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Advances in Brief

The Fhit Tumor Suppressor Protein Regulates the Intracellular Concentration of Diadenosine Triphosphate but not Diadenosine Tetraphosphate[1]

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Abstract

To determine the role of the *FHIT* tumor suppressor gene product, a diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A) hydrolase, in the regulation of the concentration of Ap_3A and diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) *in vivo*, the levels of the adenosine(5′)triphospho(5′)nucleoside (Ap_3N) and adenosine(5′)tetraphospho(5′)nucleoside (Ap_4N) families were measured by luminometry in a number of human cell lines and correlated with the expression of Fhit determined by immunoblotting. Fhit-positive cells had no Ap_3N or a very low level of Ap_3N, whereas most Fhit-negative cells had Ap_3N in the range 0.2–0.9 pmol/10^6 cells. Ap_4N (mean value, 0.17 pmol/10^6 cells) did not correlate with Fhit expression. The results suggest that Fhit efficiently metabolizes Ap_3A and Ap_3N but not Ap_4A or Ap_4N *in vivo*.

Introduction

Abnormalities in the expression or structure of the human *FHIT* gene, which spans the FRA3B fragile site at 3p14.2, have been observed in a wide variety of common carcinomas and may represent one of the earliest changes in neoplastic transformation (1–3). Such tumors and derived tumor cell lines express little or no Fhit protein. Reexpression of Fhit protein by transfection or transduction of such cells greatly reduces their tumorigenicity in nude mice, suggesting that Fhit has a tumor suppressor function (4, 5). *In vitro*, Fhit has Ap_3A[3] hydrolase activity (6). Curiously, this activity does not appear to be required for tumor suppression because a mutant Fhit that binds but does not hydrolyze Ap_3A is as effective as the wild type (4, 7). This has led to the proposal that a Fhit-nucleotide complex may be a component of a new antiproliferative signaling pathway (7, 8). Nevertheless, because Fhit binds and hydrolyzes both Ap_3A and Ap_4A (a nucleotide commonly deemed to be associated with proliferation) *in vitro* (6), any model of Fhit function should take this into account. The very few measurements of intracellular Ap_3A concentration that have been made in eukaryotes (9) suggest it to be present at a level 5–10-fold higher than that of Ap_4A; however, no correlations with Fhit expression have been made. Here, we report measurements of Ap_3N and Ap_4N made in a range of Fhit-positive and Fhit-negative cell lines and show that, in the majority of cases, Ap_3N (and thus Ap_3A) is undetectable in Fhit-positive cells but is readily assayed in Fhit-negative cells, whereas the level of Ap_4N (and thus Ap_4A) is not influenced by Fhit expression. The significance of this with regard to Fhit-mediated signaling is discussed.

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[3] The abbreviations used are: Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_3N, adenosine(5′)triphospho(5′)nucleoside; Ap_4N, adenosine(5′)tetraphospho(5′)nucleoside.

Materials and Methods

Human Cell Lines. The U2020 small cell lung carcinoma and RC8 renal carcinoma cells were generously provided by Dr. K. Huebner (Kimmel Cancer Center, Philadelphia, PA). HRT18 rectal adenocarcinoma, RT112 bladder transit cell carcinoma, 2780 ovarian carcinoma, and I407 embryonic jejunal epithelial cells were from the Oncology Research Unit, University of Liverpool (Liverpool, United Kingdom). Calu3 non-small cell lung carcinoma cells were the gift of Dr. C. Walker (Clatterbridge Cancer Research Trust, Wirral, United Kingdom). Adenovirus 5-transformed 293 kidney cells were purchased from European Collection of Animal Cell Cultures (United Kingdom), and HL60 promyelocytic leukemia cells were provided by Dr. D. M. Tidd (Department of Biochemistry, University of Liverpool, United Kingdom). All cell lines were grown in recommended media containing 10% FCS.

