Designed *FHIT* alleles establish that Fhit-induced apoptosis in cancer cells is limited by substrate binding

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The *FHIT* gene is inactivated early in the development of many human tumors, and Fhit-deficient mice have increased cancer incidence. Viral reexpression of Fhit kills Fhit-deficient cells by induction of apoptosis. Fhit, a member of branch 2 of the histidine-triad superfamily of nucleoside monophosphate hydrolases and transferases, is a diadenosine polyphosphate hydrolase, the active-site histidine of which is not required for tumor suppression. To provide a rigorous test of the hypothesis that Fhit function depends on forming a complex with substrates, we designed a series of alleles of Fhit intended to reduce substrate-binding and/or hydrolytic rates, characterized these mutants biochemically, and then performed quantitative cell-death assays on cancer cells virally infected with each allele. The allele series covered defects as great as 100,000-fold in k_{cat} and increases as large as 30-fold in K_M. Nonetheless, when mutant *FHIT* genes were expressed in two human cancer cell lines containing *FHIT* deletions, reductions in apoptotic activity correlated exclusively with K_M. Mutants with 2- and 7-fold increases in K_M significantly reduced apoptotic indices, whereas the mutant with a 30-fold increase in K_M retained little cellular function. These data indicate that the proapoptotic function of Fhit is limited by substrate binding and is unrelated to substrate hydrolysis.

The *FHIT* gene spans the most active common fragile site in the human genome, FRA3B, and is inactivated by deletion in a large proportion of human malignancies (1–3). *Fhit*-heterozygous mice suffer high rates of stomach and sebaceous tumors when exposed to levels of nitrosomethylbenzamine that do not induce carcinomas in wild-type mice (4). In addition, untreated *Fhit*-deficient and *Fhit*-heterozygous mice suffer high rates of spontaneous tumors (5), and the ability of human Fhit-deficient cells to form tumors in nude mice is blocked by reintroduction and expression of Fhit (6–8). Beyond the demonstration that Fhit is an authentic tumor suppressor via these types of assays, two additional issues have been explored. First, viral and transfection-mediated induction of Fhit indicated that tumor suppression by Fhit is accompanied by induction of apoptosis (9, 10), and subsequent studies have begun to dissect the downstream players in Fhit-dependent cell death (11–13). Second, adenoviruses and adeno-associated viruses that express Fhit were used to demonstrate that nitrosomethylbenzamine-induced stomach carcinomas in *Fhit*-heterozygous mice can be prevented by subsequent infection with *FHIT* viruses (14). Here, viral reintroduction with quantitative cell-based assays and Fhit enzymology were coupled to perform structure-function analysis on Fhit and to probe the cellular mechanism of its action.

A member of branch 2 of the histidine-triad superfamily of nucleotide hydrolases and transferases (15), Fhit encodes a diadenosine polyphosphate (Ap_nA) hydrolase (16, 17) that cleaves substrates such as diadenosine P^1,P^3-triphosphate (ApppA) and diadenosine $5',5'''$-P^1,P^4-tetraphosphate (AppppA) to AMP plus the other nucleotide. Catalysis is mediated via a covalent mechanism involving attack of His-96 on the α-phosphate of substrates (18, 19) that bears features in common with the branch 1 hydrolases including Hint (20, 21) and galactose-1-phosphate uridylyltransferase (22), a member of branch 3 of the superfamily (15). One of the first mechanistic questions asked of Fhit as it relates to cellular function was whether hydrolysis of Ap_nA is required for tumor suppression. Such K_{cat} dependence would be expected if accumulation of Ap_nA were to result in cells that fail to undergo apoptosis. On the contrary, if Fhit has a cellular mechanism more similar to GTPases (23), then reduction of Ap_nA hydrolysis might be expected to lock the enzyme into an active, signaling form. Two studies have shown that an H96N allele of Fhit is functional in qualitative tumor-suppressor assays (6, 7). Purification of this mutant protein revealed that it is 4 million-fold reduced in k_{cat} and 4-fold elevated in K_M relative to wild-type enzyme, assayed with ApppA (24). Given that the H96N substitution is qualitatively permissive for tumor suppression, these data indicated that Fhit does not function via consumption of Ap_nA *in vivo*. The mild effect on ApppA binding and the fact that wild-type and mutant Fhit proteins bound nonhydrolyzable ApppA in a nearly identical manner were taken as evidence that a Fhit-substrate complex is required for proapoptotic signaling (24). However, despite the strong conservation of the AMP-binding site in Fhit (20, 24), it could also be argued that the tumor-suppressing mechanism is entirely independent of nucleotide binding and hydrolysis.

