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REVIEW ARTICLES

The Histidine Triad Superfamily of Nucleotide-Binding Proteins

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Histidine triad (HIT) proteins were until recently a superfamily of proteins that shared only sequence motifs. Crystal structures of nucleotide-bound forms of histidine triad nucleotide-binding protein (Hint) demonstrated that the conserved residues in HIT proteins are responsible for their distinctive, dimeric, 10-stranded half-barrel structures that form two identical purine nucleotide-binding sites. Hint-related proteins, found in all forms of life, and fragile histidine triad (Fhit)-related proteins, found in animals and fungi, represent the two main branches of the HIT superfamily. Hint homologs are intracellular receptors for purine mononucleotides whose cellular function remains elusive. Fhit homologs bind and cleave diadenosine polyphosphates (Ap_nA) such as ApppA and AppppA. Fhit-Ap_nA complexes appear to function in a proapoptotic tumor suppression pathway in epithelial tissues. In invertebrates, Fhit homologs are encoded as fusion proteins with proteins related to plant and bacterial nitrilases that are candidate signaling partners in tumor suppression. J. Cell. Physiol. 181:179–187, 1999.

Modern life is littered with remnants of the RNA world. Essentially every important cellular process, including DNA replication, transcription, translation, translocation, signal transduction, and apoptosis, requires multiple factors consisting of small RNAs and/or mono- and dinucleotides. In the history of biochemistry and cell biology, some of these factors, like cAMP and NAD, were discovered and appreciated early on. Others, like cADP ribose, were discovered early and are only becoming appreciated lately. The tools of molecular biology are such that it is difficult to appreciate unusual nucleic acids without identifying the proteins that interact with them. Discovery of the histidine triad (HIT) superfamily of nucleotide-binding proteins provides the opportunity to elucidate additional signaling functions of purine nucleotides in the contexts of cancer and apoptosis. Current work suggests that the human fragile histidine triad (Fhit) protein functions as a tumor suppressor that may provide the link between increased levels of diadenosine polyphosphates (Ap_nA) and programmed cell death.

DISCOVERY AND DEFINITION OF HIT PROTEINS AS A SUPERFAMILY OF NUCLEOTIDE-BINDING PROTEINS

HIT proteins fall into two branches, the Fhit branch that is found only in animals and fungi and the ancient histidine triad nucleotide-binding protein (Hint) branch that has representatives in all cellular life. As is the case for many proteins in the genomic age, HIT proteins were recognized initially not by a functional property but by virtue of sequence alignment. Figure 1 shows that bacteria, archaea, and eukarya have predicted proteins quite similar to rabbit Hint while animals and fungi have homologs of human Fhit (Brenner et al., 1997a). Although members of each branch of the superfamily maintain substantial similarity with other members of the same branch, only six residues are absolutely conserved throughout the HIT protein superfamily. Three of the six identical amino acids are His residues in the His-ϕ-His-ϕ-His-ϕ-ϕ (ϕ is a hydrophobic amino acid) motif that gives HIT proteins their name (Seraphin, 1992). The literature on HIT proteins was scant when John Lowenstein and his coworkers purified a HIT protein from rabbit heart cytosol by affinity chromatography with N6-linked adenosine agarose (Gilmour et al., 1997). On the basis of the HIT motif and the ability of the protein to bind adenosine and AMP, the protein from rabbit heart was named Hint for histidine triad nucleotide-binding protein (Brenner et al., 1997a).

Hint is a dimeric protein conserving two purine mononucleotide-binding sites and is related to GalT

When the rabbit Hint sequence was obtained and showed no similarity to Ras or other known nucleotide-binding proteins, we were aware that at least some part of the Hint sequence would represent a new nucleotide-binding motif. Whether that motif would be coincident with the HIT protein signature, however, was an open question. As Andrew Szent-Györgyi

