**Fhit Interaction with Ferredoxin Reductase Triggers Generation of Reactive Oxygen Species and Apoptosis of Cancer Cells**

* Received for publication, November 5, 2007, and in revised form, February 28, 2008 Published, JBC Papers in Press, March 3, 2008, DOI 10.1074/jbc.M709062200

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Fhit protein is lost in most cancers, its restoration suppresses tumorigenicity, and virus-mediated *FHIT* gene therapy induces apoptosis and suppresses tumors in preclinical models. We have used protein cross-linking and proteomics methods to characterize a Fhit protein complex involved in triggering Fhit-mediated apoptosis. The complex includes Hsp60 and Hsp10 that mediate Fhit stability and may affect import into mitochondria, where it interacts with ferredoxin reductase, responsible for transferring electrons from NADPH to cytochrome P450 via ferredoxin. Viral-mediated Fhit restoration increases production of intracellular reactive oxygen species, followed by increased apoptosis of lung cancer cells under oxidative stress conditions; conversely, Fhit-negative cells escape apoptosis, carrying serious oxidative DNA damage that may contribute to an increased mutation rate. Characterization of Fhit interacting proteins has identified direct effectors of the Fhit-mediated apoptotic pathway that is lost in most cancers through loss of Fhit.

The *FHIT* gene encompasses the most active common fragile site at chromosome 3p14.2 (1, 2). Fhit expression is lost or reduced in a large fraction of most types of human tumors due to allelic loss, genomic rearrangement, promoter hypermethylation, or combinations thereof (3, 4). Fhit knock-out mice show increased susceptibility to cancer development (5, 6) and *FHIT* gene knock-out mice of normal appearing bronchial epithelial cells of smokers, prior to hyperplastic lesions, DNA damage checkpoint genes are already activated, leading to selection for mutations in checkpoint proteins and neoplastic progression (14, 15). Evidence of DNA alteration at FRA3B within the *FHIT* gene accompanies the hyperplasia and checkpoint activation. Loss of *FHIT* alleles occurs in normal appearing bronchial epithelial cells of smokers, prior to pathologic changes or alterations in expression of other suppressor genes (16–18). Fhit expression is down-regulated by exposure to DNA damaging agents (19) and Fhit plays a role in response to such agents (20, 21), with Fhit-deficient cells escaping apoptosis and accumulating mutations. To identify proteins that interact with Fhit to effect downstream apoptotic pathways, we cross-linked proteins within cells after viral-mediated Fhit overexpression in lung cancer cells, and characterized proteins associated with Fhit and the pathways affected by them.

**MATERIALS AND METHODS**

Cells, Vectors, and Antisera—A549, H1299, MKN74-E4, and A116, and HCT116 cells were maintained in RPMI 1640 medium plus 10% fetal bovine serum and penicillin/streptomycin (Sigma). HEK293 cells (Microbix) used for preparation of recombinant adenoviruses were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and penicillin/streptomycin. AdFHIT-His6 virus was prepared as described under supplemental Methods.

Full-length *FDXR* was amplified from human brain cDNAs (Clontech), subcloned into the pcDNA3.1/V5-HisTOPO TA vector (Invitrogen) and sequenced; details are as described under supplemental Methods. Cells were transfected using Lipofectamine™ (Invitrogen) following the manufacturer’s directions.

**Western Blot Analysis**—Immunoblot analyses were performed as described (13) using monoclonal anti-pentaHis (Qiagen); rabbit polyclonal anti-Fhit (Zymed Laboratories Inc.); rabbit polyclonal antisera against GFP, Hsp60, Hsp10, and...
cytochrome c (Santa Cruz Biotechnology); rabbit polyclonal anti-Fdxr (Abcam); monoclonal anti-CoxIV (Molecular Probes); anti-V5 (Sigma); anti-Parp1 (Santa Cruz Biotechnology); and anti-caspase 3 (Cell Signaling). Protein levels were normalized relative to β-actin or/and GAPDH level, detected with appropriate antisera (Santa Cruz Biotechnology).

**Mass Spectrometry Studies**—Protein pellets were solubilized and digested by trypsin as described under supplemental Methods. Peptide mixtures were injected for LC-MS/MS analysis. After protein identification by data base search, inspection of LC-MS/MS data was undertaken to assess the exclusive presence of mass peaks belonging to candidate partner proteins in samples from cells infected with AdFHIT-His6. See supplemental data for technical details.