A rigorous test of the substrate-dependent signaling hypothesis had to await development of quantitative cell-based assays of Fhit function. Guided by the crystal structure of Fhit bound to nonhydrolyzable ApppA (24), we designed a series of Fhit mutants that exhibit defects in substrate binding of 2- to 30-fold and defects in turnover rates as great as 100,000-fold. Despite the huge differences in k_{cat}, when these mutant alleles were assayed for quantitative effects on initiation of programmed cell death, losses of biological activity could be attributed entirely to losses in substrate binding. The data indicate that apoptotic indices decline as K_M rises, establishing that Fhit-substrate binding is limiting for tumor suppression.

Materials and Methods

Purification and Kinetic Characterization of Wild-Type and Mutant Fhit. Mutant *FHIT* cDNAs, cloned in bacterial expression vector pSGA02 (25), were generated via site-directed mutagenesis (26), and DNA sequences were confirmed. Each enzyme was purified from a 2-liter culture of transformed *Escherichia coli* strain BL21. Cells were aerated at 30°C in LB medium with 150 μg/ml

Abbreviations: Ap_nA, diadenosine polyphosphate; ApppA, diadenosine P^1,P^3-triphosphate; GpppBODIPY, GTP-γ-*S*-(4–4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacine-3-yl) methylaminoacetyl; CMV, cytomegalovirus.

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ampicillin until an optical density (λ = 600 nm) of 0.4 was reached, induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside, and shaken for an additional 8 h. Subsequent steps were performed at 0–4°C. Frozen cell pellets were resuspended in 30 ml of buffer A (25 mM NaHepes, pH 7.2/3 mM DTT/10% glycerol) with two tablets of complete protease inhibitor mixture (Roche, Indianapolis) and lysed by sonication. Cleared lysates were subjected to protamine sulfate precipitation (0.5 mg/ml) followed by centrifugation to remove nucleic acids. From the protamine sulfate-treated lysates, 20–60% ammonium-sulfate fractions were obtained, and pellets were resuspended into 13 ml of buffer A and dialyzed against buffer A. Samples were loaded onto a 58-ml POROS 20 PI column (Applied Biosystems) and eluted by using linear gradients of buffer A with 1 M NaCl. Homogeneous enzymes eluted at ≈0.23 M NaCl. Enzyme activity, yield, and specific activity of fractions were calculated from 20 μM GTP-γ-*S*-(4–4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacine-3-yl) methylaminoacetyl (GpppBODIPY) (17) assays with 20 mM NaHepes, pH 7.0/0.5 mM $MnCl_2$/0.2 mg/ml BSA in total volumes of 30 μl. Reactions were incubated at room temperature and stopped at 300 s by the addition of 30 μl of sodium citrate, pH 3.0. Cumulative yields of enzyme activity ranged from 18% to 40%. To determine kinetic parameters, duplicate assays were performed with six measurements of each substrate initial rate at five or six substrate concentrations ranging from 1.25 to 120 μM. To avoid exceeding 5–8% substrate consumption, the amount of enzyme varied from 30 fmol (wild type) to 2 nmol (Fhit-H96N) per assay. Reactions were quantitated with a Wallac (Gaithersburg, MD) Victor2 multilabel counter fitted with a 485-nm excitation filter and 535-nm emission filter. Reactions were calibrated against stopped, mock reactions containing substrate at the appropriate concentrations and mixtures of 95% substrate/5% product and 90% substrate/10% product at initial substrate concentrations. k_{cat} and K_M values were calculated according to the method of Eadie and Hofstee (27).

Cell Lines and Recombinant Adenoviruses. Lung cancer cell line A549 and cervical cancer cell line SiHa were purchased from the American Type Culture Collection. Cells were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated FBS (Invitrogen). Human embryonic kidney cell line HEK293 (Microbix, Toronto), maintained in DMEM with 10% FBS, was used for generation and amplification of all recombinant adenoviruses. Ad*FHIT* construction has been described (11). Ad *FHIT*-H96N, Ad *FHIT*-H96D, Ad *FHIT*-L25W, Ad *FHIT*-I10W, L25W, and Ad *FHIT*-L25W,H96D were generated with the AdenoVator system as described (28). Briefly, every *FHIT* cDNA was cloned into the transfer vector pAdenoVator-cytomegalovirus (CMV)5-GFP under the transcriptional control of a CMV5 promoter/enhancer. *FHIT* alleles were cloned upstream to internal ribosome entry and GFP sequences. Linearized, recombinant transfer vectors were recombined into BJ5183 *E. coli* with the construct AdVator DE1/E3 containing the defective adenoviral genome. Resulting vectors were linearized and transfected into HEK293 cells by the calcium-phosphate method to package viruses. Single viral plaques were isolated, expanded, and checked for Fhit expression. Viruses were purified by cesium chloride, and titration was performed as described (28). AdGFP, carrying the reporter protein GFP under the transcriptional control of a CMV promoter, was purchased from Qbiogene (Carlsbad, CA). A549 and SiHa cells were infected with seven different adenoviruses, each at a multiplicity of infection of 25, 1 day after seeding into 60-mm dishes.