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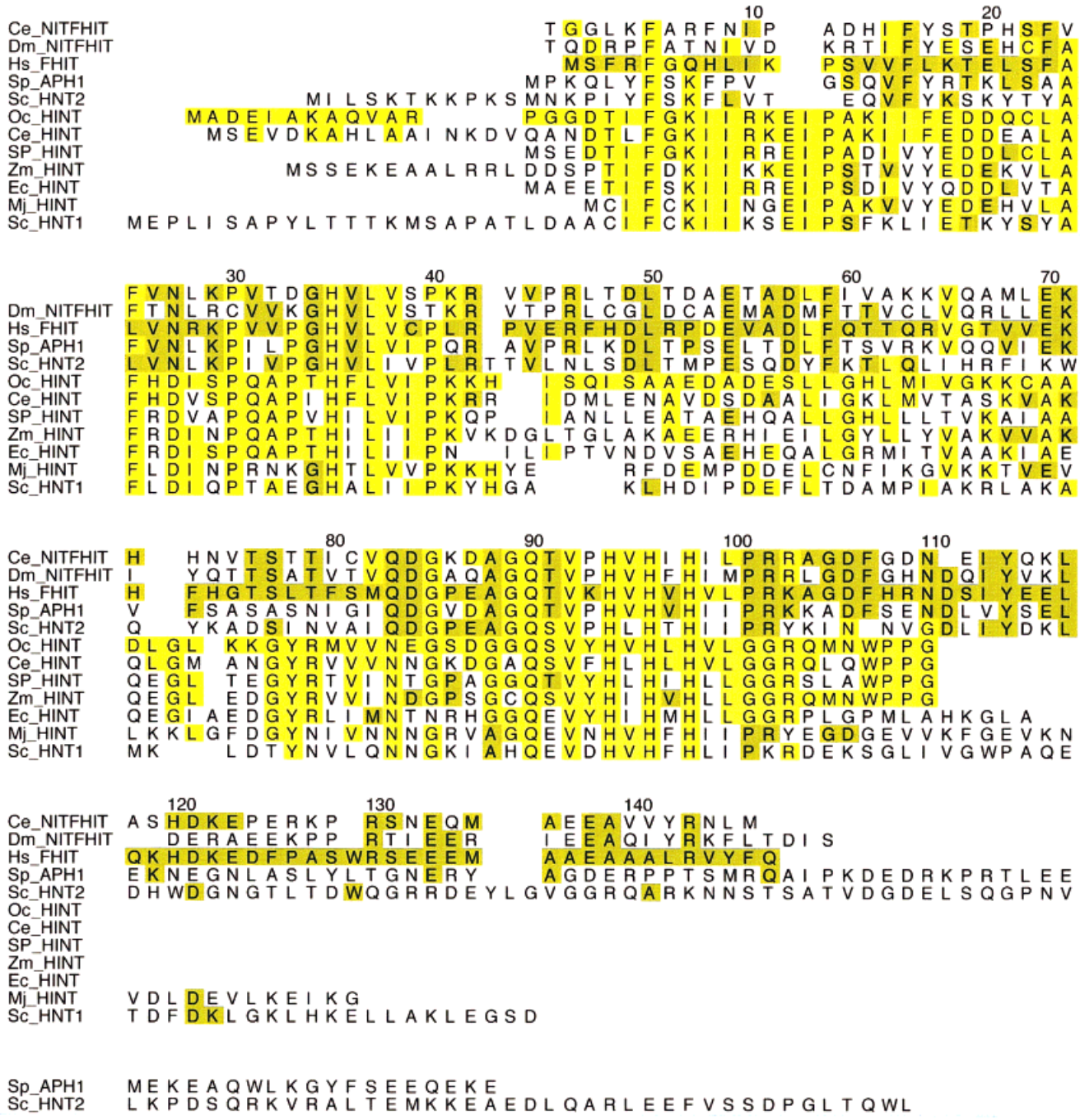

Fig. 1. Sequence alignment of HIT proteins. A multiple sequence alignment of HIT proteins illustrates the extent of sequence identity within and between the Hint and Fhit branches. Yellow shading denotes identity with rabbit (*Oryctolagus cuniculus*) Hint. Olive shading denotes identity with human Fhit at a residue that is not identical to rabbit Hint. Two-letter taxonomic identifiers are as follows: Ce, *Caenorhabditis elegans;* Dm, *Drosophila melanogaster;* Hs, *Homo sapiens;* Sp, *Schizosaccharomyces pombe;* Sc, *Saccharomyces cerevisiae;* Oc, *Oryctolagus cuniculus;* SP, *Synechococcus* PCC7942; Zm, *Zea mays;* Ec, *E. coli;* and Mj, *Methanococcus jannaschii.* Numbering corresponds to human Fhit. In the cases of *C. elegans* and *D. melanogaster* NitFhit, only the C-terminal Fhit domains are aligned.

pointed out (personal communication), if crystal structures of nucleotide-bound Hint were to demonstrate that the same residues that define the HIT protein superfamily coordinate nucleotide binding, then a structural-evolutionary argument could be made that HIT proteins are conserved as nucleotide-binding proteins.