**Protein Interaction Analyses**—Proteins were extracted in 15 mM Tris-Cl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 0.1 mM dithiothreitol, 0.5% Triton X-100, 10 mg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride. Co-immunoprecipitation experiments, with or without dithiothreitol (succinimidyl propionate) (DSP), were performed by incubating 1 mg of total proteins with Hsp60, Hsp10, Fdxr, penta-His, and V5 antisera conjugated with Sepharose for 2 h at 4 °C; after washing, beads were boiled in 1X SDS sample buffer and proteins separated on 4–20% polyacrylamide gels (Bio-Rad), transferred to a poly(vinylidene difluoride) filter (Millipore), and probed with specific antisera.

**Subcellular Localization of Fhit Protein**—Fhit was sublocalized in ponasterone A (PonA)-induced, Fhit-expressing H1299 D1 cells by indirect immunofluorescence detection using anti-Fhit serum and by detection of FhitHis6 in A549 AdFHIT-His6-infected cells in immunoelectrophoresis micrographs using anti-penta-His (see supplemental Methods for details). In fractionation studies, mitochondria were isolated with the Mitochondria/Cytosol Fractionation kit and the FractionPREPTM Cell Fractionation System was used to extract proteins from cytosol, membranes, nuclei, and cytoskeleton (Biovision Research Products). For sub mitochondrial localization according to the method of Daheron et al. (22), mitochondria were resuspended in 0.1 mM carbonate, pH 11.5, on ice for 30 min with periodic vortexing and fractionated as described under supplemental Methods.

**Flow Cytometry**—HCT116 FDXR+//+ and FDXR−/− cells were infected with AdFHIT or AdGFP at m.o.i. 50 and 100 and assessed at 48 h postinfection. PonA-induced H1299 D1 and E1 cells were treated with 0.25 and 0.5 mM H2O2 or with chemotherapeutic drugs and incubated for varying times, as indicated in the text and figures. For both experiments the cells were collected, washed with phosphate-buffered saline, and resuspended in cold 70% ethanol. For analysis, cells were spun down, washed in phosphate-buffered saline, and suspended in 100 μl of propidium iodide/Triton X-100 staining solution (0.1% Triton X-100, 0.2 mg/ml DNase-free RNase A) for 30 min at room temperature and analyzed by flow cytometry.

**Assessment of Intracellular Reactive Oxygen Species (ROS)**—Intracellular superoxide was measured through ethidium fluorescence as a result of oxidation by hydroethidine (dihydroethidium-HE; Molecular Probes). MNK74 stably Fhit expressing cells, A549 cells transiently expressing Fhit, and H1299 inducible Fhit expressing cells were treated with 0.5, 1.0, 2.0, and 4.0 mM H2O2 at 37 °C; 4 h later, hydroethidine (10 μM) was added to cells and incubated for 15 min at 37 °C. Fluorescence was measured by flow cytometry. Dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) was used in D1 cells expressing induced Fhit, stressed with H2O2 (0.1 to 1.0 mM), treated with 10 μM DCFH-DA, and incubated for 1 h at 37 °C. DCF fluorescence was measured by flow cytometry on a FACScan flow cytometer and fluorescence microscopy.

**Hsp60 and Hsp10 Silencing**—A549 lung cancer cells at 8 × 105/well (6 wells plate) were transfected with Lipofectamine 2000 reagent (Invitrogen) and 6 μg of Hsp60 and/or Hsp10 siRNAs (Dharmacon catalog numbers NM_002156 and NM_002157, respectively); 48 h later cells were infected with AdFHIT at m.o.i. 1 and collected for cytosol/mitochondria protein fractionation 24 h later. Proteins were analyzed by SDS-PAGE and immunoblotting; filters were probed with Hsp60, Hsp10, and Fhit antisera. Protein loading was normalized with GAPDH and CoxIV. 1 × 106 H1299 D1 and E1 lung cancer cells were transfected as described above and at 24 h after transfection the cells were PonA-induced; 48 h after induction a cycloheximide (CHX) (10 μg/ml) chase at 1, 4, 6, and 12 h was performed and the protein lysates were analyzed as previously described.

**Real-time RT-PCR**—Total RNA isolated with TRIzol reagent (Invitrogen) was processed after DNase treatment (Ambion) directly to cDNA by reverse transcription using SuperScript First-Strand (Invitrogen). Target sequences were amplified by qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems). FDXR primers were: forward, 5′-TCGACCAAGGC- GTCGCCCTTTT-5′; reverse, 5′-TGTCGCCAGGAGGCAGA- GCATC-5′. Samples were normalized using Actin and GAPDH genes.

**Chemotherapeutic Drug Treatment**—Paclitaxel (Sigma) was dissolved in DMSO as a 10 mmol/liter stock solution and stored at −80 °C. Cisplatin (Sigma) was dissolved in water and freshly prepared before use. H1299 D1 and E1 cells were seeded (1 × 105 cells/well) in 96-well culture plates, PonA-induced, and after 24 h treated with paclitaxel (50, 100, and 500 ng/ml) or cisplatin (0.05, 0.1, and 0.2 mm). H1299 D1 and E1 cells PonA-induced were incubated for 24, 48, and 72 h and assessed for viability with an MTS kit (Cell Titer 96 Aqueous MTS kit, Upstate Biotechnology, Lake Placid, NY), as recommended by the manufacturer.