Western Analysis. Samples were extracted in 15 mM Tris·Cl, pH 7.5/120 mM NaCl/25 mM KCl/2 mM EGTA/0.1 mM DTT/0.5% Triton X-100/10 mg/ml leupeptin/0.5 mM PMSF. Total protein (10 μg) from each sample was separated on a 4–20% polyacrylamide gel (Bio-Rad) and transferred to a poly(vinylidene difluoride) filter (Millipore). The filter was blocked in 5% nonfat dry milk, incubated with a rabbit primary antibody against human Fhit (Zymed), washed, probed with goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and developed with enhanced chemiluminescence (Amersham Pharmacia). Filters were reprobed with enzyme-conjugated antibodies to GFP and β-actin (Santa Cruz Biotechnology).

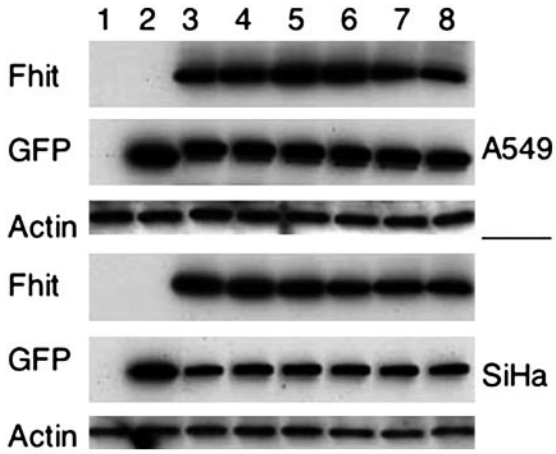

Fig. 1. Western analysis of infected cells. Signals due to anti-Fhit, anti-GFP, and the anti-β-actin antibody used as a protein loading control are indicated. Lanes for A549 and SiHa cells: 1, uninfected; 2, AdGFP; 3, Ad*FHIT*; 4, Ad*FHIT-H96D*; 5, Ad*FHIT-H96N*; 6, Ad*FHIT-L25W*; 7, Ad*FHIT-L25W,H96D*; and 8, Ad*FHIT-I10W,L25W*. All *FHIT*-expressing adenoviruses express GFP from an internal ribosome entry sequence downstream of *FHIT*.

Flow Cytometry. Cells were harvested 96 h after infection. To sort cells by DNA content, cells were fixed with 70% ethanol for 10 min, incubated with RNase A, and stained with propidium iodide as described (29). For quantitation of cells positive for mature caspase-3, cells were washed, fixed, permeabilized, and stained with phycoerythrin-conjugated monoclonal anti-mature caspase-3 (Becton Dickinson) according to the manufacturer's procedure. Analysis was performed with a FACScan cytometer (Becton Dickinson).

Results

Enzymology of Fhit Mutants. Crystal structures of wild-type and H96N forms of Fhit bound to nonhydrolyzable ApppA were used to design five additional mutants selectively defective in substrate binding and/or hydrolysis. Alterations of the nucleophilic His-96 (16, 18, 24) are expected to result in reduced k_{cat}, whereas bulky substitutions for conserved hydrophobic residues involved in adenine recognition were predicted to result in increased K_M values (24). Wild-type and five mutant enzymes were expressed in *E. coli* and purified by following GpppBODIPY hydrolytic activity (17). Kinetic characterization of homogeneous Fhit enzymes is summarized in Table 1. The two mutants with only His-96 substitutions were subject to nearly identical 1.8- and 2.0-fold increases in K_M. Assayed with GpppBODIPY, the H96N substitution cost the enzyme 5 orders of magnitude in the k_{cat} term, whereas the alternative nucleophilic substitution of H96D cost the enzyme >5,000-fold in k_{cat}. The designed K_M mutant L25W produced a 7-fold increase in K_M with no loss in k_{cat}. The I10W,L25W mutant, designed to produce a larger K_M defect, resulted in a 30-fold increase in K_M with no significant loss in turnover rate. Mutation of H96D in the context of the L25W K_M

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Table 1. Kinetic parameters for wild-type and mutant Fhit enzymes

Enzyme	$K_M \pm$ SE, μM	$k_{cat} \pm$ SE, s^{-1}	k_{cat}/K_M, $s^{-1} \cdot M^{-1}$
Fhit	1.71 ± 0.17	0.599 ± 0.261	3.5×10^5
Fhit-H96D	3.39 ± 0.13	0.000106 ± 0.000025	3.12×10^1
Fhit-H96N	3.06 ± 0.56	0.00000434 ± 0.00000356	1.42
Fhit-L25W	11.2 ± 0.7	0.685 ± 0.055	6.12×10^4
Fhit-L25W, H96D	12.0 ± 0.8	0.00394 ± 0.00264	3.28×10^2
Fhit-L25W, I10W	51.9 ± 1.5	0.444 ± 0.052	8.55×10^3

