC9 relieves transcription repression by interacting with TEL transcription factor [30], and activates receptor-dependent transcription by interacting with the androgen receptor [31]. UBC9 also catalyses conjugation of the small ubiquitin-like molecule-1 (SUMO-1) to Ran GTPase-activating protein (RanGAP1) [32]. SUMO-1 (also known as sentrin, UBL1, GMP1 or PIC1) modifications have been implicated in the targeting of proteins to the nuclear envelope and intranuclear structures, and in converting proteins that are resistant to ubiquitin-mediated degradation [33–36].

Several possible outcomes may result from the Fhit–hUBC9 interaction. First, hUBC9 may be involved directly in the ubiquitin-dependent proteolysis of Fhit protein. Secondly, hUBC9 may modulate Fhit activity by conjugating the latter with SUMO-1 or through other pathways. Given that Fhit–Ap_3A complexes have been suggested as the active signalling form of Fhit [13], such a modulation may have a significant role in regulating the tumour suppressive activity of Fhit. Indeed, hUBC9 has been implicated in interacting with the negative regulatory domain of WT1 to repress transcription [21,37]. Furthermore, it appears that UBC9 could have dual functional roles in regulating target proteins under different situations. As a case in point, UBC9 has been shown to be involved in the degradation of IκBα through ubiquitination, resulting in NF-κB activation [28]; on the other hand, UBC9-dependent SUMO-1 modification of IκBα inhibits NF-κB activation [36]. Lastly, Fhit might modulate the activity of hUBC9. Although there is no solid evidence yet supporting any of the aforementioned hypotheses, the involvement of UBC9 in a broad range of intracellular processes, such as cell cycle control, apoptosis and transcription, indicates that the interaction of Fhit and hUBC9 may be biologically significant.

REFERENCES

1 Ohta, M., Inoue, H., Cotticelli, M. G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T. et al. (1996) The *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell **84**, 587–597

2 Sozzi, G., Veronese, M. L., Negrini, M., Baffa, R., Cotticelli, M. G., Inoue, H., Tornielli, S., Pilotti, S., De Gregorio, L., Pastorino, U. et al. (1996) The *FHIT* gene at 3p14.2 is abnormal in lung cancer. Cell **85**, 17–26

3 Negrini, M., Monaco, C., Vorechovsky, I., Ohta, M., Druck, T., Baffa, R., Huebner, K. and Croce, C. M. (1996) The *FHIT* gene at 3p14.2 is abnormal in breast carcinomas. Cancer Res. **56**, 3173–3179

4 Tanaka, H., Shimada, Y., Harada, H., Shinoda, M., Hatooka, S., Imamura, M. and Ishizaki, K. (1998) Methylation of the 5′ CpG island of the *FHIT* gene is closely associated with transcriptional inactivation in esophageal squamous cell carcinomas. Cancer Res. **58**, 3429–3434

5 Zou, M. J., Shi, Y., Farid, N. R., Al-Sedairy, S. T. and Paterson, M. C. (1999) *FHIT* gene abnormalities in both benign and malignant thyroid tumors. Eur. J. Cancer **35**, 467–472

6 Virgilio, L., Shuster, M., Gollin, S. M., Veronese, M. L., Ohta, M., Huebner, K. and Croce, C. M. (1996) *FHIT* alterations in head and neck squamous cell carcinomas. Proc. Natl. Acad. Sci. U.S.A. **93**, 9770–9775

7 Sozzi, G., Alder, H., Tornielli, S., Corletto, V., Baffa, R., Veronese, M. L., Negrini, M., Pilotti, S., Pierotti, M. A., Huebner, K. and Croce, C. M. (1996) Abberant *FHIT* transcripts in Merkel cell carcinoma. Cancer Res. **56**, 2472–2474

8 Seraphin, B. (1992) The HIT protein family: a new family of proteins present in prokaryotes, yeast and mammals. J. DNA Sequencing Mapping **3**, 177–179