**RESULTS**

**Isolation of a Fhit Protein Complex**—To identify Fhit-interacting proteins, we generated an adenovirus carrying Fhit cDNA modified at its 3′ end with a sequence encoding a His6 epitope tag (AdFHIT-His6). The biological activity of this tagged Fhit protein expressed in A549 cells was comparable...
**Fhit Interaction with Fdxr Enhances ROS Production**

**TABLE 1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession no.</th>
<th>Molecular mass</th>
<th>Function/category</th>
<th>Subcellular localization</th>
<th>No. identified peptides</th>
<th>Peptide sequences</th>
<th>Protein Mascot score</th>
<th>Sequence coverage</th>
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<tr>
<td>Hsp60</td>
<td>NP_002147</td>
<td>60 kDa</td>
<td>Heat shock protein</td>
<td>Cytosol/mitochondria</td>
<td>6</td>
<td>VGEVYVVVK LSKVAVVLK IGIIIIKRR VDIATANTR TVITIQWUHSPK VGQISDVEVREK</td>
<td>239</td>
<td>10%</td>
</tr>
<tr>
<td>Malate dehydrogenase (Mdh)</td>
<td>NP_005909</td>
<td>33</td>
<td>Catalyzes the reversible oxidation of malate to oxaloacetate</td>
<td>Mitochondrial matrix</td>
<td>8</td>
<td>ANTFYAVELK IGQAEITEVIK VNPVISOHAGK IFPGVTTLIDV FVFFRLDAMKX GCDVVFPIAQYVR AGAAGSALSMAYAGAR GYLGPEQCLPDCLK EIDDGLETTLR VETTEDLVAK LESLISVDEPPQR</td>
<td>193</td>
<td>28%</td>
</tr>
<tr>
<td>Electron transfer flavoprotein (Etfb)</td>
<td>NP_001976</td>
<td>28</td>
<td>Specific electron acceptor for mitochondrial dehydrogenases</td>
<td>Mitochondrial matrix</td>
<td>3</td>
<td>EEIOPSVK VLGQTVAVGSGK VLVLDKFLFR</td>
<td>96</td>
<td>12%</td>
</tr>
<tr>
<td>Hsp10</td>
<td>AAC96332</td>
<td>10</td>
<td>Heat shock protein</td>
<td>Cytosol/mitochondria</td>
<td>3</td>
<td>GCIEQPGSVK VLGQTVAVGVSGK VLVLDKFLFR</td>
<td>92</td>
<td>34%</td>
</tr>
<tr>
<td>Mitochondrial aldehyde dehydrogenase 2 (Adh2)</td>
<td>NP_000681</td>
<td>55</td>
<td>Second enzyme of the major oxidative pathway of alcohol metabolism</td>
<td>Mitochondrial matrix</td>
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<td>LAGL18R LQPALATONVNVVK</td>
<td>75</td>
<td>4%</td>
</tr>
<tr>
<td>Ferredoxin reductase (Fdxr)</td>
<td>P22570</td>
<td>54</td>
<td>First electron transfer protein in all the mitochondrial p450 systems</td>
<td>Mitochondrial matrix</td>
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<td>FQVAPDHPDEVK</td>
<td>47</td>
<td>2%</td>
</tr>
</tbody>
</table>

with wild-type Fhit activity (supplemental Fig. S1). A549 lung cancer-derived cells, which are susceptible to Fhit-induced apoptosis (10), were infected with AdFHIT or AdFHIT-His₆ and treated with DSP, a cross-linker that crosses membranes and fixes proteins in complex in vivo. Cells were lysed and proteins isolated with nickel beads avid for the His₆ epitope tag. Purified proteins were treated with dithiothreitol to cleave DSP and dissociate the complex, and digested by trypsin; protein constituents were identified by LC-MS/MS (Table 1 and supplemental Fig. S2). Six proteins were identified, all with mitochondrial localization: Hsp60 and 10, ferredoxin reductase (Fdxr), malate dehydrogenase, electron-transfer flavoprotein, and mitochondrial aldehyde dehydrogenase 2; Hsp60 and Hsp10 are also distributed in the cytosol (23).

