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Purification and Characteristics of Functional Properties of Soluble Nucleoside Triphosphatase (Apyrase) from Bovine Brain

V. F. Sivuk[1], I. M. Rusina[1,2], and A. F. Makarchikov[1,2]*

[1]*Laboratory of Biochemical Toxicology and Narcology, Institute of Pharmacology and Biochemistry, National Academy of Sciences of Belarus, BLK 50, 230030 Grodno, Belarus; fax: +375 (152) 434-121; E-mail: office@biochem.unibel.by*
[2]*Technological Faculty, Grodno State Agricultural University, ul. Tereshkovoi 28, 230008 Grodno, Belarus; fax: +375 (152) 721-365; E-mail: a_makarchikov@yahoo.com*

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Abstract—Soluble NTPase, differing in its properties from known proteins exhibiting NTPase activity, was purified from bovine brain to homogeneity. The enzyme has pH optimum at 7.5 and shows absolute dependence on bivalent cations and broad substrate specificity towards nucleoside-5′-tri- and -diphosphates, characteristics of apyrases. The NTPase follows Michaelis–Menten kinetics in the range of investigated substrate concentrations, the apparent K_m values for UTP, ITP, GTP, CTP, CDP, and ATP being 86, 25, 41, 150, 500, and 260 μM, respectively. According to gel-filtration and SDS-PAGE data, the molecular mass of the enzyme is 60 kD. The NTPase is localized in the cytosol fraction and expressed in different bovine organs and tissues. Total NTPase activity of extracts of bovine organs and tissues decreases in the following order: liver > heart > skeletal muscle > lung > brain > spleen > kidney ≈ small intestine. The enzyme activity can be regulated by acetyl-CoA, α-ketoglutarate, and fructose-1,6-diphosphate acting as activators in physiological concentrations, whereas propionate exhibits an inhibitory effect.

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In addition to the universal adenylate system of energy storage and transformation, a number of different nucleoside-5′-triphosphates are involved in biosynthesis pathways and cell signaling [1]. In biological systems, along with ATPases of P-, F-, and V-types and cytoskeletal ATPases, several different phosphatases capable of nucleoside-5′-tri(di)phosphate hydrolyses have been found. One group of these proteins, classified as NTPases (nucleoside triphosphatases), has ordinal number 3.6.1.15 in the International Enzyme Classification. NTPases include a number of unrelated enzymes encoded by genomes of eukaryotes and prokaryotes, as well as by viral genomes [2-4]. It was shown that NTPases are localized in different cell compartments and are evidently involved in different aspects of vital activity [5-8]. Nevertheless, biological functions of most eukaryotic NTPases, except for the enzyme of nuclear envelope, have not been identified. Also, little is known about the molecular structure of these enzymes, which makes it impossible to assign them to a certain protein family. For example, some phosphatases like ectoapyrase [9] or NTPase from *Toxoplasma gondii* [10], formerly considered as NTPases, were later identified as apyrases (EC 3.6.1.5) by the presence in them of apyrase conserved regions (ACR) [11].

This work deals with purification and determination of the kinetic and regulatory properties of soluble NTPase discovered in bovine brain. The enzyme exhibits broad substrate specificity towards nucleoside-5′-tri- and -diphosphates with CTP as the preferred substrate, and it clearly differs from all known soluble proteins exhibiting NTPase activity. Unlike another soluble NTPase, expressed mainly in liver, kidneys, and small intestines [12], the enzyme under investigation is widespread in bovine tissues. We have also shown the cytosolic localization of the NTPase. Since non-coupled NTP hydrolysis should be considered as an undesirable process, we suppose that, based on the effects of fructose-1,6-diphosphate, propionate, and acetyl-CoA, the enzyme might be involved in the regulation of gluconeogenesis rate by changing local concentrations of GTP (ITP), the substrates of phosphoenolpyruvate carboxykinase.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

The following reagents were used in this work: Sephadex G-100 and Sephacryl S-200 (Amersham Pharmacia Biotech, Sweden); hydroxyapatite (Fluka, Germany); DEAE-Toyopearl 650 M and Toyopearl HW-60 (Toyo-Soda Co., Japan); protein standards (Serva, Germany); nucleoside phosphates, pyruvate, and propionate (Sigma-Aldrich, USA); fructose-1,6-diphosphate, glucose-6-phosphate, oxaloacetate, and phosphoenolpyruvate (Reanal, Hungary); all other reagents were of analytical grade (Russia).

