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Fhit, a Putative Tumor Suppressor in Humans, Is a Dinucleoside 5′,5‴-P^1,P^3-Triphosphate Hydrolase[†]

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ABSTRACT: Human Fhit (**f**ragile **hi**stidine **t**riad) protein, encoded by the *FHIT* putative tumor suppressor gene, is a typical dinucleoside 5′,5‴-P^1,P^3-triphosphate (Ap_3A) hydrolase (EC 3.6.1.29) on the basis of its enzymatic properties we report here. Ap_3A is the preferred substrate among Ap_nA ($n = 3-6$), and AMP is always one of the reaction products. Mn^{2+} and Mg^{2+} are equally stimulatory, while Zn^{2+} is inhibitory with Ap_3A as the substrate. Values of the K_m for Ap_3A and Ap_4A are 1.3 and 4.6 μM, respectively. Values of the specificity constant, k_{cat}/K_m, for Ap_3A and Ap_4A are 2.0×10^6 and 6.7×10^3 s^{-1} M^{-1}, respectively, for a glutathione *S*-transferase (GST)−Fhit fusion protein. Site-directed mutagenesis of *FHIT* demonstrated that all four conserved histidines are required for full activity, and the central histidine of the triad is absolutely essential for Ap_3A hydrolase activity. This putative tumor suppressor is the first evidence for a connection between dinucleotide oligophosphate metabolism and tumorigenesis. Also, Fhit is the first HIT protein in which the histidine residues have been demonstrated by mutagenesis to be critical for function.

FHIT[1] is a putative human tumor suppressor gene at chromosome 3p14.2 that was identified recently by positional cloning (Ohta et al., 1996). Homozygous deletions within the *FHIT* locus have been observed in cell lines derived from cancers of the esophagus, stomach, colon, breast, kidney, and lung, and aberrant transcripts were observed in several types of primary tumors (Ohta et al., 1996; Sozzi et al., 1996). This gene also encompasses the site of the t(3;8) translocation breakpoint of familial renal clear cell carcinoma and the fragile site locus *FRA3B* (Ohta et al., 1996). Fhit is a 147 amino acid protein that has 52% identity and 69% similarity in a core region of 109 amino acids to diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) hydrolase from the fission yeast *Schizosaccharomyces pombe* (Ohta et al., 1996; Huang et al., 1995; Robinson et al., 1993). The latter enzyme is a 182 amino acid protein that catalyzes the hydrolysis of

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[1] Abbreviations used: Fhit, fragile histidine triad; HIT, histidine triad; Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_nA, diadenosine 5′,5‴-P^1,P^n-polyphosphate ($n = 2-6$); DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GST, glutathione *S*-transferase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; PBT, 1% bovine serum albumin, 0.1% Tween 20 in PBS.

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dinucleoside polyphosphates with Ap_4A as the preferred substrate (Robinson et al., 1993; Huang et al., 1995). Both the Fhit protein and *S. pombe* Ap_4A hydrolase are related by sequence to the HIT proteins, a group of unknown function that is characterized by four conserved histidines, three of which make up a histidine triad (HIT) sequence, H × H × H (Séraphin, 1992; Huang et al., 1995; Ohta et al., 1996).

Dinucleoside 5′,5‴-P^1,P^n-polyphosphates (Np_nN', where N and N′ are purine or pyrimidine nucleosides and $n = 3-6$) are generally present in prokaryotic and eukaryotic organisms at basal concentrations of 10 nM to 1 μM (Garrison & Barnes, 1992). The adenosine-containing dinucleoside tri- and tetraphosphates are largely the result of a side reaction during amino acid activation catalyzed by some tRNA synthetases (Plateau & Blanquet, 1992). Ap_4A and Ap_3A have been proposed to have various intracellular functions, including regulation of DNA replication and signaling stress responses. However, none of these hypotheses is supported by strong evidence. The dinucleotides are known to be secreted along with other nucleotides from platelets and neural secretory tissues and to have a variety of effects as extracellular mediators. The proposed functions of Ap_nA ($n = 3-6$) have been reviewed (Kitzler et al., 1992; Remy, 1992; Ogilvie, 1992; Plateau & Blanquet, 1994; Baxi & Vishwanatha, 1995).

The similarity in sequences of the Fhit protein and *S. pombe* Ap_4A hydrolase suggested that the former may be an enzyme. Here we report that the human Fhit protein is a dinucleoside 5′,5‴-P^1,P^3-triphosphate hydrolase and that the histidine residues of the HIT sequence are critical for the Ap_3A hydrolase activity of Fhit. The results suggest that Ap_3A or similar dinucleotides may be a factor in tumorigenesis.

EXPERIMENTAL PROCEDURES

Materials. Ap_3A and Ap_4A were custom-labeled with tritium by Moravek Biochemicals, Inc. and Amersham, respectively. [^{3}H]Ap_3A was purified by paper chromatography and was 99% pure by HPLC (Moravek Biochemicals, Inc.). [^{3}H]Ap_4A was purified by chromatography on boronate resin (Barnes et al., 1985) and was 99% pure by TLC (Guranowski et al., 1987). Unlabeled nucleotides were purchased from Sigma except for Ap_3G, Ap_4G, Gp_3G, and the mRNA cap analogues. Ap_3G was purchased from P-L Biochemicals. Ap_4G was kindly donated by A. Shatkin, and the mRNA cap analogues and Gp_3G were donated by E. Darzynkiewicz, J. Stepinski, and M. Jankowska. The Mono Q HR5/5 HPLC column, glutathione-Sepharose 4B, and pGEX-5X-3 vector were from Pharmacia. Factor Xa was purchased from New England Biolabs.

