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Communication

Human diadenosine triphosphate hydrolase: Preliminary characterisation and comparison with the Fhit protein, a human tumour suppressor✪★

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Human platelets diadenosine triphosphatase was characterised and compared with the Fhit protein, a human tumour suppressor with diadenosine triphosphatase activity. Both enzymes exhibit similar K_m, are similarly activated by Mg^{2+}, Ca^{2+} and Mn^{2+}, and inhibited by Zn^{2+} and suramin. However, they are differentially inhibited by Fhit antibodies and exhibit differences in gel-filtration behaviour.

In the last decade diadenosine polyphosphates (Ap_nA) have emerged as a family of signalling dinucleotides involved in a great diversity of cellular functions [1, 2]. Although the biosynthesis of Ap_nA appears to be rather non-specific, highly specific intracellular

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Abbreviations: Ap_3Aase, diadenosine triphosphate hydrolase (EC 3.6.1.29); Ap_4Aase, asymmetrical diadenosine tetraphosphate hydrolase (EC 3.6.1.17); Ap_nA, diadenosine 5′,5‴- P^1,P^n-polyphosphates; Ap_3A, diadenosine 5′,5‴- P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴- P^1,P^4-tetraphosphate; Ap_5A, diadenosine 5′,5‴-P^1,P^5-pentaphosphate; ε-(Ap_nA), di(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^n-polyphosphates; ε-(Ap_2A), di(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^2-pyrophosphate; ε-(Ap_3A), di(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^3-triphosphate; ε-(Ap_4A), di(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^4-tetraphosphate; ε-AMP, ε-ADP and ε-ATP refer to the 1,N^6-etheno derivatives of AMP, ADP and ATP respectively; ε stands for "etheno". Fhit and *FHIT* are fragile histidine triad protein and the gene coding for it, respectively.

Ap_nA cleaving enzymes, in addition to non-specific phosphodiesterases, are involved in Ap_nA degradation [3, 4]. Diadenosine triphosphate hydrolase or Ap_3Aase (EC 3.6.1.29) is an enzyme that specifically cleaves dinucleoside triphosphates; Ap_3A is the preferred substrate, the products of its cleavage being AMP and ADP. This enzyme, a 30–35 kDa polypeptide in mammals, was first discovered in rat liver [5, 6]; later the enzyme from various eukaryotic organisms was investigated [4]. In spite of those and other studies, the biological significance of the enzymatic cleavage of Ap_3A and related dinucleotides has not been clarified. Ap_3A appears to be involved in intracellular regulation of ATP-gated K^+ channels [7], in interferon action [8], and in the mechanisms of differentiation and apoptosis [9]. A series of recent findings has established that human Fhit protein, the product of the tumour suppressor gene *FHIT*, is a homodimeric enzyme of about 32 kDa displaying Ap_3Aase activity [10–12], and is able to suppress tumorigenicity in cancer cells lacking endogenous Fhit protein [13]. These results strongly suggest that Ap_3A and/or related dinucleotides are involved in tumour suppression signalling, which has markedly renewed interest in Ap_3A and Ap_nA-interacting proteins. The similarity between the catalytic properties of the previously described non-human Ap_3Aases and the Fhit protein [11, 14] suggests a close relationship among them, or even that they could be the same protein. However, the lack of data on a human Ap_3Aase hinders its comparison with Fhit. In this communication we present a preliminary characterisation of partially purified human platelet Ap_3Aase, and compare its properties with those of the Fhit protein. Fluorogenic derivatives of Ap_nA [15, 16] were used as enzyme substrates. To the best of our knowledge this is the first study reporting a characterisation of a diadenosine triphosphate hydrolase isolated from human material.

MATERIALS AND METHODS

Materials. Concentrates of human platelets from healthy donors were obtained from the Servicio de Hematología, Hospital de La Candelaria (Santa Cruz de Tenerife). Human Fhit protein expressed in *E. coli*, a gift from Dr. C. Brenner, was obtained as described [17]. Fhit protein was at least 95% pure as checked by SDS/PAGE under reducing and non-reducing conditions; in both cases a major band corresponding to the 16.8 kDa monomer was observed. The fluorogenic substrates ε-(Ap_nA) n = 2–4 were prepared by chemical modification of Ap_nA (Sigma, St. Louis, MO, U.S.A.) and their purity checked as described [18]. *Crotalus durissus* phosphodiesterase was from Boehringer (Manheim, Germany). Sephacryl S-100 HR, Sephacryl S-200 HR and DEAE-Sephacel were from Pharmacia Biotech. (Uppsala, Sweden). Suramin was from RBI (Natick, MA, U.S.A.). Molecular mass markers for gel filtration chromatography were from Sigma. Polyclonal anti-Fhit antibodies (ZR44 and ZP54) were from Zymed (San Francisko, CA, U.S.A.). All salts and acetonitrile HPLC grade were from Merck (Darmstadt, Germany).