NTPase activity was determined by P_i formation. The standard reaction mixture contained 50 mM Tris-HCl, pH 7.0, 0.5 mM UTP, 10 mM $MgCl_2$, 20 μg BSA, and the enzyme sample in final volume 0.2 ml. The reaction was carried out for 10-30 min at 37°C and stopped by addition of 1 ml reagent for P_i determination according to Lanzetta et al. [13]. The enzyme amount that catalyzed formation of 1 μmol P_i in 1 min under the standard experimental conditions was taken as 1 activity unit (U).

The molecular mass of the enzyme was determined by electrophoresis [14] and by gel filtration on a Sephacryl S-200 column (2.2 × 46 cm) calibrated using standard proteins. Electrophoresis was carried out in vertical plates of 10% polyacrylamide gel in a phosphate buffer system with staining by Coomassie Brilliant Blue R-250. Molecular mass of denatured NTPase was calculated using a calibration plot based on protein standards. Gel filtration was carried out at 4°C in 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl. Samples were applied onto the column in the volume of 3 ml and eluted at flow rate 5 cm/h.

To study NTPase distribution in tissues, samples of bovine organs stored at –20°C were homogenized in a glass homogenizer in four volumes of 50 mM Tris-HCl, pH 7.3, 0.15 M KCl, 0.2 mM EDTA, and centrifuged at 105,000*g* for 90 min. Erythrocytes were isolated from blood diluted with 5% sodium citrate buffer, pH 6.0, at ratio 4 : 1. Red blood cells were washed twice with three volumes of isotonic NaCl solution and disintegrated by freeze–thawing, and then membranes were removed by high-speed centrifugation. To determine molecular mass of components, corresponding to peaks of NTPase activity, extracts were chromatographed on a Sephacryl S-200 column as described above.

For isolation of subcellular fractions, samples of fresh brain tissue were homogenized for 10 cycles in a glass homogenizer with Teflon plunger at 600 rpm in nine volumes of 10 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 0.5 mM EDTA. The homogenate was filtered through eight layers of wet gauze and fractionated by differential centrifugation to obtain nuclear (1000*g*, 10 min), mitochondrial (10,000*g*, 20 min), microsomal (105,000*g*, 60 min), and cytosol (final supernatant) fractions. Each pellet was washed twice using the isolation medium. Parts of the pellets were suspended in 50 mM Tris-HCl, pH 7.3, 0.15 M KCl, 0.2 mM EDTA and frozen to –20°C. Then thawed suspensions were homogenized in a glass homogenizer and centrifuged for 60 min at 105,000*g* to separate membrane and soluble fractions. Before determination of NTPase activity, pellets were suspended in initial buffer containing 0.32 M sucrose.

Protein was determined according to Bradford [15] with BSA as standard and by absorption at 280 nm or at 215/225 nm [16].