The crystal structure of Hint-GMP, shown in the top of Figure 2, is dominated by a 10-stranded anti-parallel β-sheet, five strands contributed by each monomer

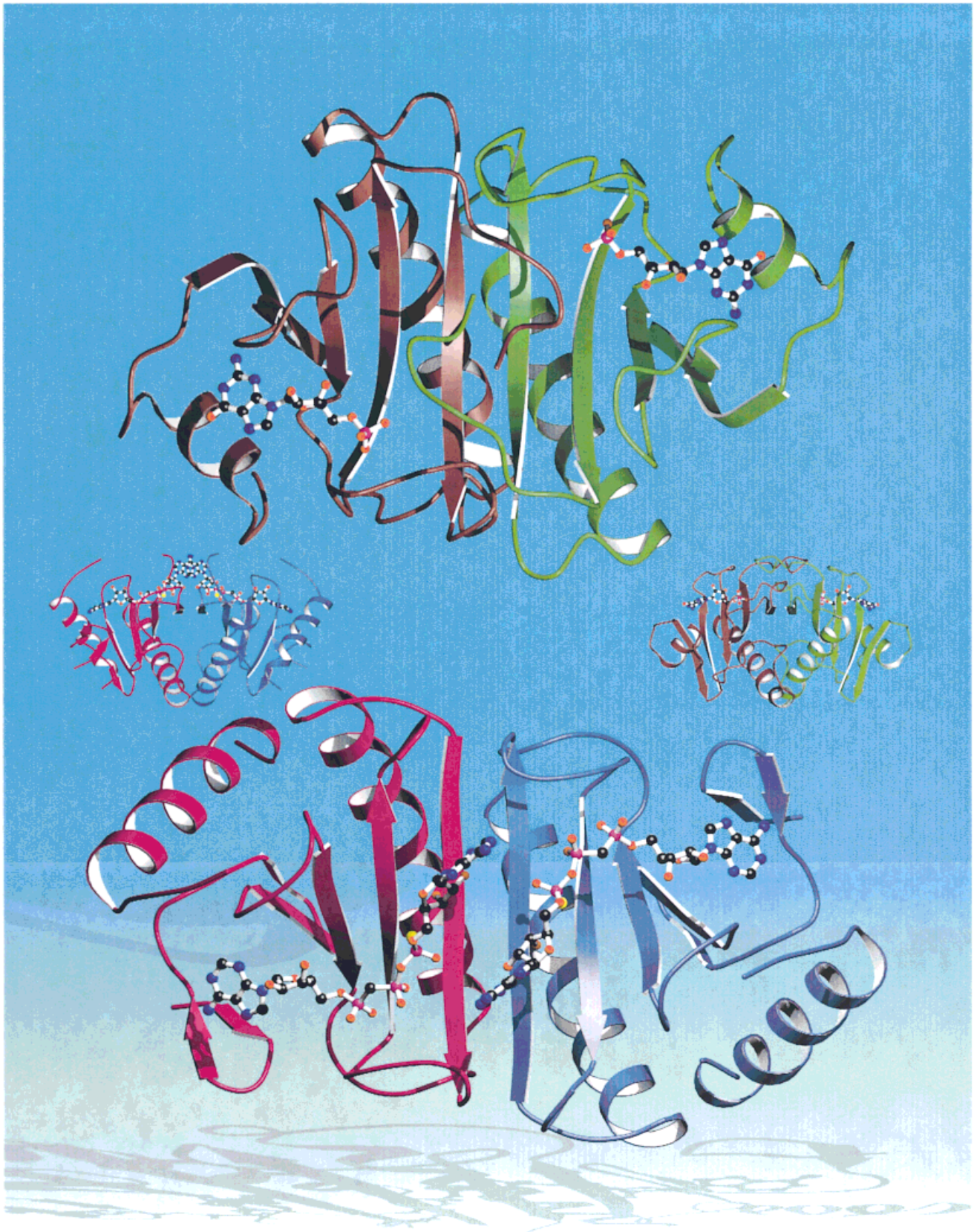

Fig. 2. Three-dimensional structures of nucleotide-bound HIT proteins. The upper dimer is Hint-GMP (Brenner et al., 1997a). The lower dimer is Fhit bound to a nonhydrolyzable ApppA analog (Blackburn et al., 1998; Pace et al., 1998). The half-barrel protein folds, first observed in GalT (Wedekind et al., 1995), are created by 10-stranded β-sheets containing two α-helices in their interiors. The half-barrels can be appreciated readily in the side views of each protein dimer (Hint on the right, Fhit on the left).

(Brenner et al., 1997a). Two identical nucleotide-binding sites are found on the top of the sheet. A set of conserved hydrophobic residues create the binding site for the purine base. Conserved nonpolar and polar residues form the binding site for the ribose. Conserved polar residues, including His 110 and His 112 from the HIT motif, create the binding site for the α-phosphate. Crystal structures of Hint-GMP, Hint-adenosine, and Hint-8Br-AMP allowed the definition of a 102-amino acid core region of Hint that contains all seven conserved secondary structural elements. Comparing the sequences of 17 HIT proteins within this region, only six residues were identical in every HIT protein and 26 nonglycine, nonproline residues were identical in a majority of proteins. Five of the six absolutely conserved residues and 14 of the 26 highly conserved residues make direct contact with the nucleotide. Thus, in advance of any functional information about HIT proteins, we made the argument that HIT proteins constitute a new superfamily of nucleotide-binding proteins (Brenner et al., 1997a).