Cell Culture. The COS1 cell line was obtained from ATCC. Cells were kept at 50%−70% confluence in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Sigma) and 15 μg of gentamycin/mL in a humidified atmosphere at 37 °C with 5% CO_2.

Construction of the FHIT Vectors. For preparation of the bacterial expression vector, the human *FHIT* cDNA (Ohta et al., 1996) was used as a template for PCR amplification using a primer pair, 5′GTGGGATCCACATGTCGTTCAGATTTGGC3′ (nucleotides −2 to 18 of the *FHIT* sequence) and 5′CCGCTCGAGTCACTGAAAGTAGACCCG3′ (nucleotides 445−427). The PCR product was cleaved with *Bam*HI and *Xho*I enzymes and subcloned into *Bam*HI and *Xho*I sites of the GST fusion protein expressing vector pGEX-5X-3. In the resulting construct, pGEX-*FHIT*, the full-length *FHIT* coding frame was placed in-frame downstream of the glutathione *S*-transferase gene.

The eukaryotic expression vector, pRc/CMV, containing enhancer−promoter sequences from the immediate early gene of the human cytomegalovirus (CMV) and a neomycin resistance gene, was purchased from Invitrogen. A DNA fragment containing the *FHIT* cDNA was obtained by PCR amplification of the human *FHIT* cDNA plasmid, pFHIT (Ohta et al., 1996) using oligonucleotides HITF, 5′TGAAGCTTGGATCCATGTCGTTCAGATTTGG3′ (*FHIT* nucleotides 1−17), and HITR, 5′TTTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCCTGAAAGTACACCCGCAGAG3′ (*FHIT* nucleotides 444−422). The second primer includes a FLAG octapeptide coding sequence (underlined) allowing production of a carboxy-terminal FLAG fusion protein (Eastman Kodak). Amplification was performed in 100 μL final volume of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 5 ng of pFHIT DNA, 0.5 mM primers, and 2.5 units of Taq DNA polymerase (Boehringer Mannheim), using a Perkin-Elmer Cetus thermal cycler for 30 cycles, consisting of 94 °C for 1 min of denaturation, 44−64 °C for 1 min (44 °C annealing temperature for the first 5 cycles for partly complementary primers and 64 °C for 25 cycles for fully complementary oligonucleotides), and an extension step at 72 °C for 45 s. The PCR-amplified product was introduced into the *Xba*I/*Hin*dIII-digested pRc/CMV vector, and recombinant clones containing the *FHIT* gene were sequenced using Taq DyeDeoxy Terminator Cycle Sequencing kits (Applied Biosystems Inc.). The resulting plasmid was designated pRcCMV-*FHIT*.

Transfection and Transformation Procedures. COS1 cells were seeded 24 h before transfection at a density of 10^6 cells per 100 mm dish. On the day of transfection the cells were washed twice with DMEM, and then DMEM containing 10% FBS, 0.5 mg of DEAE-dextran/mL, and 100 μM chloroquine was added. pRcCMV-*FHIT* was added to a concentration of 1.5 μg/mL, and the cells were incubated for 3.5 h at 37 °C. Afterward, the cells were washed once with DMEM and incubated 2 min in 10% DMSO diluted into PBS. After the shock the cells were washed twice with DMEM and incubated for 36 h before preparation of cell lysates. *Escherichia coli* strain BL21 was transformed with pGEX-5X-3 or pGEX-*FHIT* according to Sambrook et al. (1989).

Cell Lysate Preparation and Western Blot Analysis. For analysis of Fhit protein expressed in eukaryotic cells, the transfected COS1 cells were washed twice with PBS and scraped into 400 μL of lysis buffer (LB: 150 mM NaCl, 1% NP40, 2 mM EDTA, 1 mM DTT, 50 mM Tris, pH 8.0, 10 μg/mL each of chymostatin, leupeptin, aprotinin, and pepstatin, 1 mM PMSF), sonicated for 2 × 30 s pulses at a setting of 6.0 using Sonicator XL2020 (Heat Systems, Inc.), and centrifuged 10 min at 15 000*g*. The supernatant was treated with SDS-reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, 0.7% 2-mercaptoethanol), boiled for 5 min, and subjected to SDS−PAGE (Laemmli, 1970). After SDS−PAGE the gel was blotted and the membrane was blocked in 5% dried milk in PBS for 1 h at room temperature, followed by overnight incuba-

tion at 4 °C in 1 μg/mL antiFLAG M2 monoclonal antibody (Eastman Kodak Co.) diluted into PBS, 1% BSA, 0.1% Tween 20 (PBT). After three washes of 5 min each in PBT, the blot was incubated in 1/15 000 HRP-conjugated goat anti-mouse IG into PBT for 1 h. The signal was detected using the ECL system (Amersham) as described by the manufacturer. For assay of dinucleoside polyphosphate hydrolase activity, cells were scraped into 400 μL of hypotonic buffer (50 mM Hepes, 10% glycerol, 0.5 mM PMSF, 0.5 μg/mL of both pepstatin A and leupeptin), incubated for 10 min at 4 °C, sonicated, and centrifuged for 10 min at 15 000*g*. The supernatant was used to assay the Fhit protein for dinucleoside polyphosphate hydrolase activity.