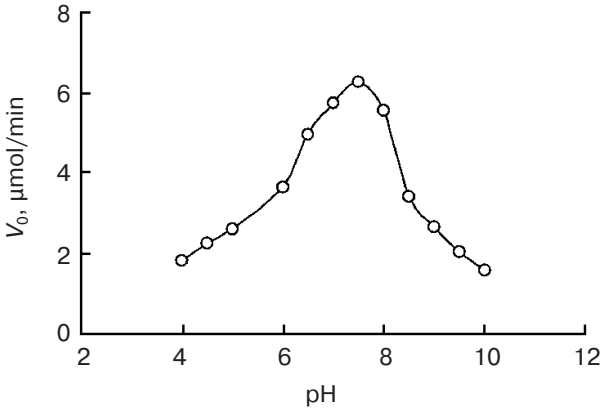
The NTPase was purified at 4-6°C. A sample of bovine brain (200 g) stored at –80°C was thawed and homogenized in 400 ml 50 mM Tris-HCl, pH 7.4, 0.15 M KCl, 1 mM EDTA in an RT-1 tissue disintegrator at 4000 rpm for 2 min. The homogenate was centrifuged for 60 min at 5000*g*. The supernatant was filtered through 10 layers of wet gauze, pH was adjusted to 7.4 using 1 M Tris-HCl, pH 8.9, and ammonium sulfate was added to 35% saturation (208.4 g/liter). After stirring for 30 min, the precipitate was removed by centrifugation at 5000*g* for 60 min. The supernatant was passed through a Toyopearl HW-60 column (3.4 × 19 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.2 mM EDTA, and ammonium sulfate (35% saturation) at the flow rate 10 cm/h. The column was washed with four volumes of buffer, and adsorbed protein was eluted in a linearly decreasing (from 35 to 0% saturation) gradient of $(NH_4)_2SO_4$ (250 ml in each chamber) in the same buffer at a rate of 10 cm/h. Fractions with specific activity 10-15-fold exceeding that at the previous stage were combined and precipitated with ammonium sulfate (390 g/liter), and then the precipitate was dissolved in 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.2 mM EDTA and chromatographed on a Sephadex G-100 column (3.6 × 67 cm) at flow rate 9 cm/h. Fractions whose specific activity increased 3-4 times were combined, and glycerol was added to 20% concentration. Then the protein solution was applied onto a DEAE-Toyopearl 650 M column (1.5 × 19 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 50 mM KCl, and 20% glycerol. The carrier-bound protein was eluted in a linearly increasing (from 50 to 400 mM) KCl gradient (150 ml in each chamber) at flow rate 10 cm/h. Fractions eluted at 78-96 mM salt concentration were combined and applied onto a hydroxyapatite column (1.7 × 7.5 cm) equilibrated with 1 mM K-phosphate buffer, pH 7.2. Contaminant proteins were eluted by a linearly increasing KCl gradient (100 ml in each chamber) from 0 to 0.5 M; NTPase was desorbed in a linearly increasing gradient (100 ml in each chamber) of K-phosphate buffer, pH 7.2, from 1 to 300 mM at flow rate 5 cm/h. Fractions with high specific activity eluted in 135-160 mM K-phosphate buffer were combined, concentrated using Centriplus 10 (Amicon, USA) centrifuge filters, and chromatographed on a Sephacryl S-200 column (2.2 × 46 cm) in 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.2 mM EDTA at flow rate

5 cm/h. At the final stage of purification concentrated fractions were applied onto a column (0.8 × 3.0 cm) of Blue Sepharose equilibrated with 20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, and 50 mM NaCl. Adsorbed protein was eluted in a linearly increasing pH and glycerol gradient (from 7.5 to 8.5 and from 0 to 40%, respectively) (20 ml in each chamber). Fractions of equal specific activity were combined, concentrated to 0.5 ml, frozen, and used for analysis.

RESULTS

The purified enzyme preparation had specific activity 639 U/mg protein, which 4915-fold exceeds that in the extract (Table 1). In the course of gel filtration on the Sephacryl S-200 column (the penultimate stage of purification), the NTPase from bovine brain eluted as a symmetrical activity peak corresponding to a 58.6 kD protein. SDS-PAGE of the purified protein revealed a single protein band of 60.2 kD (data not shown).

Kinetic properties and substrate specificity of the NTPase. The effect of hydrogen ion concentration on NTPase activity was studied in the pH range from 4.0 to 10.5 under standard reaction conditions, but to maintain the pH acetate (20 mM, pH 4.0-5.5), maleate (25 mM, pH 6.0-6.5), Tris-maleate (50 mM, pH 7.0), Tris-HCl (50 mM, pH 7.5-8.9), or glycine (50 mM, pH 9.2-10.5) buffers were used. The data in the figure show that the optimum pH for the enzyme activity is 7.5.



Effect of pH on activity of NTPase from bovine brain

No NTPase activity was observed in the absence of bivalent metal ions. As seen in Table 2, Mg^{2+} exhibits the maximal activating effect among the studied cations. Other metal cations like Mn^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, and Zn^{2+} are less efficient, and Ba^{2+} is not able to activate the enzyme.