Preston Garrison compared the secondary structure of the Hint dimer to the secondary structure of the core of GalT (Wedekind et al., 1995) and observed that the 10-stranded Hint dimer was similar in topology to the central 9-stranded sheet within a GalT monomer (Brenner et al., 1997a). This remarkable observation was made with the benefit of no bioinformatic algorithm and, initially, no computer graphics. By least-squares superposition of the manual alignment, residues 49–302 of GalT were shown to overlay the HIT protein dimer (GalT has a helix that connects two subdomains of its "half-barrel" protein fold). Coincident with the alignment of GalT β-strands with Hint β-strands, the UMP bound to GalT superimposed almost perfectly with the GMP bound to Hint and allowed us to identify the remains of sequence identity between GalT and HIT proteins (Brenner et al., 1997a). Independently, structural similarity between GalT and HIT proteins was discovered by a sensitive bioinformatic search (Holm and Sander, 1997a,b).

DISCOVERY OF A FUNGAL AND ANIMAL-SPECIFIC BRANCH OF THE HIT SUPERFAMILY: FHIT-RELATED PROTEINS

Two independent lines of research led to the discovery of the Fhit branch of the HIT protein superfamily: purification of an AppppA hydrolase from *Schizosaccharomyces pombe* (Huang et al., 1995; Robinson et al., 1993) and positional cloning of a tumor suppressor gene on the short arm of chromosome 3 (Ohta et al., 1996). Much of our ongoing research continues to address the potential links between Ap_nA binding and/or hydrolysis and tumor suppression.

Ap_nA and the proteins that cleave them

Ap_nA were discovered more than 30 years ago by Paul Zamecnik and coworkers (Zamecnik et al., 1966). Their most likely biological source is from the aminoacyl-adenylate intermediates of certain tRNA synthetases. tRNA synthetases transfer amino acids to cognate tRNAs via aminoacyl-adenylate intermediates. Under certain conditions, tRNA synthetases adenylate compounds such as ATP rather than produce aminoacyl-tRNA. Adenylation of ATP, GTP, and ADP produces AppppA, AppppG, and ApppA, respectively. Accumulation of these compounds has been reported to be sensitive to environmental signals and stresses in animal cells (Vartanian et al., 1996, 1997), reviving interest in the "alarmone" hypothesis of AppppA from earlier years (Varshavsky, 1983).

Larry Barnes and his co-workers purified aph1 protein, the AppppA hydrolase from *S. pombe* (Robinson et al., 1993), and observed sequence similarity with HIT proteins (Huang et al., 1995). Discovery that HIT proteins are nucleotide-binding proteins suggested that the manner of mononucleotide binding by Hint might suggest how members of the Fhit branch of the superfamily would bind Ap_nA (Brenner et al., 1997a).

Cloning the *FHIT* gene and evidence that it is a tumor suppressor

Twenty years ago, clear cell renal carcinoma, normally a disease of the aged, was observed to be frequent in a family with a hereditary balanced chromosomal translocation between the short arms of chromosomes 3 and 8 (Cohen et al., 1979). Cancer occurred among carriers of the translocation with early onset, in both kidneys and in multiple sites per kidney, and was accompanied by loss of genetic information from the short arm of chromosome 3. Kay Huebner and coworkers demonstrated that expression of a 1.1-kb mRNA was disrupted by the t(3;8) translocation and by frequent deletions in cancers of the gastrointestinal tract (Ohta et al., 1996), lung (Sozzi et al., 1996), and other tissues. The small message is transcribed from a huge gene, spanning greater than 1 Mb of DNA and including the most fragile site in the human genome, FRA3B (Inoue et al., 1997). Because the predicted protein is encoded at the fragile site and encodes a HIT protein, it was named Fhit (Ohta et al., 1996).

By inspection of sequences, it became apparent that Fhit is the human ortholog of the *S. pombe* aph1 protein. Partial purification of a glutathione-S-transferase-Fhit fusion protein expressed in *Escherichia coli* revealed that Fhit possesses Ap_nA hydrolase activity (Barnes et al., 1996). As shown in Figure 1, members of the Fhit branch of the HIT protein superfamily are found in animals and fungi. To this date, the *S. pombe* (Robinson et al., 1993), human (Barnes et al., 1996; Pace et al., 1998), *Saccharomyces cerevisiae* (Chen et al., 1998), *Drosophila melanogaster* (Pekarsky et al., 1998), and *Caenorhabditis elegans* (S. C. Hodawadekar, A. Draganescu, and C. Brenner, unpublished data) enzymes have been characterized biochemically. All of these enzymes possess Ap_nA hydrolase activity, producing AMP as one of the two mononucleotide products.