Nucleotide Assays. The assay used detects mixed nucleotides of the form Ap_3N and Ap_4N, where N is any base, and not just the diadenosine nucleotides. For each determination, six 90-mm dishes of cells were grown to about 80% confluence. Cells were counted in two dishes, and cells in the remaining four dishes were extracted separately as described below. Cell layers were washed briefly with 4 ml of warm serum-free medium, the medium was removed rapidly, and 3 ml of ice-cold 0.4 M trichloroacetic acid were added. Cells were scraped into a cold tube, the dishes were rinsed with two 1-ml portions of trichloroacetic acid, and the combined 5-ml extract was left at 4°C for 15 min. Five ml of 0.6 M tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane were added, and the tube was shaken for 5 min and then centrifuged at 1,000 × *g* for 5 min. The upper aqueous layer (4.4 ml) was mixed with 110 μl of 2 M Tris-HCl (pH 8.5), 0.2 M magnesium acetate, and 10 units of shrimp alkaline phosphatase (Boehringer, Germany) and incubated for 60 min at 37°C to hydrolyze mononucleotides. Next, 100 μl of a 50% (v/v) DEAE-Sephacel suspension in 20 mM Tris-HCl (pH 7.6) were added to adsorb the remaining nucleotides. After a 10-min shaking, the suspension was centrifuged for 1 min at 10,000 × *g*. After discarding the supernatant, the pellet was washed with three 1.5-ml portions of water and then shaken for 5 min with 0.5 ml of 1.0 M triethylammonium bicarbonate (pH 7.5) to elute the dinucleotides. After centrifugation for 1 min, the supernatant was removed, the pellet was reextracted in the same manner, and the combined supernatants were freeze-dried. After redissolving in 400 μl of 25 mM HEPES-NaOH (pH 7.8), 5 mM magnesium acetate and 3 units of shrimp alkaline phosphatase were added, and the tubes were incubated for 30 min at 37°C, followed by a 15-min incubation at 65°C. Each sample was then split in two, and the Ap_3N and Ap_4N content was measured in each by luminometry as described previously (10). In cases where no Ap_3N was detected by the above-mentioned procedure, results were confirmed by repeating the extraction with a greater number of cells (six 140-mm dishes) and using appropriately adjusted reagent volumes. For calculations, the recovery of nucleotides from cell extracts was assumed to be 100%.

Western Blot Analysis. Analysis of Fhit protein expression was carried out by Western blotting. Cell lysates were obtained essentially as described previously (6). Samples of lysate containing 15 μg of protein were then subjected to SDS-PAGE in a 15% gel. The gel was blotted onto nitrocellulose, and the membrane was blocked overnight at 4°C in PBS containing 0.03% (v/v) Tween 20 and 3% (w/v) dried milk (blocking buffer). Fhit protein expression was detected with a polyclonal rabbit antibody raised against a synthetic COOH-terminal peptide of Fhit (ZP54; Zymed, South San Francisco, CA). The blot was incubated in primary antibody at a concentration of 0.25 μg/ml for 3 h at room temperature, washed with blocking buffer, and then incubated for 1 h in a 1:5000 dilution of peroxidase-conjugated goat antirabbit IgG (Bio-Rad). The blot was then washed in PBS containing 0.03% (v/v)

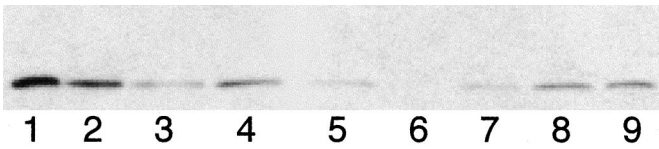

Fig. 1. Immunoblot detection of Fhit protein in different human cell lines. Cell identification and experimental procedures are described in "Materials and Methods." *Lane 1*, 293; *Lane 2*, U2020; *Lane 3*, RC8; *Lane 4*, HRT18; *Lane 5*, HL60; *Lane 6*, RT112; *Lane 7*, 2780; *Lane 8*, I407; *Lane 9*, Calu3.

Tween 20, and the Fhit protein was visualized using the enhanced chemiluminescence detection reagent (Amersham, Little Chalfont, United Kingdom).

Results and Discussion

The level of Fhit protein expression was determined in a number of tumor cell lines and in nontumor 293 kidney cells by Western blotting using an antibody raised against a COOH-terminal Fhit peptide. This shows Fhit as a M_r 16,800 polypeptide. Because inactivating point mutations have rarely been described in the *FHIT* gene, the presence of the full-length Fhit protein is taken to indicate an active Ap_3A hydrolase. This analysis revealed levels of expression varying from strong to moderate (293, U2020, RC8, HRT18, I407, and Calu3 cells) to low or undetectable (HL60, RT112, and 2780 cells; Fig. 1). High expression of Fhit protein in 293, U2020, and Calu3 cells is in agreement with previous data (11, 12).