mutant produced the expected result in the sense that K_M was not increased further (12 μM for L25W,H96D compared with 11 μM for L25W), and k_{cat} was specifically depressed. The 150-fold drop in k_{cat} associated with the change from L25W to L25W,H96D was 40-fold less severe than would have been expected from the 5,000-fold reduction in k_{cat} due to H96D, which suggests that some of the strain associated with fitting a substrate into the crowded AMP-binding site of an L25W mutant can be used productively to ameliorate the catalytic defect associated with H96D. Although this one interaction between a k_{cat} mutation and a K_M mutation was not anticipated, we succeeded in creating a series of Fhit mutants encoding stable proteins that vary in K_M by 30-fold in three intervals and vary in k_{cat} by 5 orders of magnitude in three intervals (Table 1).

Apoptotic Activities of *FHIT* Alleles. For physiological evaluation of the effect of Fhit-nucleotide kinetics, a cellular assay in which nearly all Fhit-deficient cells could be converted to $FHIT^+$ was required. To this end, we exploited the Adenovirus5 system (28) in which *FHIT* genes were expressed under transcriptional control of the *CMV5* promoter/enhancer and followed by an internal ribosome entry sequence driving expression of the GFP from *Aequoria victoria* (11). We constructed recombinant adenoviruses containing wild-type *FHIT* and the five mutant *FHIT* genes. For physiological analysis, we focused on two previously investigated Fhit-deficient lung and cervical cancer cell lines, A549 and SiHa, respectively (9, 13, 30). These cells were infected with each *FHIT* recombinant adenovirus and with Adenovirus5 expressing GFP under *CMV5* control and were examined by fluorescence microscopy to ascertain the efficiency of infection. At a multiplicity of infection of 25, both cell lines exhibited ≈90% of GFP-positive cells (data not shown). Twenty-four hours after infection, total protein was extracted to explore levels of expression of the transgenes. Fig. 1 shows that all infected samples exhibited equivalent expression of GFP and that each *FHIT*-infected sample expressed an equivalent amount of Fhit protein.

Ninety-six hours after infection, we stained cells with propidium iodide and analyzed cell populations by flow cytometry (29). A representative experiment in Fig. 2*A* shows that after infection with Ad*FHIT*, A549 cells show a dramatic appearance of cells with subG$_1$ DNA content (43%) compared with noninfected and Ad*GFP*-infected cells (1% and 3%, respectively). A substantial fraction of cells infected with Ad*FHIT-H96N* and Ad*FHIT-H96D* (32% and 29%, respectively) had subG$_1$ DNA content, although the effect was not as pronounced as in wild-type *FHIT*-infected cells. Cells expressing Fhit-L25W, Fhit-H96D,L25W, and Fhit-I10W,L25W enzymes were more impaired in promoting cell death, with 14%, 17%, and 10%, respectively, of total cells counted with subG$_1$ DNA contents. These results were paralleled qualitatively in SiHa cells, although over a smaller dynamic range (Fig. 2*B*). Wild-type Fhit promoted death in 26% of infected cells against a background of 3% of dead cells in uninfected and 4% of cells in GFP-infected cells. Active-site mutant Fhit proteins, Fhit-H96N and Fhit-H96D, each killed 14% of infected cells at the 96-h time point, whereas the three mutants with high K_M values killed between 7% and 8% of infected cells. The A549 cells and SiHa cells were infected independently, stained, and sorted three times. Data representing the percentage of subG$_1$ cells in both cell lines with each treatment are presented in Table 2.

To assess whether Fhit expression-dependent cell death is apoptotic and to establish a second quantitative assay for structure-function analysis of Fhit, we sorted for cells containing activated caspase-3 (31–33), the detection of which indicates cells committed to death before the full range of apoptotic phenotypes (34). This assay demonstrated a tremendous dynamic range. As shown in Fig. 3*A*, 96 h after A549 cells were infected, 84% of the cells were mature caspase-3-positive versus 2% of uninfected cells and 7% of GFP-infected cells. The two Fhit mutants with active-site mutations depressed mature caspase-3 positivity to 61–63%, whereas the three mutants with substantially increased K_M values depressed caspase-3 positivity to 26–28%. Caspase-3 data from SiHa-infected cells followed predictable patterns (Fig. 3*B*). First, detection of mature caspase-3 is a more sensitive assay for cell death than is subG$_1$ DNA content. Second, SiHa cells are somewhat more resistant to Ad*FHIT* at this time point and multiplicity of infection. Third, the allele specificity of *FHIT* as an inducer of apoptosis is essentially identical to that observed in A549 cells. The loss of caspase-3 positivity was one-third in going from wild-type Fhit to the first interval of K_M defects. Two-thirds and three-quarters of caspase-3 positivity was lost from wild-type Fhit to the second and third interval of K_M defects. The weakness of the I10W,L25W allele notwithstanding, our data show conclusively that this allele has some residual apoptotic activity compared with GFP alone. The data presented in Fig. 3 and summarized in Table 2 are qualitatively similar to the effect of the same set of *FHIT* alleles on Fhit-deficient esophageal and pancreatic cancer cells lines (data not shown). Thus, the structure-activity