For analysis of GST−Fhit fusion protein, *E. coli* strain BL21, transformed with the plasmid pGEX-*FHIT* or pGEX-5X-3, was induced to express the protein by incubating for 2 h at 37 °C in the presence of 1.0 mM IPTG. The cell pellets were resuspended in 1/20 volume of PBS containing 0.5 mM PMSF, 0.5 μg of pepstatin A/mL, and 0.5 μg of leupeptin/mL. After lysis by sonication, the crude extract was incubated for 60 min at 4 °C by mixing gently in the presence of 1% Triton X-100 and centrifuged at 10 000*g* for 10 min at 4 °C. The GST−Fhit fusion protein was purified from the supernatant by elution from glutathione Sepharose 4B affinity matrix with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Unconjugated Fhit protein was purified by incubating GST−Fhit fusion protein bound to the glutathione-Sepharose 4B affinity matrix with factor Xa protease in 50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ overnight at room temperature. Fhit protein was eluted from the matrix in 50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ buffer. The unpurified supernatants or the proteins purified using the affinity matrix were subjected to electrophoresis on a 12% SDS−PAGE gel.

Site-Specific Mutagenesis. Site-specific mutants were isolated in two steps by PCR amplification using a full-length *FHIT* cDNA plasmid, pFHIT (Ohta et al., 1996), as template. During the first step, *FHIT* coding DNA fragments were obtained using specific mutant oligonucleotides and primer HITR. After purification individual DNA fragments were used in a second PCR step to obtain full-length *FHIT* genes containing the selected point mutations. The PCR reactions were performed using UlTma DNA polymerase as described by the manufacturer (Perkin Elmer). To enrich for mutant *FHIT* gene fragments in the final amplification step, oligonucleotides complementary to the FLAG coding sequence and HITF primer were added. *Hin*dIII−*Xba*I fragments were introduced into the pRcCMV vector, and mutations were verified by sequencing. The oligonucleotides used for site-directed mutagenesis were as follows: H35N (*FHIT* nucleotides 91−116), 5′GTGGTACCAGGAAATGTCGTTGTGTG3′; H94N (268−293), 5′CAGACTGTGAAGAACGTTCACGTCCA3′; H96N (274−299), 5′GTGAAGCACGTTAACGTCCATGTTCT3′; and H98N (280−305), 5′CACGTTCACGTCAATGTTCTTCCCAG3′. Constructs containing mutant *FHIT* genes were used in transient transfection of COS1 cells.

Assay of Dinucleoside Polyphosphate Hydrolase Activity. HPLC analysis was used to determine the substrate specificity of the GST−Fhit fusion protein expressed in *E. coli* and to identify reaction products. Potential nucleotide substrates, each at 20 μM, were incubated in the absence or presence of GST−Fhit protein in 50 mM Hepes-NaOH, pH 6.8, and 0.5 mM $MnCl_2$ at 37 °C for 30 min in a volume of 100 μL. The reaction was stopped by freezing on dry ice. Subsequently, the entire assay solution was injected onto a Mono Q HPLC column and eluted with a linear gradient of ammonium bicarbonate, pH 8.5, from 50 to 600 mM (Guranowski et al., 1994). Nucleotides were detected at 254 nm with a Beckman model 160 detector. Nucleotides were identified by retention time, and peak areas were integrated using an Altex model C-R1A integrator−recorder. The percent hydrolysis of a substrate was calculated by dividing the peak areas of the substrate in the presence and absence of the protein.

Hydrolase activity also was assayed by separation of [^{3}H]-Ap_3A from the reaction products, [^{3}H]AMP and [^{3}H]ADP, by thin-layer chromatography (Guranowski et al., 1987). The standard incubation mixture (50 μL total volume) contained 50 mM HEPES-NaOH buffer (pH 6.8), 0.5 mM $MnCl_2$, 0.1 mM [^{3}H]Ap_3A (300 000 cpm), 2% glycerol, 0.2 mg of bovine serum albumin/mL, and the GST−Fhit fraction. The incubation was carried out at 37 °C. The initial velocity of Ap_3A hydrolysis was determined by taking 5 μL aliquots after 5, 10, and 15 min of the incubation and spotting onto aluminium plates precoated with silica gel containing fluorescent indicator (E. Merck). An ADP standard was added and the chromatogram developed in dioxane:ammonia:water (6:1:4, v/v/v). After drying, ADP spots were cut out and the radioactivity was counted. The R_f values for Ap_3A, AMP, and ADP were 0.75, 0.64, and 0.24, respectively. The pH optimum was estimated using one of the following 50 mM buffers: sodium actetate, MES-NaOH, HEPES-NaOH, or Bis-Tris-propane-HCl, covering the pH range between 5.0 and 9.6. The other components were as in the standard mixture. Effects of divalent cations, as the chloride salts, were assayed in the standard incubation mixture in which the cation tested was substituted for 0.5 mM $MnCl_2$.