Enzyme purification. Concentrates of human platelets (700 ml) were processed to eliminate other contaminating blood cells and to fractionate platelet protein between 40% and 80% of ammonium sulphate saturation according to [19]. The protein pellet after 80% ammonium sulphate precipitation was redissolved in 50 mM Hepes KOH, pH 7.0, 0.1 M KCl, 5% glycerol (26 ml), divided into three aliquots and each subjected to gel-filtration chromatography on a Sephacryl S-200 column (26 mm × 900 mm) equilibrated in the same buffer and eluted at 2.0 ml/min. Fractions of 6.0 ml were collected. The column was previously calibrated with Blue dextran, bovine serum albumin, carbonic anhydrase and cytochrome *c*. Fractions were assayed by non-continuous fluorimetric assays to localise

those containing Ap_3Aase and asymmetrical Ap_4Aase activity. Fractions containing Ap_3Aase and Ap_4Aase were pooled (180 ml) and concentrated to 20 ml, the buffer being changed to 20 mM phosphate, pH 7.5, 10% glycerol, using an Amicon ultrafiltration cell with a 10 kDa cut-off membrane. Two 10 ml aliquots of the concentrated Sephacryl extract were applied onto a DEAE-Sephacel column (16 mm × 150 mm) equilibrated with 20 mM phosphate, pH 7.5, 10% glycerol, and the column eluted at a flow rate of 0.5 ml/min with 75 ml of the same buffer, and then with a linear gradient of 200 ml 0–0.3 M KCl in the equilibrating buffer. The column was washed with 25 ml of 1.0 M KCl and re-equilibrated with the initial buffer. Fractions of 4.0 ml were collected and assayed for Ap_3Aase and Ap_4Aase activity using the non-continuous fluorimetric assay. Active fractions containing Ap_3Aase and Ap_4Aase were pooled separately, concentrated in 50 mM Hepes buffer, pH 7.0, 10% glycerol and stored in 250 μl aliquots at –40°C.

Enzymatic assays. Non-continuous fluorimetric assays were used to detect Ap_3Aase, Ap_4Aase and non-specific phosphodiesterase in the chromatographic eluates. Sample mixtures (300 μl) containing, 50 mM Hepes/NaOH, pH 7.0, 4 mM $MgCl_2$, 0.2 mg/ml BSA, protein extract (5–50 μl) and 2 μM substrate (ε-(Ap_3A), ε-(Ap_4A) or ε-(Ap_2A) to detect Ap_3Aase, Ap_4Aase or phosphodiesterase, respectively) were prepared in Eppendorf tubes and incubated at 37°C for 10–60 min. Reactions were stopped by cooling in an ice-water bath and fluorescence was immediately measured using a Hitachi F-2000 spectrofluorimeter (λ_{ex} 305 nm, λ_{em} 410 nm) to detect hydrolase activity.

Continuous fluorimetric assays for quantitative purposes were performed in quartz microcuvettes to record time-dependent fluorescence increase associated with ε-(Ap_3A) hydrolysis as previously detailed [14]. Assay mixtures (250 μl) contained 50 mM Hepes/NaOH, pH 7.0, 4 mM $MgCl_2$, 0.2 mg/ml BSA, the required concentration of substrate and enzyme (Ap_3Aase or Fhit) and were incubated at 37°C under stirring for up to 5 min. To determine K_m for ε-(Ap_3A), initial reaction rates were measured at fixed substrate concentrations ranging between 0.1 and 20 μM. Ap_3Aase and Fhit protein concentrations were adjusted to obtain initial linear time-dependent fluorescence increases and total substrate consumption below 10%.

HPLC assays using ion-pair conditions used to identify reaction products and quantify reaction rates were performed as previously detailed [14].

Protein assays. Protein concentration was determined according to the Bradford method [20] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Protein extracts obtained by ammonium sulphate fractionation (40–80%) of platelet homogenates exhibit hydrolase activity on ε-(Ap_3A) and ε-(Ap_4A) due to Ap_3Aase and asymmetrical Ap_4Aase, respectively. The presence of asymmetrical Ap_4Aase in human blood cells has previously been reported [19, 21]. Hydrolase activity on ε-(Ap_2A), the substrate used to detect non-specific phosphodiesterase activity, was negligible. The specific activity of Ap_3Aase was 0.15 mU/mg. This is a low figure if compared to equivalent rat brain extracts with specific activities about 60 times higher (our own results, not shown and [4]).