Because Fhit loss is a frequent event in carcinogenesis and Fhit is encoded at a fragile site, it was important to determine whether deletions in the *FHIT* gene are contributing causes to epithelial cancer or, alternatively, are consequences of the genome instability of cancer. Fhit was shown to be an authentic tumor suppressor with the result that stable re-expression of Fhit in cancer cell lines with Fhit deletions suppressed their ability to form tumors in mice (Siprashvili et al., 1997). Analysis of preneoplastic and neoplastic lesions from the lungs of smokers has indicated that events that lead to lack of expression of Fhit are the *earliest* and the *most frequent* identified genetic changes in lung

TABLE 1. Three models for function of Fhit with respect to Ap_nA[1]

Model	Enzymatic requirement	Function of Ap_nA
1. Catabolic: clear cell of Ap_nA	k_{cat}/K_m	Stimulate S
2a. Enzyme-substrate complex-dependent signaling	K_m	Alarmone via Fhit
2b. Enzyme-AMP complex-dependent signaling	1st step competence	Alarmone via Fhit
3. Nucleotide-independent	None	None

[1] Model 1 requires Fhit to clear the cell of Ap_nA, lest Ap_nA stimulate DNA replication. Models 2 require Fhit to bind Ap_nA and transmit an antiproliferative or proapoptotic signal as an enzyme-substrate complex (Model 2a) or as an enzyme-AMP complex (Model 2b). Model 3 requires Fhit to function in tumor suppression in a nucleotide-independent manner. Current evidence favors Model 2a.

cancer (Sozzi et al., 1998b). The molecular genetics of human *FHIT* have been reviewed elsewhere (Huebner et al., 1997, 1998, 1999; Sozzi et al., 1998a).

Probing the connections between Ap_nA and Fhit function in tumor suppression

Given the observations that Fhit possesses Ap_nA hydrolase and tumor suppressor activity, it was necessary to determine what is the connection, if any, between the biochemical and physiological phenomena of Fhit. The literature on Ap_nA was such that multiple models could be proposed. On the one hand, Ap_nA had been reported to be associated with initiation of DNA replication (Baril et al., 1985). On the other hand, Ap_nA had also been reported to be associated with stress (Vartanian et al., 1996). As summarized in Table 1, three mutually exclusive models were proposed. If Ap_nA compounds stimulate DNA replication, then it would be expected that loss of Fhit Ap_nA *hydrolase* activity would promote accumulation of Ap_nA and inappropriate entry into S phase. Alternatively, if Ap_nA are not replication-associated molecules but stress-related molecules, then loss of Fhit enzyme activity would not explain tumorigenesis. According to the second models, the Fhit protein is seen as a *receptor* for transmission of an Ap_nA-mediated cell-cycle arrest or cell death signal. Third, it was possible to posit that Ap_nA is unrelated to an antiproliferative or proapoptotic function of Fhit.

Biochemically, the models were distinguishable because the first model requires Fhit to *cleave* Ap_nA, the second models require Fhit to *bind* Ap_nA, and the third model does not require Fhit to cleave or bind Ap_nA. A physically stable, mutant Fhit protein that binds Ap_nA well but cleaves Ap_nA poorly would be necessary to distinguish between these models.

Crystal structures of Hint bound to GMP and 8-Br-AMP suggested that such a mutant could be constructed (Brenner et al., 1997a). Because the mostly hydrophobic residues recognizing the adenosine moiety are distinct from the polar groups recognizing the α-phosphate, it was reasonable to test whether alteration of conserved His residues to Asn would leave Fhit competent to fold and bind substrates but cripple catalytic ability. Indeed, the His96Asn allele of Fhit had less than 0.1% of wild-type activity as a glutathione-S-transferase fusion protein (Barnes et al., 1996). Remarkably, in the tumor suppressor assay, His96Asn Fhit was as active as wild-type Fhit (Siprashvili et al., 1997). Kinetic analysis of purified His96Asn Fhit indicated that k_{cat} had been reduced more than a million fold by this mutation, eliminating model 1 (Pace et al., 1998). Because the His96Asn mutation increased K_m by less than fourfold, function of Fhit as an Ap_nA receptor was entirely consistent with the observed biochemical phenotype of the His96Asn allele.

Given that Fhit has not only Ap_nA-*binding* but Ap_nA-*hydrolase* activity, it could be envisioned that Fhit might function to signal the presence of Ap_nA in two different ways. A Fhit-substrate complex might be the active, signaling form of Fhit (model 2a) or a Fhit-catalytic intermediate might be the active, signaling form (model 2b). Indeed, if the several million-fold catalytic defect of the His96Asn mutant were confined to a step after formation of an enzyme-intermediate complex, then this mutant would not have distinguished between models 2a and 2b. Perry Frey and coworkers demonstrated that, like GalT, Fhit proceeds through a covalent nucleotidylated enzyme intermediate (Abend et al., 1999). Single turnover assays with the His96Asn allele of Fhit demonstrated that the mutant is as defective in the adenylation part of the reaction as it is in the overall reaction (Pace et al., 1998). Because the His96Asn mutant is functional in tumor suppression (Siprashvili et al., 1997) but not in any measurable chemical step of ApppA cleavage, models 1 and 2b were eliminated.