The hydrolase activities of the GST−Fhit fusion protein and Fhit protein were assayed as a function of Ap_3A and Ap_4A concentrations by formation of tritiated mononucleotides from [^{3}H]Ap_3A and [^{3}H]Ap_4A, respectively. Protein was incubated with substrate in 50 mM Hepes-NaOH, pH 6.8, and 0.5 mM $MnCl_2$ at 37 °C for 5 or 10 min in a volume of 100 μL. Reaction products were separated from residual substrate by chromatography on a boronate-derivatized resin as described in detail (Barnes et al., 1985). The hydrolase activity of the GST−Fhit fusion protein also was assayed as a function of Ap_3A concentration using the TLC system described above. Enzymic activity was expressed as nmol of Ap_3A hydrolyzed/min/mg of protein. Activity was a linear function of the time of incubation and the mass of protein. All experiments were done at least twice with duplicates and controls in each experiment. Two different preparations each from COS1 cells and the *E. coli* expression system were analyzed. Values of K_m and V_{max}, the latter for calculation of k_{cat}, were calculated from a hyperbolic fit to the Michaelis−Menten equation with required corrections whenever the hydrolysis of substrate exceeded 10% (Lee & Wilson, 1971). Protein concentrations were determined using either a colorimetric method with Coomassie Blue (Bradford, 1976) or a turbidimetric method with tannic acid (Mejbaum-Katzenelenbogen, 1955).

RESULTS

Expression of Fhit Fusion Proteins in E. coli and COS1 Cells. To characterize the enzymatic activities of the Fhit protein, two expression systems were prepared. For expression in *E. coli*, the *FHIT* cDNA (Ohta et al., 1996) was expressed as an N-terminal fusion with glutathione *S*-transferase. GST−Fhit protein was estimated to be at least 95% pure after chromatography on glutathione-Sepharose 4B affinity matrix (Figure 1A). The estimated molecular weight of 46 000 for the fusion protein was in good agreement with the expected value of 42 800. For expression in mammalian cells, the *FHIT* cDNA was cloned into the eukaryotic expression vector pRc/CMV with a C-terminal FLAG octapeptide added to allow antibody detection. Expression of the Fhit−FLAG fusion protein in COS1 cells after transient transfection was detected on a Western blot using antiFLAG M2 monoclonal antibody (Figure 1B).

Initial assays on crude supernatants from the *E. coli* and COS1 expression systems indicated that the Fhit protein is an Ap_3A hydrolase which requires Mg^{2+}, Mn^{2+}, Ca^{2+}, or Co^{2+}. It also has weaker activity on Ap_4A. The Ap_3A hydrolase activity expressed in *E. coli* was 55 times the cellular background (89 ± 22 vs 1.6 ± 0.3 nmol of Ap_3A hydrolyzed/min/mg of protein; $n = 3$) while activity expressed in COS1 cells increased at least 100 times (see Table 2).

The affinity-purified GST−Fhit protein also exhibited Ap_3A hydrolase activity. Treatment of this protein with factor Xa protease yielded only small quantities of unconjugated Fhit protein; consequently, we determined the kinetic properties of the purified GST−Fhit fusion protein. The K_m values for Ap_3A hydrolysis by unconjugated Fhit and the GST−Fhit fusion were indistinguishable (see below), and the pH optima were close (see below), providing validation for studying the fusion protein. Samples of pure GST−Fhit were free of detectable phosphodiesterase I, adenylate kinase, 5′-nucleotidase, apyrase, and phosphatase activities under conditions for hydrolysis of the dinucleoside oligophosphates as determined by TLC (Guranowski et al., 1987) and HPLC (Guranowski et al., 1994) systems. These systems permit detection of nonspecific degradation of substrates and reaction products.

Substrate Specificity. The relative activity of the GST−Fhit protein with different mono- and dinucleotides as potential substrates is shown in Table 1. The preferred substrate is Ap_3A. The relative activity decreases as the number of phosphates in Ap_nA ($n = 3-6$) increases from 3 to 6. AMP is always one of the reaction products with Ap_nA, $n = 3-6$, as substrates (Figure 2). Adenine dinucleotides are preferred over the corresponding guanine dinucleotides as substrates. Ap_3G and Ap_4G are preferentially cleaved to yield AMP and GDP or GTP, respectively. N^7-methylation of both guanine moieties in Gp_3G decreases the relative hydrolysis (Table 1).

Kinetic Properties with Ap_3A as Substrate. The GST−Fhit protein exhibited one-third of its maximal activity observed at 2 mM $MnCl_2$ when incubated in the absence of an exogeneous divalent cation and no activity was observed in the presence of 0.1 mM EDTA or 2 mM 1,10-*o*-phenanthroline. The Ap_3A hydrolase activity of GST−Fhit protein exhibited a broad plateau between 0.5 and 5 mM each of $MnCl_2$, $MgCl_2$, and $CaCl_2$. $CoCl_2$, however, was active within a very narrow range of concentration with a maximum at about 0.05 mM. Relative velocities at 2 mM $MnCl_2$, $MgCl_2$, and $CaCl_2$ and at 0.05 mM $CoCl_2$ were 1:1:0.8:0.8, respectively. $ZnCl_2$, $CdCl_2$, and $NiCl_2$ did not support hydrolysis of Ap_3A, and each one inhibited the reaction when added to the standard incubation mixture. The I_{50} values for $ZnCl_2$, $CdCl_2$, and $NiCl_2$ were 30, 20, and 40 μM, respectively.