Gel-filtration chromatography of platelet extracts on Sephacryl S-200 demonstrates the presence of two well differentiated but partially overlapping peaks of Ap_3Aase and Ap_4Aase activities, eluting as expected for proteins of molecular sizes of about 30 and 20 kDa, respectively (Fig. 1A). Fractions containing Ap_3Aase and Ap_4Aase were concentrated by ultrafiltration (Ap_3Aase specific activity was 1.4 mU/mg at this stage) and subjected to ion-exchange chromatography on DEAE-

Sephacel. A complete separation of both activities was observed: Ap_3Aase is clearly retarded in comparison with non-retained proteins but elutes at very low ionic strength, while Ap_4Aase elution requires higher salt nificant activity in the absence of added divalent cations is completely eliminated by 1 mM EDTA. Suramin and Zn^{2+} are inhibitory, with IC_{50} values of 2 and 20 μM, respectively (Fig. 2B), while F^- is not an inhibitor. The histidine

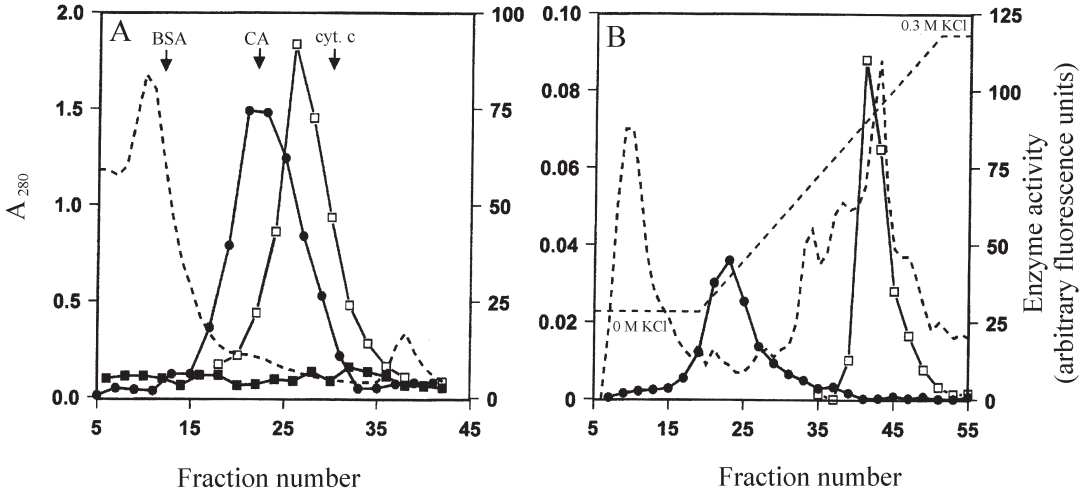

Figure 1. Chromatographic purification of Ap_3Aase and Ap_4Aase from human platelet extracts.

A. Gel filtration chromatography. A 9.0 ml sample of platelet protein fractionated between 40–80% of ammonium sulphate saturation was applied onto a Sephacryl S-200 column (26 mm × 900 mm). Fractions containing Ap_3Aase and Ap_4Aase were pooled, then concentrated. B. Ion-exchange chromatography. Concentrated eluate from gel-filtration was applied onto a DEAE-Sephacel column (16 mm × 150 mm). Fractions containing Ap_3Aase and Ap_4Aase were separately pooled, and concentrated. See Methods for details. Protein elution profile, A_{280} (- - -). Enzymatic activity profiles (—) are given in arbitrary fluorescence units: Ap_3Aase • , Ap_4Aase • , non-specific phosphodiesterase • . Arrows indicate the elution positions of the molecular mass markers bovine serum albumin (BSA, 67 kDa), carbonic anhydrase (CA, 29 kDa) and cytochrome *c* (cyt. c, 12.4 kDa).

concentration (Fig. 1B). Due to the scarcity of Ap_3Aase obtained and to the repeatedly reported instability of Ap_3Aases, further purification attempts, e.g. by affinity chromatography, were postponed and biochemical characterisation studies were performed with the enzyme obtained after the ion-exchange step. This preparation presents a specific activity of 34.0 mU/mg, with a purification factor of about 250 and 17% yield relative to the ammonium sulphate extract.