**Fhit Subcellular Localization**—Because candidate Fhit interactors are mitochondrial proteins, we determined if Fhit, which lacks a mitochondrial localization signal, localized in these organelles. Fhit negative H1299 lung cancer cells carrying an inducible FHIT cDNA (D1 cells) were treated with the inducer, PonA for 48 h and indirect immunofluorescence detection of Fhit subcellular location was assessed using anti-Fhit serum and MitoTracker Red 580, a marker of mitochondria; Fhit fluorescent signal (green staining, Fig. 1A) was cytoplasmic and partly co-localized (yellow staining, Fig. 1A, lower right) with MitoTracker Red dye, indicating that exogenous Fhit localized to mitochondria and cytosol. Anti-Fhit specificity was confirmed by absence of green fluorescence in the Fhit negative H1299 clone E1 cells (not shown). To confirm mitochondrial localization, A549 cells infected with AdFHIT-His₆ or AdFHIT at m.o.i. 20 were examined by immunoelectron microscopy 48 h later, by anti-pentaHis staining; FhitHis6-transduced cells demonstrated significant numbers of gold particles in mitochondria (Fig. 1B, right panel), whereas AdFHIT-transduced cells showed sparse reactivity (Fig. 1B, left panel). To assess Fhit submitochondrial localization, mitochondria were purified from A549 cells infected with AdFHIT m.o.i. 1, as described above. The sodium carbonate procedure is a nondestructive approach that allows effective release in the supernatant of both soluble proteins and peripheral membrane proteins from intracellular membranes after inducing the generation of open sheets of membranes; furthermore, it allows recovery of integral proteins with the membranes (pellet) (24). Fig. 1E shows that Fhit was only detectable in the soluble fraction. To further define Fhit submitochondrial localization, mitochondria were treated with 0.10 and 0.15% digitonin to selectively disrupt mitochondrial outer membrane, releasing proteins contained in the intermembrane space and the matrix; as shown in Fig. 1F, gradual disruption of outer and inner membranes releases increasing amounts of Fhit protein, suggesting that Fhit is mainly distributed either at the luminal side of the inner membrane or in the matrix of mitochondria. Mitochondrial localization was confirmed in gastric cancer-derived MKN74A116 cells stably expressing exogenous Fhit (9) and in HCT116 colon cancer cells expressing endogenous Fhit (Fig. 1, G and H).

**Fhit Interacts with Hsp60, Hsp10, and Fdxr**—Among candidate interactors proteins, we focused on Hsp60 and Hsp10 as possible chaperonins and on Fdxr, a mitochondrial respiratory chain protein transactivated by p53 and involved in responses to therapeutic drugs (25). To validate interactions, A549 cells were infected with AdFHIT or AdFHIT-His₆ at m.o.i. 20, with or without DSP. Fhit complexes were purified through the His₆ tag and co-purified proteins were detected with antisera against Hsp60, Hsp10, and Fdxr; Hsp60 and Fdxr were detected only in lyses of cells exposed to DSP (Fig. 2, A and C), whereas Hsp10
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As shown in Fig. 3B, to determine whether the Fhit-Hsp60/10 interaction is important for the stability of the Fhit protein, H1299 with inducible Fhit gene and siRNA were transfected with the F5 tagged FDXR-V5 and FHT plasmids, and proteins were precipitated with monoclonal anti-V5; co-precipitated Fhit was detectable only after DSP cross-linking (Fig. 2E). To determine whether these proteins also interact with endogenous Fhit, we immunoprecipitated each endogenous candidate interactor protein from DSP-treated Fhit-positive HCT116 cells and looked for co-precipitation of endogenous Fhit (Fig. 2F). Endogenous Fhit co-precipitated with Hsp10 and Fdxr, confirming the presence of endogenous Fhit in mitochondria and its interaction with endogenous chaperones and respiratory chain protein in the absence of stress.