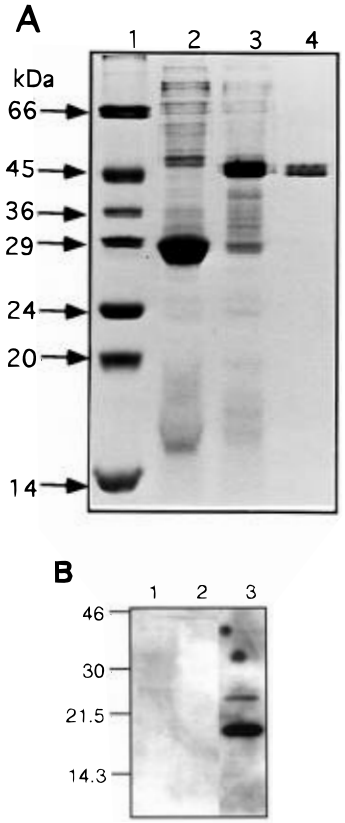

FIGURE 1: Expression of Fhit fusion proteins. A. Expression of the GST−Fhit fusion protein in *E. coli*. Lane 1, standard proteins (2.5 μg each) of the indicated molecular weights; lanes 2 and 3, 13 μg of each homogenate from bacteria transformed with pGEX-5X-3 or pGEX-*FHIT* plasmids, respectively; lane 4, 0.7 μg of GST−Fhit fusion protein after purification on glutathione-Sepharose 4B affinity matrix. The proteins were stained with Coomassie Blue. B. Expression of the Fhit−FLAG fusion protein in COS1 cells. Immunoblot analysis of Fhit in COS1 cell crude supernatants: lane 1, untransfected COS 1 cells; lane 2, COS1 cells transfected with pRc/CMV plasmid; lane 3, COS1 cells transfected with recombinant pRcCMV-*FHIT* vector. The migration of molecular size standards is indicated to the left. Samples were subjected to electrophoresis on a 12% SDS−polyacrylamide gel.

The highest activities of the GST−Fhit and Fhit proteins were observed at pH 6.5 and 6.8, respectively, and half of the activities were reached at pH 5.3 and 7.8 in sodium acetate and Bis-Tris-propane-HCl buffers, respectively. Fluoride, a specific inhibitor of most asymmetrical Ap_4A hydrolases (Guranowski, 1990), had no significant effect on the hydrolase activity of GST−Fhit protein at concentrations up to 10 mM.

Values of the K_m with Ap_3A and Ap_4A as substrates were 1.3 ± 0.4 μM ($n = 4$) and 4.6 ± 1.7 μM ($n = 3$), respectively, for the GST−Fhit fusion protein. For the unconjugated Fhit protein the value of the K_m for Ap_3A was 1.2 ± 0.1 μM ($n = 2$). Values of the catalytic constant, k_{cat}, for GST−Fhit protein with Ap_3A and Ap_4A as substrates were 2.7 ± 1.5 s^{-1} ($n = 4$) and 0.033 ± 0.015 s^{-1} ($n = 3$),

Table 1: Substrate Specificity of Human GST−Fhit Fusion Protein[a]

substrate (20 μM)	relative hydrolysis (%)[b]	reaction products
Ap_3A	100	AMP + ADP
Ap_4A	11	AMP + ATP
Ap_5A	7.9	AMP + p_4A
Ap_6A	2.3	AMP + p_5A[c]
Ap_3G	44	AMP + GDP (81%)[d]
		GMP + ADP (19%)
Ap_4G	15	AMP + GTP (85%)
		GMP + ADP (15%)
Gp_3G	36	GMP + GDP
Gp_4G	8.5	GMP + GTP
m^7Gp_3G	35	GMP + m^7GDP (51%)
		m^7GMP + GDP (49%)
$m^7Gp_3Gm^7$	17	m^7GMP + m^7GDP
AMP, ADP, ATP, p_4A, Ap_2A	<0.5	not detected

[a] GST−Fhit was incubated in 50 mM Hepes-NaOH, pH 6.8, and 0.5 mM $MnCl_2$ with the indicated nucleotides for 30 min at 37 °C in 100 μL. The entire assay solution was analyzed by HPLC as described under Experimental Procedures. [b] The percent hydrolysis of a substrate was calculated by dividing the peak areas of the substrate in the presence and absence of the protein. The percent hydrolysis of each substrate per μg of GST−Fhit was expressed relative to the hydrolysis of Ap_3A. Data are representative values of a minimum of two assays of each substrate with an experimental difference of 15% or less. [c] p_5A was not detected and is a presumed product. [d] Percentage values represent the distribution of the indicated nucleotides as products.

respectively. Values of k_{cat} are based on an estimated molecular mass of 42.8 kDa for the GST−Fhit protein (Ohta et al., 1996; Smith & Johnson, 1988). Thus, the values of the specificity constants, k_{cat}/K_m, were $2 \pm 1 \times 10^6$ s^{-1} M^{-1} ($n = 4$) and $6.7 \pm 2.1 \times 10^3$ s^{-1} M^{-1} ($n = 3$) for Ap_3A and Ap_4A, respectively.