Table 2. Physiological analysis of cells infected by wild-type and mutant *FHIT* viruses

	A549		SiHa	
Adenovirus5	% subG$_1$ DNA	% Mature caspase-3	% subG$_1$ DNA	% Mature caspase-3
None	1.34 ± 0.09	1.59 ± 0.37	2.63 ± 0.37	3.54 ± 0.74
GFP	3.04 ± 0.77	7.73 ± 1.41	3.88 ± 0.39	7.81 ± 1.61
FHIT	43.4 ± 3.7	84.7 ± 4.3	26.2 ± 2.1	61.1 ± 4.3
FHIT-H96D	29.7 ± 2.9	60.8 ± 3.4	13.8 ± 1.4	40.8 ± 4.2
FHIT-H96N	31.0 ± 2.8	62.8 ± 3.5	14.0 ± 1.7	44.6 ± 2.6
FHIT-L25W	14.7 ± 2.3	27.9 ± 2.4	7.92 ± 1.08	23.8 ± 2.1
FHIT-L25W,H96D	15.8 ± 1.7	26.0 ± 3.3	7.24 ± 0.96	17.3 ± 2.7
FHIT-I10W,L25W	11.0 ± 1.3	26.3 ± 2.9	7.57 ± 0.77	14.4 ± 2.6