The level of Ap_4N determined in these cells varied from 0.029 pmol/10^6 cells (U2020) to 0.55 pmol/10^6 cells (RC8), with an average of 0.17 pmol/10^6 cells (Table 1). These values are similar to previous data, which typically measure Ap_4A or Ap_4N in unstressed cultured cells within the range of 0.1–0.5 pmol/10^6 cells with extreme values of 0.05 and 7.5 pmol/10^6 cells (9). The differences are largely a function of cell volume; for example, U2020 cells are very small, whereas RC8 cells are large. Given a typical cell volume of 2 pl, 1 pmol/10^6 cells equates roughly to a concentration of 0.5 μM. There was no indication from our results that the level of Ap_4N was affected in any way by the degree of Fhit expression. In contrast, the level of Ap_3N was markedly affected by Fhit status in the majority of the cells. Fhit-negative cells (HL60, RT112, and 2780 cells) had readily detectable Ap_3N (0.2–0.9 pmol/10^6 cells) at a level above that of Ap_4N (Table 1); however, in most Fhit-positive cells, Ap_3N was either undetectable (293, U2020, and RC8 cells) or very low (HRT18 cells), indicating that Fhit is an effective Ap_3N hydrolase *in vivo* and that it normally maintains a very low level of intracellular Ap_3A and Ap_3N.

At first sight, two cell lines did not conform to this pattern. Calu3 cells at passage 29 were Fhit positive and showed a single band corresponding to normal Fhit mRNA when examined by reverse transcription-PCR (data not shown), yet they had a low but significant level of Ap_3N (0.15 pmol/10^6 cells). Surprisingly, as these cells were passaged in culture, the level of Ap_3N increased with passage number, whereas the level of Ap_4N remained constant (Fig. 2; Table 1). The most probable explanation is that before passage 29, these cells were Fhit positive (no Ap_3N) with one normal and one defective *FHIT* allele and that during culture, loss of the second allele occurred spontaneously in one cell. The growth advantage conferred by this loss allowed a more rapid clonal expansion of the homozygous deletant so that it gradually replaced the original cells during subculture. Thus, the proportion of Fhit-negative (high Ap_3N) cells increased with passage number. Unfortunately, this could not be tested directly because the culture was lost at passage 37, and the finding could not be repeated. Although it is unusual to report an irreproducible result, such a lack of reproducibility would not be surprising if the underlying cause was a random mutational event. It is highly improbable that the trend of increasing Ap_3N seen in Fig. 2 could be due to experimental error; therefore, these data are included. Fhit-positive I407 cells also had a high level of Ap_3N (Table 1). In this case, no change in the Ap_3N:Ap_4N ratio was noted with increased passage number. These cells may have been particularly responsive to a serum factor in the growth medium that stimulated Ap_3N synthesis. IFN is one such factor that is known to increase intracellular Ap_3A (13).

The general conclusion from these results is that Ap_3N in Fhit-positive cells is normally maintained below the level of 0.01 pmol/10^6

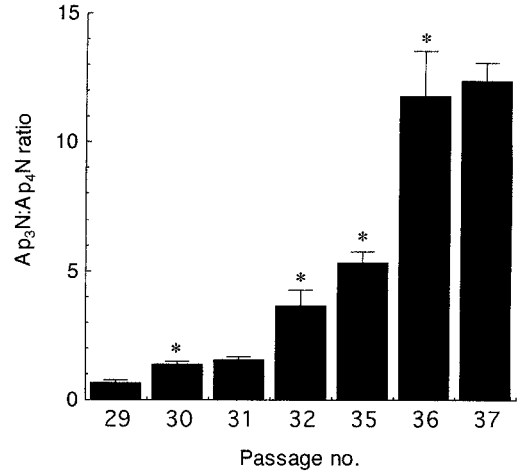

Fig. 2. Change in Ap_3N:Ap_4N ratio in Calu3 cells with passage number. Cells were grown in DMEM containing 10% FCS. They were split 1:3 every 3–4 days and grown to confluence, and nucleotide levels were determined as described in "Materials and Methods." Each value is the mean ± SE (n = 4). *, the statistical significance determined by Student's t test ($P < 0.05$) of the difference in the ratio at one passage number compared to the previous one. The mean ± SE concentration of Ap_4N for all passage numbers was 0.25 ± 0.02 pmol/10^6 cells (n = 28).