Histidine Mutants of Fhit. In Fhit, amino acid H35 corresponds to the conserved histidine present in the N-terminal region of HIT proteins and H94, H96, and H98 correspond to the conserved histidines in the triad, H × H × H (Ohta et al., 1996; Huang et al., 1995; Séraphin, 1992). Each of these four histidine residues was changed to an asparagine by site-directed mutagenesis. Each change resulted in a decrease in Ap_3A hydrolase activity, but histidine 96 was essential for activity (Table 2).

DISCUSSION

The Fhit putative tumor suppressor from humans is a typical dinucleoside 5′,5‴-P^1,P^3-triphosphate hydrolase based on its substrate specificity, regiospecificity of hydrolysis of Ap_nA ($n = 3-6$) to yield AMP, inhibition by Zn^{2+}, and absence of inhibition by F^-. These properties are generally characteristic of Ap_3A hydrolases from *Saccharomyces cerevisiae* (Brevet et al., 1991), rat tissues (Sillero et al., 1977; Costas et al., 1984), yellow lupin seeds (Jakubowski & Guranowski, 1983), *E. coli* (Hurtado et al., 1987), *Artemia* (Prescott et al., 1992), and bovine adrenal medulla (Ramos & Rotlán, 1995). There have been no previous reports of a specific Ap_3A hydrolase from human tissues, but a nonspecific, plasma nucleotide phosphodiesterase I active on Ap_3A has been described (Lüthje & Ogilvie, 1987). The sequence similarity between the human Fhit protein and the *S. pombe* Ap_4A hydrolase (EC 3.6.1.17) suggested the possibility that the former is an Ap_4A hydrolase also (Ohta et al., 1966). The present data, particularly the values of the specificity constants for Ap_3A and Ap_4A, clearly demonstrate that the human Fhit protein should be classified as an Ap_3A hydrolase (EC 3.6.1.29). As reported above, the value of the specificity constant, k_{cat}/K_m, for the GST−Fhit fusion protein is $2 \pm 1 \times 10^6$ s^{-1} M^{-1} ($n = 4$) with Ap_3A as substrate. For comparison, the values of k_{cat}, K_m, and k_{cat}/K_m for the *S. pombe* Ap_4A hydrolase with Ap_4A as substrate are 1.1 s^{-1}, 22 μM, and 5×10^4 s^{-1} M^{-1}, respectively [based on data presented in Robinson et al. (1993)]. This is a minimum value for k_{cat}/K_m due to the heterogeneity of the *S. pombe* Ap_4A hydrolase preparation, so the value for homogeneous enzyme may be 10-fold larger. A human Ap_4A asymmetrical hydrolase has been described, but its sequence is unrelated to Fhit (Thorne et al., 1995). The known Ap_3A hydrolases have molecular masses between 30 and 55 kDa except for the enzyme from *Artemia* (Prescott et al., 1992). Since *FHIT* encodes a polypeptide of 16.8 kDa (Ohta et al., 1996), Fhit may be a dimer in its native state based on comparison with

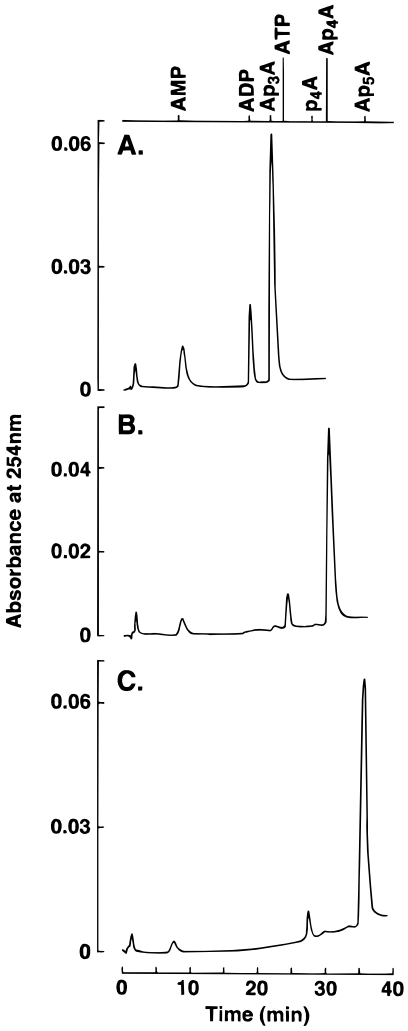

FIGURE 2: HPLC identification of the products of hydrolysis of Ap_3A, Ap_4A, and Ap_5A catalyzed by the GST−Fhit protein. 20 μM of each dinucleotide was incubated with GST−Fhit protein in 50 mM Hepes-NaOH, pH 6.8, and 0.5 mM $MnCl_2$ for 30 min at 37 °C. Nucleotide standards and assay solutions were subjected to chromatography on a Mono Q column eluted with a gradient from 50 to 600 mM ammonium bicarbonate, pH 8.5, at 1 mL/min. Nucleotides were detected spectrophotometrically at 254 nm. Panels A−C show the reaction profiles after partial hydrolysis of Ap_3A, Ap_4A, and Ap_5A, respectively. Assays with Ap_4A and Ap_5A contained five times more GST−Fhit protein than assays with Ap_3A. The peak at 1.65 min represents the solvent front. The time of elution is below panel C, and the elution positions of nucleotide standards are above panel A.