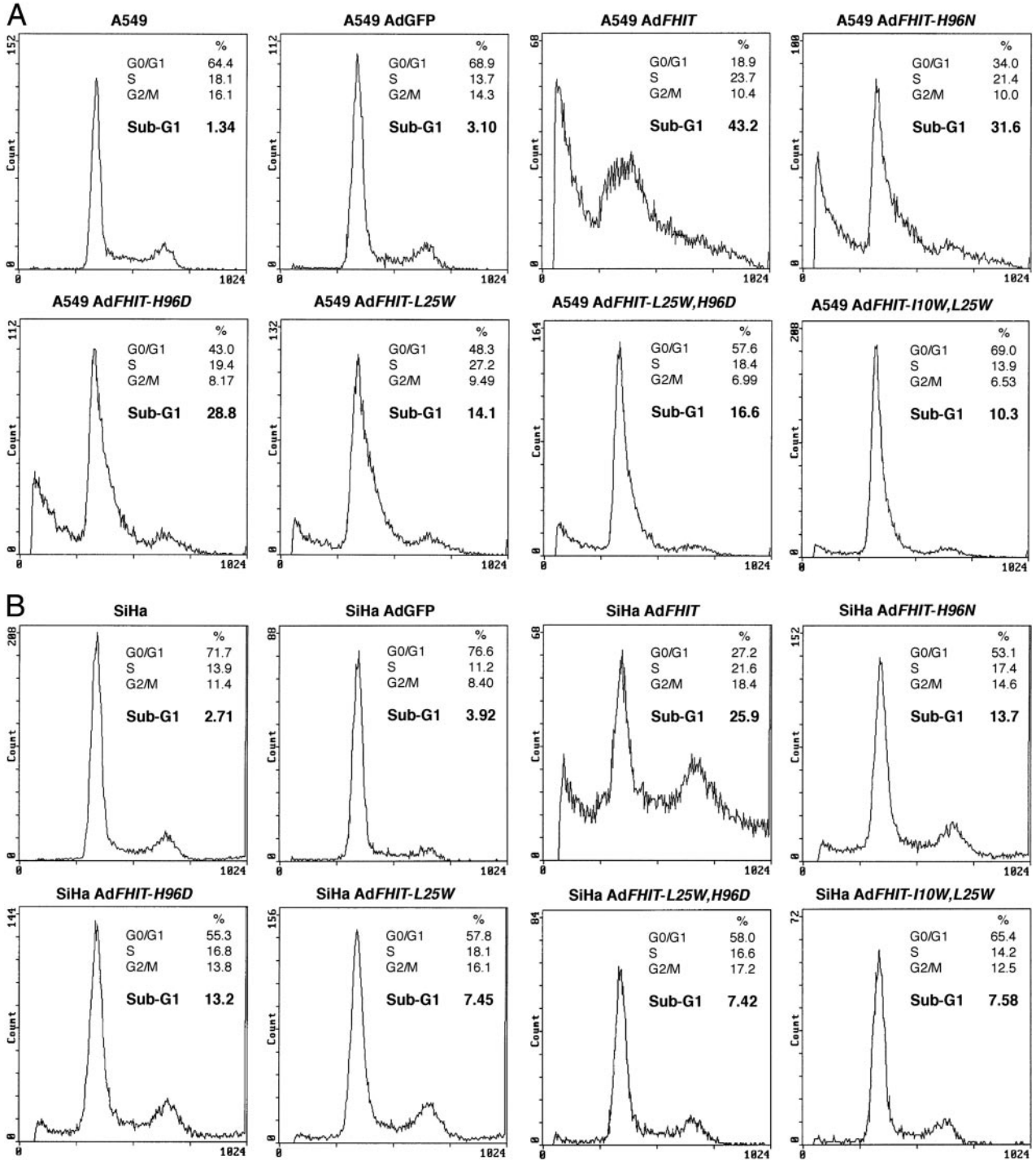

Fig. 2. SubG$_1$ DNA content of wild-type and mutant *FHIT*-infected cancer cells. Flow-cytometric analysis of A549 (*A*) and SiHa (*B*) cells stained with propidium iodide as a function of recombinant adenoviral infection is shown. The rank order of cells with subG$_1$ DNA content is Ad*FHIT* > Ad*FHIT-H96N* ≈ Ad*FHIT-H96D* > Ad*FHIT*-L25W ≈ Ad*FHIT*-H96D,L25W > Ad*FHIT*-I10W,L25W > AdGFP ≈ uninfected.

relationship of *Fhit* alleles is fundamental to the tumor-suppressing function of Fhit and is not a cell type-specific phenomenon.

Discussion

Earlier, *FHIT-H96N* was scored as functional in qualitative tumor-suppressor assays (6, 7). On the basis of the huge depression of k_{cat} and mild increase in K_M relative to wild-type enzyme (24), we hypothesized that the active, signaling form of Fhit is bound to Ap_nA substrates and that mutations that increase K_M for nucleotide substrates would result in loss of function (24). This turns out to be the case: His-96 mutants, which slightly increase K_M, are slightly loss-of-function, whereas designed K_M mutants, which more substantially increase K_M, are substantially loss-of-function. The mutant forms of Fhit examined differed, additionally, >5 orders of magnitude in k_{cat}, but there was no correlation between k_{cat} and biological activity; all the differences in proapoptotic activity between the alleles could be