Table 3 shows data on the substrate specificity of the NTPase. As seen, the enzyme catalyzes hydrolysis of a broad spectrum of nucleoside-5′-tri- and diphosphates with CTP as the preferred substrate. With nucleoside-5′-monophosphates like UMP and IMP as substrates, the amount of liberated P_i was less than 10% compared to CTP, whereas no reaction took place with GMP, AMP, or CMP.

We studied the effect of increasing substrate concentrations on initial rate of the NTPase reaction at fixed 10 mM concentration of Mg^{2+}. CTP, UTP, ITP, GTP, ATP, and CDP were used as substrates. In all cases the reaction followed Michaelis–Menten kinetics in the studied concentration intervals, except for UTP for which increase in concentration to 0.6 mM caused partial inhibition of the enzyme. Apparent K_m values calculated using Hanes plots were 150 ± 20, 86 ± 2, 25 ± 1, 41 ± 3, 260 ± 30, and 500 ± 40 μM, respectively, for CTP, UTP, ITP, GTP, ATP, and CDP (data not shown).

Effect of different compounds on the NTPase activity. We studied a number of subcellular compounds, including some glycolysis and Krebs cycle metabolites (glucose-6-phosphate, fructose-1,6-diphosphate, α-ketoglutarate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, citrate, isocitrate, succinate, and oxaloacetate), as well as propionate and acetyl-CoA as possible enzyme modulators. All these compounds were used at 0.5 mM concen-

Table 1. Purification of NTPase from bovine brain

Fraction	Volume, ml	Total protein, mg	Specific activity, U/mg
Extract	385	4080	0.13
Toyopearl HW-60	100	336	1.7
Sephadex G-100	35	63.0	6.1
DEAE-Toyopearl 650 M	15	24.6	14.8
Hydroxyapatite	3	3.4	69
Sephacryl S-200	5	1.0	211
Blue Sepharose	0.5	0.07	639

Table 2. Effect of bivalent metal ions on activity of NTPase from bovine brain

Cation (5 mM)	Relative activity, %
—	0
Mg^{2+}	100
Mn^{2+}	73 ± 6
Co^{2+}	60 ± 5
Ca^{2+}	52 ± 11
Cu^{2+}	46 ± 4
Zn^{2+}	34 ± 9
Ba^{2+}	0

Table 3. Substrate specificity of NTPase from bovine brain

Substrate (0.5 mM)	Relative activity, %
CTP	100 ± 3
UTP	66 ± 10
ITP	58 ± 3
CDP	41 ± 3
IDP	41 ± 3
XTP	40 ± 5
ATP	32 ± 2
UDP	32 ± 1
GTP	30 ± 5
ADP	25 ± 3
GDP	19 ± 2
UMP	8.2 ± 1.0
IMP	5.3 ± 1.5
GMP	0
AMP	0
CMP	0

Table 4. Effect of different compounds on activity of NTPase from bovine brain

Compound	Concentration, mM	Activity, %
None	–	100
Acetyl-CoA	0.5	148 ± 1
«	0.05	127 ± 1
Citrate	0.5	80 ± 1
α-Ketoglutarate	0.5	148 ± 2
«	0.05	128 ± 6
Succinate	0.5	45 ± 1
«	0.05	98 ± 3
Fructose-1,6-diphosphate	0.5	155 ± 4
«	0.05	126 ± 1
Propionate	0.5	39 ± 2
«	0.05	63 ± 6

tration. Intermediates of glycolysis had no noticeable effect on the NTPase activity, except for fructose-1,6-diphosphate that increased the rate of GTP hydrolysis by 55%. α-Ketoglutarate and acetyl-CoA activated the enzyme to practically the same extent, although other Krebs cycle metabolites like citrate and succinate are inhibitors. Moreover, the enzyme activity decreases in response to propionate (Table 4). These effects of fructose-1,6-diphosphate, α-ketoglutarate, acetyl-CoA, and propionate are also clearly revealed at 0.05 mM concentration.