Structural consequences of Fhit binding to Ap_nA

Model 2a requires Fhit not only to bind stress-induced Ap_nA compounds but, additionally, to transmit a signal to a cellular effector that such compounds have been produced. In the case of the Ras p21 oncoprotein, binding GTP is accompanied by a protein conformational change that mediates altered protein-protein interactions (Campbell et al., 1998; Wittinghofer, 1998).

Christopher Lima, Wayne Hendrickson, and coworkers determined the crystal structures of Fhit bound to adenosine (Lima et al., 1997a) and adenosine mononucleotides (Lima et al., 1997b). When we prepared stable, crystalline complexes of Fhit (Brenner et al., 1997b) with nonhydrolyzable ApppA analogs (Blackburn et al., 1998), we were able to determine how binding Ap_nA analogs alters the surface properties of Fhit (Pace et al., 1998). As shown in Figure 2, Fhit binds two Ap_nA molecules per protein dimer in a manner that fills a deep, positively charged groove with all of the phosphates of both Ap_nA molecules (Pace et al., 1998). Although a 21-residue segment that is disordered in all Fhit crystal structures could potentially have a role in transmitting the signal that Fhit is bound to Ap_nA, the primary signal appears to be the presentation of surface phosphates and adenosine moieties of Ap_nA by the protein dimer. Much as protein function can be altered by covalent protein phosphorylation, binding of Ap_nA substrates to Fhit appears to be an alternative means to modify protein function by reversible phosphorylation (Brenner, 1999). The intrinsic Ap_nA hydrolase activity of Fhit would be expected to return Fhit to the ground state (Pace et al., 1998).

NitFhit, a Fhit-associated protein and candidate signaling partner

When proposed, the Fhit-Ap_nA signaling model required two types of information that were not available at the time. First, because Ap_nA would have to compete for Fhit with more abundant mononucleotides such as ATP, it was important to determine the binding constants for individual Ap_nA species and for related compounds that would compete for Fhit active sites. Second, if Fhit-Ap_nA is a signaling complex, then the effector to which Fhit-Ap_nA signals would have to be identified.

Fluorescent and fluorigenic Ap_nA analogs have been used to determine how well Fhit binds Ap_nA vis a vis competing compounds. While Fhit exhibits little K_m discrimination between ApppA and AppppA (2.0 μM vs. 2.6 μM), binding to purine mononucleotides is almost 100-fold weaker. Surprisingly, inorganic pyrophosphate was a more effective inhibitor than purine mononucleotides by ~10-fold (A. Draganescu, S.C. Hodawadekar, K.R. Gee, and C. Brenner, unpublished data). These data suggest a hierarchical means for forming Fhit-Ap_nA complexes. The ground state of the enzyme is likely bound to pyrophosphate to the exclusion of ATP. Upon elevation of Ap_nA levels (Vartanian et al., 1996, 1997), the enzyme is predicted to exchange pyrophosphate for Ap_nA (A. Draganescu, S.C. Hodawadekar, K.R. Gee, and C. Brenner, unpublished data).

In flies and worms, Fhit is encoded as a natural fusion protein with members of the nitrilase superfamily (Pekarsky et al., 1998). Nitrilases are plant and bacterial enzymes that convert nitriles (such as indoleacetonitrile) to the corresponding acids (such as indoleacetic acid) plus ammonia by addition of two water molecules. Though invertebrates are unique in encoding Fhit as NitFhit fusion proteins, animal-type nitrilase homologs were cloned from the human and murine systems. In mouse, Nit1 and Fhit mRNAs accumulate in proportionate levels in seven of eight tissues examined (Pekarsky et al., 1998). Current evidence suggests that the mechanism of Fhit-dependent tumor suppression is the induction of apoptosis (Ji et al., 1999; Sard et al., 1999). Reflecting this information and depicted in Figure 3, the current model of Fhit function proposes that Fhit-Ap_nA stimulates a proapoptotic enzymatic activity of Nit.

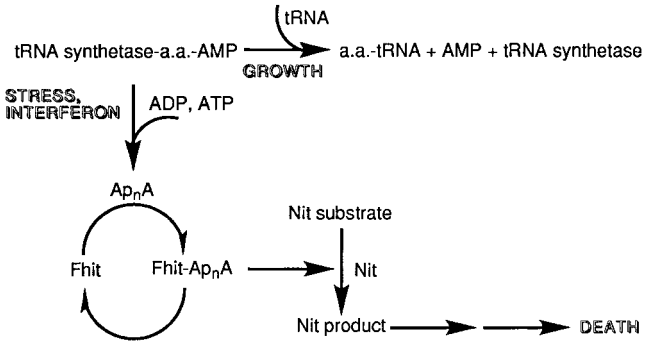

Fig. 3. A model for Fhit-Ap_nA and Nit function in proapoptotic tumor suppression. According to this model, stress signals cause tRNA synthetases to produce Ap_nA rather than deliver amino acids to tRNA. Fhit-Ap_nA complexes would then activate a proapoptotic activity of Nit proteins.