Hsp60/10 Interaction Affects Fhit Stability and/or Mitochondrial Import—Hsp60 and -10 are molecular chaperones found in complex (26) and are important for folding and import of proteins into mitochondria. We hypothesized that the Hsp60/10 complex was responsible for Fhit correct folding and mitochondrial addressing. To investigate the location of these interactions, A549 cells were infected with AdFHIT-H5, m.o.i. 5 and protein lysates were collected from cytosol and mitochondrial fractions after cross-linking. Complexes were isolated by Fhit-H6-nickel pull down, separated on a polyacrylamide gel, and filters probed with Hsp60 and Fhit antisera. At 24 and 48 h after infection interaction with Hsp60 is observed in the cytosol and mitochondria (Fig. 3A) commensurate with the increase in Fhit expression at these times (Input), as shown in Fig. 3A. To determine whether the Fhit-Hsp60/10 interaction is important for the stability of the Fhit protein, H1299 with inducible Fhit expression (D1 cells) were transfected with Hsp60 and Hsp10 was also detectable without cross-linking (Fig. 2B). A time course experiment after infection showed recruitment of Fdxr by Fhit (Fig. 2C); also, endogenous Hsp60 co-immunoprecipitated Fhit and Hsp10 in the absence of DSP (Fig. 2D). To verify specificity of interactions we generated an FDXR cDNA expression plasmid with a 3’ V5 epitope tag. A549 cells were co-transfected with FDXR-V5 and FHT plasmids, and proteins were precipitated with monoclonal anti-V5; co-precipitated Fhit was detectable only after DSP cross-linking (Fig. 2E).
both Hsps, the Fhit level was unaffected in the cytosol but reduced in mitochondria compared with control (Fig. 3B), suggesting that the Hsp60/10 complex may mediate virally transduced Fhit stabilization and mitochondrial localization. It is also true that if Hsp60 and Hsp10 are involved in Fhit stability after Fhit viral transduction, the cellular compartment with less Fhit would be affected by a decrease in Fhit stability. We also examined the Fhit complex in H1299 D1 cells expressing inducible Fhit, with Fhit negative E1 cells as control; 48 h after Fhit induction in D1 cells (Fig. 3C, left panel), distribution of the Fhit complex proteins was similar in the cytosol and mitochondria of D1 and E1 cells, with and without H$_2$O$_2$. Hsp60 was immunoprecipitated from total cell lysates of these cells at 48 h after PonA induction, with or without H$_2$O$_2$, and coprecipitated Fhit and Fdxr (Fig. 3C, right panel). Induction of Fhit expression in D1 cells does not cause biological changes in vitro; thus the Fhit complex does not form as a consequence of apoptosis. We performed a time course experiment in D1 cells after PonA-induced Fhit expression, with and without stress conditions, to determine whether there were biological changes in Fhit protein interactors. We did not detect changes in localization after Fhit expression.

**Fhit Induces Generation of Reactive Oxygen Species**—Fdxr, a 54-kDa flavoprotein, is located on the matrix side of the inner mitochondrial membrane, and is responsible for transferring electrons from NADPH, via the single electron shuttle ferrodoxin-cytochrome P450, to substrates (27). Under substrate-limiting conditions, electrons leak from this shuttling system and generate ROS (29, 30). Fdxr mediates p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells, through generation of ROS (29, 30), potent intracellular oxidants, and regulators of apoptosis (31). The discovery of the Fhit/Fdxr interaction prompted us to determine whether ROS production could be involved in Fhit-mediated apoptosis. Overexpression of Fdxr increases sensitivity of tumor cells to apoptosis on H$_2$O$_2$ treatment, through ROS production (29, 30). We examined ROS production in A549 cells, with and without H$_2$O$_2$ treatment, after transient transfection with the *FHIT* expression plasmid. Intracellular superoxide was assessed by measuring ethidium fluorescence, as a result of oxidation of hydroethidine by superoxide. Intracellular superoxide was measured 5 h after stimulation with increasing concentrations of H$_2$O$_2$. ROS generation was ~3 times higher (16.7 versus 5.4% at 0.5 mM H$_2$O$_2$ and 18.8 versus 7.7% at 1.0 mM H$_2$O$_2$) in Fhit-transfected cells. 2 mM H$_2$O$_2$ was toxic to Fhit-expressing but not to non-expressing cells (Fig. 4A).

A similar experiment was performed with p53 and Fhit negative lung cancer-derived H1299 D1 and E1 clones carrying PonA-inducible *FHIT* and empty vector expression plasmids, respectively; the cells were treated with 5 μM PonA and at 48 h treated with 0.5 and 1.0 mM H$_2$O$_2$; the % ROS-positive cells was higher in Fhit-positive D1 cells than in E1 control cells (20 versus 3.5% at 0.5 mM H$_2$O$_2$, and 78 versus 25% at 1.0 mM H$_2$O$_2$, respectively) (Fig. 4B). These results were paralleled by experiments with human gastric cancer-derived cells, MKN74A116 (supplemental Fig. S3), which express mutant p53 (32) and stably express exogenous Fhit (9).
To further study the generation of ROS after Fhit reconstitution during oxidative stress, DCFH-DA was used to measure the redox state of Fhit-overexpressing cells. Peroxidases, cytochrome c, and Fe$^{2+}$ can oxidize DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of H$_2$O$_2$; thus, DCF indicates H$_2$O$_2$ levels and peroxidase activity. Increased DCF fluorescence was detected in D1 cells compared with E1 cells under stress conditions (Fig. 4C). The decreased cell viability after H$_2$O$_2$ treatment in Fhit-expressing cells was also assessed by an MTS cytotoxicity assay 24 h after H$_2$O$_2$ treatment. H$_2$O$_2$ treatment caused reduced cell viability or growth arrest in both E1 and D1 cells, but this phenotype was more pronounced in D1 cells (Fig. 4D). To determine whether H$_2$O$_2$ treatment with or without Fhit could affect cell viability or cell cycle kinetics we performed flow cytometry (Fig. 4E); when Fhit was present under stress conditions there was a consistent increase of G$_2$/M arrest at 48 h after 0.25 and 0.5 mM H$_2$O$_2$ treatment, 45.5 and 49.5%, respectively, compared with 27.5 and 29% of E1 cells under the same conditions. To assess if the G$_2$/M arrest could affect long-term viability of the cells, a colony assay was performed (Fig. 4F). No colonies were detected in Fhit-expressing cells after exposure to 0.25 mM or higher concentrations of H$_2$O$_2$.