Subcellular localization of the NTPase and its distribution in bovine organs and tissues. Approximately 44% of the total NTPase activity was found in soluble extract obtained by high-speed centrifugation (105,000*g*, 60 min) of homogenate prepared from samples of frozen brain tissue. Independently of substrate (GTP, UTP, CTP, or ATP) used for activity determination, chromatography of the extract on a column of Sephacryl S-200 produced two NTPase peaks (data not shown). One of these peaks appeared in the column void volume, while the other, containing up to 78% of the enzyme activity, corresponded to the 60.5 kD protein. As shown by differential centrifugation, the cytosol fraction of bovine brain contains about 43% of the total NTPase activity of the initial homogenate. The rest of the activity was pelleted with subcellular particles.

NTPase activity was found in extracts of all studied bovine organs and tissues, and specific activity varied from 0.09 U/mg in erythrocytes to 0.47 U/mg in liver. Total NTPase activity per gram of tissue insignificantly differed for extracts of skeletal muscle, heart, spleen, kidneys, brain, lungs, and small intestine (2.5-4.8 U/g), whereas enzyme activity in liver extract reached 21.9 U/g. In all cases of gel filtration of different tissue extracts (except for erythrocytes) NTPase activity peaks corresponding to the 60 kD protein were eluted (data not shown). Besides, large peaks corresponding to a 146 kD protein were revealed on chromatograms of extracts of liver, kidneys, and small intestine. A significantly lower amount of high molecular weight NTPase was found in spleen, heart, and lung, whereas it was not detected in brain, skeletal muscle, and erythrocytes. Peak areas suggest the following distribution of 60 kD NTPase in bovine tissues: liver > heart > skeletal muscle > lungs > brain > spleen > kidneys ≈ small intestine.

DISCUSSION

Nucleoside-5′-triphosphates are a source of energy for various vital functions. The central role among this class of compounds belongs to ATP, which provides energy for the biosynthesis of the most important cell components, active transmembrane transport, motion processes, and gene expression. Besides, GTP, UTP, CTP, and ITP are used in some processes and biosynthesis pathways [1]. Along with energy transforming ATPases [17, 18], enzymes probably involved in uncoupled hydrolysis of nucleoside-5′-triphosphates are found in cells. Two leading groups of such enzymes, whose substrate specificity is mainly restricted to nucleoside-5′-tri- and -diphosphates, are classified as NTPases (EC 3.6.1.15) and apyrases (EC 3.6.1.5).

The distinguishing feature of apyrase family members is the presence in their structure of four homologous sequences known as apyrase conservative regions (ACR) [11]. Although apyrases were originally described as ectoenzymes like CD39 [19] or other ecto-NTPases [9, 20], they can also be observed inside cells [21-23]. It is assumed that the main role for apyrases localized at the cell surface is completion of the physiological effect of nucleosides released from cells [24, 25]; biological functions of endoapyrases are less obvious. Endoapyrases might be involved in protein glycosylation, sugar concentration control, and regulation of membrane integrity [21, 26]. Apyrases are widespread in different types of eukaryotic cells, from yeasts to mammals [9, 21, 26, 27]. Most studied apyrases are localized in cell membrane structures, but soluble proteins belonging to this family are also known [10, 28].

Several soluble NTPases of eukaryotic origin are described in the literature. Thus, it was shown that two cytosolic NTPases with pH optima 4.0-4.5 and 8.6-9.9 and molecular mass 65 and 125 kD [6, 29], respectively, were found in rat liver. A metal-independent 75 kD NTPase with pH optimum 3.0 was isolated from human blood serum [3]. Another acidic NTPase exhibiting maximal activity at pH 5.0-5.5 was found in lysosomes of rat liver and kidneys [5]. Rather recently, we have purified a soluble NTPase from bovine kidneys: it is a 146 kD protein with pH optimum 7.0 and strict specificity for GTP, UTP, and ITP. In this work, we show that another soluble enzyme exhibiting apyrase activity is present in bovine tissues.