Human platelet Ap_3Aase exhibits hyperbolic kinetics (Fig. 2A) with a K_m value of 1.4 ± 0.4 μM for ε-(Ap_3A); V_{max} measured with ε-(Ap_3A) is about 80% of V_{max} using Ap_3A. Reaction products are ε-AMP and ε-ADP. The enzyme is activated by Mg^{2+}, Ca^{2+} and Mn^{2+}, maximal activity reached at 0.5–1 mM. Its sig-reagent diethyl pyrocarbonate rapidly inactivates the enzyme. Optimum pH is 7.0–7.2 in Hepes/NaOH and Hepes/KOH buffers. These properties are similar to those of other mammalian Ap_3Aases [4, 18, 22] although the human enzyme shows a lower K_m value for ε-(Ap_3A) compared with the rat brain and bovine adrenomedullary enzymes, which exhibit K_m values around 11 μM [18, 22]. We have recently reported that the Fhit protein recognises ε-(Ap_3A) as a substrate with K_m = 2.0 μM and that activators and inhibitors affect this Fhit-catalysed reaction and the activities of other mammalian Ap_3Aases in a similar manner [14].

Up to now the major differences between the Ap_3Aase activity from human platelets and the Fhit enzyme were in their apparent molec-

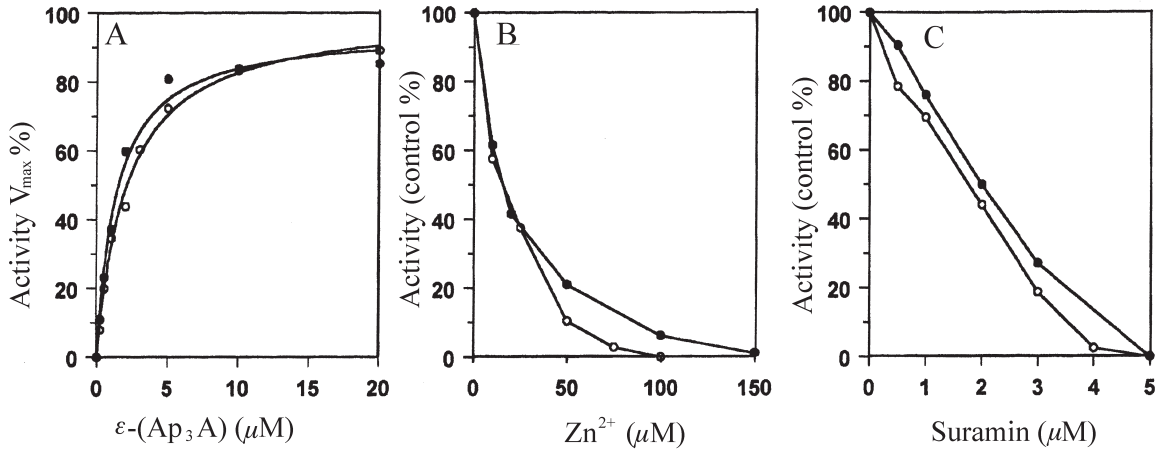

Figure 2. Comparison of the kinetics of ε-(Ap_3A) hydrolysis (A), inhibitory effects of Zn^{2+} (B) and suramin (C) on human platelet Ap_3Aase and Fhit protein activities.

Aliquots of platelet Ap_3Aase (DEAE-Sephacel step) and Fhit protein containing the same amount of enzymatic activity (0.10 mU) were assayed under identical conditions by continuous fluorimetric assays, as detailed in Methods, to determine the effects of substrate and inhibitor concentration on their hydrolase activity. In (A) reaction rates are expressed as % of V_{max}. In (B) and (C), reaction rates are expressed as % of activity measured at 20 μM substrate without inhibitor. Platelet Ap_3Aase ● , Fhit protein ● . Results are means of three experiments, each performed in duplicate. ±S.D. were less than 19 %.