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**FIGURE 4.** Fhit expression induces intracellular ROS generation after treatment of cells with peroxide. A, fluorescence-activated cell sorter (FACS) analysis for ROS assessment in A549 cells 48 h after transfection with FHIT plasmid, with and without a 5-h H$_2$O$_2$ treatment. Empty vector-transfected cells served as control. Intracellular superoxide was determined according to the fluorescence of ethidium as a result of oxidation of hydroethidine by O$_2^-$. M2 refers to the fraction of ROS positive cells. B, FACS analysis for ROS assessment by the fluorescence produced from the oxidation of hydroethidine in D1 and E1 cells; 48 h after PonA treatment, cells were treated for 5 h with 0.5 and 1.0 mM H$_2$O$_2$ and oxidative stress was measured; % positive refers to the fraction of fluorescent cells, indicating ROS. These experiments were repeated three times with similar results. C, increased green fluorescent DCF signal in H1299 Fhit-expressing cells (D1) under stress conditions. Cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate, a ROS indicator that can be oxidized in the presence of ROS to the highly green fluorescent dye DCF, at 48 h after Fhit induction and after a 5-h H$_2$O$_2$ treatment of E1 and D1 cells (magnification ×40). D, MTS cell viability assays were performed on E1 and D1 cells. Cells were treated with PonA for 48 h and then with increasing concentrations of H$_2$O$_2$ (0.125, 0.25, and 0.5 mM) for 4 h. Analysis was at 24 h after H$_2$O$_2$ treatment. Columns report the average of four experiments ± S.E. Each point was measured in quadruplicate and standard deviation calculated; p < 0.05 was considered significant. E, FACS analysis of D1 and E1 cell cycle kinetics at 48 h after oxidative stress treatment. Cells were treated with PonA for 48 h and then with increasing concentrations of H$_2$O$_2$ (0.25 and 0.5 mM) for 4 h. Analysis was at 48 h after H$_2$O$_2$ treatment. All experiments were performed twice in triplicate. F, colony formation assay of H1299/D1 and H1299/E1 cells after 5-mM PonA stimulation and a 5-h H$_2$O$_2$ treatment at the indicated concentrations.

To further study the generation of ROS after Fhit reconstitution during oxidative stress, DCFH-DA was used to measure the redox state of Fhit-overexpressing cells. Peroxidases, cytochrome c, and Fe$^{2+}$ can oxidize DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of H$_2$O$_2$; thus, DCF indicates H$_2$O$_2$ levels and peroxidase activity. Increased DCF fluorescence was detected in D1 cells compared with E1 cells under stress conditions (Fig. 4C). The decreased cell viability after H$_2$O$_2$ treatment in Fhit-expressing cells was also assessed by an MTS cytotoxicity assay 24 h after H$_2$O$_2$ treatment. H$_2$O$_2$ treatment caused reduced cell viability or growth arrest in both E1 and D1 cells, but this phenotype was more pronounced in D1 cells (Fig. 4D). To determine whether H$_2$O$_2$ treatment with or without Fhit could affect cell viability or cell cycle kinetics we performed flow cytometry (Fig. 4E); when Fhit was present under stress conditions there was a consistent increase of G$_2$/M arrest at 48 h after 0.25 and 0.5 mM H$_2$O$_2$ treatment, 45.5 and 49.5%, respectively, compared with 27.5 and 29% of E1 cells under the same conditions. To assess if the G$_2$/M arrest could affect long-term viability of the cells, a colony assay was performed (Fig. 4F). No colonies were detected in Fhit-expressing cells after exposure to 0.25 mM or higher concentrations of H$_2$O$_2$.