The use of routine methods of purification made it possible to obtain from bovine brain the soluble NTPase preparation with specific activity 639 U/mg protein (Table 1). According to gel filtration and SDS-PAGE data, the molecular mass of the enzyme is 60 kD. Kinetic studies have shown that the pH optimum of the enzyme is 7.5 (Fig. 1), and it is absolutely dependent on bivalent metal cations (Table 2). The combination of these properties allows one to differentiate between the bovine brain NTPase and all earlier described soluble NTPases of eukaryotic origin, because the latter have higher molecular mass [10, 29], different pH optimum [5, 6], or dependence on metal ions [3].

The bovine brain NTPase is characterized by broad specificity towards different nucleoside-5′-tri- and -diphosphates and it is fully indifferent towards GMP, AMP, and CMP. However, this enzyme is capable of slight acceleration of UMP and IMP hydrolysis (Table 3). A similar pattern of substrate specificity is characteristic of apyrases. To our knowledge no soluble apyrase from mammalian tissues is described in the literature. Like some membrane-bound apyrases [30, 31], the enzyme from bovine brain exhibits a high apparent affinity to nucleoside-5′-triphosphates.

Experiments using differential centrifugation of brain homogenates point to cytosolic localization of the studied enzyme. In this connection, it can be supposed that it is involved in nucleoside diphosphate hydrolysis not coupled with any work. It is obvious that such a process is of no use for a cell. To obtain any information concerning a possible biological role of the NTPase, we studied the effects of a number of intracellular compounds related to energy metabolism. Some of them appeared to influence the enzyme activity (Table 4). We consider as especially interesting the fact that the NTPase is sensitive to fructose-1,6-diphosphate and propionate in physiological concentrations, and the first of these is an activator while the other exhibits inhibitory effect.

It is known that propionate is a precursor in glucose biosynthesis, and this process is of special importance for ruminants [32]. Despite the fact that bacteria of the rumen are capable of efficient cleavage of the vegetable fodder cellulose, the formed glucose does not immediately enter the bloodstream. Instead, it is fermented to volatile fatty acids (VFA), among which propionate plays a noticeable role [33]. Propionate transported into the blood is mainly metabolized in liver, while a part of it is spread into tissues, where it can be transformed to glucose during gluconeogenesis. It was shown that the VFA transfer through cell membranes in the gastrointestinal tract is mediated by monocarboxylate transporter 1 of the SLC16 gene family [34]. The same protein is widely expressed in different organs and tissues, including the central nervous system, where together with monocarboxylate transporter 2 it is involved in VFA transport in endothelial cells of the blood–brain barrier, astrocytes, and neurons [35, 36]. Evidently, the increase in intracellular propionate concentration should stimulate glucose synthesis. On the other side, propionate inhibits NTPase. This may result in increase in local concentrations of GTP (ITP), the substrates of phosphoenolpyruvate carboxykinase, catalyzing phosphoenolpyruvate formation in the pyruvate kinase shunt. At the same time, the increase in fructose-1,6-diphosphate level under energy deficiency conditions could cause an increase in NTPase activity, accelerated GTP (ITP) hydrolysis, and as a result a decrease in the rate of glucose synthesis. It seems that opposite effects of fructose-1,6-diphosphate and propionate allow us to suppose as a working hypothesis that the enzyme under investigation can be involved in reciprocal regulation of the glycolysis and gluconeogenesis rates. It should be noted in this connection that although brain metabolism is extremely dependent on the blood glucose, nevertheless cells of nerve tissue are able to synthesize their own glucose from non-carbohydrate precursors, but to much lesser extent compared to liver or kidneys [37].

In the light of this hypothesis, it would be possible to explain the activating effect of acetyl-CoA (Table 4). In some states (like lipolysis), increased intracellular concentration of acetyl-CoA is observed, which should undergo accelerated metabolism in the Krebs cycle. This requires a continuous supply of regenerating substrate,

oxaloacetate, synthesized from pyruvate by pyruvate carboxylase. Thus, acetyl-CoA, a positive effector of NTPase, is able to retard phosphoenolpyruvate formation from oxaloacetate (gluconeogenesis), which in the situation under consideration should stimulate an increase in glycolysis rate.

It should be said in conclusion that whatever the function of NTPase under investigation is, the enzyme is probably not related to the specificity of the nerve cell functions because it is characterized by wide distribution in organs and tissues.

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