9 Huang, Y., Garrison, P. N. and Barnes, L. D. (1995) Cloning of the *Schizosaccharomyces pombe* gene encoding diadenosine 5′-5‴-P1,P4-tetraphosphate (Ap4A) asymmetrical hydrolase: sequence similarity with the histidine triad (HIT) family. Biochem. J. **312**, 925–932

10 Weinmann-Dorsch, C., Grummt, I., Albert, W., Ferdinand, F. J., Friss, R. R., Pierron, G., Moll, W. and Grummt, F. (1984) Drastic rise of intracellular adenosine (5′) tetraphospho (5′) adenosine correlates with onset of DNA synthesis in eukaryotic cells. Eur. J. Biochem. **138**, 179–185

11 Baxi, M. D., McLennan, A. G. and Vishwanatha, J. K. (1994) Characterization of the Hela cell DNA polymerase α associated Ap_4A binding protein by photoaffinity labeling. Biochemistry **33**, 14601–14607

12 Barnes, L. D., Garrison, P. N., Siprashvili, Z., Guranowski, A., Robinson, A. K., Ingram, S. W., Croce, C. M., Ohta, M. and Huebner, K. (1996) Fhit, a putative tumor suppressor in humans, is a dinucleoside 5′,5‴-P1,P3-triphosphate hydrolase. Biochemistry **35**, 11529–11535

13 Pace, H. C., Garrison, P. N., Robinson, A. K., Barnes, L. D., Draganescu, A., Rosler, A., Blackburn, G. M., Siprashvili, Z., Croce, C. M., Huebner, K. and Brenner, C. (1998) Genetic, biochemical and crystallographic characterization of Fhit-substrate complexes as the active signaling form of Fhit. Proc. Natl. Acad. Sci. U.S.A. **95**, 5484–5489

14 Panagopoulos, I., Thelin, S., Mertens, F., Mitelman, F. and Aman, P. (1997) Variable FHIT transcripts in non-neoplastic tissues. Genes Chromosomes Cancer **19**, 215–219

15 Bieche, I., Latil, A., Becette, V. and Lidereau, R. (1998) Study of FHIT transcripts in normal and malignant breast tissue. Genes Chromosomes Cancer **23**, 292–299

16 Huebner, K., Garrison, P. N., Barnes, L. D. and Croce, C. M. (1998) The role of the FHIT/FRA3B locus in cancer. Annu. Rev. Genet. **32**, 7–31

17 Siprashvili, Z., Sozzi, G., Barnes, L. D., McCue, P., Robinson, A. K., Eryomin, V., Sard, L., Tagliabue, E., Greco, A., Fusetti, L. et al. (1997) Replacement of Fhit in cancer cells suppresses tumorigenicity. Proc. Natl. Acad. Sci. U.S.A. **94**, 13771–13776

18 Ji, L., Fang, B., Yen, N., Fong, K., Minna, J. D. and Roth, J. A. (1999) Induction of apoptosis and inhibition of tumorigenicity and tumor growth by adenovirus vector-mediated fragile histidine triad (*FHIT*) gene overexpression. Cancer Res. **59**, 3333–3339

19 Shi, Y., Zou, M. J., Ahring, P., Al-Sedairy, S. T. and Farid, N. R. (1995) Thyrotropin internalization is directed by a highly conserved motif in the seventh transmembrane region of its receptor. Endocrine **3**, 409–414

20 Seufert, W., Futcher, B. and Jentsch, S. (1995) Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. Nature (London) **373**, 78–81

21 Wang, Z.-Y., Qiu, Q.-Q., Seufert, W., Taguchi, T., Testa, J. R., Whitmore, S. A., Callen, D. F., Welsh, D., Shenk, T. and Deuel, T. F. (1996) Molecular cloning of the cDNA and chromosome localization of the gene for human ubiquitin-conjugating enzyme 9. J. Biol. Chem. **271**, 24811–24816

22 Scheffner, M., Smith, S. and Jentsch, S. (1998) The ubiquitin-conjugation system. In Ubiquitin and the Biology of the Cell (Peters, J.-M., Harris, J. R. and Finley, D., eds.), pp. 65–98, Plenum, New York