IF FHIT IS A RECEPTOR FOR AP_NA, THEN WHAT IS HINT?

The universal conservation of Hint orthologs suggests that Hint performs a fundamental function in all cells. However, deletion of the single Hint homolog, Hnt1, in *S. cerevisiae* has yet to shed light on this function. Laboratory yeast strains devoid of Hnt1 can grow, divide, mate, sporulate, undergo pseudohyphal development, and survive all stresses they have been challenged with in a manner indistinguishable from wild-type strains (P. Bieganowski and C. Brenner, unpublished data). It would appear likely that yeasts know how to do things that yeast geneticists have yet to assay. Beyond the structure-based prediction that Hint homologs are conserved as nucleotide-binding proteins (Brenner et al., 1997a), we cannot yet determine their function in living cells. Despite our own commitment of several person-years of effort to assign a function to Hint and Hint homologs, we are frequently surprised to find unsubstantiated references and database annotations identifying Hint as a protein kinase C (PKC) inhibitor and a zinc-binding protein. Here, in an effort to clarify what has become a misleading literature, we review how these references came about and try to set the record straight.

Hint is not a PKC inhibitor

The sequence of bovine Hint first entered protein databases, misidentified as PKC inhibitor-1 (PKCI-1; McDonald and Walsh, 1985; Pearson et al., 1990). Although no one ever reported rabbit Hint to be a PKC inhibitor and work on rabbit Hint refuted the PKCI-1 designation (Brenner et al., 1997a; Gilmour et al., 1997), the misidentification is so pervasive that releases 35 through 37 of SWISSPROT annotate rabbit Hint as though it were a PKC inhibitor. Remarkably, the same annotations are associated with Hint homologs from bacteria, archaea, and protista, organisms that do not have homologs of PKC.

In an attempt to identify heat-stable protein fractions from brain cytosol that inhibited PKC, two chromatographic fractions were purified to homogeneity (McDonald et al., 1987). All fractions contained EGTA and thus, in retrospect, it is not surprising that the fractions exhibited heat-stable PKC inhibitory activity. Nonetheless, two fractions, named PKCI-1 (Pearson et al., 1990) and PKCI-2 (Mozier et al., 1990), for PKC inhibitors 1 and 2 were obtained in high yield and sequenced by Edman degradation. The sequences of these fractions are, in fact, bovine Hint (Brenner et al., 1997a) and FKBP12 (Albers et al., 1991; Walsh, 1991).

The fraction formerly known as PKCI-2, shown by Stuart Schreiber and coworkers to be the 12-kDa FK506-binding protein (Albers et al., 1991), was shown to be chromatographically separable from PKC inhibitory activity and the PKCI-2 designation was withdrawn in 1991 (Walsh, 1991). In the same year, the claim that PKCI-1 was a PKC inhibitor was withdrawn with the observation that the major PKC inhibitory activity from bovine brain was in a heat-labile rather

than a heat-stable fraction (Fraser and Walsh, 1991). Unfortunately, the retraction of the PKCI-1 sequence as a bona fide PKC inhibitor (Fraser and Walsh, 1991) has not been noticed in some circles and references to Hint as PKCI persist to this day (Juengel et al., 1998; Klein et al., 1998). Adding to the confusion, there is a widely used molecule named PKCI, consisting of the pseudosubstrate sequence from residue 19 to 31 of PKC, that is an authentic PKC inhibitor (De Zeeuw et al., 1998). Hint has been tested repeatedly for PKC inhibitory activity and it has none (Gilmour et al., 1997; Klein, 1997). The scientific community would be well served to limit the use of the term PKCI to authentic PKCI (De Zeeuw et al., 1998) and to refrain from referring to polypeptides as PKCI that are not PKCI (Fraser and Walsh, 1991; Walsh, 1991).

The reason that Hint re-emerged as "PKCI-1" in the mid-1990s after its sequence was withdrawn as a PKC inhibitor (Fraser and Walsh, 1991) relates to two findings with the yeast two-hybrid interaction trap. Two groups cloned partial Hint cDNAs in independent two-hybrid screens and, motivated by still extant references to PKCI-1 in protein databases and the potential to implicate a putative PKC inhibitor with the bait proteins, named these clones human PKCI-1 (Brzoska et al., 1995; Lima et al., 1996). These classifications occurred prior to the adoption of the Hint nomenclature (Brenner et al., 1997a) and seemed to make sense at the time, especially when one considers that references to discredited PKCI-1 literature persist to this day. The bait proteins in these two interaction traps were Atdc, an early candidate (but not authentic) Ataxia-Telangiectasia complementing gene product (Brzoska et al., 1995) and the amino-terminus of PKC β (Lima et al., 1996).