Table 2: Ap_3A Hydrolase Activity of Histidine Mutants of Fhit[a]

crude supernatant fraction from untransfected cells (control) or cells transfected with vector carrying wild-type or mutant *FHIT*	specific activity[b] (nmol of Ap_3A hydrolyzed/min/mg)
COS 1 control	<0.1
wild-type Fhit	15.8 ± 2.0
H35N Fhit	9.3 ± 0.8
H94N Fhit	4.9 ± 1.0
H96N Fhit	<0.1
H98N Fhit	0.38 ± 0.18

[a] Site specific mutations in *FHIT* were generated as described under Experimental Procedures. Constructs containing mutant *FHIT* gene were used in transient transfection of COS1 cells. Crude supernatant fractions were prepared from control and transfected COS1 cells and assayed for Ap3A hydrolase activity as described under Experimental Procedures except the incubation time was extended up to 5 h. [b] Values are the average of two determinations.

other Ap_3A hydrolases and preliminary results on gel filtration chromatography of non-tagged Fhit expressed in *E. coli*.[2] Also, other HIT proteins are believed to be dimers (Pearson et al., 1990; Robinson et al., 1995), and the human PKCI HIT protein is known to be a dimer from the crystal structure (Lima et al., 1996).

The Fhit Ap_3A hydrolase (Ohta et al., 1996), the *S. pombe* Ap_4A hydrolase (Huang et al., 1995; Robinson et al., 1993), and a hypothetical protein from *S. cerevisiae* (Ohta et al., 1996), which is likely to be the *S. cerevisiae* Ap_3A hydrolase (Brevet et al., 1991), constitute a subclass of HIT proteins which are more closely related to each other than to the other HIT proteins. None of the other HIT proteins is known to have Ap_3A or Ap_4A hydrolase activity. The prototype HIT protein, from cattle, was originally detected as a protein kinase C inhibitor (PKCI-1); it was also shown to bind Zn^{2+} at the histidine triad and undergo a large conformational change (Pearson et al., 1990; Mozier et al., 1991). The *in vivo* significance of the PKCI activity has been questioned (Fraser & Walsh, 1991). Lima *et al.* (1996) found that the human PKCI-1 HIT protein binds to but does not inhibit PKC, lacks Ap_4A hydrolase activity, but possesses a hydrolytic activity which they did not specify. It appears likely that the non-Fhit-type HIT proteins interact with nucleotides in some fashion and that the conserved histidines, especially the central one in the triad, will be critical for function (Huang et al., 1995; Séraphin, 1992; Pearson et al., 1990; Lima et al., 1996). The inhibition of enzyme activity by Zn^{2+} in Fhit and other Ap_3A hydrolases suggests that Zn^{2+} binding may be conserved, but it is actually antagonistic to function.

The results presented here suggest that loss of Ap_3A hydrolase activity and resulting elevated levels of Ap_3A or similar compounds may contribute to carcinogenesis. Mechanisms by which this might occur are difficult to predict, partly because dinucleoside triphosphates have not been studied as extensively as dinucleoside tetraphosphates. As mentioned above, the dinucleotides of both types are known to have a variety of effects as extracellular mediators (Remy, 1992; Ogilvie, 1992; Baxi & Vishwanatha, 1995). However, Fhit is apparently not an extracellular enzyme, making it more likely that an intracellular mechanism must be sought. The activity of Fhit on mRNA cap analogs raises the possibility that failure of a decapping function might be tumorigenic. However, the properties of Fhit are quite different from those of enzymes known to decap mRNA, making this an unlikely mechanism (Nuss & Furuichi, 1977; Nuss et al., 1982; Stevens, 1988; Kumagai et al., 1992).

Sanchez et al. (1994) found that introduction of chromosome 3p14-p12 into renal carcinoma cells with a t(3;8) translocation in that region resulted in partial suppression of tumor growth in nude mice. Tumor suppression was associated with rapid cell death in the tumor core. They suggested that a tumor suppressor in 3p14-p12 restored growth factor dependence on cells and that cell death was caused by low growth factor levels in the tumor core. If *FHIT* is the gene responsible for these effects, then a straightforward hypothesis is that elevated Ap_3A levels stimulate a growth enhancing signal transduction pathway or block an inhibitory or apoptotic pathway. One mechanism for such effects could be inhibition of a protein kinase by Ap_3A functioning as an ATP analog (Levy et al., 1983). Insight into this or other possible mechanisms may be obtained by the phenotypes of gene disruptions in yeast.

[2] A. Guranowski, A. K. Robinson, P. N. Garrison, and L. D. Barnes, unpublished observations.