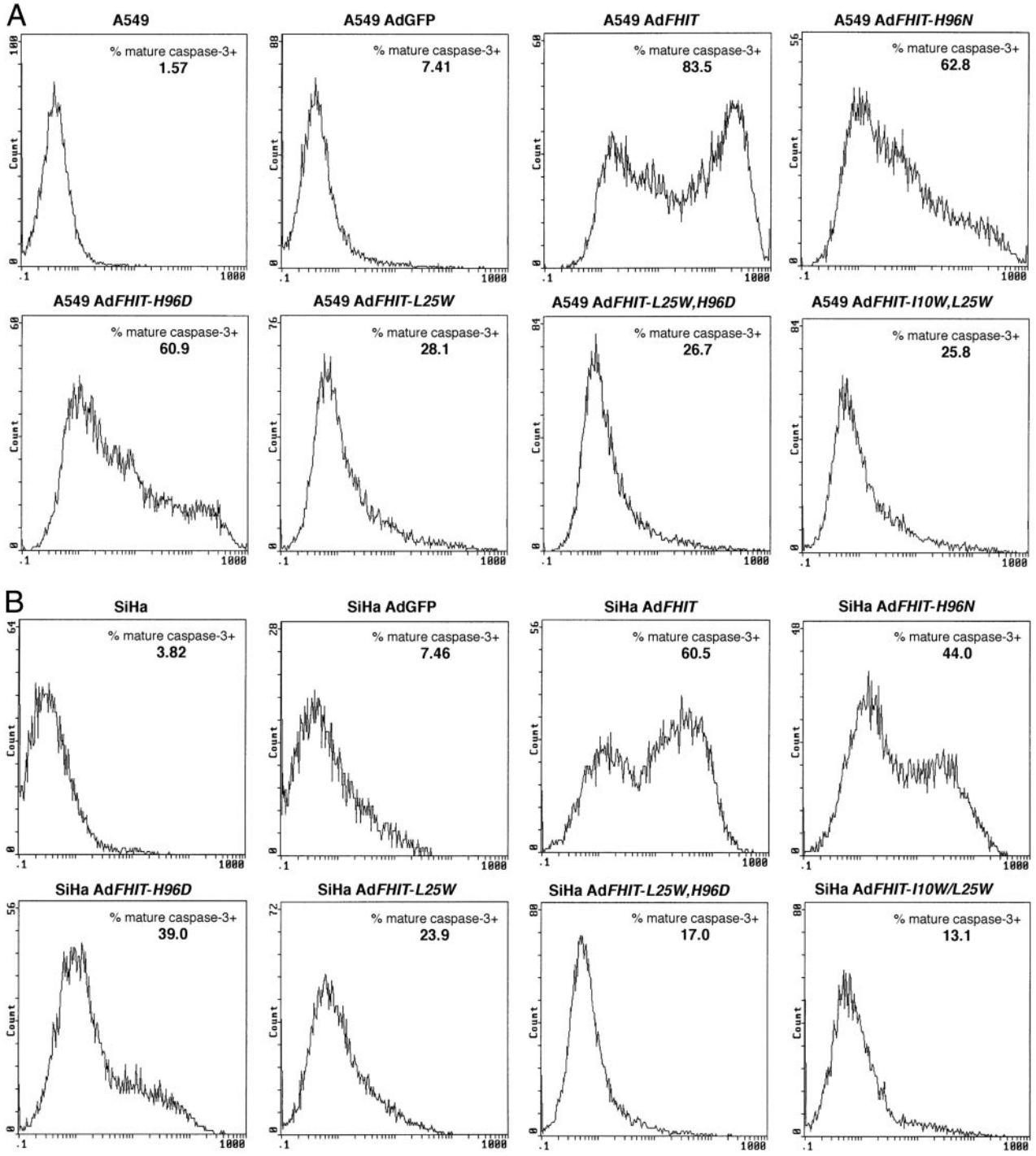

Fig. 3. Mature caspase-3 in wild-type and mutant *FHIT*-infected cancer cells. Flow-cytometric analysis of A549 (*A*) and SiHa (*B*) cells stained for mature caspase-3 as a function of recombinant adenoviral infection is shown. The rank order of cells with mature caspase-3 is Ad*FHIT* > Ad*FHIT-H96N* ≈ Ad*FHIT-H96D* > Ad*FHIT*-L25W ≈ Ad*FHIT*-H96D,L25W ≈ Ad*FHIT*-I10W,L25W > AdGFP > uninfected.

accounted for by changes in K_M. Thus, although Fhit signaling depends on substrate binding, the *in vitro* lifetimes of Fhit-substrate complexes do not predict biological activity. In the case of signaling by low molecular GTPases, mutations that reduce intrinsic and GTPase-activating protein-catalyzed rates are said to lock the enzymes in their active substrate-bound conformations and promote signaling (23). However, because the catalytic mechanism of Fhit requires leaving-group exit and water entry at the substrate-exposed surface of the dimeric enzyme, polypeptides that bind to the Fhit–Ap_nA complex are expected to stabilize the complex and retard turnover (24), whereas many proteins that bind to substrate-bound forms of GTPases accelerate the low intrinsic rate of GTP hydrolysis (23, 35, 36).

Loss of apoptotic activity with loss of substrate binding also establishes that Fhit substrates are limiting for Fhit biological activity. One key to the hypothesis that the active, signaling form of Fhit is bound to Ap_nA substrates is the ability of Fhit to bind Ap_nA substrates in the presence of high concentrations of

nucleoside triphosphates, such as ATP, that exist in cells. We have shown that Fhit exhibits an ≈100-fold preference for Ap_nA over nucleoside triphosphates and that the likely competitor in cells is inorganic pyrophosphate, the K_i value of which, 20 μM, is only ≈10-fold in excess of the K_M values of Ap_nA substrates (17). Genetic and pharmacological dissection of the proapoptotic events immediately downstream of Fhit is likely to produce new drug targets and novel strategies for inducing and blocking programmed cell death. In addition, compounds targeted against Fhit (37–39) may now be assayed quantitatively for cell-directed activities.

This paper is dedicated to the memory of Eraldo Antonini, eminent biochemist, who died prematurely 20 years ago on March 19, 1983. We thank Zurab Siprashvili, Byron Feig, and Hannes Alder for colleagueship and assistance. This work was supported by National Institutes of Health Grants CA75954 (to C.B.), CA77738 (to K.H.), and CA56036 (to C.M.C.).