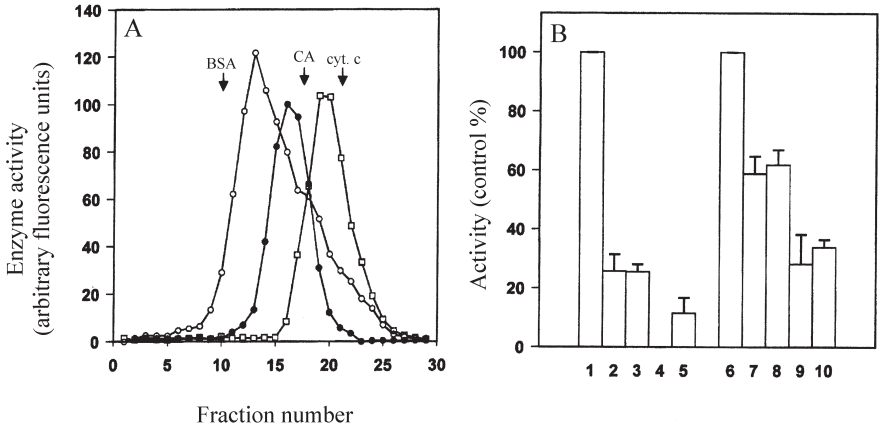

Figure 3. Elution profiles of human platelet Ap_3Aase and Fhit protein in gel-filtration chromatography (A) and immunoinhibition of human platelet Ap_3Aase and human Fhit protein enzymatic activities by Fhit antibodies (B).

In (A), samples of platelet Ap_3Aase (DEAE-Sephacel step) and Fhit protein were separately applied to a Sephacryl S-100 HR column (16 mm × 660 mm) previously calibrated with BSA, CA and cyt. c as indicated by the arrows. Elution profile of platelet Ap_4Aase (DEAE-Sephacel step) is also included. Enzyme activities were detected by non-continuous fluorimetric assays, as detailed in Methods, and are expressed in fluorescence units. The chromatographic profile shown for each enzyme is one of three independent, essentially superimposable profiles obtained. Ap_3Aase ● , Ap_4Aase ● , Fhit protein ● . In (B) aliquots of Ap_3Aase (DEAE-Sephacel step) and Fhit protein containing the same amount of enzymatic activity (0.05 mU) were incubated overnight at 4°C in the standard assay mixture (substrate omitted) with Fhit antibodies ZR44 or ZP54 each at 0.5 and 1.0 μg/ml as indicated, in a final volume of 300 μl. After adding 20 μM substrate, enzymatic activities were measured by continuous fluorimetric assays as detailed in Methods. Activities expressed as % of activity measured in samples incubated without antibody. 1, Fhit control; 2 and 4, + anti-Fhit ZR44 at 0.5 and 1 μg/ml, respectively; 3 and 5, + anti-Fhit ZP54 at 0.5 and 1.0 μg/ml, respectively; 6, Ap_3Aase control; 7 and 9, + anti-Fhit ZR44 at 0.5 and 1 μg/ml, respectively; 8 and 10, + anti-Fhit ZP54 at 0.5 and 1.0 μg/ml, respectively. Results are means ±S.D. of three experiments, each performed in duplicate.

ular masses and the response to Fhit antibodies. In gel-filtration chromatography, human Ap_3Aase elutes as a well-shaped peak corresponding to a polypeptide of about 32 kDa (Fig. 3A) similarly to the previously studied rat and bovine Ap_3Aases [4, 18]. SDS/PAGE indicates that this enzyme is a monomeric protein of about 30 kDa in mammals and 41 kDa in plants [23, 24]. Although it has been suggested that the Fhit protein functions *in vivo* as a homodimer of two 16.8 kDa monomers [11, 12], the Fhit elution profile (Fig. 3A) suggests the presence of other larger enzymatically active forms in addition to the dimer. It is worth noting that soluble aggregates of the Fhit protein, larger than dimers, were detected by gel-filtration during purification of human Fhit protein expressed in *E. coli* [17]. Such observations suggest that after purification as a homodimer, the protein undergoes aggregation/dissociation. Polyclonal anti-Fhit antibodies differentially inhibit enzymatic activities of Fhit and platelet Ap_3Aase. Indeed, as shown in Fig. 3B, the inhibitory effect of Fhit antibodies is stronger on Fhit than on platelet Ap_3Aase. These results indicate that human platelet Ap_3Aase and the Fhit protein are kinetically and immunochemically closely related enzymes, but they may differ in some structural aspects which deserve further investigation.

We have also partially purified Ap_3Aase activities from human leukocytes and placenta, initial data indicating that there are no significant differences in kinetic properties and molecular mass relative to the platelet Ap_3Aase. The distinction between Fhit enzyme and other Ap_3A-cleaving enzymes is a crucial point with basic and clinical implications. It has recently been demonstrated that human Fhit enzyme and lupin Ap_3Aase (probably a plant homologue of human Ap_3Aase) may be distinguished by using new selective inhibitors [25]. It follows from this fine observation that these inhibitors should now be tested to verify whether human Fhit enzyme and human Ap_3Aase may be similarly distinguished.

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