Despite the fact that full-length human Hint cDNA sequences are abundant in expressed sequence tag databases, in neither case was a two-hybrid interaction obtained with full-length Hint cDNA. In the former screen, sequences encoding residues 47–126 of Hint were cloned (Brzoska et al., 1995). In the latter, residues 32–126 were cloned (Lima et al., 1996). Given what is appreciated today about two-hybrid artifacts, cloning fragments of a single domain protein in two-hybrid screens would suggest cautious interpretation. A lack of functional assays for Atdc has prevented validation of the reported Atdc-Hint interaction. However, extensive analysis of PKC β activity and localization failed to provide evidence that Hint has any interaction with PKC and showed that only truncated forms of Hint produced the two-hybrid artifact (Klein, 1997). For reasons that are not clear, these investigators continue to refer to Hint as PKCI (Klein et al., 1998). Although the two-hybrid interaction with PKC does not reflect an authentic interaction (Klein, 1997), the article that reported it was significant in providing the crystal structure of human Hint (Lima et al., 1996).

Persistent errors in the scientific literature have economic consequences. For example, scientists from Incyte Pharmaceuticals, citing literature that claimed Hint as PKCI, were issued U.S. Patent 5,773,580 for "Human Protein Kinase C Inhibitor Homolog" (Au-Young et al., 1998).

Hint is not a zinc-binding protein and the HIT motif is not a zinc-binding site

It was reported that "bovine PKCI-1" dried onto nitrocellulose filters binds $^{65}Zn^{2+}$ (Pearson et al., 1990). Subsequently, peptides containing the HIT motif (His-ϕ-His-ϕ-His-ϕ-ϕ, in which ϕ specifies a hydrophobic amino acid) were synthesized, spotted on nitrocellulose, and probed with radioactive zinc (Mozier et al., 1991). Unfortunately, it was never clearly stated that Hint is insoluble in the presence of zinc (C. Brenner and J.M. Lowenstein, unpublished data; published data in the methods sections of Pearson et al., 1990, and Lima et al., 1996 bear this out). Thus, no amount of zinc associated with filter-bound peptides should be sufficient to convince a reviewer that Hint is a zinc-binding protein. The frequently cited observation that synthetic, nitrocellulose-bound peptides derived from Hint bind zinc (Mozier et al., 1991) is not wrong but neither does it inform one about the behavior of Hint in solution. So penetrating was the impression created by this observation that a crystal structure of the "zinc-form" of Hint was published that had no zinc electron density and no change in structure from the nonzinc form of the protein (Lima et al., 1996). Whether zinc is a specific or nonspecific denaturant of Hint remains a matter of speculation because, as Peter Medawar said, "research is surely the art of the soluble" (Medawar, 1964). In any case, X-ray crystallography has made it abundantly clear that the HIT motif forms the conserved α-phosphate binding site in Hint (Brenner et al., 1997a) and Fhit (Lima et al., 1997b; Pace et al., 1998) and it is hoped that protein database annotations are revised to reflect these facts.

FUTURE DIRECTIONS FOR RESEARCH ON HIT PROTEINS

Biochemical studies indicate that Hint possesses enzymatic activity, cleaving ADP to AMP plus inorganic phosphate (Lima et al., 1997b), and suggest that Hint-nucleotide complexes (Brenner et al., 1997a) may represent enzyme-product complexes. The specificity constant for this reaction, 8.5 s^{-1} M^{-1} (Lima et al., 1997b), is >4,000,000-fold lower than that of Fhit cleaving ApppA (Pace et al., 1998), suggesting that ADP may not be the biologically important substrate. It is of great interest to determine the nucleotide specificity of Hint and to establish under what conditions such nucleotides are produced in cells.

To bring research on Hint and Fhit to the next level, it is of utmost importance to establish genetic systems to assay their function and to continue to probe the requirements for nucleotide binding and hydrolysis for function (Pace et al., 1998; Siprashvili et al., 1997). Manipulation of cellular nucleotide levels may prove to be more challenging, as introduction of any polyanionic species through lipid bilayers is problematic. Frameworks for nonhydrolyzable substrates that retain good binding to HIT proteins are in place (Blackburn et al., 1998; Liu et al., 1999; Pace et al., 1998). Whether these compounds can be elaborated with unmaskable esters to allow cell penetration and intracellular activation remains to be seen. Finally, it is expected that the Fhit-Ap_nA complex will regulate proteins, some of which may emerge as specific drug targets for the high

fraction of human epithelial cancers that are derived from early inactivation of the *FHIT* gene (Huebner et al., 1999).

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