Table 1 *Intracellular concentration of Ap_3N and Ap_4N in different human cell lines*

Cells	Ap_4N[a] (pmol/10^6 cells)	Ap_3N[a] (pmol/10^6 cells)	Ap_3N/Ap_4N[b]	Fhit status[c]
293	0.16 ± 0.01 (n = 12)	ND[d] (n = 12)		+++
U2020	0.029 ± 0.005 (n = 11)	ND (n = 11)		++
RC8	0.55 ± 0.04 (n = 20)	ND (n = 20)		+
HRT18	0.082 ± 0.012 (n = 8)	0.071 ± 0.006 (n = 8)	0.97 ± 0.13	++
HL60	0.12 ± 0.01 (n = 10)	0.22 ± 0.05 (n = 10)	1.86 ± 0.39	+/−
RT112	0.095 ± 0.016 (n = 10)	0.23 ± 0.08 (n = 10)	2.38 ± 0.48	−
2780	0.12 ± 0.02 (n = 12)	0.91 ± 0.14 (n = 12)	9.35 ± 1.04	+/−
I407	0.097 ± 0.019 (n = 10)	0.75 ± 0.07 (n = 10)	11.8 ± 2.6	+
Calu3	0.25 ± 0.02 (n = 28)	0.15–1.7[e]	0.6–12.4[e]	+

[a] Nucleotides were measured luminometrically as described in "Materials and Methods." Each value is the mean ± SE. Number of determinations (n) in brackets.
[b] Each value is the mean of the individual ratios from each determination (± SE) rather than the ratio of the averaged Ap_3N and Ap_4N concentrations for all determinations.
[c] Fhit status was determined visually from the immunoblot in Fig. 1.
[d] ND, not detected. The detection limit of the assay is 0.01 pmol/10^6 cells.
[e] See Fig. 2.

cells by the hydrolase activity of the Fhit protein and that loss of Fhit leads to an increase in intracellular Ap_3N of at least 2 orders of magnitude. Loss of Fhit does not significantly affect Ap_4N, the level of which is presumably dependent on the unrelated Ap_4A hydrolase, a member of the nudix hydrolase (MutT motif protein) family (14). In addition, stimulation of Ap_3N synthesis, for example, by induction or activation of tryptophanyl-tRNA synthetase (13, 15), may lead to a significant level of Ap_3N in Fhit-positive cells. These results do not exclude the possibility that in particular circumstances, Ap_3N may also be metabolized by other proteins, *e.g.*, nudix hydrolases (14) or other Ap_3A-binding proteins (16).

How do these results impact on proposed models for Fhit-mediated signaling? Reexpression of Fhit in Fhit-negative tumor cells leads to an accumulation of cells in S phase and an increase in apoptotic cells (5, 17), whereas an increase in intracellular Ap_4A may also induce apoptosis (18, 19). Brenner *et al.* (8) have recently proposed that the ground state of Fhit is a Fhit-PPi complex and that an increase in the level of diadenosine 5′,5‴-P^1,P^n-polyphosphate displaces PPi, generating a Fhit-diadenosine 5′,5‴-P^1,P^n-polyphosphate complex that signals cell death. Our results would suggest that this complex is most likely to be a Fhit-Ap_4A complex. Although increased Ap_4A is associated with proliferating cells, there is no direct evidence that it stimulates or is otherwise required for proliferation *per se* as is often assumed; indeed, one of us (A. G. M.; Ref. 20) has recently proposed its involvement in S-phase checkpoint control, regulating replicon initiation and apoptosis after DNA damage in a manner reminiscent of p53. Ap_4A is better suited as a positive effector of Fhit function than is Ap_3A due to the 80-fold lower catalytic constant of Fhit for the former substrate and the consequent longer lifetime of the proposed Fhit-Ap_4A signaling complex (6). Thus, an increase in Ap_4A above the normal level would promote the still poorly defined Fhit-mediated responses, which may include proliferation, checkpoint control, or apoptosis, whereas an increase in the more rapidly degraded Ap_3A would counter these responses not by positive signaling but by occupation of Fhit in a futile turnover reaction, thus favoring cessation of proliferation and differentiation (13, 18).

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