**Fhit-induced ROS Generation Is Fdxr-dependent**—To evaluate the role of Fdxr in Fhit-mediated ROS generation, we examined the Fdxr level in D1 cells after Fhit induction and observed a 2.4-fold increase of its expression compared with E1 cells (Fig. 5A), an increase that was not due to increased transcription as determined by real time RT-PCR (Fig. 5F). We next measured the Fdxr level, with or without Fhit expression, in the presence of MG132, an inhibitor of proteasome degradation; 4 h after MG132 treatment a significant increase of Fdxr protein was observed in D1 cells compared with E1 cells (Fig. 5B), suggesting that Fhit protects Fdxr from proteasome degradation. The rate of Fdxr degradation in the presence or absence of Fhit protein was evaluated by the 4–12-h CHX chase (Fig. 5C); the rate of Fdxr degradation was higher in Fhit-negative E1 cells.
Fhit Interaction with Fdxr Enhances ROS Production

FIGURE 5. Apoptosis triggered by Fhit viral transduction can be mediated by its interaction with Fdxr. A, immunoblot analysis with antisera against Fdxr, Fhit, and GAPDH. Proteins were extracted from E1 (control) and D1 cells 48 h after treatment with PonA. B, immunoblot analysis of Fdxr expression in D1 and E1 cells after a 4-h treatment with 25 μM MG132, a proteasome inhibitor. GAPDH detection shows equal protein loading. C, immunoblot analysis of Fdxr, Fhit, and GAPDH in D1, expressing Fhit, and E1 cells, showing Fdxr level after CHX chase (30 μmol/l) for 4 – 12 h. Densitometry based on GAPDH levels shows enhanced stability of Fdxr in the presence of Fhit. D, FACS analysis of FDXR+/−/+ and FDXR+/−/− cell cycle kinetics after infection with AdFHIT m.o.i. 50 and 100. The experiment was performed 48 h after infection and was repeated three times with similar results. Profiles of AdGFP-infected cells were similar to those of non-infected cells (not shown). E, immunoblot analysis showing expression of Fdxr, Fhit, and GAPDH after infection of FDXR+/−/+ and FDXR+/−/− with AdFHIT m.o. 50 and 100. The PCR product was normalized to GAPDH and Actin expression and each point was repeated in quadruplicate; differences between control and Fhit positive samples were not significant. F, caspase 3 and Parp1 activation. Immunoblot analysis, using Fhit, caspase 3, Parp1 antisera, of total cell lysates from HCT116 FDXR+/−/+ cells 48, 72, and 96 h after infection with AdFHIT and AdGFP at m.o.i. 50. GAPDH and CoxIV served as internal protein markers. H, immunoblot analysis, using Fhit and cytochrome c antisera, of cytosol/mitochondria fractions from HCT116 FDXR+/−/+ cells 48, 72, and 96 h after infection with AdFHIT and AdGFP at m.o.i. 50. GAPDH and β-actin served as internal protein markers.

HCT116 colon cancer cells, which express endogenous wild-type p53 and Fhit and carry three FDXR alleles (FDXR+/−/+), and HCT116 FDXR+/−/− cells with two alleles knocked-out (28), were used to determine whether AdFHIT-induced apoptosis is influenced by the Fdxr expression level; the FDXR null condition was not compatible with viability (29). These cells were infected with AdFHIT m.o.i. 50 or 100 and assessed for apoptosis at 48 and 72 h post-infection. Wild-type HCT116 cells (FDXR+/−/+ and FDXR+/−/−) were susceptible to exogenous Fhit-mediated apoptosis in a dose-dependent manner, as the fraction of sub-G1 cells was 12.1 and 18.8% at m.o.i. 50 and 100, respectively; FDXR+/−/− cells were refractory at 48 and 72 h (data not shown) to Fhit-induced cell death, with a sub-G1 population of 4.7 and 4.3% at m.o.i. 50 and 100 (Fig. 5D). Fhit overexpression led to increased Fdxr protein levels in both FDXR+/−/+ and FDXR+/−/− cells (Fig. 4E) and FDXR+/−/− cells were committed to Fhit-mediated apoptosis by 72 h after infection. The Fhit-mediated increase of Fdxr expression was not at the transcriptional level, as determined by real time RT-PCR (Fig. 5F) and thus not related to the p53 transcriptional activation. To better determine whether the sub-G1 peak detected in FDXR+/−/+ cells after AdFHIT infection was related to apoptosis induction, a time course experiment at 48, 72, and 96 h for caspase 3 and Parp1 cleavage was performed and compared with AdGFP-infected cells (Fig. 5G). Caspase 3 cleavage and related Parp1 cleavage were observed at 48, 72, and 96 h after virus-mediated Fhit overexpression. The time course of cytochrome c release from mitochondria into cytosol was assessed after infection of HCT116 cells with AdFHIT m.o.i. 100 (Fig. 5H); progressive cytochrome c release was observed in HCT116 FDXR+/−/+ cells compared with GFP-infected cells, indicating initiation of the apoptotic cascade in Fhit overexpressing HCT116 FDXR+/−/+ cells.