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REFERENCES

Barnes, L. D., Robinson, A. K., Mumford, C. H., & Garrison, P. N. (1985) *Anal. Biochem. 144*, 296−304.
Baxi, M. D., & Vishwanatha, J. K. (1995) *J. Pharmacol. Toxicol. Methods 33*, 121−128.
Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
Brevet, A., Chen, J., Fromant, M., Blanquet, S., & Plateau, P. (1991) *J. Bacteriol. 173*, 5275−5279.
Costas, M. J., Montero, J. M., Cameselle, J. C., Sillero, M. A. G., & Sillero, A. (1984) *Int. J. Biochem. 16*, 757−762.
Fraser, E. D., & Walsh, M. P. (1991) *FEBS Lett. 294*, 285−288.
Garrison, P. N., & Barnes, L. D. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 29−61, CRC Press, Boca Raton, FL.
Guranowski, A. (1990) *FEBS Lett. 262*, 205−208.
Guranowski, A., & Sillero, A. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 81−133, CRC Press, Boca Raton, FL.
Guranowski, A., Biryukov, A., Tarussova, N. B., Khomutov, R. M., & Jakubowski, H. (1987) *Biochemistry 26*, 3425−3429.
Guranowski, A., Brown, P., Ashton, P. A., & Blackburn, G. M. (1994) *Biochemistry 33*, 235−240.
Huang, Y., Garrison, P. N., & Barnes, L. D. (1995) *Biochem. J. 312*, 925−932.
Hurtado, C., Ruíz, A., Sillero, A., & Sillero, M. A. G. (1987) *J. Bacteriol. 169*, 1718−1723.
Jakubowski, H., & Guranowski, A. (1983) *J. Biol. Chem. 258*, 9982−9989.
Kitzler, J. W., Farr, S. B., & Ames, B. N. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 135−149, CRC Press, Boca Raton, FL.
Kumagai, H., Kon, R., Hosino, T., Aramaki, T., Nishikawa, M., Hirose, S., & Igarashi, K. (1992) *Biochim. Biophys. Acta 1119*, 45−51.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lee, H.-J., & Wilson, I. B. (1971) *Biochim. Biophys. Acta 242*, 519−522.
Levy, B. T., Sorge, L. K., Drum, C. C., & Maness, P. F. (1983) *Mol. Cell. Biol. 3*, 1718−1723.

Lima, C. D., Klein, M. G., Weinstein, I. B., & Hendrickson, W. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5357−5362.
Lüthje, J., & Ogilvie, A. (1987) *Eur. J. Biochem. 169*, 385−388.
Mejbaum-Katzenelenbogen, W. (1955) *Acta Biochim. Polon. 2*, 279−296.
Mozier, N. M., Walsh, M. P., & Pearson, J. D. (1991) *FEBS Lett. 279*, 14−18.
Nuss, D. L., & Furuichi, Y. (1977) *J. Biol. Chem. 252*, 2815−2821.
Nuss, D. L., Altschuler, R. E., & Peterson, A. J. (1982) *J. Biol. Chem. 257*, 6224−6230.
Ohta, M., Inoue, H., Cotticelli, M. G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., Croce, C. M., & Huebner, K. (1996) *Cell 84*, 587−597.
Ogilvie, A. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 229−273, CRC Press, Boca Raton, FL.
Pearson, J. D., DeWald, D. B., Mathews, W. R., Mozier, N. M., Zürcher-Neely, H. A., Heinrikson, R. L., Morris, M. A., McCubbin, W. D., McDonald, J. R., Fraser, E. D., Vogel, H. J., Kay, C M., & Walsh, M. P. (1990) *J. Biol. Chem. 265*, 4583−4591.
Plateau, P., & Blanquet, S. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 229−273, CRC Press, Boca Raton, FL.
Plateau, P., & Blanquet, S. (1994) *Adv. Microb. Physiol. 36*, 82−109.
Prescott, M., Thorne, N. M. H., Milne, A. D., & McLennan, A. G. (1992) *Int. J. Biochem. 24*, 565−571.
Ramos, A., & Rotlán, P. (1995) *Biochim. Biophys. Acta 1253*, 103−111.
Remy, P. (1992) in *Ap_4A and Other Dinucleoside Polyphosphates* (McLennan, A. G., Ed.) pp 151−204, CRC Press, Boca Raton, FL.
Robinson, A. K., de la Peña, C. E., & Barnes, L. D. (1993) *Biochim. Biophys. Acta 1161*, 139−148.
Robinson, K., Jones, D., Howell, S., Soneji, Y., Martin, S., & Aitken, A. (1995) *Biochem. J. 307*, 267−272.
Sambrook, J., Fritsch, E. G., & Maniatis, T. (1989) *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanchez, Y., El-Naggar, A., Pathak, S., & Killary, A. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3383−3387.
Séraphin, B. (1992) *DNA Sequence 3*, 177−179.
Sillero, M. A. G., Villalba, R., Moreno, A., Quintanilla, M., Lobatón, C. D., & Sillero, A. (1977) *Eur. J. Biochem. 76*, 331−337.
Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31−40.
Sozzi, G., Veronese, M. L., Negrini, M., Baffa, R., Cotticelli, M. G., Inoue, H., Tornielli, S., Pilotti, S., De Gregorio, L., Pastorino, U., Pierotti, M. A., Ohta, M., Huebner, K., & Croce, C. M. (1996) *Cell 85*, 17−26.
Stevens, A. (1988) *Mol. Cell. Biol. 8*, 2005−2010.
Thorne, N. M. H., Hankin, S., Wilkinson, M. C., Nuñez, C., Barraclough, R., & McLennan, A. G. (1995) *Biochem. J. 311*, 717−721.

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