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) *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., Pastorini, U., *et al.* (1996) *Cell* **85,** 17–26.
3. Croce, C. M., Sozzi, G. & Huebner, K. (1999) *J. Clin. Oncol.* **17,** 1618–1624.
4. Fong, L. Y. Y., Fidanza, V., Zanesi, N., Lock, L. F., Siracusa, L. D., Mancini, R., Siprashvili, Z., Ottey, M., Martin, S. E., Dolsky, R., *et al.* (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4742–4747.
5. Zanesi, N., Fidanza, V., Fong, L. Y., Mancini, R., Druck, T., Valtieri, M., Rudiger, T., McCue, P. A., Croce, C. M. & Huebner, K. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 10250–10255.
6. 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) *Proc. Natl. Acad. Sci. USA* **94,** 13771–13776.
7. Werner, N. S., Siprashvili, Z., Fong, L. Y. Y., Marquitan, G., Schroder, J. K., Bardenheuer, W., Seeber, S., Huebner, K., Schutte, J. & Opalka, B. (2000) *Cancer Res.* **60,** 2780–2785.
8. Ishii, H., Dumon, K. R., Vecchione, A., Fong, L. Y., Baffa, R., Huebner, K. & Croce, C. M. (2001) *J. Am. Med. Assoc.* **286,** 2441–2449.
9. Ji, L., Fang, B., Yeh, N., Fong, K., Minna, J. D. & Roth, J. A. (1999) *Cancer Res.* **59,** 3333–3339.
10. Sard, L., Accornero, P., Tornielli, S., Delia, D., Bunone, G., Campiglio, M., Colombo, M. P., Gramegna, M., Croce, C. M., Pierotti, M. A. & Sozzi, G. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8489–8492.
11. Ishii, H., Dumon, K. R., Vecchione, A., Trapasso, F., Mimori, K., Alder, H., Mori, M., Sozzi, G., Baffa, R., Huebner, K. & Croce, C. M. (2001) *Cancer Res.* **61,** 1578–1584.
12. Dumon, K. R., Ishii, H., Vecchione, A., Trapasso, F., Baldassarre, G., Chakrani, F., Druck, T., Rosato, E. F., Williams, N. N., Baffa, R., *et al.* (2001) *Cancer Res.* **61,** 4827–4836.
13. Roz, L., Gramegna, M., Ishii, H., Croce, C. M. & Sozzi, G. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 3615–3620.
14. Dumon, K. R., Ishii, H., Fong, L. Y. Y., Zanesi, N., Fidanza, V., Mancini, R., Vecchione, A., Baffa, R., Trapasso, F., During, M. J., *et al.* (2001) *Proc. Natl. Acad. Sci. USA* **98,** 3346–3351.
15. Brenner, C. (2002) *Biochemistry* **41,** 9003–9014.
16. Barnes, L. D., Garrison, P. N., Siprashvili, Z., Guranowski, A., Robinson, A. K., Ingram, S. W., Croce, C. M., Ohta, M. & Huebner, K. (1996) *Biochemistry* **35,** 11529–11535.
17. Draganescu, A., Hodawadekar, S. C., Gee, K. R. & Brenner, C. (2000) *J. Biol. Chem.* **275,** 4555–4560.
18. Lima, C. D., Klein, M. G. & Hendrickson, W. A. (1997) *Science* **278,** 286–290.
19. Abend, A., Garrison, P. N., Barnes, L. D. & Frey, P. A. (1999) *Biochemistry* **38,** 3668–3676.
20. Brenner, C., Garrison, P., Gilmour, J., Peisach, D., Ringe, D., Petsko, G. A. & Lowenstein, J. M. (1997) *Nat. Struct. Biol.* **4,** 231–238.
21. Bieganowski, P., Garrison, P. N., Hodawadekar, S. C., Faye, G., Barnes, L. D. & Brenner, C. (2002) *J. Biol. Chem.* **277,** 10852–10860.
22. Wedekind, J. E., Frey, P. A. & Rayment, I. (1996) *Biochemistry* **35,** 11560–11569.
23. Bollag, G. & McCormick, F. (1991) *Nature* **351,** 576–579.
24. 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. & Brenner, C. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5484–5489.
25. Ghosh, S. & Lowenstein, J. M. (1997) *Gene* **176,** 249–255.
26. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
27. Fersht, A. (1985) *Enzyme Structure and Mechanism* (Freeman, New York).
28. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. & Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2509–2514.
29. Milner, A. E., Levens, J. M. & Gregory, C. D. (1998) *Methods Mol. Biol.* **80,** 347–354.
30. Druck, T., Hadaczek, P., Fu, T. B., Ohta, M., Siprashvili, Z., Baffa, R., Negrini, M., Kastury, K., Veronese, M. L., Rosen, D., *et al.* (1997) *Cancer Res.* **57,** 504–512.
31. Fernandes-Alnemri, T., Litwack, G. & Alnemri, E. S. (1994) *J. Biol. Chem.* **269,** 30761–30764.
32. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S. & Dixit, V. M. (1995) *Cell* **81,** 801–809.
33. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., *et al.* (1995) *Nature* **376,** 37–43.
34. Hengartner, M. O. (2000) *Nature* **407,** 770–776.
35. Joneson, T. & Bar-Sagi, D. (1997) *J. Mol. Med.* **75,** 587–593.
36. Vetter, I. R. & Wittinghofer, A. (2001) *Science* **294,** 1299–1304.
37. Blackburn, G. M., Liu, X. H., Rosler, A. & Brenner, C. (1998) *Nucleosides Nucleotides* **17,** 301–308.
38. Liu, X., Brenner, C., Guranowski, A., Starzynska, E. & Blackburn, G. M. (1999) *Angew. Chem. Int. Ed. Engl.* **38,** 1245–1247.
39. Varnum, J. M., Baraniak, J., Kaczmarek, R., Stec, W. J. & Brenner, C. (2001) *BMC Chem. Biol.* **1,** 3.