Fhit Enhances ROS-related Effects of Chemotherapeutic Agents—Generation of intracellular ROS is an early event in the apoptosis of lung cancer cells induced by treatment with paclitaxel (33). We tested paclitaxel on H1299 D1 and E1 cells with or without induced Fhit expression. After induction of Fhit expression, D1 cells were more sensitive to paclitaxel than E1 cells (Fig. 6A) as measured by the MTS cell viability test. Cisplatin induces Fdxr expression and the cisplatin-induced apoptotic pathway is associated with ROS generation (34). Fhit expressing D1 cells were more sensitive than E1 cells to cisplatin, measured by MTS assay at 24 and 48 h (Fig. 6B). To examine cell viability after drug treatment, we performed flow cytometry analysis (Fig. 6, C and D); PonA-induced D1 and E1 cells treated with increasing paclitaxel concentra-
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DISCUSSION

Earlier searches for Fhit-interacting proteins pointed to several candidate proteins, none of which we could confirm as interactors by co-immunoprecipitation experiments, including Ubc9, α-tubulin, and Mdm2 (35–37). To readdress the question of Fhit protein interactors, we used adenovirus-transduced Fhit-His6 for Fhit complex purification after cross-linking, and Fhit-linked proteins, Hsp60, Hsp10, and Fdxr, were identified; subcellular location of these proteins suggested that mitochondria might be foci of Fhit activity. Hsp “stress proteins” as molecular chaperones perform functions such as protein translocation, folding, and assembly (38). The finding that Fhit interacts with Hsp60/Hsp10 after AdFHIT infection suggests that the Hsp complex may be important for Fhit stability, and possibly for its correct folding to import it into mitochondria, prior to activation of the apoptotic pathway, a suggestion we investigated by knocking down expression of Hsp60, Hsp10, or both in AdFHIT-infected lung cancer cells; Fhit stability was assessed after CHX chase in H1299 D1 cells, the lung cancer cell line expressing inducible Fhit. The level of Fhit protein in isolated mitochondria after knockdown of both Hsp60 and -10 was reduced, strengthening the proposal that Fhit-Hsp60/10 interaction is involved in Fhit stabilization and/or in correct folding for importation into mitochondria.

Targeted disruption of the FDXR gene in HCT116 colon cancer cells showed that it was essential for viability; reduction of the gene copy number resulted in decreased sensitivity to 5-fluorouracil-induced apoptosis (29) and FDXR is a target gene of the p53 family (30). Overexpression of Fdxr-sensitized colon cancer cells to H2O2, 5-fluorouracil, and doxorubicin-induced cell death, indicating that Fdxr contributes to p53-mediated apoptosis through generation of oxidative stress in mitochondria. Thus, activated p53 induces apoptosis in response to cellular stresses in part through ROS, and simultaneously p53 increases transcription of the FDXR gene, which in turn enhances p53 function by increasing ROS-induced apoptosis (29, 30).

4 K. Huebner, unpublished data.
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We have shown the presence of Fhit in the mitochondrial fraction; when Fhit is overexpressed or Fhit-expressing cells are stressed, Fhit can protect Fdxr from proteosomal degradation, leading to an increase in the Fdxr protein level, which is associated with generation of ROS and followed by apoptosis. Fhit does not affect the FDXR transcriptional level but may affect stability of the protein. In H1299 cells, missing both Fhit and p53, Fdxr overexpression increases sensitivity to ROS-induced cell death, and H1299 cells expressing inducible Fhit or p53 are sensitive to ROS-induced cell death; cancer cells missing Fhit, p53, or both would lack ways to increase Fdxr expression, and would be less sensitive to oxidative damage and would survive.

Discovery of the mitochondrial function of Fhit in apoptosis through interaction with Fdxr is interesting because it extends functional parallels of the important tumor suppressors, Fhit and p53, lost sequentially in most cancers and involved in response to DNA damage, and illuminates their differences, with p53 acting as a transcriptional and Fhit a post-transcriptional Fdxr regulator. Delineation of direct downstream effectors of the Fhit suppressor pathway will lead to mechanistic studies of Fhit function that may influence preventive and therapeutic strategies to activate the Fhit pathway. The finding that ROS generation is crucial for Fhit-mediated apoptosis emphasizes the importance of Fhit loss as a negative prognostic factor in various clinical settings; for example, assessment of Fhit status in preneoplastic or neoplastic conditions may be predictive of responses to antioxidant treatments.

Acknowledgment—We are grateful to Bert Vogelstein for providing HCT116 FDXR+/−/− parental cells.

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Fhit Interaction with Ferredoxin Reductase Triggers Generation of Reactive Oxygen Species and Apoptosis of Cancer Cells
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doi: 10.1074/jbc.M709062200 originally published online March 3, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M709062200

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