23 Hershko, A. and Ciechanover, A. (1992) The ubiquitin system for protein degradation. Annu. Rev. Biochem. **61**, 761–807

24 Firestein, R. and Feuerstein, N. (1998) Association of activating transcription factor 2 (ATF2) with the ubiquitin-conjugating enzyme hUBC9. J. Biol. Chem. **273**, 5892–5902

25 Kho, C.-J., Huggins, G. S., Endege, W. O., Hsieh, C.-M., Lee, M.-E. and Haber, E. (1997) Degradation of E2A proteins through a ubiquitin-conjugating enzyme, UbcE2A. J. Biol. Chem. **272**, 3845–3851

26 Wright, D. A., Futcher, B., Ghosh, P. and Geha, R. (1996) Association of human Fas (CD95) with a ubiquitin-conjugating enzyme (UBC-FAP). J. Biol. Chem. **271**, 31037–31043

27 Kovalenko, O. V., Plug, A. W., Haaf, T., Gonda, D. K., Ashley, T., Ward, D. C., Radding, C. M. and Golub, E. I. (1996) Mammalian ubiquitin-conjugating enzyme Ubc9 interacts with Rad51 recombination protein and localizes in synaptonemal complexes. Proc. Natl. Acad. Sci. U.S.A. **93**, 2958–2963

28 Tashiro, K., Pando, M. P., Kanegae, Y., Wamsley, P. M., Inoue, S. and Verma, I. M. (1997) Direct involvement of the ubiquitin-conjugating enzyme Ubc9/Hus5 in the degradation of IκBα. Proc. Natl. Acad. Sci. U.S.A. **94**, 7862–7867

© 2000 Biochemical Society

29 Hateboer, G., Hijmans, E. M., Nooij, J. B. D., Schlenker, S., Jentsch, S. and Bernards, R. (1996) mUBC9, a novel adenovirus E1A-interacting protein that complements a yeast cell cycle defect. J. Biol. Chem. **271**, 25906–25911
30 Chakrabarti, S. R., Sood, R., Ganguly, S., Bohlander, S., Shen, Z. and Nucifora, G. (1999) Modulation of TEL transcription activity by interaction with the ubiquitin-conjugating enzyme UBC9. Proc. Natl. Acad. Sci. U.S.A. **96**, 7467–7472
31 Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J. J. and Janne, O. A. (1999) Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. J. Biol. Chem. **274**, 19441–19446
32 Lee, G. W., Melchior, F., Matunis, M. J., Mahajan, R., Tian, Q. and Anderson, P. (1998) Modification of Ran GTPase-activating protein by the small ubiquitin-related modifier SUMO-1 requires Ubc9, an E2-type ubiquitin-conjugating enzyme homologue. J. Biol. Chem. **273**, 6503–6507
33 Mahajan, R., Delphin, C., Guan, T., Gerace, L. and Melchior, F. (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. Cell **88**, 97–107
34 Mahajan, R., Gerace, L. and Melchior, F. (1998) Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association. J. Cell. Biol. **140**, 259–270
35 Okura, T., Gong, L., Kamitani, T., Wada, T., Okura, I., Wei, C. F., Chang, H. M. and Yeh, E. T. (1996) Protection against Fas/APO-1 and tumor necrosis factor-mediated cell death by a novel protein, sentrin. J. Immunol. **157**, 4277–4281
36 Desterro, J. M., Rodriguez, M. S. and Hay, R. T. (1998) SUMO-1 modification of IκBα inhibits NF-κB activation. Mol. Cell **2**, 233–239
37 Wang, Z.-Y., Qiu, Q.-Q., Gurrieri, M., Huang, J. and Deuel, T. F. (1995) WT1, the Wilms' tumor suppressor gene product, represses transcription through an interactive nuclear protein. Oncogene **10**, 1243–1247

Received 16 June 2000/14 August 2000; accepted 20 September 2000

© 2000 Biochemical Society